WNT SIGNALING AT THE PLASMA MEMBRANE: ACTIVATION, REGULATION AND DISEASE CONNECTION

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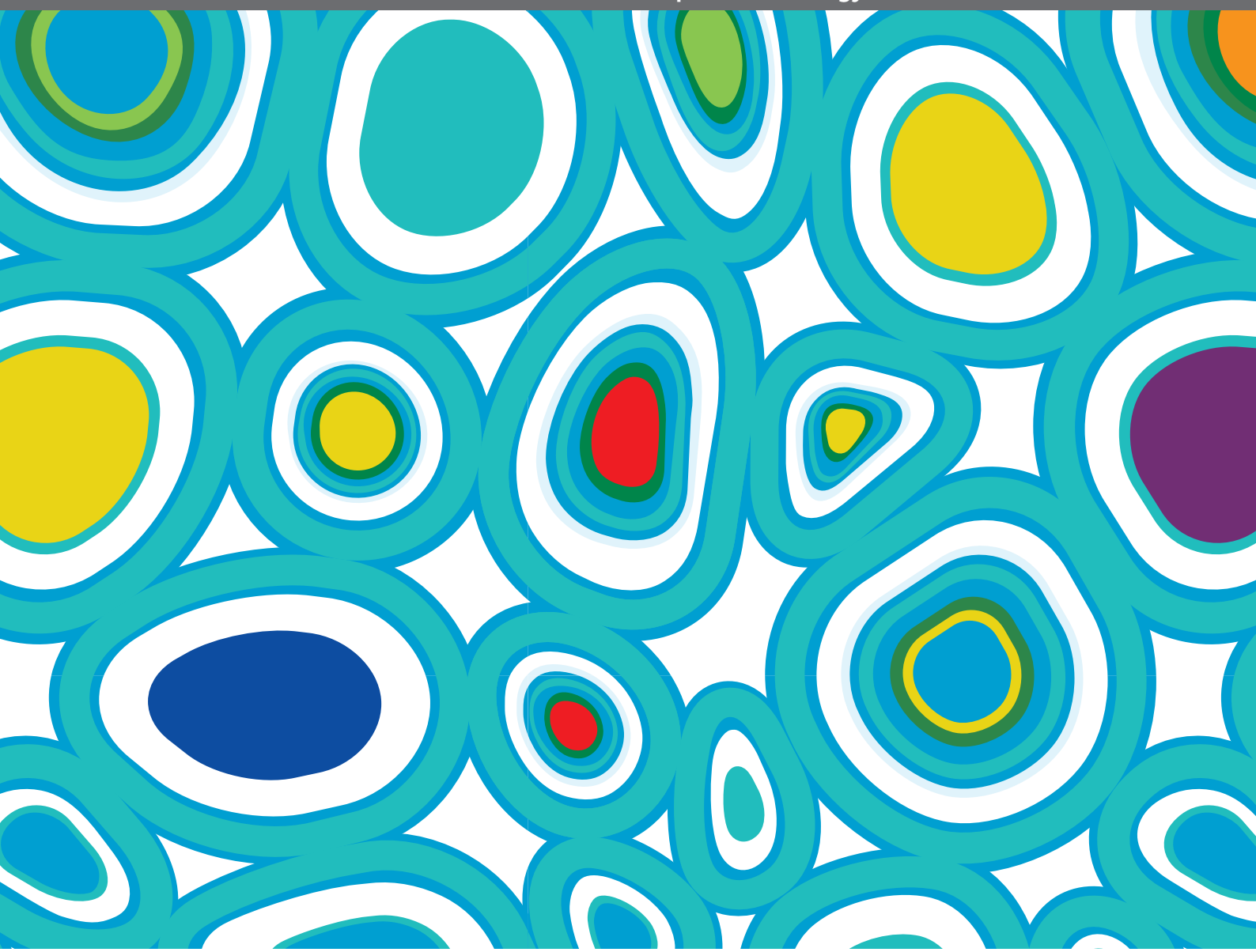

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WNT SIGNALING AT THE PLASMA MEMBRANE: ACTIVATION, REGULATION AND DISEASE CONNECTION

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EDITORIAL
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Editorial: Wnt Signaling at the Plasma Membrane: Activation, Regulation and Disease Connection

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Editorial on the Research Topic

Wnt Signaling at the Plasma Membrane: Activation, Regulation and Disease Connection

Wnt signaling pathways constitute evolutionarily conserved signaling pathways that control a wide range of cellular processes including proliferation, migration, differentiation and morphogenesis during development, maintenance of adult tissue homeostasis, and organ regeneration. Thus, it is not surprising that dysregulation of the Wnt pathway has been associated with many human diseases including cancer, congenital malformations, degenerative diseases, and metabolic disorders. At this point, detailed understanding of underlying Wnt-mediated cellular responses is essential for the development of effective therapies against Wnt-related diseases. The plasma membrane (PM) plays a key role in the regulation of cell signaling and thus has been frequently investigated for its molecules as drug targets. Owing to its essential roles in activation of Wnt-receptor complex and initiation of Wnt signaling, the PM has been investigated thoroughly for the regulatory role of its composition and lipid order in pathway regulation. A prominent feature of unhealthy cells such as cancer cells is that they significantly differ from healthy cells with respect to their PM composition and lipid organization. Since the complex interactions of Wnt proteins and their receptors are tightly dependent on the composition and organization of the PM, a better understanding of the molecular mechanisms underlying Wnt-receptor complex formation is essential for identification and testing of novel attractive drug targets for Wnt-related human diseases including cancer. In this Research Topic (RT) with 15 papers, we aimed to collect the review and research articles that provide an overview of the recent developments that enlighten the molecular interaction mechanisms of the Wnt-receptor complex components and pathway modulators as well as how misregulation of these interactions associate with signaling activation and human diseases. We have also included recent scientific progress that we believe that understanding these mechanisms will greatly contribute to the development of novel therapies that target Wnt pathways at the PM.

For this RT, we have received several papers that present detailed analyses of the structure and functional roles of various Wnt ligands, receptors and co-receptors in development and disease. In their review, Suthon et al. have provided a comprehensive view of the mechanistic roles of the non-canonical ligand WNT5B on general development, bone physiology, adipogenesis, pancreas development, heart development, central nervous system (CNS development, physiology of the mammary gland and the lung, hematopoiesis, and the lymphatic system. The review also summarized how Wnt5B is involved in bone diseases including osteoarthritis and osteoporosis, Type II diabetes, neuropathological conditions, chronic obstructive pulmonary disease and various cancers that include breast, pancreas, lung, colorectal, ovary and brain cancers as well as oral squamous cell carcinoma, osteosarcoma, hepatocellular carcinoma, and leukemia. Wei et al. have

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reviewed the broad biological functions of another Wnt ligand WNT6 in embryonic development. In specific, the authors have focused on decidualization- transformation of the endometrial cells into specialized secretory decidual cells during pregnancy-, kidney development, differentiation of human dental papilla cells, adipogenesis, and osteoblastogenesis. Furthermore, the review provides a detailed overview of the association of WNT6 with various pathologies that include cardiac injury, kidney fibrosis, lung tuberculosis, Rett syndrome, glioblastoma, colorectal cancer, and gastric cancer. In their mini-review on Wnt ligand dependencies in pancreatic cancer, Aguilera and Dawson have discussed the activation of canonical and non-canonical Wnt signaling pathways in pancreatic ductal adenocarcinoma (PDAC). They also review the potential use of therapeutic approaches that functionally antagonize Wnt signaling at the level of ligand secretion or receptor binding. Dhasmana et al., have presented the results of their elegant work on characterization of the role of lipid modifications of the canonical Wnt ligand Wnt3 in membrane organization, secretion, interaction, and signaling activity by exploiting the zebrafish model. By substituting the homologous cysteine and serine residues of zebrafish Wnt3 with alanine, they have revealed that Wnt3 is lipidated at both residues. Lipidation at either of the residues is sufficient for its secretion and proper interaction with the membrane, while the lipid modification at serine is indispensable for receptor interaction and signaling.

Pascual-Vargas and Salinas have elaborately reviewed the molecular function of Frizzled receptors in the mammalian CNS with a special focus on its roles in axon guidance, dendritic morphogenesis, synapse formation and postnatal hippocampus development. The authors have also discussed in detail the post-translational modifications, namely N-linked glycosylation, phosphorylation, and ubiquitination/deubiquitination, of Frizzled receptors regarding their modulation of the receptor function in neuronal connectivity through control of its localization and stability at the PM. Another review by Ren et al. has systematically examined the similarities and divergences between the canonical Wnt co-receptors LRP5 and LRP6 with respect to their expression patterns in human tissues and organ development. They highlight that, in contrast to the high homology between LRP5 and LRP6, they are expressed differentially in diverse tissues and organs in embryonic development and adulthood and that LRP6 is functionally more potent and unique *via* coupling both Wnt with Hippo signaling pathways. The authors have also discussed how Wnt signaling is activated after kidney injury and in renal tissues in diabetes. In their brief research report, Haack et al. have analyzed whether and how the quantitative ratio between membrane receptors and membrane microdomains (rafts) influence LRP6 phosphorylation and WNT/β-catenin pathway activation. By using a computational modeling approach and considering microdomains as single compartments in the membrane, they have found that LRP6 phosphorylation and downstream Wnt signaling activity decreases when the number of raft compartments exceeds the number of pathway specific membrane proteins. This reveals that pathway specific targeting and sorting are critical to restrict the receptor/raft ratio and signaling activation. Jeong and Jho have reviewed activation of LRP6 and its influence on β-catenin-dependent and -independent signaling pathways. They have also discussed in detail how LRP6 dysregulation is associated with diseases including cancer, neurodegeneration, metabolic syndrome, inflammation, and skeletal disease. Shi has thoroughly examined the functional roles of Disheveled in Wnt signaling pathways in gastrulation and neurulation processes by focusing on the vertebrates mouse, *Xenopus*, and zebrafish. The author specifically emphasizes the influence of Disheveled on maternal contribution for axis patterning, cell fate specification and morphogenetic movements.

A comprehensive review by Azbazdar et al. summarizes how membrane composition and lipid order tightly controls formation of the Wnt-receptor complex, its interactions with the pathway modulators and activation of Wnt signaling pathways. Moreover, the study gives a detailed overview of the involvement of the ligands, receptors, coreceptors and extracellular or membrane-bound modulators of Wnt pathways in progression of lung, colorectal, liver, and breast cancers that have been associated with abnormal activation of Wnt signaling.

In their original research, Yong et al. have unraveled the stimulatory role of adiponectin, a secreted protein produced by adipocytes, on the expression of adiponectin receptors as well as β-catenin and MAPK signaling pathways in cementoblasts in the presence of compressive forces. They have also demonstrated that β-catenin is blocked by MAPK inhibition and rescued by adiponectin.

In addition to its well-known roles in development, growth and tissue patterning, Wnt signaling in mammals is necessary for regulation of energy homeostasis. In their elegant review, Azar and Lim have summarized the individual roles of the Wnt pathway components, namely Wnt ligands, β-catenin, destruction complex component APC and transcription factor TCF7L2 in development of metabolic tissues. Moreover, they have discussed the contributions of these components to the whole-body metabolism and potential roles in development of metabolic diseases such as diabetes and obesity.

In their study to understand the role of proliferative stress, Hachim et al. have found that canonical Wnt signaling is activated and required for the asthmatic bronchial epithelial cells to respond to this stimulus. These cells have activated senescence mechanisms in a Wnt-dependent manner. Interestingly, fibroblasts from asthmatic patients were not responsive to inhibition of Wnt signaling.

Finally, Karabicici et al. have shown that the multi-kinase inhibitor regorafenib activates Wnt/β-catenin signaling selectively in hepatoblast-like HCC cell lines. Strikingly, Wnt/β-catenin signaling protects these cells from regorafenib-induced apoptosis. In contrast, long-term regorafenib-treated resistant cells have reduced Wnt/β-catenin activity and enhanced TGF-β activity with a concomitant increase in expression of cancer stem cell markers. Importantly, regorafenib treatment and TGFβ-R1 inhibition have collectively decreased colony formation ability and promoted cell death in resistant spheroids. These results demonstrate that HCC

tumors with aberrantly activated Wnt/β-catenin pathway are more resistant to regorafenib, suggesting that regorafenib administration in combination with pathway inhibition can enhance the drug-induced cell death. On the other hand, acquired regorafenib resistance in HCC can be overcome with the combined use of TGF-β pathway inhibitors together with regorafenib.

To conclude, this RT provided us with the opportunity to collect recent data and perspectives from researchers whose studies on Wnt signaling pathways span over a wide range of disciplines. By enabling us to review the recent findings and advances in activation and regulation of Wnt signaling in health and disease, we believe that this RT will greatly contribute to our understanding of pathway mechanisms and the development of new therapeutic approaches for Wnt-related human diseases.

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GO has written the Editorial manuscript.

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MINI REVIEW
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Decoding Dishevelled-Mediated Wnt Signaling in Vertebrate Early Development

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Dishevelled proteins are key players of Wnt signaling pathways. They transduce Wnt signals and perform cellular functions through distinct conserved domains. Due to the presence of multiple paralogs, the abundant accumulation of maternal transcripts, and the activation of distinct Wnt pathways, their regulatory roles during vertebrate early development and the mechanism by which they dictate the pathway specificity have been enigmatic and attracted much attention in the past decades. Extensive studies in different animal models have provided significant insights into the structure-function relationship of conserved Dishevelled domains in Wnt signaling and the implications of Dishevelled isoforms in early developmental processes. Notably, intra- and inter-molecular interactions and Dishevelled dosage may be important in modulating the specificity of Wnt signaling. There are also distinct and redundant functions among Dishevelled isoforms in development and disease, which may result from differential spatiotemporal expression patterns and biochemical properties and post-translational modifications. This review presents the advances and perspectives in understanding Dishevelled-mediated Wnt signaling during gastrulation and neurulation in vertebrate early embryos.

Keywords: Wnt signaling, Dishevelled, Wnt/ß-catenin, Wnt/PCP, convergence and extension, mouse, *Xenopus*, zebrafish

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INTRODUCTION

Wnt signaling plays critical roles in a wide variety of biological processes, including embryonic axis formation, cell proliferation, differentiation and migration, polarity establishment, and stem cell self-renewal (Steinhart and Angers, 2018; Wiese et al., 2018). Upon stimulation by Wnt ligands, membrane receptors (Frizzled) and co-receptors (LRP5/6, glypican-3/4, ROR, and RYK) assemble into complexes to activate divergent pathways (Niehrs, 2012; Green et al., 2014; Stricker et al., 2017). The activity of Frizzled receptors is further controlled by an auxiliary regulatory system involving RSPO1-4, LGR4/5/6 and ZNRF3/RNF43 (Jiang and Cong, 2016; Lehoczky and Tabin, 2018). The canonical Wnt pathway (Wnt/ß-catenin) regulates target gene transcription through stabilization and nuclear accumulation of ß-catenin by inhibition of its destruction complex, consisting of Axin-GSK3ß-APC, whereas the non-canonical Wnt pathway (Wnt/planar cell polarity or Wnt/PCP) is implicated in polarized cellular orientation and asymmetric cell movements through activation of major regulators of the cytoskeleton. Aberrant signaling of both pathways leads to tumorigenesis and metastasis of multiple cancer types, as well as human birth defects (Clevers and Nusse, 2012;

Butler and Wallingford, 2017; Humphries and Mlodzik, 2018), but how they are regulated in development and disease remains elusive.

Dishevelled (Dvl or Dsh in *Drosophila*) is a family of proteins that function as common intracellular conductors of both Wnt/ß-catenin and Wnt/PCP pathways (Boutros and Mlodzik, 1999; Wallingford and Habas, 2005; Gao and Chen, 2010). *Drosophila dsh* alleles were first identified in genetic mutants with disruptions of hair and bristle polarity (Wallingford and Habas, 2005). Vertebrates possess three highly conserved *Dvl* genes. Extensive studies in mouse, *Xenopus* and zebrafish have revealed their critical roles in germ layer specification and morphogenetic movements, which require Wnt/ß-catenin and Wnt/PCP signaling, respectively. There is accumulating evidence that Dvl isoforms display both distinct and redundant functions (Gentzel and Schambony, 2017). However, a number of important questions regarding Dvl-mediated Wnt signaling during development remain enigmatic (Mlodzik, 2016), such as Dvl-regulated switch of distinct Wnt pathways, the specific functions of Dvl isoforms in Wnt signaling and development, the post-translational modifications of Dvl functions, and the maternal contributions of Dvl to early developmental events. Fortunately, structure-function and mutational analyses have significantly advanced our understanding of Dvl-regulated Wnt signaling in development and disease. This review focuses on progresses made in this fascinating research field by using complementary vertebrate animal models.

Dvl FUNCTIONAL DOMAINS IN Wnt SIGNALING

Dvl proteins contain several highly conserved domains required for activating different Wnt pathways, including in particular the N-terminal DIX (Dishevelled and aXin) domain, the central PDZ (Post-synaptic density protein-95, Disk large tumor suppressor, Zonula occludens-1) domain, and the C-terminal DEP (Dishevelled, Egl-10 and Pleckstrin) domain (**Figure 1**). The DIX domain is involved in Wnt-induced dynamic Dvl homo- and hetero-oligomerization that is important for Wnt/ß-catenin signaling (Kishida et al., 1999; Kan et al., 2020; Ma et al., 2020). The PDZ domain interacts with a conserved KTxxxW motif located immediately after the seventh transmembrane domain of Frizzled receptors (Umbhauer et al., 2000; Wong et al., 2003), and with a wide variety of binding partners that function either as agonists or antagonists of Wnt signaling (Wallingford and Habas, 2005; Sharma et al., 2018). Biochemical and functional analyses suggest that it participates in both Wnt/ß-catenin and Wnt/PCP signaling (Habas et al., 2001; Lee et al., 2015). The DEP domain plays a major role in Dvl membrane recruitment by Frizzled receptors (Axelrod et al., 1998; Rothbächer et al., 2000; Wong et al., 2000; Pan et al., 2004; Park et al., 2005). It functions in the Wnt/PCP pathway either with the PDZ domain to activate Rho/ROCK or by direct interaction with Rac to trigger JNK activation. However, more recent evidence suggests that Dvl dimerization triggered by the N-terminal region of DEP domain is required for Wnt/ß-catenin signaling (Gammons et al., 2016a,b; Paclíková et al., 2017).

The C-terminal region beyond the DEP domain also shows evolutionary conservation but displays unique features among Dvl isofroms. Specifically, Dvl3 contains histidine-single amino acid repeats required for Wnt5a-stimulated activation of nuclear factor of activated T cells (NF-AT) and possesses proline-rich domains likely involved in the interaction with other Dvl isoforms (Ma et al., 2010; Wang and Malbon, 2012). The extreme 13 amino acids that are conserved in all Dvl isoforms bind to the third intracellular loop of Frizzled receptors and stabilize Frizzled-Dvl interaction in Wnt/ß-catenin signaling (Tauriello et al., 2012). Moreover, the last 3 residues represent a type II PDZ-binding (PDZ-B) motif that can occupy the peptide-binding pocket of the PDZ domain, inducing Dvl to adopt a closed conformation and an auto-inhibited state (Lee et al., 2015; Qi et al., 2017). Dvl variants with an opened conformation show efficient membrane recruitment and reduced activity in Wnt/ß-catenin signaling but display increased activity in Wnt/PCP signaling (Qi et al., 2017; Harnoš et al., 2019). The function of Dvl C-terminal region in Wnt signaling is further demonstrated in autosomal-dominant Robinow syndrome caused by *de novo* frameshift mutations in human *DVL1* and *DVL3* genes, which delete and replace the C-terminal region after the DEP domain (Bunn et al., 2015; White et al., 2015, 2016; Danyel et al., 2018). *In vitro* analysis suggests that DVL1 lacking the C-terminal region displays reduced activity in Wnt/ß-catenin signaling (Bunn et al., 2015). These findings suggest an importance of the C-terminus in intra- or inter-molecular interaction, which may be subjected to regulation by other partners to switch pathway specificity. Indeed, recent studies show that casein kinase 1ε (CK1ε) and NIMA-related kinase 2 (NEK2) function as scaffold proteins and regulate the dynamics of Dvl conformational changes by phosphorylation of the PDZ domain and modulation of its interaction with the extreme C-terminal tail (Harnoš et al., 2019; Hanáková et al., 2019).

Dvl DOSAGE EFFECT ON MORPHOGENETIC MOVEMENTS AND CELL FATE SPECIFICATION DURING DEVELOPMENT IN MICE

The three *Dvl* genes (*Dvl1*, *Dvl2,* and *Dvl3*) in mice are broadly expressed throughout early development. Extensive analyses of mutant phenotypes have uncovered both unique and redundant functions for these genes. Mice deficient in *Dvl1* show reduced social interaction and abnormal sensorimotor gating (Lijam et al., 1997). This abnormal behavior is caused by defective Wnt/ß-catenin signaling that may impair central nervous system functions (Belinson et al., 2016). Mice deficient in *Dvl2* exhibit more severe phenotypes, with defective cardiac morphogenesis, somite segmentation, and neural tube closure (Hamblet et al., 2002). *Dvl3* functions redundantly with *Dvl1* and *Dvl2* in several processes, including cardiac outflow tract, cochlea and neural tube development (Etheridge et al., 2008). These works reveal

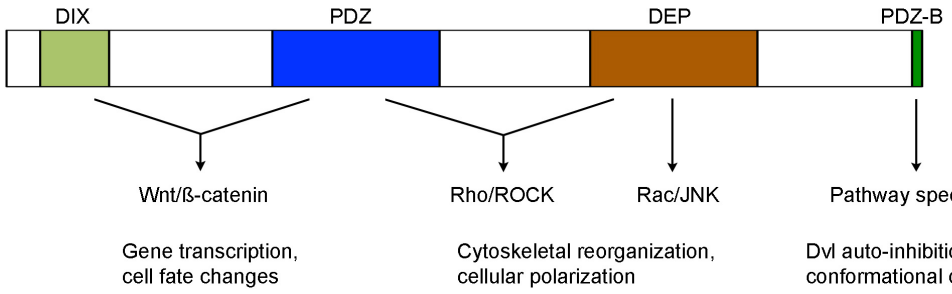

FIGURE 1 | Structure-function relationship of conserved Dvl domains in the activation of Wnt/ß-catenin and Wnt/PCP signaling (see text for details).

a sensitivity of Wnt/PCP signaling to *Dvl* dosage because most defective phenotypes in *Dvl* mutants are related to impaired Wnt/PCP signaling, in particular the defective outflow tract morphogenesis (Sinha et al., 2012). Thus, Dvl isoforms are critically required for morphogenetic movements. Particularly, they mediate Wnt/PCP in CE movements during neurulation. Dvl2 plays a predominant role in neural tube closure, but Dvl1 and Dvl3 are also involved in this process (Wang et al., 2006). The functional importance of Dvl isoforms in neural tube formation has been confirmed by the identification of rare mutations in all three human *DVL* genes, which disturb normal functions of DVL isoforms in non-canonical Wnt signaling and cause neural tube defects (De Marco et al., 2013; Liu et al., 2020). By comparison, low levels of Dvl expression from a single allele may be sufficient to normally support those developmental processes triggered by the Wnt/ß-catenin pathway (Soares et al., 2005; Wynshaw-Boris, 2012). However, deletion of all six *Dvl* alleles causes absence of mesoderm gene expression and mesoderm formation that are dependent on Wnt/ß-catenin signaling (Ngo et al., 2020).

DISTINCT AND REDUNDANT Dvl FUNCTIONS DURING *XENOPUS* DEVELOPMENT

Dvl function in vertebrates was first studied during *Xenopus* development. Overexpression of Dvl2 (Xdsh) in the ventral region of early embryos induced the formation of a complete secondary axis reminiscent of activation of maternal Wnt/ß-catenin signaling (Sokol et al., 1995). However, dorsal overexpression of Xdd1, a truncated form of Dvl2 that lacks the PDZ domain and interferes with Wnt/ß-catenin signaling triggered by Wnt ligands, did not affect dorsoventral axis formation (Sokol, 1996). Because maternal Wnt/ß-catenin signaling is required for dorsal fate specification by activating the transcription of target genes in the Spemann organizer (Carron and Shi, 2016), the absence of an inhibitory effect by Xdd1 implies that Dvl function may be dispensable for the activation of maternal Wnt/ß-catenin signaling. Consistently, simultaneous depletion of maternally expressed *Dvl2* and *Dvl3* from oocytes did not affect the expression of maternal Wnt/ß-catenin target genes and the formation of dorsal axis (Tadjuidje et al., 2011). However, it is possible that low levels of Dvl proteins are still present in the oocytes due to incomplete depletion of maternal *Dvl* mRNA. Thus, the requirement of maternal *Dvl* for dorsal axis formation in *Xenopus* requires a complete loss-of-function study. Nevertheless, a recent study suggests that activation of maternal Wnt/ß-catenin pathway and formation of dorsal axis may be achieved through a Dvl-independent mechanism (Yan et al., 2018). During organogenesis, it seems that Dvl isoforms display less functional redundancy in developmental processes that involve Wnt/ß-catenin signaling, which may be due to their differential expression patterns. For example, Dvl1 and Dvl2 are required for neural crest cell specification and somite segmentation, while Dvl3 maintains gene expression in the myotome (Gray et al., 2009).

Maternal Dvl2 and Dvl3 likely display distinct and redundant functions during CE movements. In these coordinated processes, lateral cells converge toward the dorsal region to narrow the germ layers, while dorsal midline cells undergo mediolateral intercalation by polarized protrusive behaviors to lengthen the embryo along the anteroposterior axis (Keller and Sutherland, 2020). Previous studies show that Dvl regulates CE movements through Wnt/PCP signaling (Djiane et al., 2000; Tada and Smith, 2000; Habas et al., 2001, 2003; Wallingford and Harland, 2001). Moreover, inhibition of maternal Dvl2 or Dvl3 function suggests that they exhibit a non-redundant but an additive effect on CE movements (Tadjuidje et al., 2011). More recent works reveal that Dvl1, but not Dvl2 or Dvl3, activates the Wnt/Ca^{2+} pathway (another branch of the non-canonical pathway) during CE movements (Gentzel et al., 2015), further supporting the distinct functions of Dvl isoforms in morphogenetic movements. As in mice, Dvl function is also required for neural tube closure by regulating CE movements of the midline and coordinating polarity among epithelial cells (Wallingford and Harland, 2002; Seo et al., 2017). In addition, Dvl2-mediated activation of Rac1 through the DEP domain also controls the protrusive activity of neural crest cells during migration (Kratzer et al., 2020). Because cellular polarization is tightly dependent on the asymmetric activation of Wnt/PCP signaling, both reduced and increased levels of Dvl disrupts asymmetric movements. However, the cellular behaviors are completely different. Increasing the activity of Dvl perturbs cell polarity by randomizing the formation of cellular protrusions, whereas reducing the activity of Dvl prevents cellular protrusions (Wallingford et al., 2000; Cheng et al., 2017).

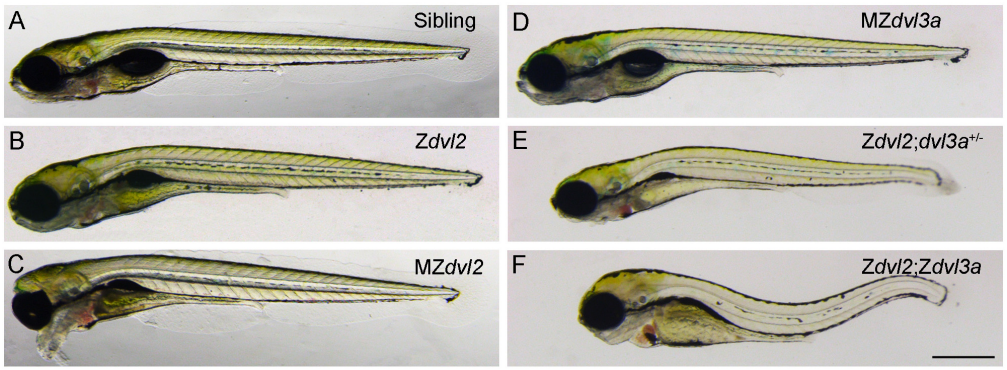

FIGURE 2 | Maternal and zygotic Dvl dosage effect on embryonic axis elongation in zebrafish embryos at 5 days post-fertilization (Xing et al., 2018). **(A)** Wild-type sibling embryo. **(B)** Zygotic *dvl2* mutant. **(C)** The "bulldog" facial phenotype of maternal-zygotic *dvl2* mutant with cyclopia. **(D)** Maternal-zygotic *dvl3a* mutant. **(E)** Triallelic *dvl2* and *dvl3a* mutant. **(F)** Zygotic *dvl2* and *dvl3a* double mutant. Scale bar **(A–F)**: 400 μm.

MATERNAL CONTRIBUTIONS OF Dvl PROTEINS TO AXIS PATTERNING AND MORPHOGENETIC PROCESSES DURING ZEBRAFISH DEVELOPMENT

The zebrafish genome contains five *Dvl* paralogs: *dvl1a*, *dvl1b*, *dvl2*, *dvl3a*, and *dvl3b*. In the early embryos, *dvl2* and *dvl3a* are maternally expressed and represent about 98% of the total pool, whereas the transcript levels of the other *Dvl* genes are negligible (Harvey et al., 2013). Knockout of *Dvl* genes reveal both distinct and redundant functions in embryonic axis specification and morphogenetic movements (Xing et al., 2018). Maternal-zygotic mutants for *dvl1a*, *dvl1b*, *dvl3a*, and *dvl3b* are phenotypically normal and fertile. In sharp contrast, maternal-zygotic *dvl2* mutants display strongly impaired CE movements during gastrulation and develop severe craniofacial defects with a "bulldog" facial phenotype, reminiscent of impaired Wnt/PCP signaling in midline structures (Kimmel et al., 2001). Zygotic *dvl2* mutant embryos are essentially normal, but only about half of them can survive to adulthood, and all male individuals show absence of courtship behavior. This suggests that there may be defects in central nervous system functions as mice *Dvl1* mutants (Lijam et al., 1997). The highest level of *dvl2* expression during early development may explain at least partly the most severe phenotypes of *dvl2* mutants.

The specification of dorsal axis in zebrafish also requires maternal ß-catenin signaling to trigger the expression of organizer genes (Kelly et al., 2000; Bellipanni et al., 2006; Fuentes et al., 2020). However, the involvement of upstream regulators has not been conclusively established. Importantly, maternal-zygotic *dvl2* and *dvl3a* double mutants, which are unresponsive to stimulation by Wnt ligands, show normal specification of dorsal cell fate, suggesting that components of Wnt signaling upstream of ß-catenin may be dispensable for its stabilization. The activation of maternal Wnt/ß-catenin signaling independent of Dvl activity is further confirmed in zebrafish *huluwa* mutants. Maternal depletion of *huluwa* impairs Wnt/ß-catenin signaling and causes loss of dorso-anterior structures. Mechanistically, Huluwa protein accumulates in the cell membrane at the dorsal region and functions independently of Wnt ligands and Frizzled receptors to promote tankyrase-mediated degradation of Axin, thereby stabilizing ß-catenin (Yan et al., 2018).

Mutational analyses of Dvl functions also reveal a major contribution of maternal Dvl to zygotic events and confirm the importance of Dvl dosage in Wnt/PCP signaling (**Figure 2**). This dosage effect is particularly reflected by the requirement of Dvl2 and Dvl3a for CE movements. Although Dvl2 plays a predominant role, Dvl3a exerts a strong synergistic effect on the loss of Dvl2 function, and progressive removal of Dvl2 and Dvl3a maternal or zygotic products increasingly aggravates CE defects and reduces the elongation of anteroposterior axis. Furthermore, zygotic *dvl2* and *dvl3a* double mutants only display a shortened body length. However, maternal-zygotic *dvl2* and *dvl3a* double mutants show most strongly impaired CE movements and completely lack axis extension. They also develop severe trunk and posterior deficiencies associated with down-regulation of zygotic Wnt/ß-catenin target genes (Xing et al., 2018). Because zygotic Wnt/ß-catenin signaling has an opposite effect with respect to maternal Wnt/ß-catenin signaling and functions to specify the posterior region (Carron and Shi, 2016), these findings highlight the importance of maternal Dvl in setting up zygotic morphogenetic and patterning processes. They support the view that maternally expressed gene products perform essential functions after zygotic genome activation (Marlow, 2020; Solnica-Krezel, 2020). Thus, both maternal and zygotic Dvl dosages are important for proper cell movements and embryonic axis patterning that occur during gastrulation.

PERSPECTIVES

Dvl conserved domains in Wnt pathways have attracted much attention. Although the function of DIX, PDZ, and DEP domains is relatively understood, how they cooperate to switch pathway specificity remains elusive. Detailed analysis of other conserved domains or isoform-specific regions, such as the basic region

preceding the PDZ domain, the proline-rich region and histidine-single amino acid repeats in the C-terminal region beyond the DEP domain, and the extreme C-terminus, may provide insights into Dvl-mediated signal transduction. Because Dvl post-translational modifications, in particular phosphorylation and ubiquitination, and Dvl interaction partners are important for subcellular localizations and specific functions of Dvl proteins (Sharma et al., 2018; Harrison et al., 2020), it is of interest to understand how these modulate Dvl activity and dictate signaling outcomes in key developmental processes. Indeed, dysregulation of Dvl phosphorylation impairs both Wnt/ß-catenin and Wnt/PCP signaling during zebrafish and *Xenopus* embryogenesis (Shimizu et al., 2014; Rauschenberger et al., 2017). The specificity of Dvl isoforms also merits investigations because tissue-specific expression patterns and differential biochemical properties may contribute to their particular functions. Another intriguing question is the sensibility of Wnt/PCP signaling, but not Wnt/ß-catenin signaling, to Dvl dosage, which is observed in all vertebrates. It suggests that Wnt/PCP-dependent developmental processes critically require Dvl function to activate downstream effectors. Consequently, moderate diminution of Dvl dosage could significantly affect polarized cellular behaviors and cell polarity. By comparison, ß-catenin may be stabilized independently of upstream Wnt signaling components, and target genes of Wnt/ß-catenin signaling may be regulated by other factors, at least during dorsal fate specification in zebrafish and *Xenopus* (Li et al., 2015; Yan et al., 2018). Thus, tissue morphogenesis regulated by Wnt/PCP signaling is more sensitive to Dvl dysfunction, as a result, many human congenital disorders, such as neural tube defects and Robinow syndrome, are associated with mutations in *DVL* genes.

AUTHOR CONTRIBUTIONS

D-LS performed the literature analysis, prepared the figures, and wrote the manuscript.

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Expression and Function of WNT6: From Development to Disease

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WNT family member 6 (WNT6) is a member of the highly conserved WNT protein family. It plays an essential role in the normal development process, not only in embryonic morphogenesis, but also in post-natal homeostasis. WNT6 functions in mice and humans. This review summarizes the current findings on the biological functions of WNT6, describing its involvement in regulating embryogenesis, decidualization, and organ development. Aberrant WNT6 signaling is related to various pathologies, such as promoting cancer development, lung tuberculosis, and kidney fibrosis and improving the symptoms of Rett syndrome (RTT). Thus, due to its various functions, WNT6 has great potential for in-depth research. This work not only describes the signaling mechanism and function of WNT6 under physiological and pathological conditions, but also provides a theoretical basis for targeted therapy.

Keywords: disease, development, differentiation, organ formation, Wnt6

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INTRODUCTION

WNT family member 6 (WNT6), a member of the Wingless/integrase 1 (WNT) family comprising at least 19 members in mammals, is a secreted glycoprotein. The WNT signaling family is highly conserved and in several species play essential roles in various physiological processes during which it is either stimulated or inhibited. WNT signals mediate various cellular functions by binding to different receptors on the cell membrane including 10 members of human Frizzled (FZD) receptors, low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) receptor, and many non-class FZD receptors (Baarsma et al., 2013). The WNT signaling pathways are classified into the canonical WNT or WNT/β-catenin pathway, and the non-canonical WNT or β-catenin-independent pathways (**Figure 1**).

The WNT/β-catenin pathway is stimulated by an extracellular WNT ligand that binds to a FZD receptor and a LRP5/6 co-receptor (FZD and LRP5/6) on the cell membrane and recruits disheveled (DVL) proteins to promote the dissociation of the β-catenin destruction complex, leading to an intracellular signaling cascade. The β-catenin destruction complex consists of the scaffold protein Axin, Adenomatous Polyposis Coli (APC), Casein Kinase-1 (CK-1), and Glycogen Synthase Kinase-3β (GSK-3β). In the absence of WNT signaling, the β-catenin destruction complex phosphorylates β-catenin, leading to subsequent proteasomal degradation of the protein. However, in the presence of WNT signaling, WNTs inactivate the β-catenin destruction complex and prevent β-catenin degradation, thereby allowing β-catenin accumulation in the cytoplasm and translocation into the nucleus. Then β-catenin binds to the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors, mediating the activation of downstream targets (Willert and Jones, 2006; MacDonald et al., 2009; Baarsma et al., 2013) such as Cyclin D1 (CCND1, a G1 phase cyclin),

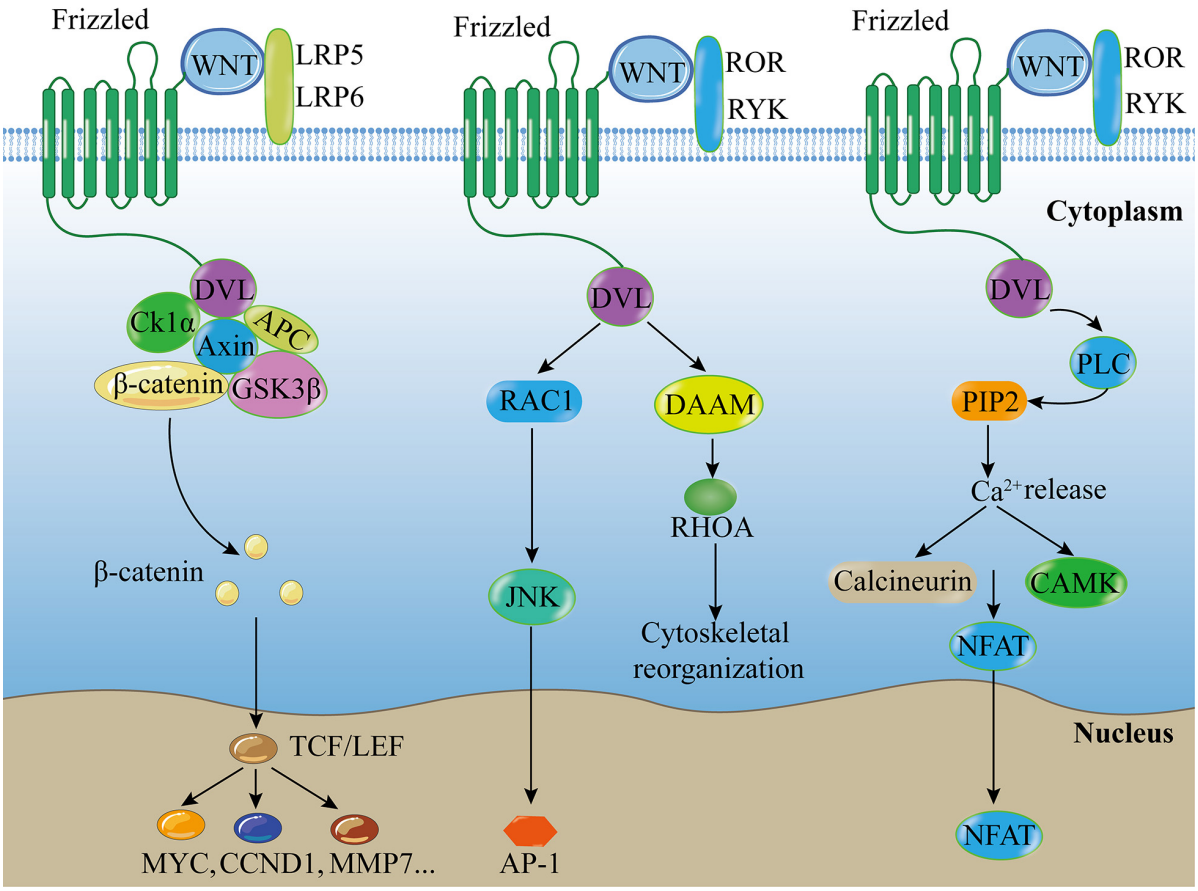

FIGURE 1 | Canonical and non-canonical WNT pathways. Canonical WNT pathway: Extracellular WNT ligands bind to FZD and LRP5/6 on the cell membrane, and recruit DVL protein to promote dissociation of the β-catenin destruction complex, followed by accumulation of β-catenin, which translocates to the nucleus to interact with target downstream signaling molecules, activating intracellular signaling cascades. WNT/PCP pathway: Extracellular non-canonical WNT ligands bind to FZD receptor and/or ROR/RYK co-receptors at the cell membrane, and recruit DVL protein to promote downstream effector components, such as RAC and RHOA signaling cascades, regulating tissue polarity and cell movement. WNT/Ca^{2+} pathway: Extracellular WNT ligands bind to FZD receptor and/or ROR/RYK co-receptors at the cell membrane, and recruit DVL protein to promote PLC, leading to the formation of IP3 and DAG from PIP2, resulting in intracellular Ca^{2+} accumulation. Ca^{2+} can activate CAMK and NFAT transcription factor to regulate downstream cascades. Abbreviations: PCP, planar cell polarity; LRP, low-density lipoprotein receptor-related proteins; ROR, receptor tyrosine kinase-like orphan receptor; RYK, receptor-like tyrosine kinase; DVL, disheveled; CK1α, Casein Kinase-1α; APC, adenomatous polyposis coli; GSK3β, glycogen synthase kinase-3β; DAAM, disheveled associated activator of morphogenesis; PLC, phospholipase C; PIP2, phospholipid phosphatidylinositol 4,5-bisphosphate; JNK, Jun-N-terminal kinase; RHOA, ras homolog family member A; CAMK, calmodulin-dependent protein kinase; NFAT, nuclear factor of activated T cells; TCF/LEF, T-cell factor/lymphoid enhancer factor; CCND1, Cyclin D1; MMP7, matrix metallopeptidase 7; AP-1, activator protein 1.

c-Myc (a proto-oncogene), and matrix metallopeptidase 7 (MMP7, a Zn2 + -dependent proteolytic enzyme) (Li et al., 2019).

In the β-catenin-independent WNT pathways, most WNT ligands bind to FZD receptors, whereas others interact with receptor tyrosine kinases of the ROR and RYK families (Angers and Moon, 2009; Clevers et al., 2014; Schulte, 2015; Shi et al., 2017). The β-catenin-independent WNT pathways are primarily classified into the WNT/Planar cell polarity (PCP) and WNT/Ca^{2+} pathways. The WNT/PCP pathway is associated with the downstream activation of c- Jun N-terminal kinase (JNK) and ras homolog family member A (RhoA) (Hwang and Kelly, 2012; Baarsma et al., 2013). The WNT/PCP signaling pathway regulates cell adhesion, migration, and polarity (Golenia et al., 2017; Shi et al., 2017; Villasenor et al., 2017). Conversely, the WNT/Ca^{2+} pathway is associated with the activation of phospholipase C (PLC) and leads to the formation of inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG), thereby increasing Ca^{2+} level and activating downstream targets. The WNT/Ca^{2+} pathway is associated with muscle contraction, gene transcription, and enzyme activation (Foulquier et al., 2018) and activates both β-catenin-dependent and β-catenin-independent pathways (Villasenor et al., 2017).

WNT6, a WNT family member that is highly conserved in various species, mainly considered to be a member of the β-catenin-dependent WNT signaling pathway (Krawetz and Kelly, 2008; Hwang and Kelly, 2012). However, it also functions through the β-catenin-independent pathways (Li et al., 2014; Schmeckpeper et al., 2015). WNT6 plays a critical role in the early development of embryos, and promotes the normal formation of various organs during the late development phase. It regulates post-natal tissue homeostasis and pathological disorders throughout the lifespan of organisms.

Because WNT6 is closely associated with many diseases, understanding its biology is important. Our review discusses the functions of WNT6 and its various signaling pathways, identified through studies of various *in vivo* mouse models, *in vitro* mouse and human cell systems, and clinical data in humans.

BASIC INFORMATION ON WNT6

WNT signaling components are highly conserved with respect to both structure and function (Lavery et al., 2008; Foulquier et al., 2018). The term WNT is a fusion of the wingless (Wg) gene and the homologous vertebrate oncogene, intergrase-1 (int-1) (Baarsma et al., 2013). WNT ligands are a family of secreted glycoproteins consisting of at least 19 members in mammals (Cawthorn et al., 2012). WNT6, an evolutionarily conserved morphogenetic factor, belongs to the WNT family of secreted, hydrophobic glycoprotein (Yuan et al., 2013). The *wnt6* gene is located in the 2q35 region of the human chromosome, and encodes a 365 amino-acid polypeptide with an N-terminal signal peptide, a WNT core domain and a RGD motif. *Wnt6* is fused with *wnt10a* in a head-to-tail manner, with an interval less than 7.0 kb (Kirikoshi et al., 2001); therefore, WNT6 and WNT10a have some similar functions. For example, both WNT6 and WNT10a inhibit adipogenesis and stimulate osteoblastogenesis (Cawthorn et al., 2012). However, among the WNT proteins, WNT1 is the most homologous to WNT6 in the human genome (Kirikoshi et al., 2001); They have synergistic effects in mice (Otto et al., 2008; Hitchins et al., 2013; Huang et al., 2019) and antagonistic effects in humans and mice (Otto et al., 2008; Scheller et al., 2008; Hitchins et al., 2013; Li et al., 2014) at different time points.

WNT6 plays a critical role in several aspects of mouse embryonic development. Furthermore, WNT6 expression is crucial for post-natal tissue homeostasis as well as pathological disorders during the lifespan of mice and humans (**Figure 2**).

FUNCTIONS OF WNT6

Decidualization

In both mice and humans, with the initiation of attachment, endometrial stromal cells surrounding the implanting blastocysts undergo decidualization to embed the embryo into the antimesometrial endometrial bed. The process of decidualization includes stromal cell proliferation and stromal decidual transformation with polyploidization. *Wnt6* mRNA is overexpressed in the stromal cells during decidualization, which occurs between days 5 and 8 of pregnancy. Mice without WNT6 expression have compromised term pregnancy with substantially reduced litter size, demonstrating that WNT6 signaling is essential for normal pregnancy (Wang et al., 2013). WNT6 deficiency markedly blocks stromal cell proliferation by prolonging the stromal cell cycle, which might be associated with decreased cyclin B1 (CCNB1), a key regulatory protein controlling cell cycle progression. Cyclin B1 is also highly expressed in the proliferative and secretory endometrium in humans (Tang et al., 2009; Wang et al., 2013). It is possible that WNT6 signaling plays a similar role during decidualization in humans. However, the loss of WNT6 has no significant effect on the differentiation of stromal cells into polyploidy decidual cells, another main process of decidualization (Wang et al., 2013). In summary, WNT6 plays a critical role in stromal cell proliferation during decidualization, thereby affecting normal embryonic development in mice, indicating that WNT6 begins to function during embryo formation, at least in mice.

Kidney Development

Renal development is initiated following the formation of the epithelial ureter bud from the nephric duct and invasion of the metanephric blastema in mice (Beaton et al., 2016). Among them, the ureter tip region is the source of the primary inducer of kidney tubule differentiation (Itaranta et al., 2002). In the mouse embryo, *wnt6* mRNA is expressed in the early ureter bud and throughout the invading ureter bud when the epithelial ureter bud grows into the nephric mesenchyme. It is also expressed in the branching ureter tips where new branches are formed during the early stage of kidney development in mouse embryo. However, *wnt6* mRNA expression is reduced during growth of the ureter, possibly because of the maturing nephrogenic mesenchyme that releases inhibitory factors to decrease WNT6 expression. WNT6 protein aids in the establishment of tubular epithelial structures, increases the number of differentiated tubules, and plays a role in further maturation. Simultaneously, the expression of paired box gene (Pax2), Pax8, and E-cadherin is markedly increased in the mesenchyme (Itaranta et al., 2002). Pax2 is a marker of induction (Dressler et al., 1990) and Pax8 is a marker of pre-tubular cell aggregation (Plachov et al., 1990). All genes are markers for pre-tubular aggregates. These results indicate that WNT6 induces pre-tubular aggregation in the mesenchyme by upregulating these genes. However, WNT6 cannot induce ureter tubule growth and branching (Lin et al., 2001; Itaranta et al., 2002). In summary, WNT6 expression stimulates the interaction between the epithelium and mesenchyme and regulates mesenchyme development to induce kidney tubule formation rather than auto-regulating the epithelium (Itaranta et al., 2002).

Differentiation of Human Dental Papilla Cells

Human dental papilla cells (hDPCs), the precursors of odontoblasts in human dental papilla tissue, have the potential

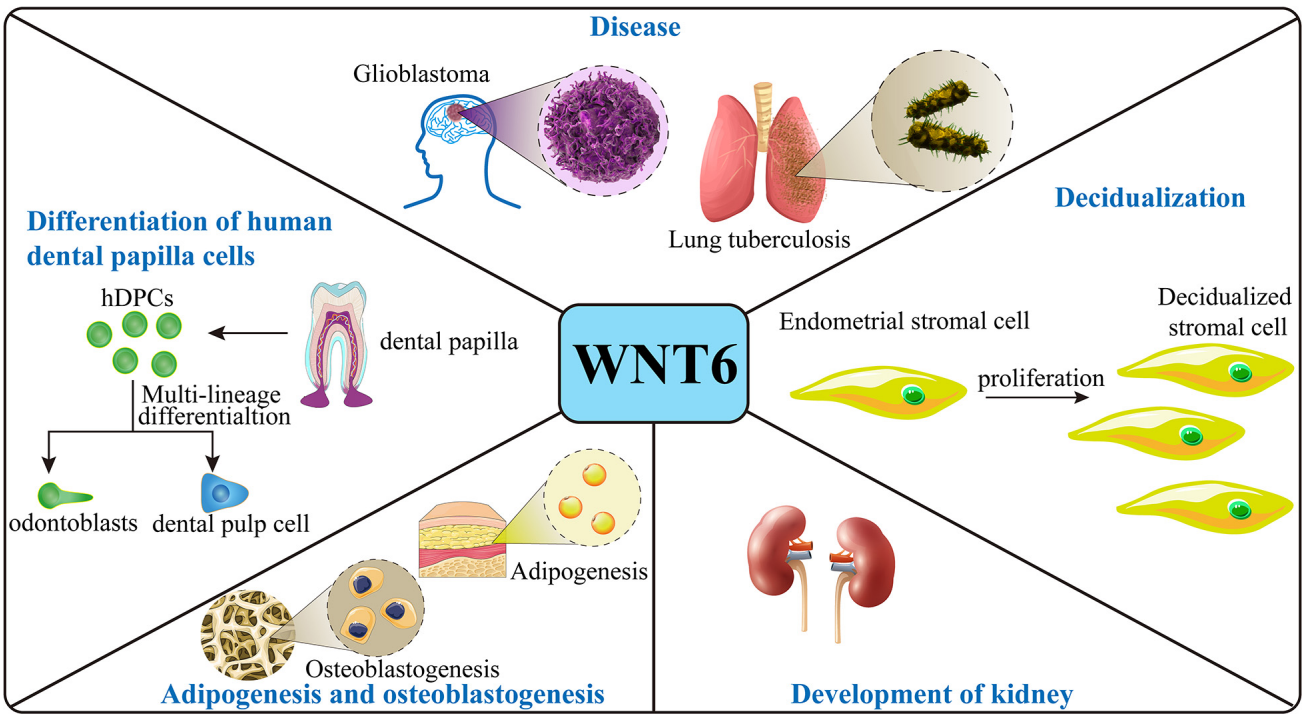

FIGURE 2 | Function of WNT6. In the mouse embryo, WNT6 is expressed and promote stromal cell proliferation during decidualization and induces pre-tubular aggregation in the mesenchyme during formation of the renal tubule. WNT6 also prevents adipogenesis and stimulates osteoblastogenesis in mice. With respect to disease, in mice, loss of WNT6 correlates with the development of kidney fibrosis, and WNT6 can maintain the integrity of epithelium. WNT6 expression promotes the development of lung tuberculosis and GBM. In human cell models, WNT6 expression promotes the differentiation and migration of hDPCs and promotes the formation and development of tumors.

to differentiate into odontoblasts and dental pulp cells (Huang et al., 2008), indicating that they play a critical role in tooth formation and development. WNT6 has been detected in the oral and dental epithelium and in the primary and secondary enamel knots, and low levels have been observed in mouse dental papilla cells (Wang et al., 2010). WNT6 has also been found in hDPCs *in vitro* (Wang et al., 2010; Li et al., 2014). The BrdU incorporation assay and flow cytometry have shown that the percentage of proliferating hDPCs in the WNT6 protein expression group is similar to that of the control group, indicating that WNT6 protein does not promote cell cycle progression in hDPCs. Thus, WNT6 does not affect the proliferation of hDPCs. However, WNT6 increases the number and size of calcified nodules in hDPCs during the tooth mineralization process, and upregulates expression of the alkaline phosphatase gene and dentin matrix protein 1 (DMP-1), which are important markers of biomineralization (Wang et al., 2010; Li et al., 2014). It also increases the expression of osteogenic marker genes such as osteonectin (ON) and osteopontin (OPN). Moreover, *wnt6* mRNA increases the expression of type I collagen (Col I), an extracellular matrix protein in the connective tissue of dentin that is primarily released by fibroblasts and osteoblasts (Dalgleish, 1997). WNT6 protein also activates and upregulates phosphorylated JNK and c-Jun mRNA in hDPCs to induce migration and differentiation (Li et al., 2014). These results indicate that although WNT6 is not involved in the proliferation of hDPCs, it is involved in the specific differentiation and migration of hDPCs through the β-catenin-independent WNT pathway.

Adipogenesis and Osteoblastogenesis

Mesenchymal stem cells (MSCs) differentiate into various cell types including adipocytes and osteoblasts. The dysregulation of these two cell types strongly correlates with obesity, type 2 mellitus diabetes, and osteoporosis. Therefore, maintenance of the normal functioning of MSCs is important. WNT/β-catenin signaling is an important regulator of mesenchymal stem cells (MSCs). Cawthorn et al. (2012) found that the mRNA expression of *wnt6, wnt10a*, and *wnt10b* was markedly reduced in adipocytes both *in vitro* and in mice. The ectopic mRNA expression of *wnt6, wnt10a*, and *wnt10b* suppresses the expression of two adipogenic transcription factors, peroxisome proliferator-activated receptor-γ (PPARγ), and CCAAT/enhancer binding protein-α (C/EBPα) in 3T3-L1 pre-adipocytes and completely inhibits neutral lipid accumulation (Cawthorn et al., 2012; Guo et al., 2019), indicating that all three WNTs suppress adipogenesis, even prior to the induction of the process. By contrast, loss of the three WNTs promotes adipogenesis. Knockdown of β-catenin prevents this inhibitory event, and also increases the expression of PPARγ. Thus, β-catenin is an essential factor for the inhibition of adipogenesis (Cawthorn et al., 2012). Thus, during adipogenesis, WNT6, WNT10a,

and WNT10b suppress adipogenic transcription factors to inhibit adipogenesis through the β-catenin-dependent WNT pathway. As for the upstream molecular mechanism of WNT6 during adipogenesis WNT6 is associated with C/EBPβ and lysine-specific demethylase 5A (KDM5A). C/EBPβ is a key early adipogenic factor that is expressed in 3T3-L1 pre-adipocytes. KDM5A is the transcriptional target of C/EBPβ. The overexpression of C/EBPβ and KDM5A decrease *wnt6* mRNA expression in 3T3-L1 pre-adipocytes during differentiation. Knockdown of C/EBPβ and KDM5A results in an increase in *wnt6* mRNA expression; however, KDM5A and C/EBPβ are not affected by WNT6. This suggests that KDM5A and C/EBPβ are upstream molecules of WNT6. Binding of C/EBPβ and KDM5A to the promoter of WNT6 results in its inhibition. The specific mechanism of inhibition includes the recruitment of KDM5A to the promoter of WNT6 in a C/EBPβ-dependent manner, which results in the inhibition of WNT6 transcription. Then the expression of C/EBPα and PPARγ is upregulated, which stimulates adipogenesis (Guo et al., 2019). This indicates that the inhibition of WNT6 can promote adipogenesis. Additionally, ribosomal protein S6 kinase 1 (S6K1), another molecule that stimulates adipogenesis, is activated and translocates into the nucleus to suppress the transcription of WNT6, WNT10a, and WNT10b genes by phosphorylating histone H2B (Yi et al., 2016). This indicates that the S6K1-WNT pathway is a potential therapeutic target for obesity and other related metabolic disorders. In summary, a complex network of signaling molecules is involved in the inhibition of adipogenesis by WNT6.

In addition to inhibiting adipogenesis, WNT6, WNT10a, and WNT10b also stimulate osteoblast differentiation. The ectopic expression of each *wnts* mRNA not only upregulate the expression of Twist1, a transcription factor involved in the regulation of osteoblastogenesis, but also increases the expression of alkaline phosphatase, an osteoblast marker. Moreover, they also increase the calcium content in cells, thereby promoting the differentiation of stem cells (ST cells) into osteoblasts. However, β-catenin loss prevents this stimulation. Similarly, a block by short hairpin RNA of three WNT expression suppresses osteoblastogenesis. In summary, WNT6, WNT10a, and WNT10b inhibit adipogenesis and induce osteoblastogenesis by activating the WNT/β-catenin pathway (Cawthorn et al., 2012). Although the functions of these three WNT ligands are similar, the impact of the positive expression and function of both WNT10a and WNT10b is stronger than that of WNT6 in osteoblastogenesis and adipogenesis. However, the impact of the endogenous knockdown of *wnt6* in adipogenesis and osteoblastogenesis is stronger than that of endogenous knockdown of both *wnt10a* and *wnt10b* (Vertino et al., 2005; Cawthorn et al., 2012). This indicates that the influence of WNT6 is more than that of WNT10a and WNT10b in adipogenesis and osteoblastogenesis, at least under *in vitro* conditions; however, the exact mechanism requires further studies. Collectively, WNT6, WNT10a, and WNT10b, as endogenous regulators in mesenchymal precursors, work synergistically to suppress adipogenesis and promote osteoblastogenesis, and also compensate for each other. The cooperative function of the three WNTs might be closely related to the arrangement of their genes in adjacent positions on the same chromosome.

DISEASES

Cardiac Injury

During heart injury, several events occur to protect the heart from damage, one of which is the inhibition of WNT signaling. Inhibition of the β-catenin-dependent WNT signaling pathway decreases the infarct size during cardiac injury (Zelarayan et al., 2008; Bergmann, 2010). In adults, cardiac progenitor cells (CPCs) reside in niches and might be involved in cardiac regeneration; however, they cannot achieve functional repair of the myocardium. The secreted frizzled-related protein 2 (Sfrp2), a WNT inhibitor, is upregulated during cardiac injury and stimulates the differentiation of CPCs into cardiomyocytes in the border zone, following ischemia reperfusion injury (Kobayashi et al., 2009; Schmeckpeper et al., 2015). WNT6 protein promotes the proliferation of CPCs in mice but inhibits their differentiation via the β-catenin-dependent WNT pathway; however, Sfrp2 blocks this event. Importantly, Sfrp2 does not influence the proliferation of CPCs without the involvement of WNT6. Moreover, Sfrp2 does not have any significant effect on CPC differentiation in the absence of WNT6, indicating that the effect of proliferation suppression and differentiation promotion are a result of the interaction between WNT6 and Sfrp2. In CPCs, Sfrp2 inhibits the β-catenin-dependent pathway and activates the β-catenin-independent pathway. Moreover, it activates the β-catenin-independent JNK pathway by inhibiting the β-catenin-dependent WNT6 pathway (Schmeckpeper et al., 2015). In summary, sfrp2 combines with WNT6 to promote CPCs differentiation and inhibit proliferation, providing a theoretical basis by which WNT6 plays a role in rescuing indirect myocardial injury (**Table 1**).

Kidney Fibrosis

Studies have suggested that activation of the WNT signaling pathway is essential for the repair of renal tubular epithelium and mesangium during acute kidney injury (Terada et al., 2003; Lin et al., 2010). WNT6 expression is markedly reduced not only in diabetic nephropathy patients, but also in mice models of tubule-interstitial fibrosis (e.g., unilateral ureteral obstruction) and acute tubular injury (AKI), which are mostly caused by the deposition of extracellular matrix and the loss of epithelial integrity. Moreover, the low-expression of WNT6 protein is associated with the development of kidney fibrosis. Thus, WNT6 has a protective effect on the kidneys and prevents renal fibrosis by regulating epithelial fate. In addition, WNT6 protects the integrity of the epithelium by inhibiting transforming growth factor-β1 (TGF-β1), thereby preventing renal epithelial cells from undergoing epithelial to mesenchymal transition (EMT) through a non-canonical TGF-β1 pathway. Additionally, WNT6 also promotes *de novo* tubulogenesis under *in vitro* conditions. Renal tubular epithelial cells that express WNT6 protein form

TABLE 1 | Summary of the expression and function of WNT6 in different diseases.

Disease	Expression	Effector(s)	Consequence(s)	References
Cardiac injury	↓	β-catenin	Proliferation, Differentiation	Schmeckpeper et al., 2015
Kidney Fibrosis	↓	TGF-β1	EMT	Beaton et al., 2016
Lung Tuberculosis	↑	Arg-1, TNF-α	Polarization	Schaale et al., 2013
		c-Myc	Proliferation	
Rett Syndrome	↑	β-catenin, IGF-1, BDNF	Rescue symptom	Hsu et al., 2020
Glioblastoma	↑	Ki-67, Cyclin D1	Proliferation	Goncalves et al., 2018
		STAT3, β-catenin	Invasion, Migration	
		–	Chemoresistance	
Colorectal Cancer	↑	Bax, caspase-3	Anti-apoptosis	Zheng and Yu, 2018
		MMP2	Migration	
Gastric Cancer	↑	β-catenin	Anti-apoptosis, chemoresistance	Yuan et al., 2013

EMT, epithelial-mesenchymal transition; TGF-β1, transforming growth factor-β1; Arg-1, Arginase-1; TNF-α, tumor necrosis factor; IGF-1, insulin-like growth factor-1; BDNF, brain-derived neurotrophic factor; STAT3, signal transducer and activator of transcription 3; Bax, B-cell lymphoma 2-associated X protein; MMP2, matrix metallopeptidase 2.

the larger spheroids and new tube-like protrusions, indicating that WNT6 promotes the formation of new kidney tubules by activating the β-catenin-dependent WNT pathway (Beaton et al., 2016). In summary, the loss of WNT6 has the potential to cause the development of renal diseases, and the expression of WNT6 induces the formation of new renal tubules, suggesting that WNT6 has regenerative and repair functions. Moreover, the ability of WNT6 expression maintain the integrity of the epithelium is also an important theoretical basis for it to become a therapeutic target for renal fibrosis.

Lung Tuberculosis

Human tuberculosis (TB), caused by *Mycobacterium tuberculosis*, infects alveolar macrophages - the primary host cells of *M. tuberculosis*. WNT signaling reportedly has immunoregulatory functions in various inflammatory and infectious diseases such as tuberculosis and atherosclerosis (Schaale et al., 2013; Villasenor et al., 2017). Infection of the mouse lung with *M. tuberculosis* leads to an increase in the level of *wnt6* mRNA compared to other *wnt* genes. Furthermore, a positive correlation has been identified between the intensity of WNT6 protein expression, the number of *M. tuberculosis* colony forming units (CFUs), and the degree of inflammation induced by *M. tuberculosis*. A mutual relationship was identified between WNT6 expression and macrophages in TB infection. The level of *wnt6* mRNA in the macrophages of infected lung tissues depends on toll-like receptor (TLR), myeloid differentiation primary response88 (Myd88), and nuclear factor kappa (NFκB). Furthermore, *wnt6* mRNA increases the level of Arginase-1 (Arg-1) and mannose receptor 1 (MRC-1). Arg-1 is the mouse marker for alternatively activated macrophages (M2), and MRC-1 is a marker for alternative macrophage activation. These indicate that WNT6 promotes inflammation in the infected macrophages, and in uninfected macrophages by increasing anti-apoptotic factors such as B-cell lymphoma 2 (BCL-2). WNT6 changes macrophages into the M2-like phenotype by increasing Arg-1 and decreasing TNF-α (Schaale et al., 2013). Additionally, *wnt6* mRNA induces expression of the c-Myc gene in macrophages, through a β-catenin independent pathway, to promote macrophage proliferation (Varlakhanova and Knoepfler, 2009; Schaale et al., 2013). c-Myc regulates the expression of genes associated with the M2 phenotype (Pello et al., 2012); however, the WNT6/c-Myc-associated mechanism requires further study. These results suggest that WNT6 induces inflammatory responses. In summary, WNT6 is exclusively expressed in macrophages with granulomatous lesions, and promotes inflammation rather than eradicating *M. tuberculosis*. Moreover, the infection of human monocyte-derived macrophages with *M. tuberculosis* H37Rv increases *wnt6* mRNA expression, suggesting the regulation of a similar WNT homolog in human macrophages (Schaale et al., 2013).

Rett Syndrome

Rett syndrome (RTT) is a neurological and developmental disorder caused by a mutation in the methyl-CpG-binding protein 2 (MECP2) gene. Patients usually exhibit normal development at the infant stage; however, abnormal behaviors, such as locomotion impairment, cognitive function deficits, and other intellectual disability-related symptoms appear later in life (Amir et al., 1999; Swanberg et al., 2009). MECP2, an X chromosome-linked gene, is a transcriptional repressor. *wnt6* mRNA expression is significantly reduced in MECP2 mutant mice models, controlled by MeCP2 SUMOylation (Hsu et al., 2020). MeCP2 is suppressed in multiple RTT-associated MECP2 mutations (Tai et al., 2016). Additionally, the expression of brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) improves RTT in both patients and RTT mouse models (Li et al., 2012; Li and Pozzo-Miller, 2014; Pini et al., 2014). WNT6 overexpression increases the activity of the promoters of both IGF-1 and BDNF in a dose-dependent manner in HEK293T cells, and can also restore the level of *bdnf* and *igf-1* mRNA in mouse, and suppress the binding of cAMP-responsive element binding protein (CREB) to IGF-1 and BDNF to increase the expression of two genes. WNT6 may increase MECP2 SUMOylation by increasing the expression of IGF-1 and BDNF. Thus, the overexpression of WNT6 increases the expression

of both IGF-1, BDNF and MECP2 SUMOylation to partly improve abnormal behaviors. Additionally, in mutant mice, environmental enrichment (EE) also upregulates WNT6 protein expression by mediating the NMDA receptor and increases the expression of IGF-1, BDNF, and MECP2 SUMOylation. Thus, a positive regulatory loop exists between MECP2 SUMOylation and WNT6 expression, and this loop is composed of multiple molecules that improve RTT. However, the exact molecular mechanism requires further investigation. One signaling pathway might be a β-catenin-dependent WNT pathway, since the overexpression of WNT6 restores the level of β-catenin and GSK-3β phosphorylation in mutant mice. However, lentivirus-WNT6 transduction, which shows limited expressed in the amygdala, partially but not completely, rescues motor function deficits and social behavior deficits in MECP2 T158A mutant mice. The activated neuron in the amygdala is insufficient to recovery the full behavior. Therefore, it is possible that maybe other molecules, in addition to WNT6, have the potential to improve RTT (Hsu et al., 2020). Nevertheless, WNT6 signaling plays an essential role in improving RTT syndrome via the WNT/β-catenin pathway. A preliminary hypothesis is that the overexpression of WNT6 in the amygdala regulates dopamine release from the nucleus accumbens to regulate motor behavior; however, the hypothesis requires further investigation (Hsu et al., 2020). Collectively, WNT6 expression alleviates symptoms of RTT syndrome. Although one mechanism involves the β-catenin-dependent WNT pathway, several other pathways are also associated with WNT6, however, this requires further investigations.

Glioblastoma

In adults, glioblastoma (GBM) is the most lethal tumor of the central nervous system. Grade IV glioma has the strong ability to invade and spread (Goncalves et al., 2018; Goncalves et al., 2020). Analysis of The Cancer Genome Atlas (TCGA) has shown that in low-grade glioma, *wnt6* mRNA is not overexpressed. Analysis of TCGA and other dataset of glioma tissues has shown that certain GBM patients show high expression of *wnt6* mRNA and protein in tumor cells. Analysis of various GBM subtype patients in independent queues has revealed that *wnt6* mRNA is overexpressed in several GBM molecular subtypes (classical, mesenchymal, neural and proneural) (Goncalves et al., 2018). These results suggest that the level of WNT6 expression is associated with the glioma grade; however, no significant difference has been found in the GBM subtypes. *Wnt6* mRNA expression markedly enhances the proliferation, migration, and invasion of human GBM cells through cell proliferation, as shown by enzyme-linked immunoassay, wound healing migration, and matrigel invasion assays. Furthermore, high expression of *wnt6* mRNA and protein has been shown to decrease overall survival (OS) in both intracranial mouse models and patients. Mice with WNT6-overexpressing tumors show GBM-related neurological symptoms early on. The body weight loss in WNT6 over-expressing tumors is faster than that in low expressing tumors (Goncalves et al., 2018). These results indicate that WNT6 expression is a key oncogenic and aggressive mediator of GBM. Previous reports have shown that WNT signals are closely associated with GBM resistance (Nager et al., 2012). WNT6 silenced human GBM cells are more sensitive to temozolomide (TMZ), which is a gold-standard chemotherapy drug used in GBM patients. The sensitivity of radiation treatment is not significantly affected by the level of WNT6 expression (Goncalves et al., 2018). In addition to the aforementioned functions of WNT6 in tumors, it also regulates the functions of tumor stem cells, thereby promoting tumor development. WNT6 expression is positively correlated with typical stem cell-associated genes in GBM patients, according to TCGA analysis. The levels of NESTIN and SOX2 proteins, common markers of GBM stem cells (GSCs), are significantly decreased in WNT6-silenced human GBM cells. Moreover, WNT6 increases the capacity of GBM cells to form neurospheres and the frequency of neurosphere formation (upon culturing under GSC conditions), functionally indicating that WNT6 positively correlates with the self-renewal capacity of tumor stem cells. In addition to promoting GSC form neurosphere, WNT6 also maintains the phenotype of GSCs. This indicates that WNT6 promotes the expression of glioblastoma stem cell-associated genes and functionally impacts the self-renewal capacity of the tumor (Goncalves et al., 2018).

In WNT6-silenced human GBM cells, the activation of Src family kinases (SFK), heat shock protein (HSP) family (HSP27), and the total levels of β-catenin are inhibited or decreased, indicating that WNT6 acts through the β-catenin-dependent WNT pathway, the SFK/STAT pathway, and the PI3K/AKT/mTOR pathway. However, the WNT6 target in each pathway is different in different cell lines, but the effect is the same. In the PI3K/AKT/mTOR pathway, WNT6-silenced U373 cells directly inhibit the activation of AKT and mTOR, while WNT6-silenced SNB19 cells inhibit the downstream targets such as p70 S6K and eNOS. In the SFK/STAT pathway, WNT6-silenced U373 cells increase serine 727(S727) phosphorylation to decrease signal transducer and activator of transcription 3 (STAT3) activity. WNT6-silenced SNB19 cells decrease the phosphorylation of tyrosine 705 (Y705) to decrease STAT3 activity (Goncalves et al., 2018). In GBM, WNT6 expression is regulated by Homeobox A9 (HOXA9) to stimulate the β-catenin-dependent WNT pathway. HOXA9, a transcriptional regulator, binds to the WNT6 promoter and activates the expression of WNT6 in GBM cells. Moreover, WNT6 and HOXA9 are associated with the progress of GBM, resulting in poor clinical outcome when either show a high expression level. However, the functioning of WNT6 is independent on HOXA9. Furthermore, both WNT6 and HOXA9 are co-expressed in GBM and other diseases including leukemia, testicular germ cell tumor, melanoma, and cholangiocarcinoma (Goncalves et al., 2020), indicating that the scope of WNT6 function is broader. WNT6 expression is regulated by HOXA9 and DNA methylation. In glioma, the DNA methylation level of the WNT6 promoter and specific CpG regions on the genome is associated with the expression level of WNT6. Moreover, a high level of DNA methylation of specific CpG regions in the promoter is associated with WNT6 silencing, while gene body methylation is positively associated with its expression. The CpG sites are more frequently methylated in low-grade gliomas (LGG) compared to GBM patients (Goncalves et al., 2020). This indicates that WNT6

DNA methylation is at least partly involved in the regulation of WNT6 expression in gliomas. In summary, WNT6 accelerates the development of GBM in various aspects; therefore, it is a critical biomarker of tumor recurrence. WNT6 is also a potential therapeutic target or prognostic marker of GBM.

Colorectal Cancer

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. *Wnt6* mRNA expression is increased in HCT116 and SW480 human CRC cells compared to other three colon cancer cell lines (LoVo, SW620, and HT29) (Zheng and Yu, 2018; Li et al., 2019). The high expression of WNT6 increases the proliferative ability of CRC cells (human CRC lines) by accelerating the cell cycle. The loss of WNT6 protein inhibits the expression of caspase-3 and increases the expression of B-cell lymphoma 2-associated X protein (Bax) (an apoptosis-promoting member of the Bcl-2 family). WNT6 overexpression, however, reverses this effect. This indicates that WNT6 protein expression can regulate apoptosis-regulated genes to inhibit apoptosis and promotes tumor development. Moreover, the high level of WNT6 protein increases the expression of MMP2 and induces CRC cell migration. MMP2 is also involved in the breakdown of the extracellular matrix (Zheng and Yu, 2018). Therefore, WNT6 expression promotes the development and metastasis of CRC. In addition, the promoter region of WNT6 is bound by polymorphic adenoma-like protein 2 (PLAGL2) in the nucleus of CRC cells (Li et al., 2019). PLAGL2, a zinc finger protein derived from the PLAG gene family (Kas et al., 1998; Furukawa et al., 2001; Wezensky et al., 2010), is a proto-oncogene and a transcription factor. PLAGL2 combines with the WNT6 promoter and activates the β-catenin-dependent WNT signaling pathway, thereby stimulating various downstream target genes (such as MMP7, CCND1) and promoting tumor development (Li et al., 2019). The acceleration of tumor progression by WNT6 is not only reflected in the basic molecular mechanisms, but also in the clinical prognosis. Analysis of 106 patients with colorectal liver metastasis revealed that, compared to the low level of WNT6 protein expression, the overexpression of WNT6 protein shortens the 5-year OS rate following liver resection in colorectal liver metastasis. Moreover, the influence of WNT6 in patients with different risk levels is also different. Following liver resection for colorectal liver metastasis in low-risk patients, the 5-year OS rate was found to be lower in the group with high WNT6 protein expression level compared to the low WNT6 expression group. However, it was not significantly different in the high-risk patients. This might be attributed to the different characteristics of patients with different-risk levels. Since low-risk patients have the potential for long-term survival, they are more sensitive to WNT6 expression (Peng et al., 2019). However, these result require further study because the sample size has not been large enough. In summary, WNT6 plays a key role in promoting the development of CRC and is a potential therapeutic target.

Gastric Cancer

Gastric cancer (GC) is one of the largest causes of cancer-related deaths worldwide. WNT6 expression is low in some primary human GC cell lines compared to cell lines derives from distant metastases such as MKN45, MKN7, and NCIN87. Not all GC cells express high levels of WNT6. In GC, caveolin-1 (Cav1) can regulate the expression of WNT6. Caveolin, a plasma membrane micro-domain enriched in cholesterol and sphingolipids, is an essential structural protein (Razani et al., 2002; Parton and Simons, 2007). Cav1 is associated with lipid transport, gene regulation, and signal transduction (Anderson and Jacobson, 2002; Williams and Lisanti, 2005). MKN45/RNAi cells expressing low levels of Cav1 were found to have a low level of *wnt6* mRNA expression, while MKN45/Cav1 cells overexpressing Cav1 had a high level of *wnt6* mRNA expression. Other GC cell lines (AGS and HEK293) also had the same expression profile, indicating that Cav1 can upregulate the expression of WNT6. MKN45/RNAiCav1 contributes to chemoresistance. MKN45/RNAi cells are more sensitive to anthracycline (epirubicin) than Cav1-expressed cells might by activating apoptosis. MKN45/RNAi cells with WNT6 knockdown have a similar effect as anthracycline. When Cav1 is not expressed, in the absence or presence of *wnt6* mRNA expression, cells are not significantly different regarding their sensitivity to anthracycline (epirubicin). By contrast, the overexpression of both WNT6 and Cav1 decreases cell death, indicating that Cav1 activates WNT6 to prevent tumor cell apoptosis, and induces chemoresistance through the β-catenin-dependent WNT pathway. These data suggest that WNT6 and Cav1 are potential therapeutic targets to improve chemoresistance. A study found that cells expressing WNT6 at low levels, when treated with anthracycline, expressed increasing levels of *wnt6* mRNA and protein in a time-dependent manner, by activating the WNT/β-catenin pathway. These results indicate that Cav1 and WNT6 increase chemoresistance by activating the WNT/β-catenin pathway. Moreover, anthracycline promotes the expression of WNT6 to increase chemoresistance. This mechanism differs from that of the classical multidrug resistance transporters (Yuan et al., 2013). Additionally, in primary cancer, WNT6 suppresses rapid proliferation in the early stages of tumorigenesis, and its expression can be silenced. However, in the advanced stages of GC, WNT6 is re-upregulated to protect the GC cells (Yuan et al., 2013), due to the different functions of WNT6 dominating at different times.

CONCLUSION

WNT6 belongs to the family of WNT ligands and is a highly conserved molecule that plays various roles in mice and humans. Its expression and function begin from the early stage of embryonic formation, and play a role in several stages of life. Importantly, WNT6 has the ability to become a therapeutic target, because it plays an important role in human diseases. However, further studies are needed to elucidate the role of WNT6 in diseases. Current research has mainly used animal disease models and clinical studies to provide comprehensive evidence of the role of WNT6. Meanwhile, the research on the mechanisms underlying WNT6 in disease is are still limited; thus, additional investigations are needed to provide a better

understanding of the biology and function of WNT6 and its potential as a therapeutic target in these diseases.

AUTHOR CONTRIBUTIONS

MW, QW, XD, and HZ wrote, performed the revisions, and reviewed the manuscript. CZ and YT conducted the literature research. All authors gave the final approval of the manuscript.

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Regulation of Wnt Signaling Pathways at the Plasma Membrane and Their Misregulation in Cancer

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Wnt signaling is one of the key signaling pathways that govern numerous physiological activities such as growth, differentiation and migration during development and homeostasis. As pathway misregulation has been extensively linked to pathological processes including malignant tumors, a thorough understanding of pathway regulation is essential for development of effective therapeutic approaches. A prominent feature of cancer cells is that they significantly differ from healthy cells with respect to their plasma membrane composition and lipid organization. Here, we review the key role of membrane composition and lipid order in activation of Wnt signaling pathway by tightly regulating formation and interactions of the Wnt-receptor complex. We also discuss in detail how plasma membrane components, in particular the ligands, (co)receptors and extracellular or membrane-bound modulators, of Wnt pathways are affected in lung, colorectal, liver and breast cancers that have been associated with abnormal activation of Wnt signaling. Wnt-receptor complex components and their modulators are frequently misexpressed in these cancers and this appears to correlate with metastasis and cancer progression. Thus, composition and organization of the plasma membrane can be exploited to develop new anticancer drugs that are targeted in a highly specific manner to the Wnt-receptor complex, rendering a more effective therapeutic outcome possible.

Keywords: Wnt, frizzled, plasma membrane, cancer, lipid raft

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INTRODUCTION

The Wnt signaling pathway is an evolutionarily conserved signal transduction cascade that controls a wide range of biological events from embryonic development to tissue regeneration (Nusse, 2005; Aman et al., 2018; Steinhart and Angers, 2018). The pathway is broadly divided into two branches as the canonical (β-catenin-dependent) Wnt signaling and the non-canonical (β-catenin-independent) Wnt signaling, which is further branched into the Wnt/planar cell polarity (PCP) and the Wnt/Ca^{2+} pathways. Wnt signaling pathways play a multitude of essential roles in cell fate determination, cell polarity, cell migration and patterning during embryonic development, adult tissue homeostasis and regeneration of various tissues and organs. Consequently, misregulation of Wnt signaling has been associated with a variety of human diseases including developmental defects, degenerative diseases, and many cancers. Although numerous components of the Wnt

pathways have been characterized for their functional roles, many questions related to tight regulation and modifications of signaling remain unanswered. Besides, despite many efforts, so far no drugs have been approved to specifically target the pathway, leading to a gap in targeted therapy of Wnt-related diseases (Nusse and Clevers, 2017; Krishnamurthy and Kurzrock, 2018). A better understanding of the molecular mechanisms underlying Wnt-mediated cellular responses is especially critical for development of effective anticancer drugs that are expected to interfere with the highly activated Wnt signaling in many cancers (Jung and Park, 2020).

The plasma membrane plays a dual central role in regulation of cell signaling. On the one hand, it acts as a barrier and ensures spatial segregation between extracellular environment and the cytosolic compartment. At the same time, by harboring many cell surface receptors and regulators that are involved in cell signaling, it actively controls transmission of molecular signals from the exterior to the interior of a cell and precisely links them to downstream signaling events. The plasma membrane is likewise vital for initiation of Wnt signaling where Wnt ligands bind to their receptor complexes in specialized membrane domains that are considered as dynamic assemblies of various saturated lipids, sterols and lipid-anchored proteins (Sezgin et al., 2017a,b, 2015) (**Figure 1**). Owing to their central roles in initiation of signaling, Wnt pathway components acting at the plasma membrane have been frequently investigated as drug targets (Gurney et al., 2012; Krishnamurthy and Kurzrock, 2018; Zeng et al., 2018a). Since plasma membranes of healthy and diseased cells display major structural differences, the influence of membrane nanoenvironment on signaling function should be considered carefully when developing novel therapeutic approaches that target Wnt pathways at the membrane.

Here, we review the cellular mechanisms underlying regulation of Wnt signaling pathways at the plasma membrane domains with a specific focus on the role of lipid molecules. Next, we address how Wnt pathways are misregulated at the plasma membrane through their ligands, receptors and membrane associated pathway modulators in cancer. Since Wnt signaling has been associated with numerous cancers, here we will focus on four common cancers, i.e., lung, colorectal, liver, and breast cancers. We also discuss the therapeutic approaches that aim to inhibit aberrant signaling activity in cancer by targeting the Wnt-receptor complex at the plasma membrane.

WNT SIGNALING PATHWAYS

Wnt signaling pathway regulates proliferation, survival, polarity, and migration, differentiation of cells as well as maintenance of stem cells of various lineages during embryonic development and tissue homeostasis (Clevers et al., 2014). Examples come from the stem cells that reside in different regions of the body including the digestive tract, hematopoietic system and the nervous system (Lee et al., 2000; Willert et al., 2003; Nusse et al., 2008). The Wnt target gene Lgr5 has been identified as a stem cell marker at the crypts of small intestine and colon (Barker et al., 2007). Further studies have revealed that Lgr5 and Axin2 are expressed in the stem cells of multiple organs such as the liver, mammary gland, stomach, brain, kidney, cochlea and ovary and associated with widespread self-renewal capacity (Barker et al., 2010, 2012; van Amerongen et al., 2012; Bowman et al., 2013; Flesken-Nikitin et al., 2013; Huch et al., 2013; Jan et al., 2013). Thus, by ensuring the formation of the stem cell pool and its continuity via asymmetric divisions, Wnt signals play critical roles in establishing niches for stem cells in various tissues and organs and determining molecular programs of tissue regeneration.

The Wnts consist of a large family of protein ligands that interact with a number of receptors and co-receptors at the plasma membrane. Based on 19 Wnt ligands and 10 Fz receptors in mammals, these interactions constitute one of the most complex relationships between extracellular ligands and cell surface receptors (Clevers and Nusse, 2012). While some Wnt ligands are associated with a particular Wnt signaling pathway, others that cannot easily be attributed to a single Wnt pathway are competent to initiate signaling in more than one branch.

Wnt/β-Catenin Signaling: The Canonical Wnt Pathway

Being the most studied Wnt pathway, canonical Wnt signaling operates through cytoplasmic accumulation of β-catenin protein and is essential for embryonic development, adult homeostasis and stem cell maintenance (Nusse and Clevers, 2017). In the absence of canonical Wnt ligand, β-catenin is phosphorylated by a multiprotein destruction complex that includes the serine-threonine kinases glycogen synthase kinase 3β (Gsk3β) and casein kinase 1α (Ck1α), the scaffold protein Axin and the cytoplasmic effector proteins dishevelled (Dvl) and Adenomatous Polyposis Coli (APC), and targeted for degradation by the ubiquitin-proteasome system (Kimelman and Xu, 2006; MacDonald et al., 2009). Active canonical Wnt ligand, however, binds to its cell surface receptor frizzled (Fz) and co-receptor low-density lipoprotein receptor-related protein 5/6 (Lrp5/6) and recruits Dvl and Axin to the plasma membrane, leading to disassembly of the destruction complex (**Figure 2**). β-catenin then accumulates in the cytoplasm, translocates into the nucleus and interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to regulate the expression of target genes (Logan and Nusse, 2004; Taketo, 2004; Nusse, 2005; Angers and Moon, 2009; Clevers and Nusse, 2012).

The Non-canonical Wnt Pathways

Acting independently of β-catenin, the non-canonical Wnt/PCP pathway coordinates cell movement and tissue polarity via small GTPase RhoA or c-Jun N-terminal kinase (JNK) (Katoh, 2005). The typical non-canonical ligands Wnt5a, Wnt5b, and Wnt11 initiate the PCP signaling via interacting with their receptors Fz3 or Fz6 and co-receptors Ror1, Ror2, or Ptk7 (Humphries and Mlodzik, 2018). The human planar cell polarity proteins Vangl1, Vangl2, Prickle1, and Prickle2, Cadherin EGF LAG seven-pass G-type receptors (Celsr1, Celsr2, Celsr3), Dvl1, Dvl2, Dvl3, and Ankyrin repeat domain 6 (Ankrd6) are involved in the signaling cascade (**Figure 2**). Dvl-dependent Wnt/PCP signals

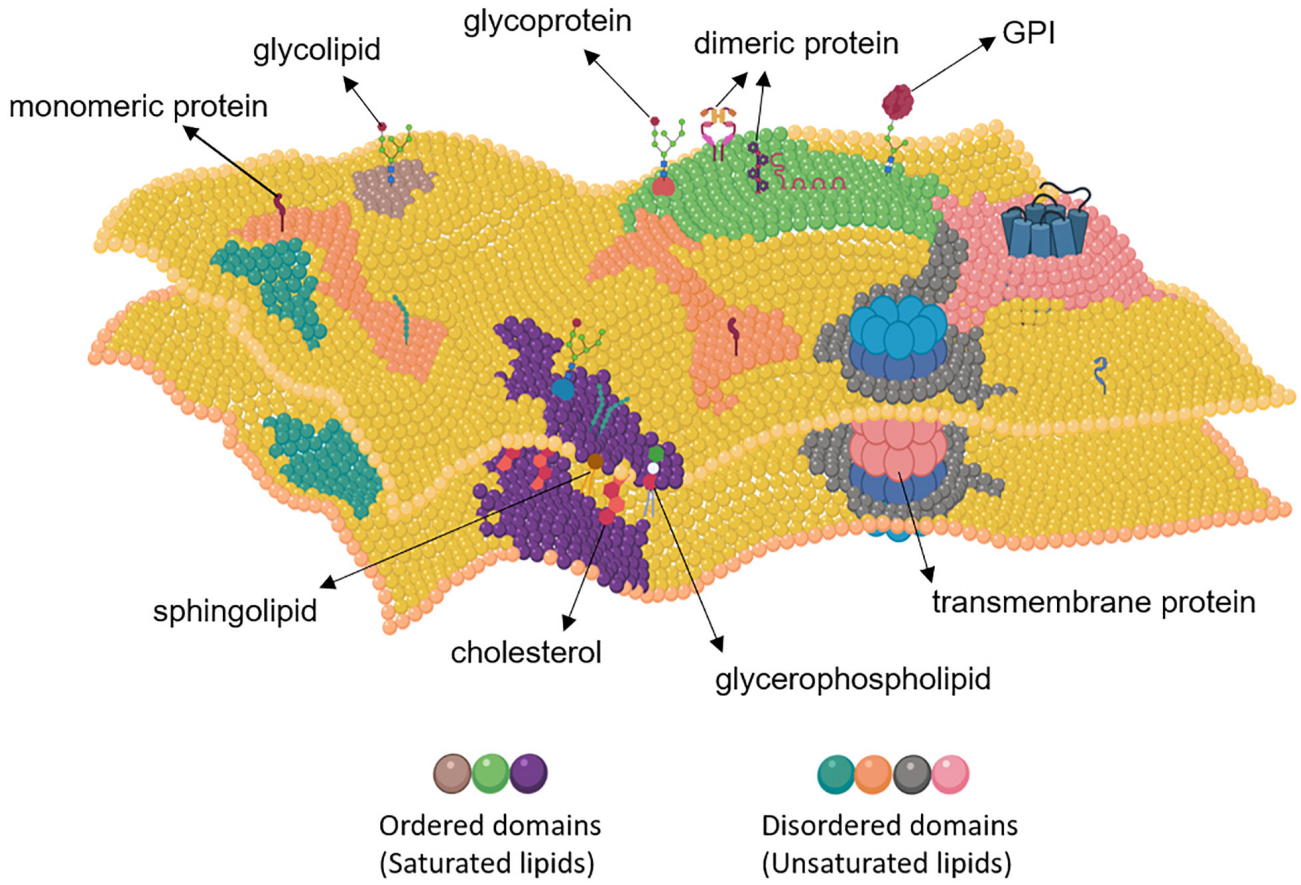

FIGURE 1 | Structure of the plasma membrane. The plasma membrane consists of ordered and disordered domains. Basically, ordered domains are enriched in cholesterol, sphingolipids, glycerophosholipids, glycosylphosphatidylinositol (GPI)-anchored proteins, glycoproteins, dimeric proteins, and saturated lipids, whereas the disordered domains usually contain monomeric and transmembrane proteins and unsaturated lipids. Created with BioRender.com.

are transduced through the Dishevelled-associated activator of morphogenesis (Daam1 and Daam2) proteins or Mitogen activated protein (MAP) kinase kinase kinase (MAPKKK) and MAPKK4/7 and activate the Ras homolog family member A-Rho-associated protein kinase (RhoA-Rock) or Rac-JNK signaling cascades, respectively. On the other hand, the Dvl-independent G protein-dependent non-canonical Wnt pathway operates through G proteins, Receptor tyrosine kinases (RTKs) and Phospholipase C (PLC), leading to intracellular calcium release, activation of the serine/threonine protein phosphatase calcineurin and accumulation of Nuclear factor of activated T cells (NFAT) in the nucleus (Katoh, 2005; Kohn and Moon, 2005; Katoh and Katoh, 2017). Elevated cytoplasmic calcium can also activate Nemo-like kinase (NLK) signaling pathway through Calcium/calmodulin dependent kinase II (CaMKII).

Regulation of Wnt Pathways at the Plasma Membrane

Both canonical and non-canonical Wnt signaling pathways are tightly regulated by a number of modulators that are evolutionarily conserved and function either extracellularly to regulate ligand-receptor interactions or intracellularly to modify cytosolic or nuclear components of the pathway. Extracellular modulators can be either secreted, membrane-bound or transmembrane proteins and are broadly classified into two groups according to their functions: (1) The agonists that include Norrin, R-spondins (Rspos), GPI-anchored membrane proteins Ly6/Plaur domain-containing 6 (Lypd6) and Reck, G protein-coupled receptors Gpr124 and Gpr37 and (2) the antagonists that include secreted Dickkopf proteins (Dkks), Secreted frizzled-related proteins (Sfrps), secreted Wnt-inhibitory factor 1 (Wif-1), secreted Wise/Sost proteins, secreted protein Cerberus, secreted insulin-like growth-factor binding protein 4 (Igfbp-4), secreted palmitoleoyl-protein carboxylesterase Notum, single-transmembrane proteins Shisa, Wnt-activated inhibitory factor 1 (Waif1/5T4), adenomatosis polyposis coli down-regulated 1 (Apcdd1), membrane-bound metalloprotease Tiki1, transmembrane E3 ubiquitin ligase Zinc and ring finger 3 (Znrf3) and its functional homolog Ring finger protein 43 (Rnf43), (Cruciat and Niehrs, 2013; Jiang and Cong, 2016; Berger et al., 2017; Eubelen et al., 2018). Owing to their critical roles in ligand or receptor modification, ligand-receptor complex formation, regulation of signaling activity in membrane

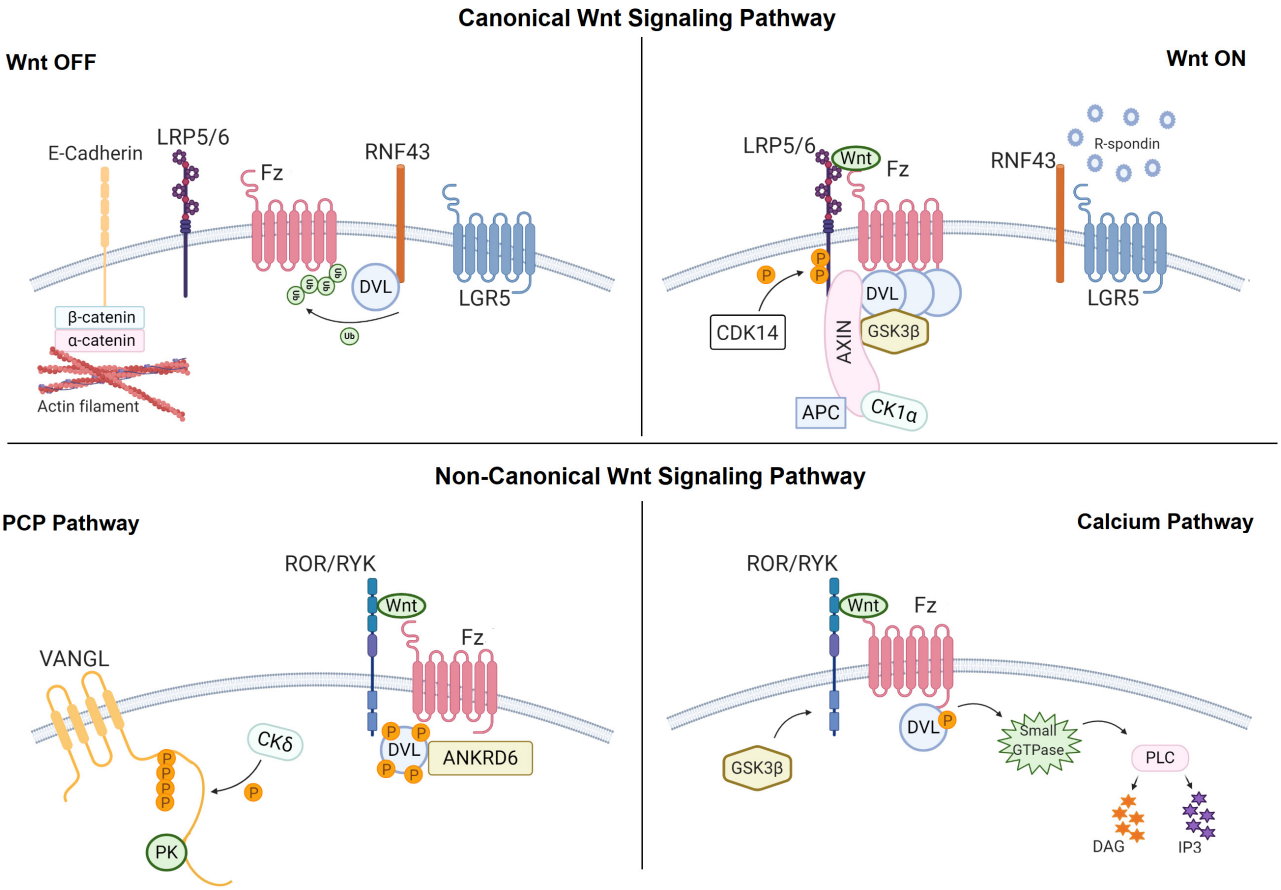

FIGURE 2 | The Wnt signaling pathways at the plasma membrane. Canonical Wnt signaling pathway: In the Wnt OFF state, defined by the absence of canonical Wnt ligand, β-catenin is phosphorylated by the destruction complex that is comprised of glycogen synthase kinase 3β (GSK3β) and casein kinase 1α (CK1α), Axin and dishevelled (DVL) and Adenomatous Polyposis Coli (APC). Ring finger protein 43 (RNF43) also inhibits the pathway by binding the Fz receptor via DVL. In the Wnt ON state, canonical Wnt ligand binds to the Frizzled (Fz) receptor and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptor. This interaction recruits DVL and AXIN to the Wnt-receptor complex and leads to disassembly of the destruction complex, causing stabilization of β-catenin in the cytosol. Binding of R-spondin to leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) enhances Wnt signaling activity by neutralizing RNF43. Non-canonical Wnt signaling pathways: In the PCP pathway, non-canonical Wnt ligands bind to the ROR/RYK-Fz receptor complex and recruit DVL. Next phosphorylation of VANGL activates the pathway. In calcium pathway, binding of non-canonical Wnt ligands to ROR/RYK-Fz recruits DVL and activated DVL binds to the small GTPase that further activates phospholipase C (PLC). Created with BioRender.com.

subdomains and receptor internalization, these secreted and membrane proteins are noteworthy candidates for therapeutic targeting of Wnt signaling pathways.

INITIATION OF WNT SIGNALING AT THE PLASMA MEMBRANE

The plasma membrane has a complex heterogeneous and highly dynamic structure that is composed of lipids and proteins with varying features and compartmentalized into numerous smaller structures called micro- or nanodomains (Eggeling et al., 2009; Owen et al., 2012; Sarmento et al., 2020). This heterogeneity leads to formation of ordered membrane domains, also known as lipid rafts or lipid nanodomains, which are assembled from saturated lipids, sphingolipids, sterols, glycolipids, glycoproteins and certain lipid-anchored proteins such as glycosylphosphatidylinositol (GPI)-anchored proteins and fatty acylated proteins, leaving the relatively disordered domains occupied by unsaturated lipids and a large fraction of membrane proteins (Sezgin et al., 2015, 2017b; Fakhree et al., 2019; Kusumi et al., 2020; **Figure 1**). The ordered domains of the plasma membrane have been shown to be essential for cell signaling events by controlling membrane-cytoskeleton communication, ligand-receptor interaction and receptor clustering (Simons and Toomre, 2000; Thomas et al., 2004; Ozhan et al., 2013; Agarwal et al., 2018; Azbazdar et al., 2019). For example, activation of epidermal growth factor receptor (EGFR) signaling by the epidermal growth factor (EGF) has been proposed to induce coalescence of different lipid rafts and formation of signaling platforms (Hofman et al., 2009; Irwin et al., 2011). After exposure to the Gram-negative bacterial cell wall component lipopolysaccharides, Toll-like receptor 4 (TLR4) of the immune system cells likewise interacts with its sorting

adaptor Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) in the lipid rafts (Barnett and Kagan, 2020).

Activation of Canonical Wnt Signaling in the Plasma Membrane Domains

Interaction of the canonical Wnt ligand with its (co)receptors triggers Lrp6 phosphorylation and endocytosis of receptor complexes, both of which appear to occur preferentially in the ordered membrane domains (Yamamoto et al., 2008; Sakane et al., 2010). While appearing in a way unexpected due to the fact that majority of the receptor Fz and the co-receptor Lrp6 are preferentially localized in the disordered membrane domains, both processes are facilitated by the GPI-anchored Lypd6 protein that interacts with Lrp6, helps it to associate with the ordered membrane domains via its GPI anchor and enhances Wnt/β-catenin signaling by promoting Lrp6 phosphorylation in these domains (Ozhan et al., 2013). Lipid raft disruption via induction of cholesterol efflux from the membrane with methyl-β-cyclodextrin significantly reduces Lrp6 phosphorylation in neuroblastoma cells, indicating that membrane order and integrity is essential for Lrp6 activity (Riitano et al., 2020). Further work has revealed that the canonical Wnts bind to their receptor complexes selectively in the ordered membrane domains (Sezgin et al., 2017a). Disruption of the membrane order *in vitro* or *in vivo* using inhibitors that specifically target lipids associated with the ordered domains significantly reduces canonical Wnt signaling activity, underscoring the influence of the plasma membrane lipid content on early interaction of the canonical Wnt with its receptors and downstream signaling activity (Sezgin et al., 2017a). Presence of canonical Wnt ligand stimulates enrichment of cholesterol, an essential component of the ordered membrane domains, in the inner membrane leaflet and receptor clustering that is likely aided by other membrane components such as heparin sulfate and phosphatidylinositol-4,5-bisphosphate (PIP2) (Mii et al., 2017; Erazo-Oliveras et al., 2018). Defects in synthesis of cholesterol thus reduces Wnt signaling and results in abnormalities in craniofacial development and neural crest cell differentiation (Sezgin et al., 2017a; Castro et al., 2020). The glycosphingolipid mannosyl glucosylceramide, which assembles with sterols into ordered membrane domains, likewise enhances presynaptic Wnt1/Wingless (Wg) signaling in the lipid rafts and promotes synaptic bouton formation at the *Drosophila* neuromuscular junction (Huang et al., 2018). Wnt/β-catenin signaling can also act in combination with Reactive Oxygen Species (ROS) signaling to regulate nuclear β-catenin levels during neural differentiation in a lipid raft dependent manner (Haack et al., 2015).

Regulation of Wnt-receptor interaction in ordered domains through membrane proteins appears not to be restricted to canonical Wnt signaling. The heparan sulfate proteoglycan Glypican-4 (Gpc4) is one such bifunctional molecule that controls canonical and non-canonical Wnt pathways by binding to Wnt3a and Lrp6 in the ordered domains and to Wnt5a and Ror2 in the disordered domains, respectively (Sakane et al., 2012). Likewise, autocrine Wnt10b has been shown to be drafted to the ordered domains by the fibroblast-derived (FD) exosomes and activate mTOR signaling that in turn promotes axonal regeneration independently of β-catenin (Tassew et al., 2017). Conversely, inhibition of fatty acid synthase (FASN), the major source of long-chain fatty acids such as palmitate, interferes with lipid biosynthesis, disrupts ordered membrane architecture and inhibits Wnt/β-catenin signaling along with PI3K-AKT-mTOR pathways, most likely also inhibiting protein lipidation that is necessary for proper signal transduction (Ventura et al., 2015).

Apart from the components of the Wnt-receptor complex, *N*-terminally dephosphorylated (dephospho) β-catenin, the *de novo* synthesized form of β-catenin that correlates with Wnt signaling activity, has been reported to colocalize at the plasma membrane with Lrp6 and two members of the destruction complex, namely APC and Axin, shortly after Wnt stimulation and independently of E-cadherin (Hendriksen et al., 2008). Routing of dephospho-β-catenin to the membrane via the Wnt receptor complex appears to constitute a key step in regulation of its transcriptional activity and Wnt signaling. The activity of β-catenin as a membrane component may be controlled by other molecules under certain ambient conditions. For example, non-muscle myosin II induces accumulation of cortical F-actin and E-cadherin to the adherens junctions, resulting in corecruitment of β-catenin to the membrane to maintain cellular contraction and inhibition of Wnt signaling due to reduced levels of cytoplasmic β-catenin (Hall et al., 2019). Cytoskeletal networks have also been reported to regulate Wnt signaling activity through plasma membrane domains during differentiation of stem cells (von Erlach et al., 2018).

Wnt Signalosome and the Role of Endocytosis

Interaction of Wnts ligands with their surface receptors within particular membrane domains activate an immediate biochemical response that triggers internalization of the Wnt signalosome, a dynamic signaling complex assembled by Dvl upon formation of the Wnt-receptor complex (Bienz, 2014; Gammons et al., 2016). Internalization of signalosome is essential for pathway activation that is balanced by degradation of excessive ligands and clearance of surface receptors, ultimately downregulating the signaling (Feng and Gao, 2015). At the same time, paradoxically, receptor-mediated endocytosis negatively regulates Wnt signaling through internalization and degradation of the receptor complex. The route of endocytosis is determined by multiple factors including the types of ligands and receptors, other molecules in the environment, feedback regulatory mechanisms and how the signal is terminated.

Canonical Wnt-mediated receptor complex is known to be endocytosed via the clathrin-independent route in the smooth invaginations of the plasma membrane, the so-called caveolae, which form as subset of the ordered membrane domains (Parton et al., 2006; Yamamoto et al., 2006; Bilic et al., 2007; Gong et al., 2008). Tyrosine-based motifs within the cytoplasmic tail of Lrp6 has been shown to be essential for determining its distribution within the membrane domains and its internalization routes, which is critical to keep signal activity under control (Liu et al., 2014). However, a large body of evidence supports that

clathrin-dependent endocytosis of the Wnt-receptor complex can also enhance β-catenin-dependent signaling and that clathrin is a prerequisite for Wnt signalosome formation (Kim et al., 2013; Hagemann et al., 2014; Gammons et al., 2016; Brunt and Scholpp, 2018). Endocytosis has been proposed to regulate Wnt/β-Catenin signaling mainly via four alternative ways that include early endosomal acidification of the co-receptor Lrp6 for pathway activation, sequestration of GSK-3 to limit β-catenin proteolysis, clearance of ubiquitin ligases that target Wnt receptors for degradation and facilitation of signaling by stabilization of Dvl (Brunt and Scholpp, 2018).

β-catenin-independent Wnt pathway operates through clathrin-dependent endocytic route that mediates uptake of PCP components together with Syndecans, the transmembrane proteoglycans (Yamamoto et al., 2008; Ohkawara et al., 2011). Wnt/PCP signaling regulates cell adhesion and migration by regulating internalization of cadherins via protocadherins during vertebrate gastrulation (Brinkmann et al., 2016; Brunt and Scholpp, 2018). Recent data obtained from plasma membrane capacitance recordings have further supported that non-canonical Wnt5a is exclusively endocytosed via clathrin-coated vesicles (Bandmann et al., 2019).

Endocytosis of the Wnt-receptor complex can also be affected by different molecules and pathways. For example, in addition to regulating proteolytic degradation of β-catenin as part of the destruction complex, APC keeps Wnt receptor internalization and pathway activation under control by forming a complex with clathrin (Saito-Diaz et al., 2018). AP2-associated kinase 1 (Aak1), which is activated by Wnt/β-catenin signaling, likewise promotes clathrin-mediated endocytosis of Lrp6 and thus negatively regulates the pathway (Agajanian et al., 2019). The mammalian target of rapamycin complex 1 (mTORC1) signaling has also been shown to inhibit Wnt/β-catenin signaling by suppressing the expression of membrane Fz through enhancing its Dvl-dependent clathrin-mediated internalization (Zeng et al., 2018b). Further mechanistic studies on the interplay between Wnt signaling and other molecules will provide a deeper understanding of the tight control of pathway activity.

Posttranslational Modifications in Wnt Proteins

Wnts are 350–400 amino acid-long cysteine-rich secreted glycoproteins that are highly conserved in metazoans. Mammals have 19 Wnt ligands that act through the canonical or non-canonical Wnt pathway and thus contributes to the pathway specificity and complexity (Clevers and Nusse, 2012).

The high number of conserved cysteine residues that Wnt proteins harbor suggest that the intra- and inter-molecular disulfide bonds are important for the proper folding, multimerization and function of Wnt proteins (Tang et al., 2012). Wnts undergo two main types of posttranslational modifications, i.e., *N*-glycosylation and lipidation/acylation at the endoplasmic reticulum (ER) and are subsequently transported to the plasma membrane via the cargo protein Wntless (Wls)/Evi, which is essential for Wnt secretion (Glaeser et al., 2018; Gradilla et al., 2018). Various mutations introduced at N-linked glycosylation sites of different Wnt proteins have shown that glycosylation is essential for proper folding and secretion of Wnt proteins but dispensable for their signaling activity (Mason et al., 1992; Komekado et al., 2007; Kurayoshi et al., 2007; Tang et al., 2012).

The Role of Palmitoylation in Wnt Function

Being a prominent mode of lipidation, acylation of Wnts takes place at conserved amino acid residues through palmitoylation and is mainly catalyzed by the protein Porcupine, a membrane-bound O-acyltransferase of the ER (Willert and Nusse, 2012; Gao and Hannoush, 2014; Nile and Hannoush, 2016). Initial mass spectrometry-based studies on mouse Wnt3a propounded two different sites for addition of palmitoyl moieties, which were a thioester-linked palmitic acid at a conserved cysteine and an oxyester-linked palmitoleic acid at a conserved serine (Willert et al., 2003; Takada et al., 2006). While the conserved serine has been confirmed as the consensus acylation site across all Wnts by high-resolution crystal structure analysis of *Xenopus* Wnt8, the conserved cysteine was found to be engaged in a disulfide bond, thus preventing it from serving as a lipidation site (Janda et al., 2012). Interestingly, canonical Wnts have been proposed to be acylated by palmitoleic acid, a monounsaturated fatty acid, which is assumed to exhibit a kinked conformation and thus fit into the cavity of the Porcupine (Takada et al., 2006; Nile et al., 2017; Lee et al., 2018, 2019). However, considering the fact that palmitoylation targets soluble proteins into ordered membrane domains, palmitoylation of Wnt by a monounsaturated fatty acid contradicts with its preferential binding in the ordered domains and ability to activate signaling therein (Zhai et al., 2004; Levental et al., 2010; Ozhan et al., 2013; Sezgin et al., 2017a). Computational structural analyses have indeed supported that canonical Wnt is likely modified by palmitic acid, a saturated fatty acid, by adopting a conformation compatible with the stereochemical features of Wnt modification (Azbazdar et al., 2019).

The role of lipidation in Wnt secretion and functionality has been investigated by mutagenesis of the conserved palmitoylation sites of various Wnt proteins (Kurayoshi et al., 2007; Franch-Marro et al., 2008; Tang et al., 2012; Luz et al., 2014; Hosseini et al., 2019; Speer et al., 2019). For example, palmitoylation mutants of mouse Wnt1 and Wnt3a were found to be secreted at varying levels while their signaling activities were significantly and consistently reduced (Takada et al., 2006; Doubravska et al., 2011; Galli and Burrus, 2011; Gao and Hannoush, 2014). Acylation mutant of Drosophila Wg was likewise found to be secreted normally but with markedly weaker signaling activity (Franch-Marro et al., 2008). Strikingly, several non-acylated mutants of Wnt1, Wnt3a, and Wnt8 from different species have been identified to vary dramatically in their rates of secretion, interactions with the receptor Fz and abilities to activate signaling *in vitro* or *in vivo* (Speer et al., 2019). Studies on the zebrafish canonical Wnt ligands have reported that Wnt palmitoylation is essential for activation of signaling but may be dispensable for secretion (Luz et al., 2014; Azbazdar et al., 2019). APT1-mediated depalmitoylation has been shown to be important for asymmetric localization of β-catenin and Wnt signaling activity during development (Stypulkowski et al., 2018). Interestingly,

Drosophila WntD does not appear to undergo any glycolysis and acylation (Ching et al., 2008). Therefore, the impact of acylation on the secretion and function of different types of Wnts appears to vary significantly. We believe that individual and context-dependent characterization of Wnt ligands will provide critical insight into the how Wnt signaling activity is modified in different types of cancers and how therapeutic approaches could be specifically designed.

The Impact: How Can We Benefit From What We Have Learned So Far?

Wnt signaling pathways control a plethora of cellular responses involved in development, homeostasis and disease. Thus, the molecular interactions underlying initiation of Wnt pathways at the plasma membrane have the potential to serve as attractive drug targets, especially for cancer where Wnt signaling is broadly misregulated. The disclosure of the functional role of plasma membrane organization in Wnt/β-catenin signaling will shed light on how the membrane background can be exploited for therapeutic approaches:

(1) The drugs can be packed in specific lipid-based drug delivery systems to directly target them to the relatively ordered domains of the membrane where Wnt-receptor complex formation occurs.
(2) The peptide drugs can be modified by introducing particular lipid moieties that help them target Wnt-receptor complex more precisely. Posttranslational lipid modifications, in particular palmitoylation, might be a good candidate to enhance domain-specific receptor targeting.
(3) A very convenient strategy would be lipid fingerprinting of cancer cells to characterize membrane lipid profiles of individual cell types. This would enable selectively inhibition of aberrant activated Wnt signaling pathway by direct modification of membrane lipid composition.

Overall, we believe that unraveling plasma membrane organization with respect to (mis)regulation of Wnt signaling in health and disease will help not only develop new strategies on targeted anticancer therapies and but also increase target specificity of existing drugs that interfere with the ligand-receptor complex at the plasma membrane.

MISREGULATION OF WNT SIGNALING PATHWAYS AT THE PLASMA MEMBRANE IN CANCER

Dysregulation of plasma membrane domains with respect to its structural organization and dynamics, disruption of membrane protein and lipid homeostasis or mutations in genes encoding for membrane proteins can cause misregulation of cell signaling events and promote oncogenic signaling activities. For example, breast cancer cell lines with high levels of epidermal growth factor receptor (EGFR) have been found to be resistant for the tyrosine kinase inhibitors (TKIs) targeting EGFR because of EGFR accumulation in the lipid rafts at the membranes of these cells and pharmacological depletion of cholesterol from the rafts decreased this resistance (Irwin et al., 2011). The constitutively active mutant form of the non-receptor tyrosine kinase Src has likewise been reported to accumulate in the lipid rafts of small cell lung cancer (SCLC) cells and stimulate oncogenic phosphoinositide 3-kinase (PI3K) signaling by facilitating the interaction of particular PI3K isoforms with Src kinases (Arcaro et al., 2007). Epithelial-to-mesenchymal transition (EMT) has also been associated with modulation of lipid raft properties strongly suggesting that alterations of membrane biophysical phenotypes are required to maintain metastatic potential of cancer cells (Tisza et al., 2016). In this section we will review how misregulation of Wnt signaling mainly in four common cancers; i.e., lung, colorectal, liver, and breast, is linked to the defective signaling at the plasma membrane and discuss the potential therapeutic approaches based on targeting the Wnt pathway at the membrane in these cancers.

Lung Cancer

Being the most common cause of cancer-related death in the world, lung cancers are histologically classified as non-small-cell lung cancer (NSCLC), which comprises about 85% of lung cancers, and small-cell lung cancer (SCLC). The main subtypes of NSCLCs are adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). Wnt signaling is frequently abnormally activated in lung cancers. Overexpression of Wnts1-3, Wnt5a, Wnt11, and Fz8 is common in NSCLC (Nakashima et al., 2012; Stewart, 2014; Huang et al., 2015; Rapp et al., 2016). Resected NSCLC samples with high levels of Wnt3 are characterized with a significantly higher Ki67 proliferation index and a significantly lower apoptotic index, resulting in a considerably lower survival rate in patients with high-Wnt tumors than in those with low-Wnt tumors (Nakashima et al., 2012). Wnt3a treatment has been found to decrease the expression of E-cadherin and increase that of N-cadherin and Vimentin, thereby promoting EMT and metastasis in NSCLC cells (Li et al., 2015). Intriguingly, overexpression of the cell surface heparan sulfate proteoglycan Glypican-5, which competitively binds to Wnt3a, inactivates Wnt/β-catenin pathway and consequently suppresses EMT and metastasis in lung ADC (Wang et al., 2016). Wnt7a and its receptor Fz9 are significantly downregulated in NSCLC compared to normal uninvolved lung tissue and, upon interaction; they likewise trigger a tumor suppressor pathway by inhibiting transformed cell growth and promoting epithelial differentiation through activation of JNK pathway but not the Wnt/ß-catenin pathway (Winn et al., 2005). On the other hand, upregulation of Ror 1, a member of the Ror family of RTKs, has been shown to promote lung carcinogenesis through activation of Wnt/PCP and Wnt/RTK signaling cascades (Katoh and Katoh, 2017).

Abnormalities in Wnt Ligands, Receptors and Pathway Modulators in Lung Cancer

In a recent analysis of correlation between expression of Wnt ligands and 23 immunosuppressive genes across all cancer types in the TCGA dataset, high levels of Wnt1 has been found to

significantly and negatively correlate with CD8 + T cells, showing that it induces immune resistance in lung adenocarcinoma cells and thus immunologically cold tumors (Kerdidani et al., 2019). A meta-analysis based on the data from 1805 NSCLC patients has reported a similar overexpression of Wnt1 and Wnt5a with an inverse correlation to the overall survival of these patients (Jin et al., 2016) (**Figure 3**).

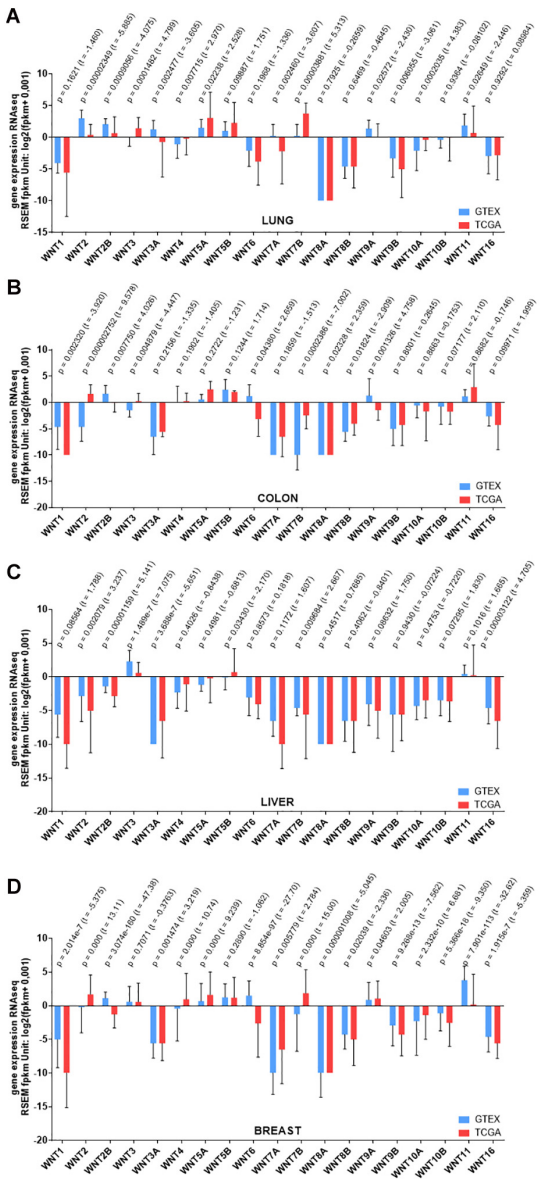

FIGURE 3 | RNAseq gene expression levels of Wnt ligands in different cancers. **(A)** Lung cancer **(B)** Colon cancer **(C)** Liver cancer **(D)** Breast cancer. The data were downloaded from https://xenabrowser.net/on August 10, 2020. For each cancer type, data were filtered from the browser extension data set on the TCGA TARGET GTEx dataset, which includes approximately 13 thousand genes. Studies were obtained from GTEx project and TCGA. Gene expressions were analyzed using RSEM fpkm method. Significance was calculated using Welch's *t*-test and graphs were generated using GraphPad Prism 7.

Another comparative study on NSCLC subtypes has reported that non-canonical Wnt5a was significantly upregulated in the SCC, while ADC was marked by a prominent expression of canonical Wnt7b (Vesel et al., 2017). In SCC, Wnt5a downregulates the ATP-binding cassette (ABC) transporter family members Abcb1 and Abcg2, which are involved in chemotherapy resistance and appear to be upregulated by canonical Wnt signaling when the cells are treated with the chemotherapeutic agent cisplatin (Vesel et al., 2017).

Aberrant pathway activation occurs as a result of mutations or polymorphisms in pathway genes, repression of pathway inhibitors or synergistic effect with other mutations such as Kras (Testa et al., 2018a). For example, Lrp6 rs10845498 polymorphism has been associated with a reduced risk of lung SCC while LRP6 rs6488507 polymorphism synergistically increased the risk of NSCLC in tobacco smokers (Deng et al., 2014). Loss of function mutations in the Wnt-feedback induced cell surface E3 ligases Rnf43 and Znrf3, which bind to Fz and target it for degradation and fusions of the Wnt agonists Rspo2 and Rspo3 have been reported in lung cancer (Katoh and Katoh, 2017). Tobacco smoking, as the main factor responsible for lung cancer, appears to activate Wnt signaling through polycomb-induced repression of the secreted Wnt antagonist Dkk1, resulting in a tumorigenic effect (Hussain et al., 2009). Moreover, concurrent activation of Wnt/β-catenin signaling and expression of the constitutively active Kras mutant *KrasG12D* in the bronchiolar epithelium of the adult mouse lung, significantly increased the tumor number and size (Pacheco-Pinedo et al., 2011).

Misregulation of Wnt signaling in lung cancers have also been associated in many reports with abnormal expression of miRNAs that regulate the membrane components of Wnt pathways. For example, smoking-induced repression of miR-487b -a tumor suppressor miRNA that normally inhibits Wnt5a, Myc, and Kras and upregulates the Wnt antagonists Dkk1, Sfrp1, Sfrp4, and Wif1 to regulate lung stem cells- results in increased proliferation, invasion and metastatic potential of lung cancer cells (Xi et al., 2013). Expression of miR-148a is likewise significantly downregulated in primary cancer tissues of NSCLC patients compared to their adjacent normal lung tissues, and negatively correlates with the expression of Wnt1, a direct target of miRNA-148a (Chen et al., 2017). In contrast, elevated levels of miR-650 in NSCLC has been associated with promotion of cell proliferation and invasion through activation of Wnt1-mediated β-catenin signaling (Tang et al., 2019). Exosomal transfer of miR-1260b has been associated with increased tumor cell invasiveness in lung ADC proliferation through activation of Wnt/β-catenin signaling in neighboring cells (Xia et al., 2020). Interestingly, miR-1260b can induce biosynthesis of ceramides, depletion of which leads to accumulation of Wnt and inhibits Wnt signaling (Pepperl et al., 2013).

Targeting Wnt Pathway at the Plasma Membrane for Lung Cancer Therapy

Several therapeutic approaches have been unraveled to suppress lung cancer progression via interfering with Wnt-receptor complex components at RNA or protein levels. For example,

aspirin-induced miR-98 expression and long non-coding RNA (lncRNA) MIR503HG have been found to suppress proliferation of lung ADC and NSCLC cells via targeting Wnt1 and thus serve as tumor suppressors (Gan et al., 2019; Lin et al., 2019). Similarly, miR-5587-3p has been reported to suppress Wnt5b, a prognostic biomarker that is highly expressed in lung ADC and positively correlates with metastasis and cancer progression (Zhang et al., 2020). lncRNA AK126698 suppresses Wnt pathway by targeting Fz8 in NSCLC cells, and prevents their proliferation and migration (Fu et al., 2016). miR-135b has been identified to directly target Fz1 at its 3'UTR in NSCLC cells and enhance the chemosensitivity of cisplatin-resistant lung cancer cell lines (Su et al., 2016). On the other hand, antimalarial compounds artemisinin, dihydroartemisinin and artesunate can specifically suppress Wnt pathway by decreasing the protein level of Wnt5a/b (Tong et al., 2016). Qiyusanlong (QYSL) decoction, a formula composed of ten different traditional Chinese medicine, likewise reduces the protein levels of Wnt1, Wnt 2, and Wnt 5a, alone or with Cisplatin (Tong et al., 2018).

Colorectal Cancer

Colorectal cancer (CRC) ranks second among cancer-related deaths worldwide and majority of CRCs arise sporadically in patients with no family history of disease (Brenner et al., 2014). Hyperactivation of the Wnt pathway due to mutational inactivation of the APC tumor suppressor is thought to be the initiating event and key oncogenic driver in most sporadic and familial CRCs (Schatoff et al., 2017). Furthermore, mutations in both the components and the modulators of Wnt-receptor complex are frequently associated with CRC (Kuipers et al., 2015; Katoh and Katoh, 2017; Testa et al., 2018b). The Cancer Genome Atlas (TCGA) consortium report reveals that Wnt signaling is altered in up to 93% of all sporadic CRCs with at least one and up to sixteen alteration(s) in Wnt pathway components including APC, CTNNB1, TCF7L2, DKK family members, AXIN2 and the pathway negative regulator FAM123B/WTX (Cancer Genome Atlas Network, 2012).

Wnt2 and its receptor Fz7 have been found to be expressed at high levels in CRC as compared to normal colonic mucosa (Holcombe et al., 2002; Kalhor et al., 2018). Wnt3, Wnt6 and Wnt11 are likewise upregulated in CRC in correlation with poor survival rate, and when downregulated proliferation and migration are suppressed and apoptosis is induced (Zheng and Yu, 2018; Gorrono-Etxebarria et al., 2019; Nie et al., 2019; Peng et al., 2019) (**Figure 3**). Polymorphic adenoma-like protein 2, a zinc finger transcription factor, is also overexpressed in CRC and can promote Wnt6 expression by binding to its promoter region (Li et al., 2019b). The tubulin acetyltransferase αTAT1 promotes CRC progression via regulating subcellular localization of β-catenin and inducing expression of Wnt1 (Oh et al., 2017). Interestingly, Wnt1 is downregulated in response to *Salmonella* infection in CRC and this inhibits cancer cell invasion and migration (Wang et al., 2018). High levels of Wnt5a and its receptor Ror2 have been associated with drug resistance in CRC by concomitant induction of non-canonical Wnt signaling and suppression of canonical Wnt signaling (Bordonaro et al., 2011). Apart from these Wnt ligands, various Fz receptors including Fz4, Fz7, and Fz10, are also upregulated in CRC and their elevated levels have been associated with increased stemness, metastasis and recurrence (Cancer Genome Atlas Network, 2012; Ye et al., 2019; Chi et al., 2020). Wnt co-receptor Lrp6 is likewise significantly upregulated in many tumoral tissues of CRC in correlation with high malignancy and poor prognosis (Rismani et al., 2017). Various Lrp6 polymorphisms such as T867A, N789S, W239L have also been associated with susceptibility to early-onset CRC (de Voer et al., 2016).

Several membrane proteins have been reported to regulate Wnt signaling activity in CRC. For example the low-density lipoprotein receptor-related protein 1B (Lrp1b), which is downregulated in CRC, can suppress the growth and migration of cancer cells via inhibiting the interaction between Dvl2 and the Axin and hence the Wnt/β-catenin signaling (Wang et al., 2017). Expression of the cystic fibrosis transmembrane conductance regulator (Cftr) gene is likewise reduced in CRC, and this reduction enhances Wnt/β-catenin signaling via promoting interaction of Dvl-2 with the plasma membrane (Strubberg et al., 2018). On the contrary, the elevated expression of the type I transmembrane protein CUB-domain containing protein 1 (CDCP1) in CRC has been associated with high metastasis via promoting nuclear localization of β-catenin and Wnt signaling activity (He et al., 2020).

The R-Spondin/Lgr5/Rnf43 Module in CRC

Wnt signaling is essential for normal intestinal function due to its roles in maintenance, proliferation and differentiation of intestinal stem cells. In particular, Lgr5 + intestinal stem cells exhibit high levels of canonical Wnt pathway activity reinforced by Rspo1-4 that drives a physical interaction between Lgr4/5 and Rnf43/Znrf3 (de Lau et al., 2014). Rspo fusions with protein tyrosine phosphatase receptor type K (PTPRK-RSPO3) and eukaryotic translation initiation factor subunit E (EIF3E-RSPO2) are frequently observed in colorectal traditional serrated adenomas and characterized by Rspo overexpression and activation of Wnt signaling (Sekine et al., 2017; Hashimoto et al., 2019). In contrast, Rspo2 appears to inhibit CRC metastasis by competing with the tumor-promoting Wnt5a for binding to Fz7 and thus antagonizing Wnt5a-driven non-canonical Wnt signaling (Dong et al., 2017). Although the majority of the studies conducted in primary clinical tissue have suggested a higher expression of the Rspo receptor Lgr5 in CRC cells relative to the adjacent normal tissue, a tumor-promoting role and enhanced chemoresistance, several studies have reported a potential tumor suppressive function of Lgr5 in CRC progression (Hsu et al., 2013; Morgan et al., 2018). *N*-terminal mutations of Rnf43, one of the most commonly mutated genes in CRC, have also been linked with enhanced Wnt/β-catenin signaling activity in colon cancer while *C*-terminal truncation mutants act similarly to the wild-type Rnf43 (Giannakis et al., 2014; Li et al., 2020).

Targeting Wnt Pathway at the Plasma Membrane for Colon Cancer Therapy

Due to their potential function as tumor suppressors mentioned above, Lrp1b and Ctfr might offer promising strategies for the treatment of colon cancer. LiCl treatment also inhibits CRC cell

proliferation via concomitant induction of the non-canonical ligand Wnt9 and suppression of β-catenin expression (Ali et al., 2016). Combination of inositol hexaphosphate and inositol can also reduce Wnt/β-catenin signaling via downregulating Wnt10b and β-catenin and suppress liver metastasis of CRC (Liu et al., 2020). Different microRNAs have been shown to affect CRC tumorigenesis as well. While miR-140-5p and miR-185 target Wnt1 and act as tumor suppressors, miR-410 targets Dkk1 and thus functions as an oncogene in CRC (Zhang et al., 2018; Wang et al., 2019b; Yeon et al., 2019). These findings reveal that miRNAs can be used as prognostic markers and to produce potential therapeutic agents for CRC patients.

Liver Cancer

Liver cancer is the fourth common cancer-related death in the world. Hepatocellular carcinoma (HCC), also referred to as hepatoma, is the most common type of liver cancer, constituting approximately 90% of all liver cancers. The molecular events that take place during multi-step initiation and progression of HCC are only partially understood. HCCs are broadly classified into the "proliferation class" and the "non-proliferation class" (Caruso et al., 2019). The proliferation class is further subdivided into the "Wnt-TGFβ subclass" with activated Wnt and TGFβ pathways and the "progenitor subclass" characterized by several features including overexpression of hepatic progenitor markers and mutations in AXIN1 (Rebouissou and Nault, 2020). On the other hand, the "non-proliferation class" of HCC includes tumors that are more heterogeneous, less aggressive, more differentiated with hepatocyte-like features, and contains at least two subclasses (Rebouissou and Nault, 2020). The most well described subclass is characterized by mutations in the β-catenin gene CTNNB1, leading to highly activated Wnt/β-catenin pathway, along with the TERT promoter and TP53 mutations (Yang et al., 2019a; Rebouissou and Nault, 2020). Thus, Wnt/β-catenin signaling is aberrantly activated in approximately 50% of HCC cases, in association with increased proliferation and inflammation, malignant tumor progression, poor prognosis, immune escape, and resistance to therapy (Yang et al., 2013; Khalaf et al., 2018; Jiang et al., 2019b; Ruiz de Galarreta et al., 2019).

Wnt-receptor complex components have been largely identified to take part in hepatocarcinogenesis. For example, several Fz receptors and the canonical Wnt ligands Wnt3 and, to a lesser extent, Wnt10b are strongly upregulated in a variety of HCC cells with different expression levels of hepatocyte lineage, epithelial and mesenchymal markers (Kim et al., 2008; Yuzugullu et al., 2009). Wnt2b, Wnt4, Wnt5a, Wnt5b, Wnt7b, Wnt8b, and Wnt9b are among the other ligands that have significantly increased expression in HCC cell lines (Yuzugullu et al., 2009) (**Figure 3**). Wnt1 is also highly expressed in HCC cell lines and has been associated with increased tumor recurrence after curative tumor resection in HBV- and HCV-related HCC patients (Lee et al., 2009; Wei et al., 2009). However, Wnt5a and Ror2 have been reported to be downregulated in HCC tissues with a poorer prognosis than HCC patients with elevated Wnt5a and Ror2 expression (Geng et al., 2012). In another study, Wnt5a overexpression was also found to decrease cell proliferation and tumor size in HCC, supporting that Wnt5a may serve as a tumor suppressor in HCC (Wang et al., 2019a). Another non-canonical ligand Wnt11a likewise decreases in HCC and its ectopic expression could suppress cell motility and migration via activation of RhoA/Rho kinase (Toyama et al., 2010). In addition to the Wnt ligands, high levels of Fz2, Fz7, Lrp5, and Lrp6 in HCC have also been found to be promote cell proliferation, migration, invasion, and EMT (Merle et al., 2004; Yuzugullu et al., 2009; Tung et al., 2012; Ou et al., 2019).

Targeting Wnt Pathway at the Plasma Membrane for Liver Cancer Therapy

In search of novel potential therapeutic targets for HCC, the tumor-promoting function of Fz7 could be effectively reverted by small interfering RNAs that suppressed proliferation and metastasis of HCC cells and enhanced their apoptosis and sensitivity to chemotherapeutic drugs (Chen et al., 2016, 2018; Xue et al., 2018). Similarly, miR-542-3p, a common tumor-suppresser that is also downregulated in HCC tissues and cell lines, has been shown to inhibit HCC cell growth by targeting Fz7 and may thus represent a novel therapeutic target for HCC (Wu et al., 2017). An anti-Wnt1-antibody has been found to inhibit Wnt/β-catenin signaling and tumor growth in a xenograft mouse model (Wei et al., 2009). In contrast, anti-Wnt1 suppressed proliferation and apoptosis, but did not affect tumor size and growth in diethylnitrosamine-induced hepatocellular adenomas (Sklavos et al., 2018). Interestingly, garlic-derived compound S-allylmercaptocysteine reduced HCC tumorigenesis by directly targeting Lrp6 at the plasma membrane (Xiao et al., 2018).

Breast Cancer

Breast cancer is the leading cause of cancer death among females worldwide (Bray et al., 2018). Breast cancer is classified into three main subtypes based on the expression of estrogen receptor (ER) or progesterone receptor (PR) and amplification of the human epidermal growth factor 2 (ERBB2, commonly referred to as HER2): hormone receptor positive/ERBB2 negative (HR+/ERBB2-), ERBB2 positive (ERBB2+; HR+ or HR-) and triple-negative (lacking all three molecular markers) (Waks and Winer, 2019). Wnt signaling activity has been reported to increase in both cell lines and patient-derived metastatic cells of breast cancer and positively correlate with the ER expression (Lamb et al., 2013). Wnt/β-catenin pathway activation, characterized by reduction of membranous β-catenin with its concomitant nuclear accumulation, appears to correlate with poor clinical outcome in triple-negative breast cancer (TNBC) and basal-like breast cancer (BLBC), an aggressive subtype of breast cancer characterized by strong expression of basal markers such as cytokeratins (Khramtsov et al., 2010; Geyer et al., 2011; Xu et al., 2015).

The role of plasma membrane in breast cancer has been unraveled in a study that characterized the role of cell adhesion protein CD44, which appears to localize preferably to the lipid rafts due to palmitoylation. Raft affiliation of CD44 was observed to be higher in non-invasive breast cell lines, while decreasing in highly invasive cell lines or in case of mutagenesis of palmitoylation sites, suggesting that lipid raft association is a key regulatory mechanism in cancer cell migration (Babina

et al., 2014). Interestingly γ-Tocotrienol, a natural isoform of vitamin E, appears to disrupt the lipid raft integrity, suppress Wnt/β-catenin signaling pathway and reduce cell motility in breast cancer (Ahmed et al., 2016). Thus, it will be very interesting to test how lipidation of Wnt and its receptors or raft association of Wnt-receptor complex influence migration and metastasis of these cells.

Misregulation of Wnt-Receptor Complex Components in Breast Cancer

In a network correlation analysis of expression of >100 Wnt pathway components in healthy and cancerous breast tissues, the strong coherence in expression levels of the Wnt ligands and Fz receptors observed in the healthy breast tissue is dramatically lost in TNBC tissue and also varies widely in TNBC and non-TNBC (Koval and Katanaev, 2018) (**Figure 3**). Wnt4 and Wnt16 have been reported to be significantly upregulated in TNBC recurrence (Tsai et al., 2015). Wnt3a, 5a, 5b, 9a, and 11 are preferentially overexpressed due to gene amplifications in BLBC (Shi et al., 2014; Jiang et al., 2019a). Among them, Wnt5b has been identified as a key regulatory factor that governs the BLBC phenotype by activating both canonical and non-canonical Wnt signaling (Jiang et al., 2019a). Interestingly, Wnt5a expression was shown to decrease at both mRNA and protein levels in TNBC in association with poor prognosis and Wnt5a signaling was able to suppress tumor growth and metastasis (Borcherding et al., 2015; Zhong et al., 2016). Wnt3a and Wnt7a are likewise upregulated in metastatic breast cancer cell lines in association with poor prognosis and inhibition of Wnt/β-catenin signaling via Wnt1 knockdown could efficiently suppress cell proliferation and tumor growth (Jang et al., 2015; Avgustinova et al., 2016). The receptors Fz2, Fz3, Fz6, Fz7, and Fz10 as well as the co-receptor Lrp6 have also been reported to significantly increase in breast cancer and contribute to mesenchymal-like stemness, invasion, metastasis, and drug resistance (Liu et al., 2010; Yang et al., 2011; Gong et al., 2014; Simmons et al., 2014; Bell et al., 2017; Corda et al., 2017; Yin et al., 2020). Loss-of-function mutations of the tumor-suppressor-like molecules Rnf43 and Znrf3 and elevated expression levels of the Wnt agonists Rspo2 and Rspo4 can also be counted among the prognostic biomarkers of breast cancer (Ciriello et al., 2015; Coussy et al., 2017; Katoh and Katoh, 2017).

Targeting Wnt Pathway at the Plasma Membrane for Breast Cancer Therapy

A number of studies have assessed the therapeutic potential of targeting Wnt signaling at the plasma membrane in TNBC. Being an important biomarker of TNBC, Fz7 is one of the well-investigated therapeutic options for breast cancer. A recombinant soluble peptide fragment (rhFzd7) has been shown to antagonize Fzd7 by competitively binding with Wnt3a and exhibit anti-tumor and anti-angiogenesis activities in TNBC (Xie et al., 2018). 2-cyano-3, 12-dioxooleana-1, 9 (11)-dien-28-oic acid-methyl ester (CDDO-Me) likewise targets Fz7 and Lrp6 and significantly inhibited tumor growth in breast cancer (Zhou et al., 2020). miR-142-3p, which is significantly down-regulated in breast cancer tissues, can also suppress Fz7 and thus serve as a tumor suppressor in breast cancer (Jia et al., 2018). A recent *in silico* study have identified several candidate molecules similar to palmitoleic acid that could potentially bind to the Fz7 transmembrane protein and inactivate the Wnt signaling pathway in TNBC cells (Alves Pinto and Freitas Da Silveira, 2020). In addition to Fz7, targeting Wnt ligands, Dkk1 or Lrp6 at the membrane may offer promising treatment options against breast cancer. For example, the antihelminthic niclosamide could sensitize TNBC cells to ionizing radiation (IR) by suppressing Wnt3a/β-catenin mediated radioresistance (Yin et al., 2016). A monoclonal anti-Wnt-1 antibody or Wnt-1 siRNA inhibit could induce apoptosis in a variety of human cancer cell lines including breast cancer (He et al., 2004). The polycomb protein chromobox homolog 7a (CBX7) likewise appears to inhibit breast tumorigenicity by enhancing the expression of the Wnt antagonist Dkk1 (Kim et al., 2015). The polyether ionophore antibiotic salinomycin, the milk thistle flavonolignan silibinin, the natural phenol echinacoside and the natural plant polyphenol rottlerin have all been shown to inhibit Wnt/β-catenin signaling by suppressing the Wnt co-receptor Lrp6 expression and exert anti-tumor effects in TNBC (Lu et al., 2012, 2014; Lu and Li, 2014; Tang et al., 2020).

Other Cancers

It is noteworthy to mention that aberrant activation of Wnt signaling pathways is obviously not limited to the four cancer types mentioned below. Various Wnt ligands, the agonist Norrin, Fz receptors and the co-receptors Lrp6 and Ror1/2 have been reported to be abnormally expressed and associated with metastatic behavior, cancer progression and chemoresistance in ovarian cancer, glioblastoma multiforme, chronic lymphocytic leukemia, melanoma, multiple myeloma, post-transplant smooth muscle tumor, prostate cancer, pancreatic cancer gastric cancer, oral squamous cell carcinoma, Ewing sarcoma, osteosarcoma, and malignant peripheral nerve sheath tumors (Derksen et al., 2004; Larue and Delmas, 2006; Dissanayake et al., 2007; Yan et al., 2016; Yu et al., 2016; Li et al., 2017, 2019a; Liu et al., 2017; Pridgeon et al., 2017; Sandsmark et al., 2017; Jiang et al., 2018; Sinnberg et al., 2018; Teiken et al., 2018; Yang et al., 2019b; Chehover et al., 2020; El-Sehemy et al., 2020; Frenquelli et al., 2020; Kotrbova et al., 2020). Thus, a thorough understanding of misregulation of Wnt signaling pathways at the plasma membrane will pave the way for new therapeutic approaches for cancer.

Inhibition of Wnt Pathway for Cancer Therapy

Biological inhibitors and small molecules related with the Wnt pathways are widely exploited in therapeutic approaches to human diseases, including cancer, that have increased Wnt signaling activity. With its pivotal role in initiation and tight control of Wnt signaling activity, components and modulators of the Wnt-receptor complex constitute promising drug targets (Katoh and Katoh, 2017; Taciak et al., 2018; Goldsberry et al., 2019) (**Figure 4**). For example, Ipafricept (OMP-54F28), a

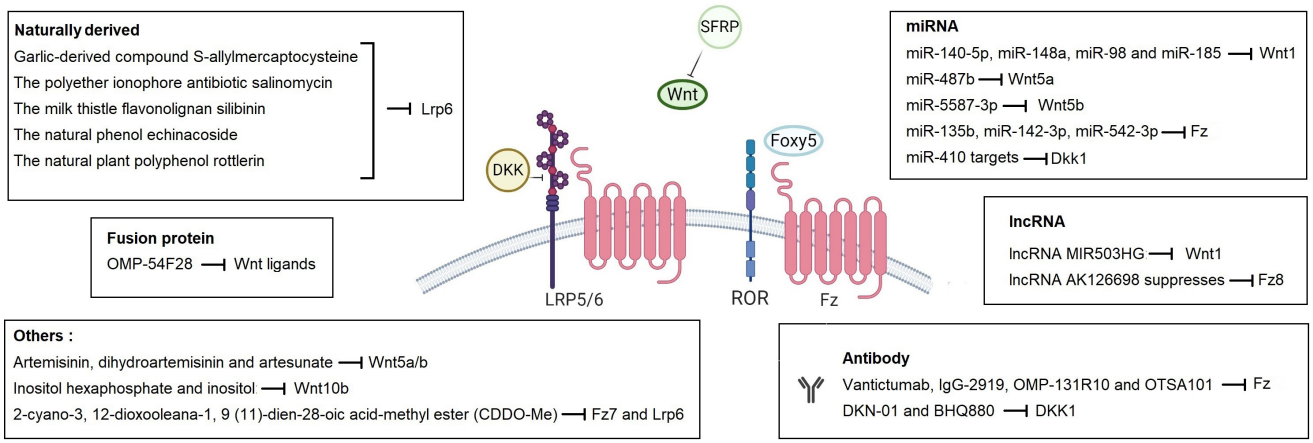

FIGURE 4 | Therapeutic molecules that target Wnt signaling pathway at the level of the plasma membrane. Different types of molecules are given with their target pathway component in boxes. Created with BioRender.com.

recombinant fusion protein comprised of the Fz8 cysteine-rich domain and human IgG1 Fc fragment, acts as decoy receptor for Wnt ligands and exhibits antitumor activity (Le et al., 2015; Jimeno et al., 2017). OTSA 101-DTPA90Y, Vantictumab (OMP-18R5) and IgG-2919 are monoclonal antibodies targeting different Fz receptors and decrease tumor growth in different cancers (Gurney et al., 2012; Nielsen et al., 2015; Steinhart et al., 2017). The monoclonal antibodies DKN-01 and BHQ880 target DKK1 and likewise exert anti-tumorigenic activity in relapsed or refractory cancers including NSCLC, multiple myeloma and gastrointestinal cancers (Fulciniti et al., 2009; Edenfield et al., 2014; Iyer et al., 2014; Bendell et al., 2016). Rosmantuzumab (OMP-131R10) targets Rspo3 and evokes favorable responses against solid tumors and CRC (Diamond et al., 2016). The small molecule inhibitor KAN0439834, antibodies Cirmtuzumab (UC-961), ROR1-CD3-DART, APVO425, and ROR1R-CAR-T cells target Ror-1 with promising effects on different types of cancers (Berger et al., 2015; Yu et al., 2016; Katoh and Katoh, 2017). LGK974, ETC-159 (ETC1922159), RXC004, CGX1321, GNF-6231, XNM7201, IWP-2, WNT974, and WNT-C59 are all Porcupine inhibitors that are in the preclinical or phase I/II stage (Katoh and Katoh, 2017; Taciak et al., 2018; Goldsberry et al., 2019). When combined with pan-PI3K inhibitor GDC-0941, porcupine inhibitor ETC-159 has been shown to potently suppress *in vivo* tumor growth in pancreatic cancer (Zhong et al., 2019). Finally, Foxy-5, a small peptide that mimics Wnt5a, is considered to disrupt the migration and invasion of epithelial cancer cells and exhibit anti-metastatic impact in metastatic breast, colorectal and prostate cancers (Canesin et al., 2017).

Clinical trials with several of these drugs have reported various adverse effects. For example, while being well tolerated by the patients with solid tumors, Ipafricept caused at least one of the treatment-emergent adverse events including dysgeusia, decreased appetite, fatigue, muscle spasms, and nausea, each of which were observed in at least 20% of patients (Jimeno et al., 2017). Different dose combinations of Vantictumab have been tested for 23 patients with advanced solid tumors, and likewise caused fatigue, nausea, vomiting, abdominal pain, constipation, and diarrhea as most common related adverse effects (Smith et al., 2013). First-in-human study of OTSA-101 on metastatic synovial sarcomna patients have reported lymphopenia, anemia, leucopenia, asthenia, hemoptysis, thrombocytopenia, neutropenia, and anorexia in some or all patients depending on the applied doses (Giraudet et al., 2018). The anti-DKK1 antibody DKN-01 also appears to cause adverse effects including cough, peripheral neuropathy, alopecia, leukopenia, neutropenia and fatigue in patients of refractory esophageal cancer or gastro-esophageal junction tumors (Bendell et al., 2016). Thus, further detailed investigations are absolutely necessary to assess the potential of Wnt inhibitors in therapeutic interventions.

CONCLUSION

The plasma membrane composition and organization play an important role in regulation of Wnt signaling by controlling ligand-receptor interaction and signal initiation. Since plasma membrane is highly dysregulated in cancer, it is essential to consider the unique organization of the Wnt-receptor complex for specific and effective targeting of the cancer cell. Understanding complex molecular interactions underlying Wnt-mediated cellular events at the plasma membrane has the potential to reveal attractive drug targets in cancers, and potentially other diseases, where Wnt signaling is extensively involved.

AUTHOR CONTRIBUTIONS

GO, YA, and MK wrote the manuscript and prepared the figures. EE contributed to the discussion. All authors contributed to the article and approved the submitted version.

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Wnt Signaling Is Deranged in Asthmatic Bronchial Epithelium and Fibroblasts

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Both canonical and non-canonical Wnt signaling pathway alterations have been documented in pulmonary disease pathogenesis and progression; therefore, they can be an attractive target for pharmaceutical management of severe asthma. Wnt/β-catenin signaling was shown to link early embryonic lung development impairment to later in life asthmatic airway remodeling. Here we explored the changes in Wnt signaling associated with asthma initiation and progression in epithelial and fibroblasts using a comprehensive approach based on *in silico* analysis and followed by *in vitro* validation. In summary, the *in silico* analysis showed that the bronchial epithelium of severe asthmatic patients showed a deranged balance between Wnt enhancer and Wnt inhibitors. A Th2-high phenotype is associated with upregulated Wnt-negative regulators, while inflammatory and neutrophilic severe asthmatics showed higher canonical Wnt signaling member enrichment. Most of these genes are regulators of healthy lung development early in life and, if disturbed, can make people susceptible to developing asthma early in life and prone to developing a severe phenotype. Most of the Wnt members are secreted, and their effect can be in an autocrine fashion on the bronchial epithelium, paracrine on nearby adjacent structural cells like fibroblasts and smooth muscles, or systemic in blood. Our results showed that canonical Wnt signaling is needed for the proper response of cells to proliferative stimuli, which puts cells under stress. Cells in response to this proliferative stress will activate the senescence mechanism, which is also dependent on Wnt signaling. Inhibition of Wnt signaling using FH535 inhibits both proliferation and senescence markers in bronchial fibroblasts compared to DMSO-treated cells. In fibroblasts from asthmatic patients, inhibition of Wnt signaling did not show that effect as the Wnt signaling is deranged besides other pathways that might be non-functional.

Keywords: asthma, Wnt/b-catenin, remodeling, *in silco* analysis, transcriptome

INTRODUCTION

The hybrid name "WNT" (for Wingless-related integration site) stands for a group of genes belonging to the INT1 (WNT1)/wingless family (Pai et al., 2017). In the animal kingdom, Wnt signaling is one of the most important regulators of development and stem cell maintenance in adult mammals (Nusse and Clevers, 2017). Wnt receptors and co-receptors are abundant in adult lung, which upon interaction with secreted Wnts can activate signaling pathways that regulate transcriptional and non-transcriptional responses (Skronska-Wasek et al., 2018). In the human lung, Wnt signaling pathways maintain lung homeostasis, and any disturbance of such pathway can cause debilitating lung diseases (Rapp et al., 2017), like fibrosis (Burgy and Konigshoff, 2018), and asthmatic airway remodeling (Hussain et al., 2017).

Both canonical and non-canonical Wnt signaling pathway alterations have been documented in pulmonary disease pathogenesis and progression; therefore, they can be an attractive target for pharmaceutical management of severe asthma (Baarsma and Konigshoff, 2017). Wnt signaling disturbance can induce antagonistic pleiotropy or developmental drift and lung aging through senescence or stem cell exhaustion (Lehmann et al., 2016). Wnt/β-catenin signaling was shown to link early embryonic lung development impairment to later in life asthmatic airway remodeling (Hussain et al., 2017).

Progenitor cells that give rise to lung epithelium use CTNNB1 to promote lung progenitor gene signature and employ Fgf (fibroblast growth factor)/Kras (Kirsten rat sarcoma viral oncogene homolog)-mediated promotion of the progenitors (Ostrin et al., 2018). It is logical then to expect that the effect on Wnt signaling early in life can lead to lung diseases like asthma and COPD (Carlier et al., 2019). The effects of maternal smoking during pregnancy on Wnt pathway gene expression and SNPs in Wnt signaling members were linked to the development of mild to moderate persistent asthma in children (Koopmans and Gosens, 2018).

Here we explored the changes in Wnt signaling associated with asthma initiation and progression in epithelial and fibroblasts using a comprehensive approach based on *in silico* analysis followed by *in vitro* validation.

MATERIALS AND METHODS

Identifying Core Differentially Expressed Genes in Asthmatic Structural Cells

To decrease the effect of technical confounding factors on the gene expression, in-house preprocessing QC, normalization, and filtering of raw CEL files extracted from well-characterized publicly available bronchial epithelium transcriptomic datasets were performed as previously described (Hachim et al., 2019, 2020a,b). In brief, the publicly available transcriptomic dataset from GEO was filtered to search for a dataset that includes asthmatic patients with defined clinical classifications of participants compared to healthy controls with different airways sampling from the same subject (central versus peripheral). The dataset GSE64913 was chosen as it fulfills the criteria. Differences between central and peripheral airways were evaluated using transcriptomic analysis (Affymetrix HG U133 plus 2.0 GeneChips) of epithelial brushings obtained from severe asthma patients (N = 17) and healthy volunteers (N = 23) as previously described (Singhania et al., 2017).

Filtering

A combination of noise and variance filtering was applied to filter out non-variant genes between severe asthmatics and healthy controls. Only probes with a value of 50 or higher in the MAS5-normalized gene expression in all 59 samples were selected. The probes that passed the first filter then are subjected to the coefficient of variation (CV) filter using their gcRMA-normalized expression. CV was calculated as the mean/standard deviation of each gene across all samples. Probes with a CV value of at least 10% across the two groups examined were considered to be variant and thus selected. Since many genes are different between males and females and should be identified, only genes that do not show a significant variance between males and female samples were passed.

Gene-Set Enrichment Analysis

After processing, normalization, and filtering of the CEL files for each dataset, the normalized probe expressions were uploaded to GSEA software using the AbsGSEA option first. Over 100,000 gene sets were downloaded from Molecular Signatures Database v7.1 of the GSEA tool[1] and gene-set libraries downloaded from the Enrichr site[2]. Gene sets where the identified genes showed significant enrichment with nominal p-value < 0.05 were selected for further analysis with classical GSEA. A signal-to-noise metric was used to generate a rank-ordered gene list based on the enrichment score of each gene in the dataset. The enriched genes were filtered based on a cutoff score ≥ 0.25 as upregulated genes in asthma; those ≤ 0.25 as downregulated genes in asthma. For each gene-set collection or library, the genes that were upregulated or downregulated in asthmatic patients compared to healthy controls in each of the significant pathways were identified and grouped. Subsequently, DEG in each dataset was intersected with the DEG from the other sets, and shared genes were identified. Consequently, genes that were enriched in more than the median value of the number of enriched pathways for each gene were selected to be the DEGs in severe asthmatic bronchial epithelium compared to healthy control. The flowchart of filtering is shown in **Figure 1**.

Primary Healthy and Asthmatic Bronchial Fibroblasts

Healthy fibroblasts and fibroblasts from asthmatic patients were grown in triplicates till they reached confluency. The description of primary cells and their preparation were previously described (Hachim et al., 2020b). In brief, primary cells from healthy and asthmatic patients were isolated from bronchial biopsies

[1]https://www.gsea-msigdb.org/gsea/msigdb/index.jsp

[2]https://amp.pharm.mssm.edu/Enrichr/#stats

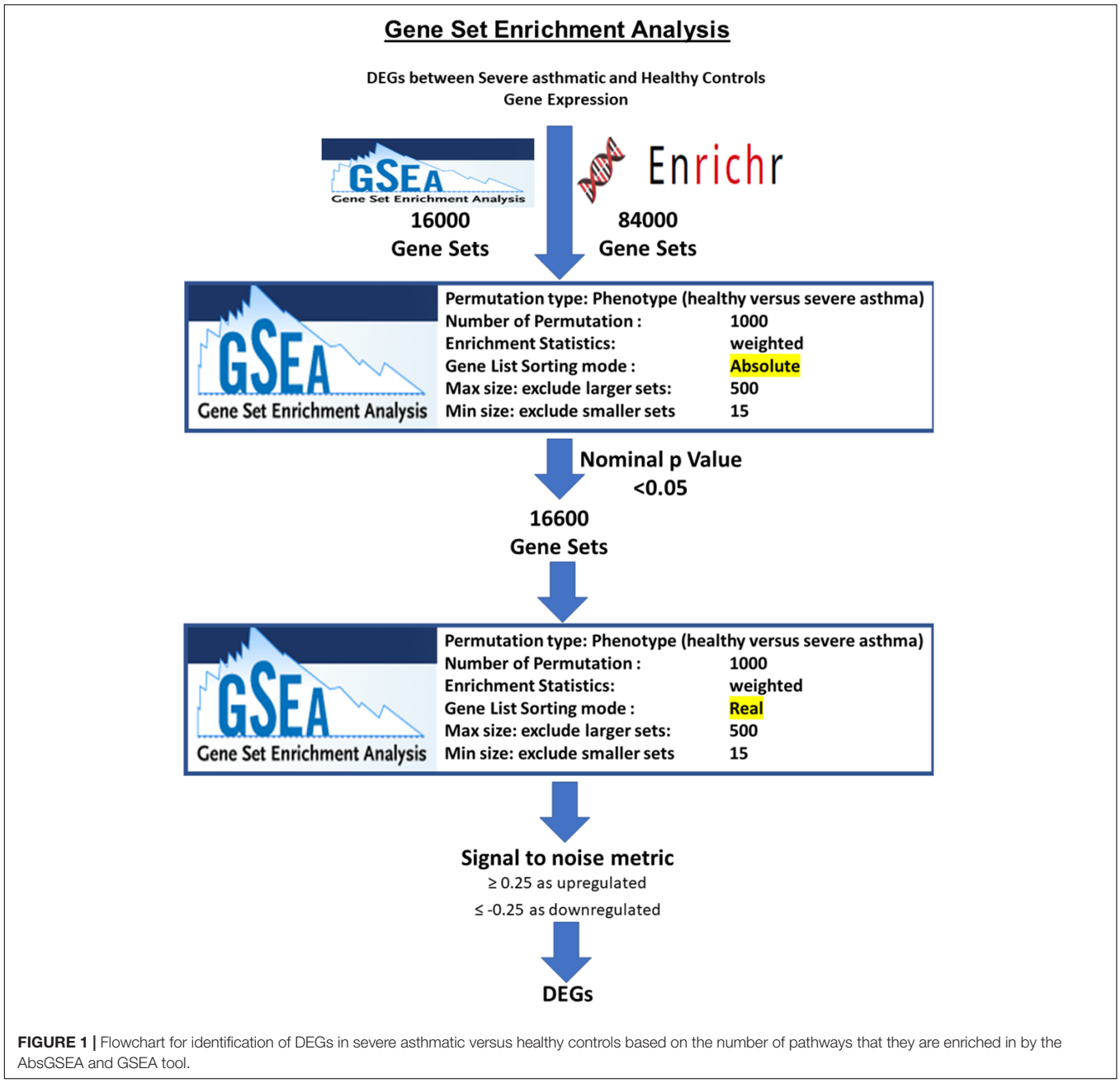


FIGURE 1 | Flowchart for identification of DEGs in severe asthmatic versus healthy controls based on the number of pathways that they are enriched in by the AbsGSEA and GSEA tool.

in Meakins-Christie Laboratories, The Centre for Respiratory Research at McGill University, and the Research Institute of McGill University Health Centre as previously described (35). In total, healthy primary fibroblasts (n = 3), and fibroblasts from asthmatic patients (n = 3). Primary fibroblasts were maintained in complete Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Germany) with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) supplemented with 100 units/mL penicillin/streptomycin (Gibco, United States). The original study was approved by the MUHC Research Ethics Board (2003–1879), and the subjects had provided written informed consent as previously described (Ramakrishnan et al., 2020).

WNT Signaling Pathway Profiling

RNA was extracted, and cDNA synthesis was performed as previously described (Hachim et al., 2020b). In brief, RNA was extracted using RNeasy mini kit (Qiagen, Germany) as per the manufacturer's instructions. The purified RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription (Applied Biosystems, United States) as per the manufacturer's instructions. The RT^2 Profiler^{TM} PCR Array Human WNT Signaling Pathway plate was used to profile the 84 Wnt signaling-related genes (Qiagen, United States) as previously described (Geng et al., 2016) and as per the manufacturer's instructions.

Calcium Mobilization

Calcium mobilization in healthy fibroblasts and fibroblasts from asthmatic patients' cells was done as previously described (Elemam et al., 2019). In brief, cells were washed two times then incubated in Ca2+ buffer containing 5 mM KCl, 145 mM NaCl, 1 mM MgCl2, 10 mM glucose, 10 mM Na/MOPS, 1 mM CaCl2, 10 mM HEPES, 0.25% BSA with a pH equal to 7.4, and 5 μM fura-2-AM (Sigma-Aldrich, St. Louis, MO, United States) for 45 min at 37°C. Consequently, the cells were washed and resuspended at a concentration of 3 × 105 cells/mL to be incubated with the 24 h healthy fibroblasts and fibroblasts from asthmatic patients or epithelium-collected supernatants. Fluorescence is measured using the fluorescence spectrometer system (LS55, Perkin-Elmer, Waltham, MA, United States), where excitation was measured at 340 and 380 nm, and the emission was determined at 510 nm. In these assays, the intensity was assessed using a photomultiplier tube system, and the fluorescence ratio of bound/free fura-2 was then calculated.

Immunofluorescence of β-Catenin

Five thousand healthy fibroblasts were seeded in a black 96-well plate along with the different treatments. After 24 h, cells were fixed using 4% paraformaldehyde, permeabilized using saponin, and then stained with rabbit anti-human CTNNB1 antibody (Abcam, United Kingdom) overnight at 4°C. On the next day, cells were incubated with Alexa-Fluor 488-anti rabbit secondary antibody (Thermo Fisher Scientific, United States) and visualized using IX53 inverted immunofluorescent microscope (Olympus, Japan).

Wnt Signaling Activation or Inhibition

Cells were seeded until they reached 80–90% confluency. Then, cells were washed with PBS and serum-starved for 1–2 h. Subsequently, cells were treated with 100 ng/ml Wnt agonist (CAS 853220-52-7, Santa Cruz, United States), a WNT agonist that mimics the effects of WNT ligand or 1 μM Wnt antagonist "FH535" (CAS 108409-83-2, Santa Cruz, United States), a β-catenin/Tcf inhibitor.

Western Blot Analysis

Fibroblast cells (healthy or asthmatic) were collected and washed with PBS, after which the proteins were extracted using the laemmli or RIPA lysis buffer (Sigma-Aldrich, Germany). All protein extracts were quantified using Bradford Protein Assay Kit, according to the manufacturer's instructions (Bio-Rad, United States). 10 μg (for fibroblasts) of protein was separated on SDS-PAGE and transferred to a nitrocellulose membrane. Expressions of β-catenin and β-actin were assessed using rabbit anti-human CTNNB1 (Cell Signaling, United States) and mouse anti-human β-actin (A5441, Sigma, Germany), respectively. Anti-rabbit and anti-mouse IgG HRP-linked

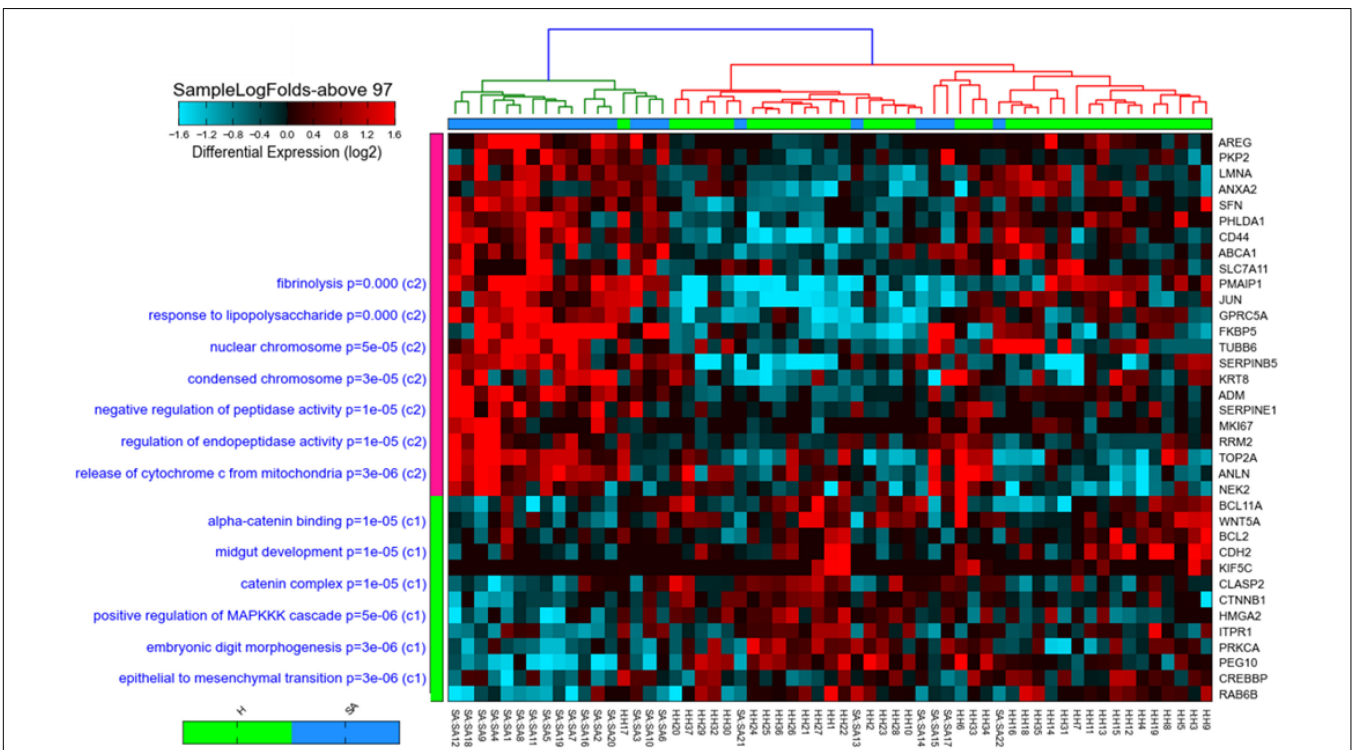


FIGURE 2 | Heat map and cosine hierarchical clustering using the expression of the 35-gene signature of samples in the GSE64913 dataset comparing healthy controls (H) to severe asthmatics (SAepithelial brushings obtained from severe asthma patients (*N* = 17) and healthy volunteers (*N* = 23). The 35 genes that show a statistically significant differential expression in severe asthmatics compared to healthy are shown on the right panel, while pathways where those genes are enriched significantly are shown on the left panel of the plot. Samples were clustered using Cosine hierarchical clustering. The graph was generated using AltAnalyze software.

antibodies (Cell Signaling, United States) were used along with Clarity Western ECL Substrate (Bio-Rad, United States) for chemiluminescent detection of protein bands. Western blot analysis of cell fractions from asthmatic bronchial fibroblast using Cell Fractionation Antibody Sampler Kit #11843 showing cytoplasmic (C.F), organellular/membrane (M.F), and nuclear/cytoskeletal localization (N.F.). Whole-cell lysates (WCL) represent total protein. The fractionation was done under two conditions, the cultivation with high glucose medium and low glucose medium.

RESULTS

Thirty-Five Core Genes in Severe Asthmatic Bronchial Epithelium

Two hundred and thirty-four genes were identified to be differentially expressed between severe asthmatic and healthy bronchial epithelia. Those 234 identified genes were further filtered to shorten the list into those that participate in more than 97 gene sets (above the median number of enrichments by the identified genes), indicating their essential role in development, progression, and response to therapy in asthmatic bronchial epithelium. Thirty-five genes fulfilled these criteria, as shown in **Figure 2**. Interestingly, the 35 genes showed specific enrichment in apoptotic signaling, TP53 downstream, and response to wounding, as shown in **Figure 2**. All the 35 genes identified earlier to be the top DEG in the bronchial epithelium were DEG between asthmatic and healthy bronchial fibroblasts as well. This highlights that those 35 genes represent DEGs in asthmatic airways irrespective of cell type.

Bronchial Epithelium Transcriptomic Signature Showed Strong Interaction at the Protein–Protein Level, and Most of Them Are Downstream of CTNNB1

To assess the protein–protein interaction between the products of the identified genes, the Protein–Protein Association Networks tool, STRING[3], was used. All genes showed a strong interaction at the protein level, and most of them were downstream of CTNNB1, as shown in **Figure 3**.

[3]https://string-db.org/

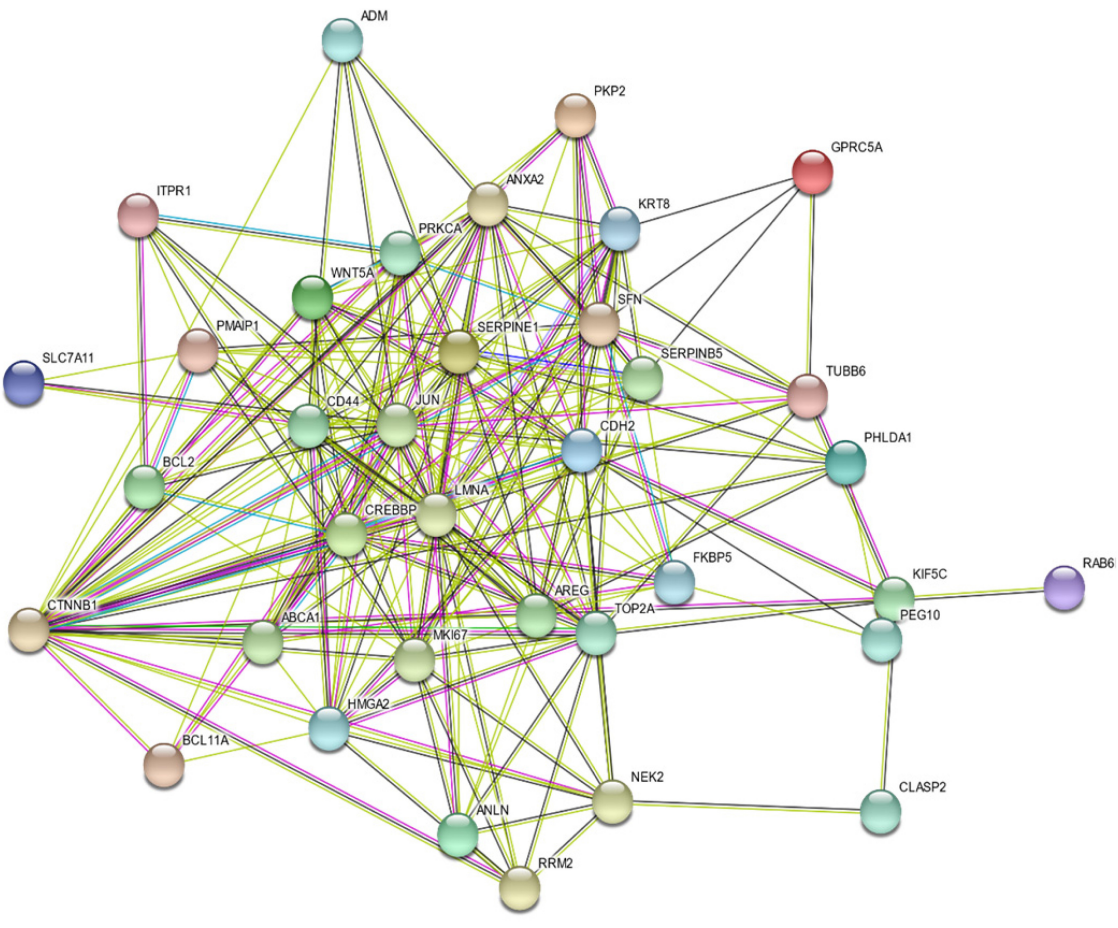


FIGURE 3 | Protein–Protein Interaction Between the Products of the Identified 35 Genes Using STRING v11: Protein–Protein Association Networks Tool.

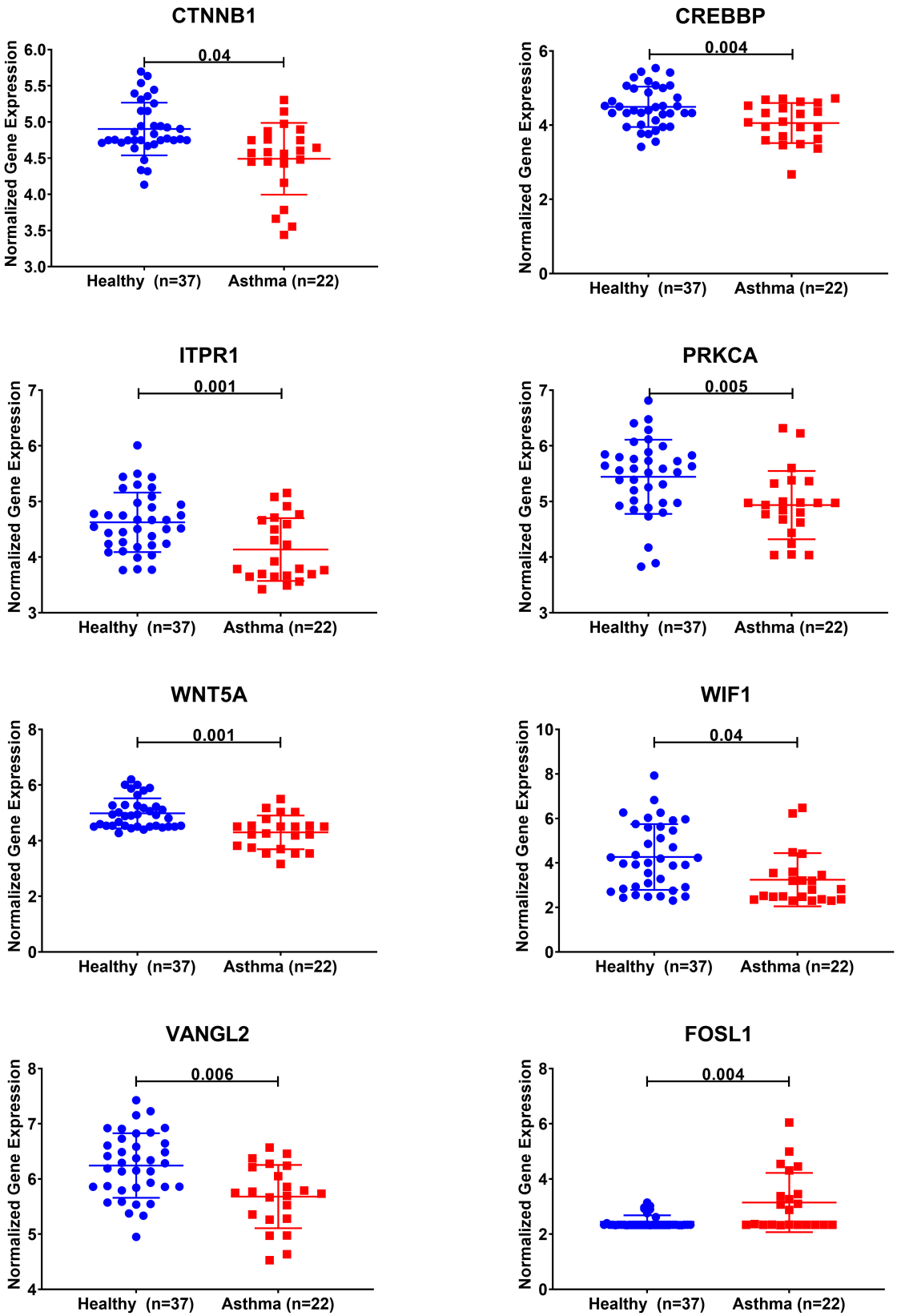
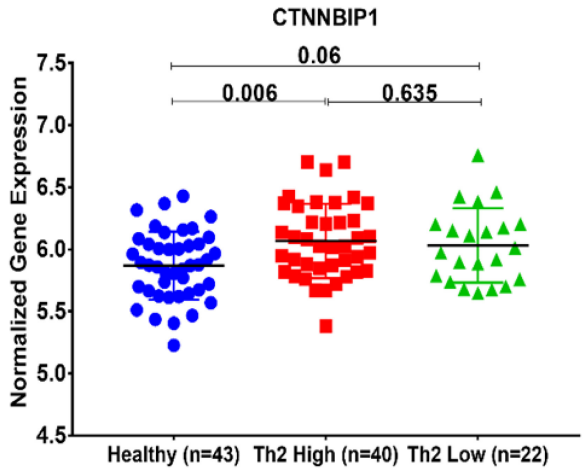
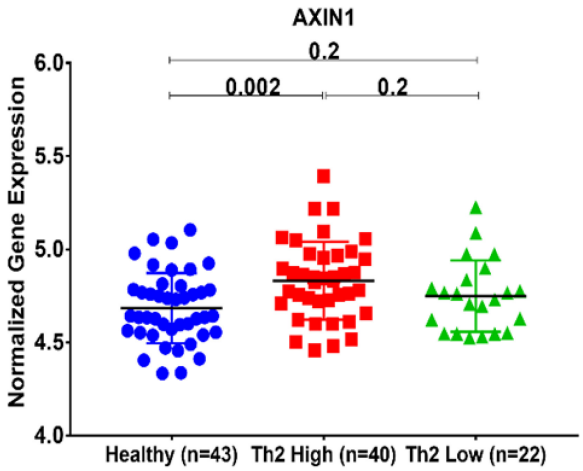
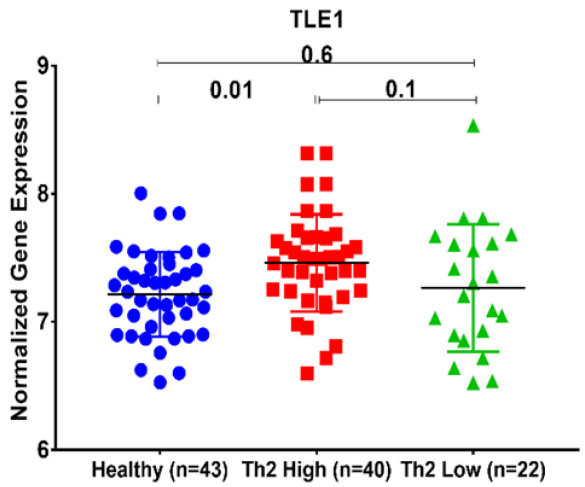
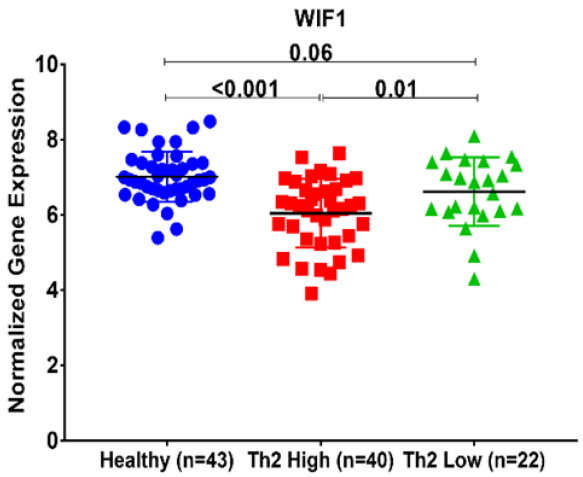


FIGURE 4 | Normalized gene expression of Wnt signaling pathway genes identified to be differentially expressed in severe asthmatic compared to healthy controls using bronchial epithelium transcriptomics data of the GSE64913 dataset.









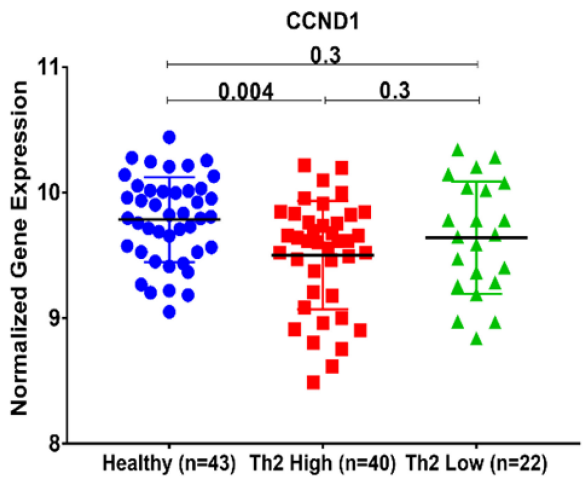


FIGURE 5 | Normalized gene expression of Wnt signaling pathways identified to be differentially expressed between Th2-high and healthy asthmatic bronchial epithelium transcriptomics data of the GSE67472 dataset to compare healthy controls (n = 43) to Th2-high asthmatics (n = 40) and Th2-low asthmatics (n = 22).

Wnt Signaling Pathway Genes Are Downregulated in Severe Asthmatic Bronchial Epithelium

Twelve genes out of the 35 were downregulated in severe asthma (CTNNB1, HMGA2, CDH2, BCL2, ITPR1, KIF5C, CLASP2, PRKCA, CREBBP, BCL11A, PEG10, and WNT5A). Interestingly, 5 out of those 12 genes are members of Wnt signaling (CTNNB1, CREBBP, ITPR1, PRKCA, and WNT5A).

Looking at other Wnt signaling pathways, DEGs in severe asthmatics' bronchial epithelium compared to healthy controls showed that three more genes (WIF1, VANGL2, and FOSL1) showed a statistically significant difference between the two groups. All identified Wnt genes were downregulated in severe asthmatic bronchial epithelia except FOSL1, as shown in **Figure 4**. Those genes were related to the non-canonical arm of Wnt signaling, which is considered to be a negative regulator of the canonical one. FOSL1 is known as a β-catenin/Wnt signaling target gene, transcribed when Wnt signaling is activated.

Wnt Signaling Pathway Genes Are Differentially Expressed in Bronchial Epithelium of Th2-High Asthmatic Compared to Healthy Controls

We investigated whether Wnt signaling members are differentially expressed in the bronchial epithelium of Th2 high, Th2 low versus healthy controls. CTNNBIP1, AXIN1, and TLE1 were upregulated in Th2-high bronchial epithelium compared to healthy controls, while WIF1 and CCND1 were significantly downregulated in Th2-high bronchial epithelium compared to healthy controls, as shown in **Figure 5**.

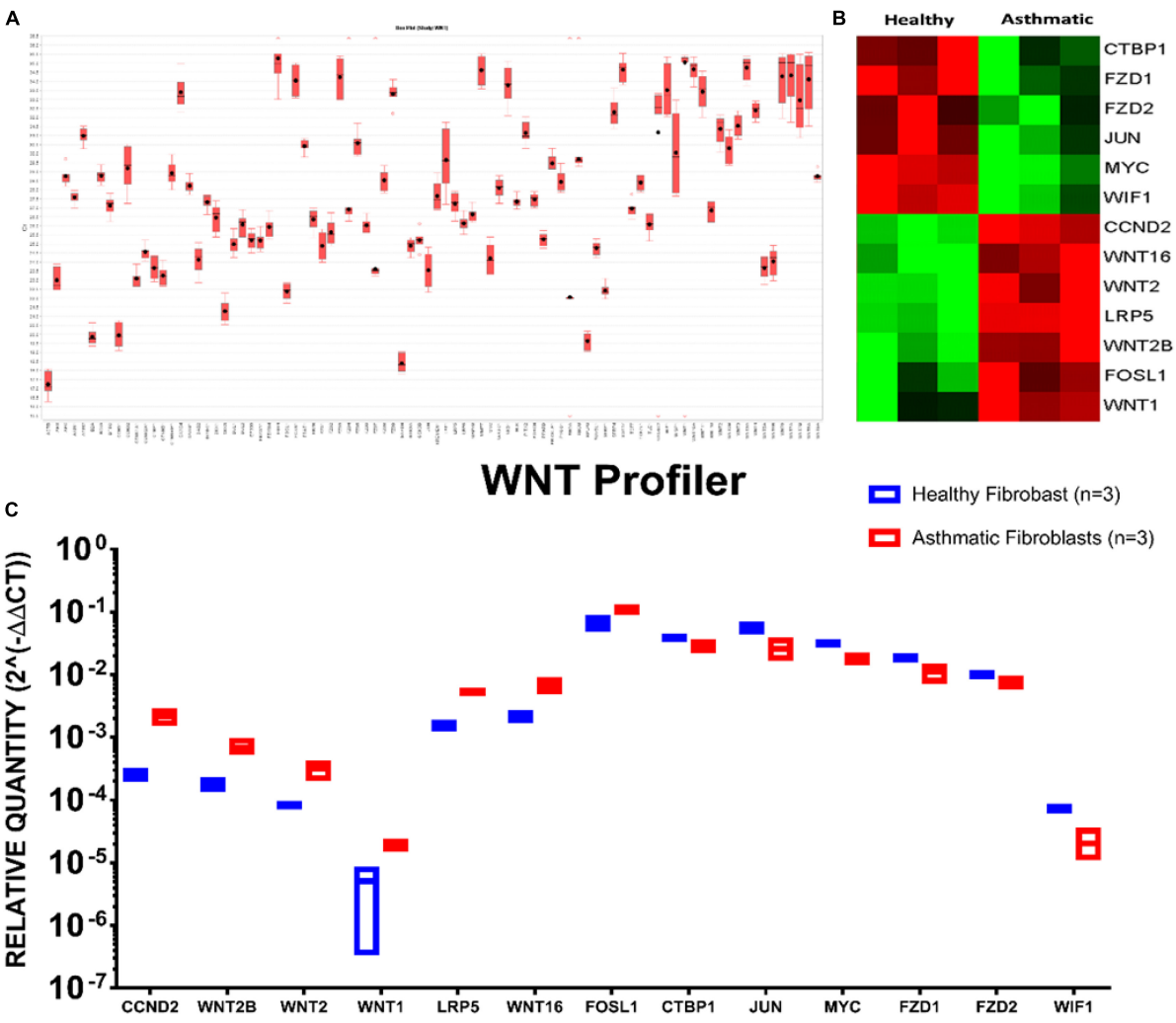


FIGURE 6 | Wnt signaling gene expression profiling in healthy versus asthmatic bronchial fibroblasts. Fibroblasts from healthy controls and asthmatic patients were seeded in triplicate, and when reaching 80–90% confluency, RNA was extracted, and Wnt genes were profiled using RT^2 Profiler^{TM} PCR Array Human WNT Signaling Pathway. **(A)** Box plot of relative expression of each gene member of the pathway. **(B)** Heat map showing the top DEGs between the two groups. **(C)** The relative quantity of the DEGs between healthy fibroblasts and asthmatic ones.

Wnt Signaling Is Aberrant in Fibroblasts From Asthmatic Patients

Aberrant Wnt signaling contributed to diverse human conditions. At the same time, its dynamics in asthma development need more attention, so we decided to dissect this pathway with a focus on its role in fibrosis and fibroblast biology. We profiled the Wnt pathway gene expression in fibroblasts taken from the lungs of healthy and non-severe asthmatic patients using RT2 Profiler PCR Arrays that profile 84 related genes simultaneously, as shown in **Figure 6**.

Interestingly, members of canonical Wnt signaling (Wnt1, FOSL1, Wnt2B, LRP5, Wnt2, Wnt16, and CCND2) were significantly upregulated in fibroblasts from asthmatic patients. On the other hand, members of the non-canonical and negative regulators of the canonical pathways were downregulated in fibroblasts from asthmatic patients like WIF1. Comprehensive mapping for the Wnt signaling members' expression in asthmatic versus healthy fibroblasts is shown in **Figure 6**. Wnt signaling gene expression profiling in healthy versus asthmatic bronchial fibroblasts is shown in **Figure 7**.

Ca2+ Mobilization Is Deranged in Fibroblasts From Asthmatic Patients

Our initial analysis showed that members of the non-canonical and negative regulators of the canonical pathways were downregulated in fibroblasts from asthmatic patients. One of the essential non-canonical Wnt pathways is the Wnt/Ca2+ signaling pathway, which is a crucial mediator in development and involved in NFκB mediated inflammatory response (De, 2011). The calcium mobilization in healthy and fibroblasts from asthmatic patients was assessed using Fura-2-AM assay in response to supernatants of healthy or

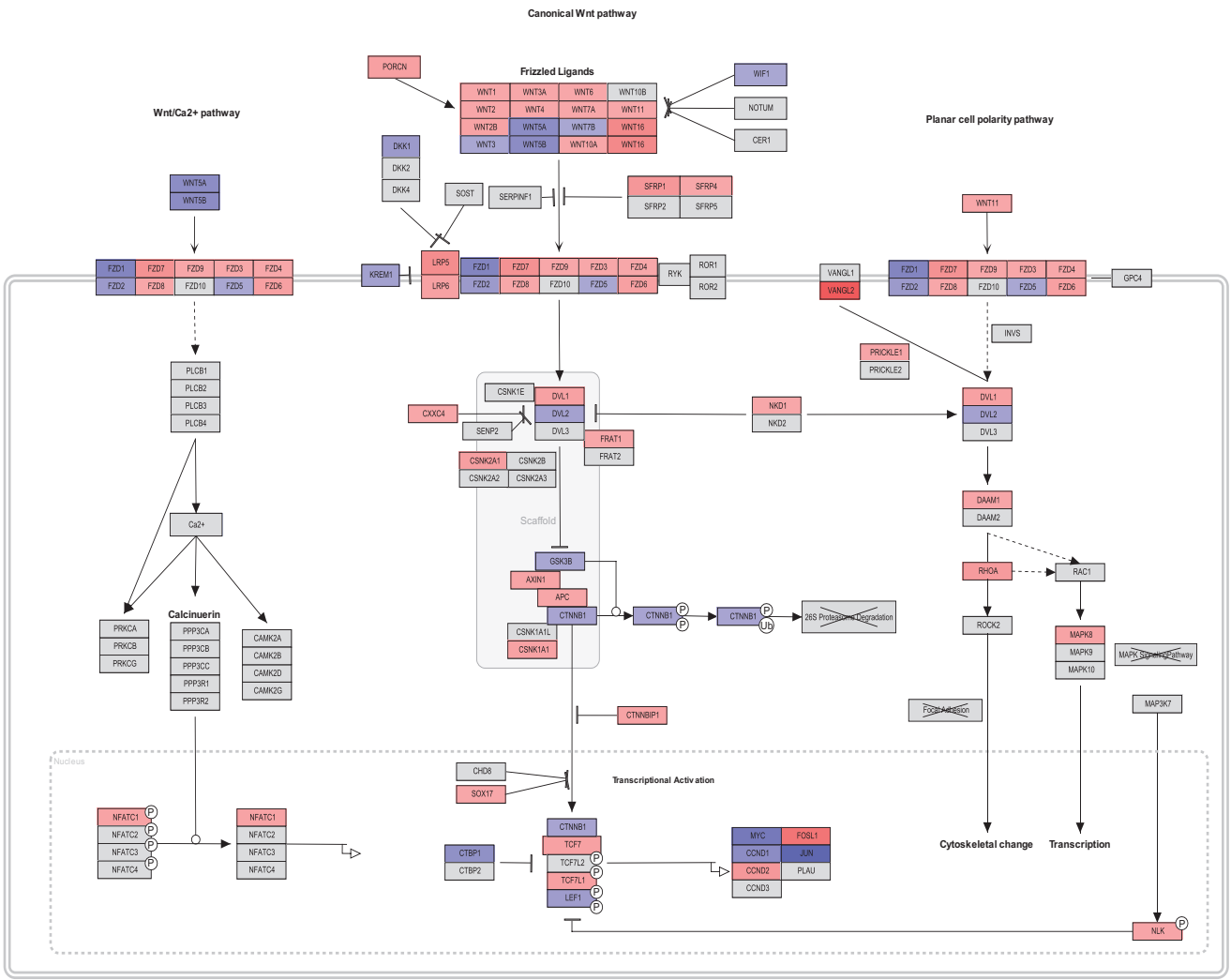


FIGURE 7 | Wnt signaling gene expression profiling in healthy versus asthmatic bronchial fibroblasts generated by PathViso pathway analysis and drawing software. The coloring scheme depends on the log fold change of asthmatic fibroblast expression versus healthy controls fibroblasts where red indicates that the gene is upregulated in asthma and blue means it is downregulated in asthma compared to healthy.

asthmatic bronchial fibroblasts and epithelium, as shown in **Figure 8**. Only asthmatic bronchial fibroblast supernatants induced Ca2+ mobilization in healthy fibroblasts with no effect on fibroblasts from asthmatic patients. On the contrary, a healthy bronchial epithelium medium induced Ca2+ mobilization in healthy fibroblasts but not in fibroblasts from asthmatic patients.

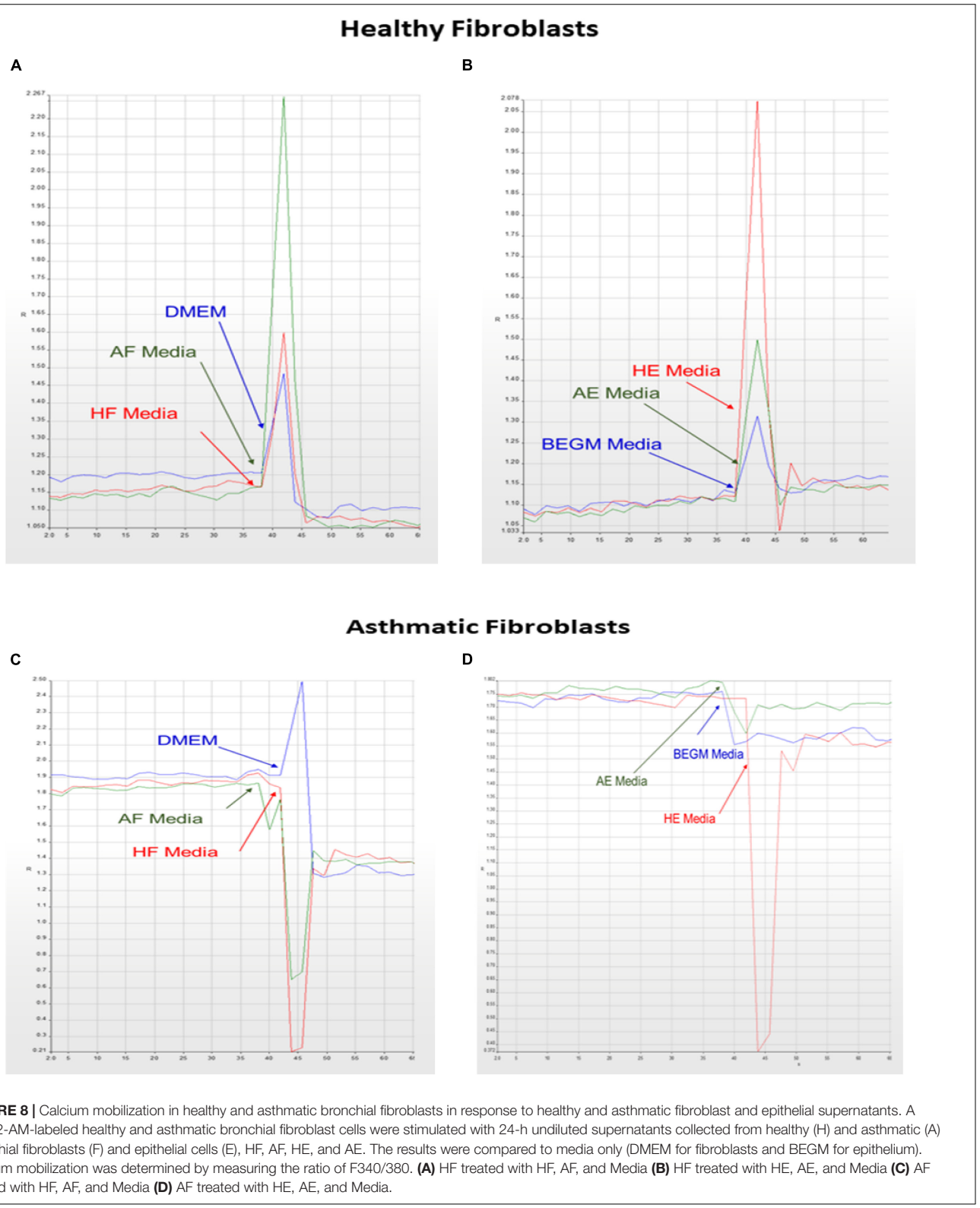


FIGURE 8 | Calcium mobilization in healthy and asthmatic bronchial fibroblasts in response to healthy and asthmatic fibroblast and epithelial supernatants. A Fura-2-AM-labeled healthy and asthmatic bronchial fibroblast cells were stimulated with 24-h undiluted supernatants collected from healthy (H) and asthmatic (A) bronchial fibroblasts (F) and epithelial cells (E), HF, AF, HE, and AE. The results were compared to media only (DMEM for fibroblasts and BEGM for epithelium). Calcium mobilization was determined by measuring the ratio of F340/380. **(A)** HF treated with HF, AF, and Media **(B)** HF treated with HE, AE, and Media **(C)** AF treated with HF, AF, and Media **(D)** AF treated with HE, AE, and Media.

This might indicate a deranged Wnt/Ca2+ signaling pathway in asthmatic bronchial fibroblasts.

Fibroblasts From Asthmatic Patients Express Less CTNNB1 Than Healthy Fibroblasts

Wnt cell signaling uses CTNNB1 protein as an essential part of relaying the signal to target genes inside the nucleus. The next step was to assess the CTNNB1 as a protein and explore its dynamics in fibroblasts from asthmatic patients. Immunoblot showed that fibroblasts from asthmatic patients express less CTNNB1 compared to healthy fibroblasts, as shown in **Figures 9A,B**. Interestingly, we noticed that later passage of healthy fibroblasts decreases CTNNB1. In contrast, later passages in diseased (asthmatics and COPD) fibroblasts increased its expression, as shown in **Figure 9C**.

CTNNB1 Is Shuttled to the Nucleus in Fibroblasts From Asthmatic Patients

Since CTNNB1 activity is based on shuttling between cell membrane, cytoplasm, and nucleus, we examined its intracellular localization using immunofluorescent and subcellular fractionation using immunoblot. Immunofluorescence detection showed that the nuclear fraction of CTNNB1 is more in fibroblasts from asthmatic patients compared to healthy controls, and the fractions are increased in later passages, as shown in **Figure 10**. Immunoblotting for subcellular localization of CTNNB1 in fibroblasts from asthmatic patitents showed a doubling of the nuclear and membrane fractions relative to the membrane abundance (**Figure 10**).

Low Glucose Increases CTNNB1 Shuttling to the Nucleus

The note of increasing CTNNB1 with increased passage necessitates further explanation to understand the mechanisms of CTNNB1 synthesis in fibroblasts. We compared high-glucose with low-glucose culture media to examine the effect of glucose concentration in media on CTNNB1 protein shuttling. Interestingly, a low-glucose medium increased the shuttling of CTNNB1 to the nucleus and membrane compartment, indicating its role in fibroblasts' response to the change in its environment, as shown in **Figure 11**. The increase was more evident in healthy fibroblasts than asthmatic cells indicating deranged Wnt response to the same stimuli.

Inhibiting the CTNNB1 Pathway Decreases Senescence in Bronchial Fibroblasts

Glucose restriction was shown to extend fibroblasts' lifespan while high glucose induced their premature senescence at any passages (Jin and Zhang, 2013). So we examined stimulation of the Wnt pathway with the WNT agonist or its inhibition with an FH535-specific inhibitor on fibroblast senescence using the beta-galactosidase staining kit. BML-284 (2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine), a potent and selective activator of Wnt signaling, and FH535, a small molecule inhibitor of β-catenin/TCF/LEF, were used for this purpose. As shown in **Figure 12**, FH535 significantly decreased the intensity and number of senescent cells compared to the Wnt agonist and DMSO, indicating the role of Wnt-CTNNB1 in regulating senescence.

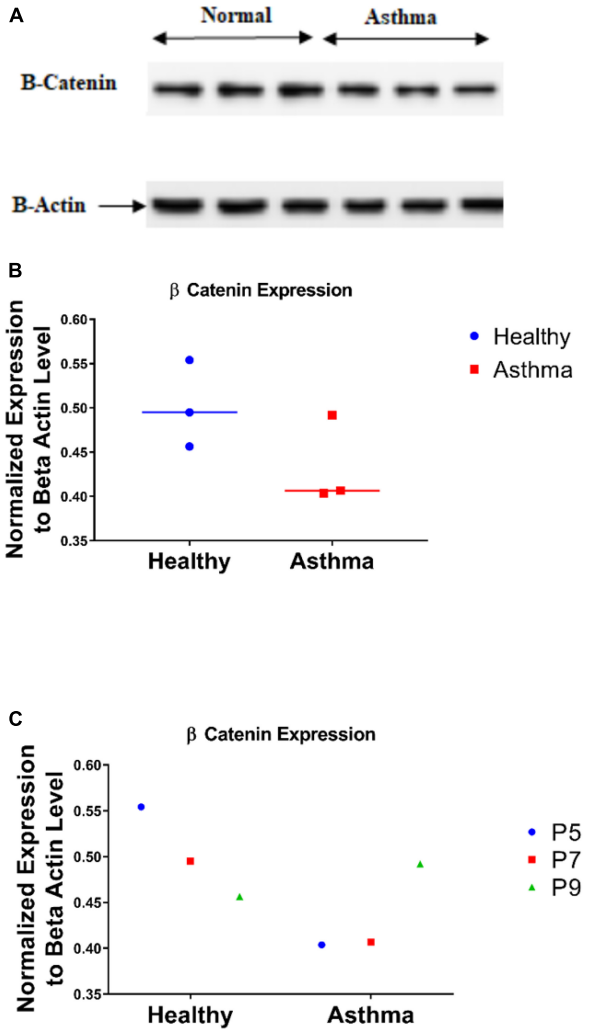


FIGURE 9 | CTNNB1 protein level in bronchial fibroblasts as measured by immunoblot **(A,B)** in primary fibroblasts from healthy, and asthmatic patients. **(C)** CTNNB1 protein expression in different passages of each group.

Wnt Signaling Inhibition Decreased Healthy Fibroblast Viability/Proliferation With No Effect on Fibroblasts From Asthmatic Patients

To evaluate the effect of Wnt signaling activation or inhibition on the fibroblast's viability/proliferation, we treated healthy fibroblasts and fibroblasts from asthmatic patients with Wnt

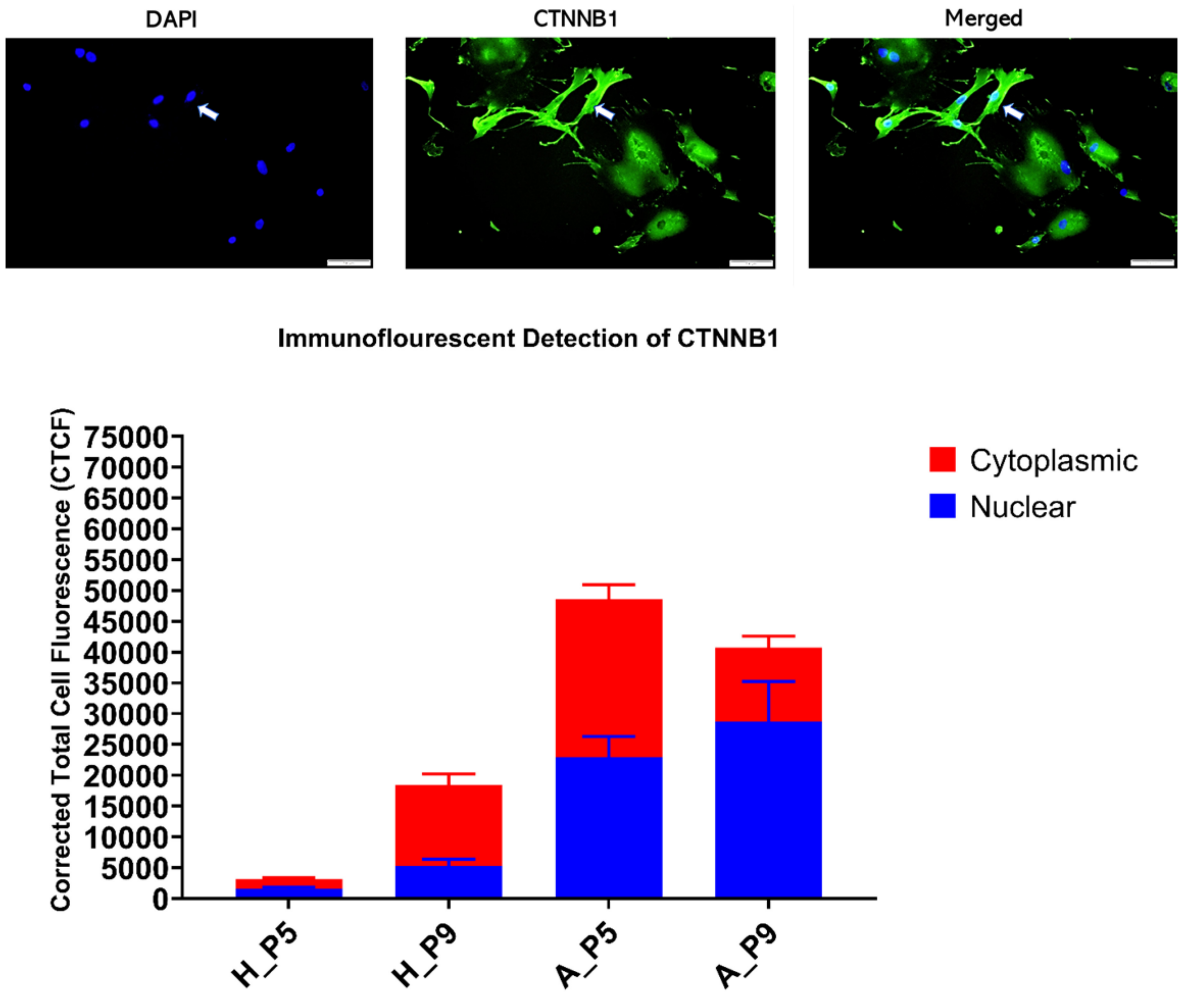


FIGURE 10 | CTNNB1 subcellular localization and fractionation in primary bronchial fibroblasts from healthy and asthmatic patients. Immunofluorescent cellular localization of CTNNB1 as corrected total cell fluorescent (CTCF) of 10 random spots in the cytoplasm compared to 10 different fields in the nucleus.

agonists and inhibitors then measured their proliferation using CellTiter 96® AQueous One Solution Cell Proliferation Assay. As shown in **Figure 13**, FH535 inhibited the growth of fibroblasts from healthy individuals with no effect on fibroblasts from asthmatic patients indicating a deranged signaling pathway in fibroblasts from asthmatic patients.

DISCUSSION

Our *in silico* analysis showed that the members of Wnt signaling are part of the core genes differentially expressed in severe asthmatic tissues compared to healthy controls. Extensive *in vitro* experiments confirmed and explained the critical role of the Wnt signaling pathway in asthma development.

Twelve genes out of the total 35 were downregulated in severe asthma, and 5 out of those 12 genes are members of Wnt signaling (CTNNB1, CREBBP, ITPR1, PRKCA, and WNT5A). Also, three more genes (WIF1, VANGL2, and FOSL1) showed statistically significant downregulation in severe asthmatic bronchial epithelium except for FOSL1. CREBBP is a known histone acetyltransferase that regulates gene expression and interacts with β-catenin to maintain cell proliferation rather than differentiation (Stefanowicz et al., 2017). It was reported that asthmatic bronchial epithelium showed a decreased gene expression of CREBBP, leading to incomplete and immature epithelium (Stefanowicz et al., 2017) while in blood monocytes, it showed increased activity during neutrophilic airway inflammation (Gunawardhana et al., 2014).

ITPR1 encodes an intracellular receptor for inositol 1,4,5-trisphosphate that mediates calcium release from the endoplasmic reticulum and plays a significant role in airway smooth muscles. Decreasing the activity of ITPR1 can make lung smooth muscle cells less reactive to contractile agonists to control asthma (Montano et al., 2018). Another member of Wnt signaling involved in calcium-related cellular activity is a member of the protein kinase C (PKC), PRKCA. PRKCA is a protein kinase that can be activated by calcium and the second

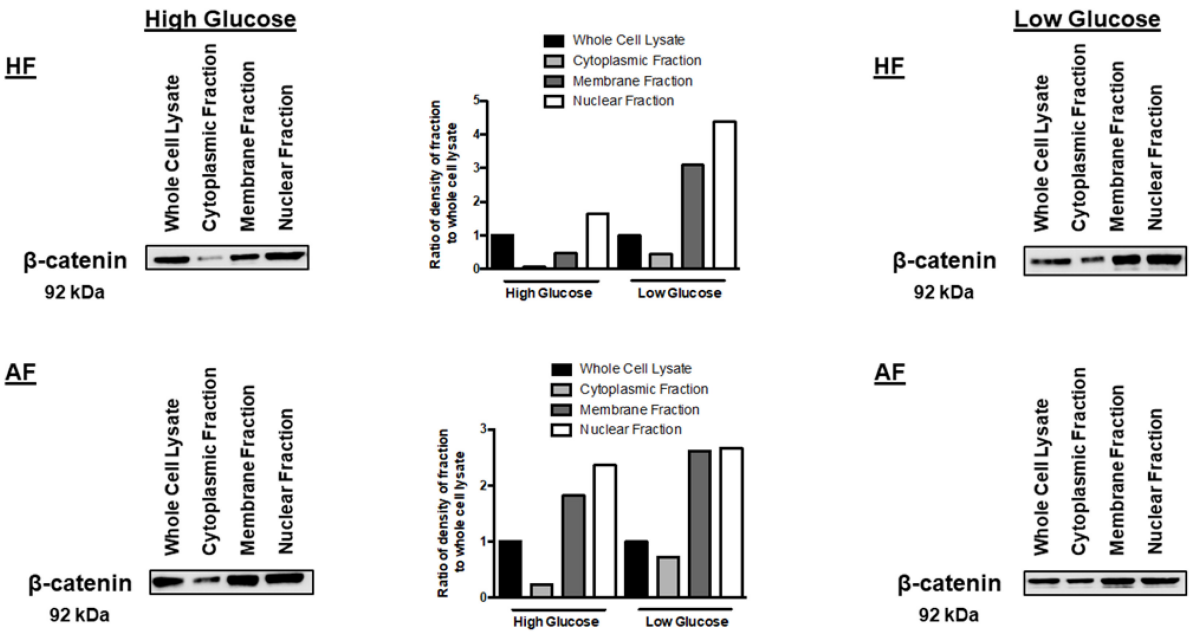


FIGURE 11 | Western blot analysis of cell fractions from healthy bronchial fibroblast (Cell Fractionation Antibody Sampler Kit #11843) showing cytoplasmic, organellular/membrane, and nuclear/cytoskeletal localization. Whole-cell lysates represent total protein. The fractionation was done under two conditions, the cultivation with high-glucose medium and a low-glucose medium.

messenger diacylglycerol. PRKCA is associated with both BMI and asthma simultaneously (Murphy et al., 2009), along with other genes with pleiotropic effects like leptin (LEP), and tumor necrosis factor (TNF)(Melen et al., 2010).

Wnt5a, a prototype of a non-canonical Wnt signaling axis with known cross talk with TGFβ1 during repair and remodeling, was elevated in the airway epithelium of Th17 asthma patients and steroid-resistant asthma (Daud et al., 2016; Dietz et al., 2017). In asthmatic airway smooth muscles cells, autocrine Wnt5a signaling regulates TGFβ1-induced ECM production (Kumawat et al., 2011). The WIF1 gene encodes a protein that binds to Wnt proteins and inhibits their activities. WIF1 expression can discriminate alveolar type 2 (AT2) cells into two groups: a high-WIF1 subgroup which is quiescent and the other low-WIF1 subgroup which selectively expresses detoxification genes and act as alveolar stem cells (Travaglini et al., 2020). Interestingly, WIF1 is linked to intrauterine airway development and lung function impairment which make neonates prone to asthma in the future (Sharma et al., 2010). Asthmatic patients with Wnt regulator (WIF1, WNT5B, and DKK3) enrichment were atopic, had early-onset, long duration, and had severe asthma with the inflammatory profile (Koopmans and Gosens, 2018).

VANGL Planar Cell Polarity Protein 2 (VANGL2) is involved in the control of early morphogenesis and planar cell polarity and is required for fetal lung development, precisely in normal lung branching morphogenesis (Yates et al., 2010). VANGL2 is significantly downregulated in lung tissue from patients with emphysema (Poobalasingam et al., 2017). In bronchial epithelium, IL4 and IL13 activation was shown to downregulate VANGL2 expression (Ladjemi et al., 2018). FOSL1 is known as a β-catenin/Wnt signaling target gene, transcribed when Wnt signaling is activated. As part of the FOSL1/AP-1 transcription factor, it regulates gene expression in human lung epithelia (Elangovan et al., 2018). mRNA expression of FOSL1 was shown to be decreased in PBMCs of aspirin-intolerant asthma (Kacprzak et al., 2014; Wieczfinska et al., 2015).

In human asthmatic airways, multiple Wnt ligand genes showed differential expression in signature between Th2-high and Th2-low asthmatics (Baarsma and Konigshoff, 2017). Our results showed that CTNNBIP1, AXIN1, and TLE1 were upregulated in Th2-high bronchial epithelium compared to healthy controls while WIF1 and CCND1 were significantly downregulated in Th2-high bronchial epithelium compared to healthy controls.

CTNNBIP1 encodes a protein that binds CTNNB1 to prevent CTNNB1 and TCF and control the downstream signaling and is a selectively enriched cluster of alveolar epithelium cells (AT2-s alveolar stem cells) (Travaglini et al., 2020). It is one of the genes upregulated by IL13 in eosinophilic conditions (Zuo et al., 2010).

AXIN1, when binding CTNNB1, acts as a negative regulator of the Wnt signaling pathway. Since β-catenin can block the overproduction of inflammatory cytokines in LPS-induced inflammatory responses, disturbance of the AXIN1 augments LPS effect (Lee et al., 2012). LPS-challenged human bronchial epithelial cells showed a decreased level of Axin

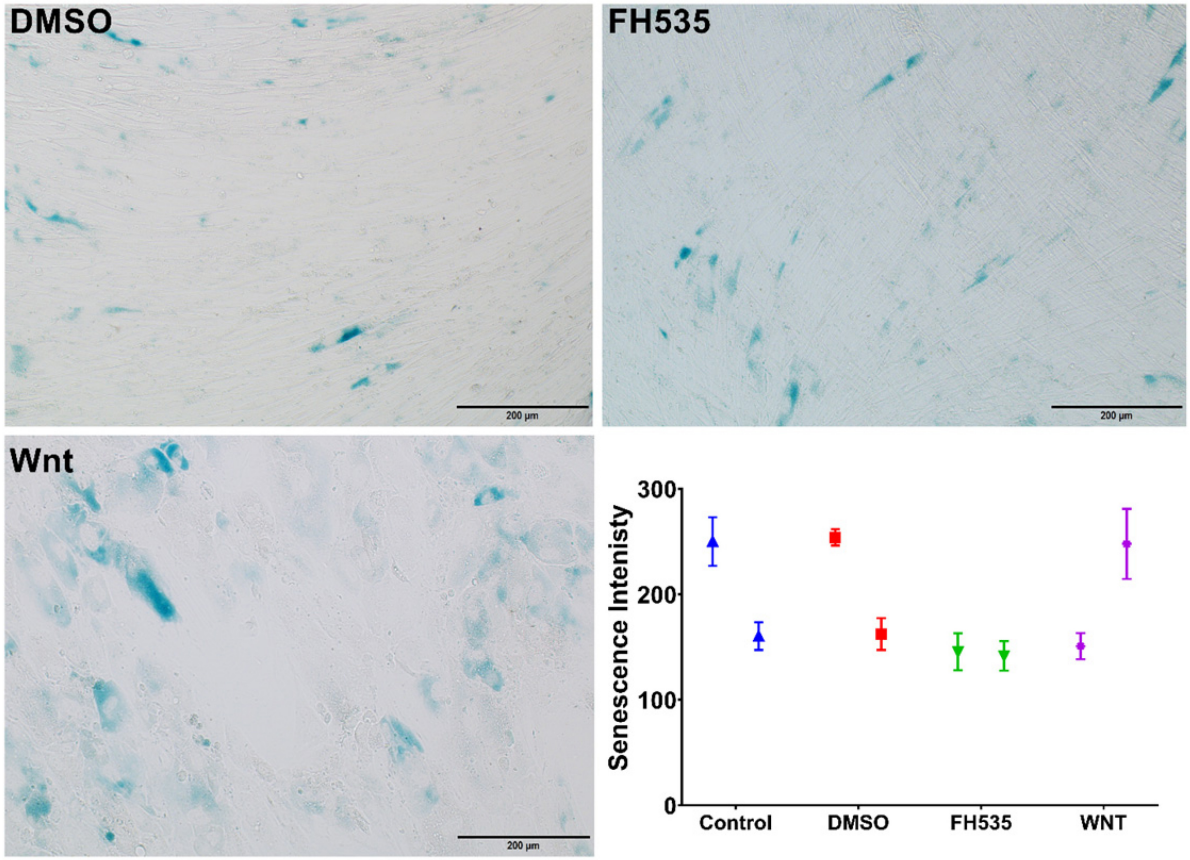


FIGURE 12 | Assessment of senescence using β-galactosidase. Healthy fibroblasts treated with DMSO, Wnt agonist, and inhibitor (FH535) then senescence signal were measured using beta-galactosidase staining kit.

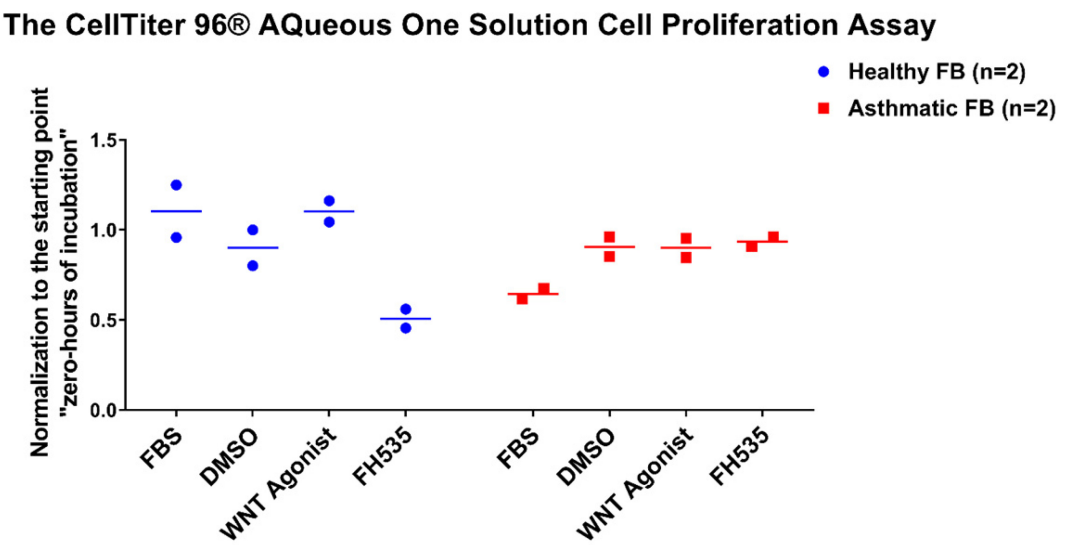


FIGURE 13 | Fibroblast cell proliferation under the effect of Wnt signaling activation or inhibition. Cells were seeded in triplicate at a density of 10,000 cells/well in opaque 96-well plates for 24 h. The CellTiter 96® Aqueous One solution cell proliferation assay was used to measure the viability of healthy and fibroblasts from asthmatic patients after 24 h of treatment with selective Wnt agonist and FH535, a small-molecule inhibitor of β-catenin/TCF/LEF. The 24-h values were normalized to their corresponding starting value (0-h incubation).

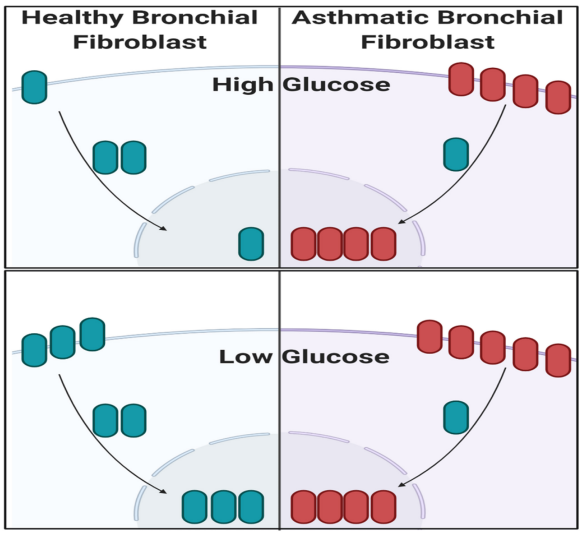


FIGURE 14 | Schematic summary of CTNNB1 intracellular localization in healthy and asthmatic bronchial fibroblasts under high- versus low-glucose culture conditions as measured by immunofluorescence and immunoblot. Created with Biorender.com.

(Jang et al., 2017). It was one of the Wnt signaling pathway genes that were attenuated by neutrophil elastase and cigarette smoke (Baarsma and Konigshoff, 2017). TLE1 (TLE Family Member 1, Transcriptional Corepressor) encodes a protein that suppresses major transcription factors like NF-kappa-B-and Wnt signaling. TLE1 was linked to dysregulation of epithelial–mesenchymal signaling in l asthmatic human bronchial epithelial cells (Loffredo et al., 2017). TLE1 was found to be in the susceptibility locus for childhood asthma as it interacts with RUNX3 to inhibit dendritic cell maturation (Modena et al., 2017).

In summary, the *in silico* analysis showed that the bronchial epithelium of severe asthmatic patients possess a deranged balance between Wnt enhancer and Wnt inhibitors. The Th2-high phenotype is associated with upregulated Wnt-negative regulators, while inflammatory and neutrophilic severe asthmatics showed higher canonical Wnt signaling member enrichment. Most of these genes are the regulator of healthy lung development early in life and, if disturbed, can make people susceptible to develop asthma early in life and prone to develop severe phenotype. Most of Wnt members are secreted, and their effect can be in an autocrine fashion on the bronchial epithelium, paracrine on nearby adjacent structural cells like fibroblasts and smooth muscles, or systemic in blood.

Wnt signaling is essential in T cell development, maturation, and hematopoiesis regulation (van Loosdregt and Coffer, 2018). T cell factor (the major transcription factor in Wnt signaling) directly blocks Th17 cell differentiation while Wnt-negative regulators (WIF1) enhance such differentiation (van Loosdregt and Coffer, 2018). Wnt6 was shown to be positively correlated with Th2-high asthmatic phenotype (Choy et al., 2011). In the infected lung, Wnt6 is produced mainly by foamy macrophage-like cells (Schaale et al., 2013). Alveolar macrophages increase the production of Wnt6 during induced lung damage (Pandit et al., 2019). On the other hand, Wnt coreceptors, Lrp5 and Lrp6, were found to be highly expressed in PBMC after lung injury (Scheraga and Thannickal, 2014). This might indicate the disturbance of CTNNB1 regulators rather than its expression. This so-called goldilocks phenomenon proposes that only the optimal amount of TCF activity can result in the desired outcome (van Loosdregt and Coffer, 2018). β-Catenin blocks inflammatory mediators to induce dendritic cells (DC) with tolerance phenotypes (Orme et al., 2016). Also, activation of canonical and non-canonical Wnt signaling can induce immune tolerance by promoting T regulatory responses (Staal and Arens, 2016). TCF/β-catenin and Foxp3 share common transcriptional targets; Wnt signaling negatively modulates Foxp3 transcriptional activity (van Loosdregt et al., 2013). Increasing Wnt signaling can inhibit such Treg cell-mediated suppression (van Loosdregt and Coffer, 2018). We can speculate that deranged Wnt in PBMCs of severe asthmatics might lead to higher but inactive T regulatory cells.

Wnt Signaling Is Aberrant in Fibroblasts From Asthmatic Patients

In asthmatic airways exposed to frequent injury and repair, fibrosis eventually will develop by the critical player "myofibroblasts," which activate three integrated pathways: TGFβ, Wnt, and YAP/TAZ signaling (Piersma et al., 2015). We decided to decipher the role of Wnt signaling on bronchial fibroblast biology. Our Wnt signaling transcriptomics profiling of fibroblasts from asthmatic patients compared to healthy ones confirmed our *in silico* analysis, as members of canonical Wnt signaling (Wnt1, FOSL1, Wnt2B, LRP5, Wnt2, Wnt16, and CCND2) were significantly upregulated in fibroblasts from asthmatic patients. On the other hand, members of non-canonical and the negative regulators of the canonical pathways were downregulated in fibroblasts from asthmatic patients like WIF1.

Non-severe fibroblasts from asthmatic patients express less CTNNB1 compared to healthy fibroblasts in early passages, but in later passages, fibroblasts from asthmatic patients start to produce more CTNNB1. Fibroblasts with decreased Wnt activation can undergo regeneration, whereas β-catenin activation can reduce regeneration in wounds (Rognoni et al., 2016); nevertheless, β-catenin loss in fibroblasts can reduce fibrosis (Xiang et al., 2017). A nuclear fraction of CTNNB1 is more abundant in fibroblasts from asthmatic patients compared to healthy controls, and that fraction is increased in later passages. Low-glucose medium increased the shuttling of CTNNB1 to the nucleus and membrane compartment. These data confirm the previous reports that β-catenin is a critical player of fibroblast activation and tissue fibrosis (Beyer et al., 2012) by controlling their expression of ECM components and myofibroblast differentiation (Baarsma and Konigshoff, 2017). A schematic summary of CTNNB1 intracellular localization in healthy and asthmatic bronchial fibroblasts under high- versus low-glucose

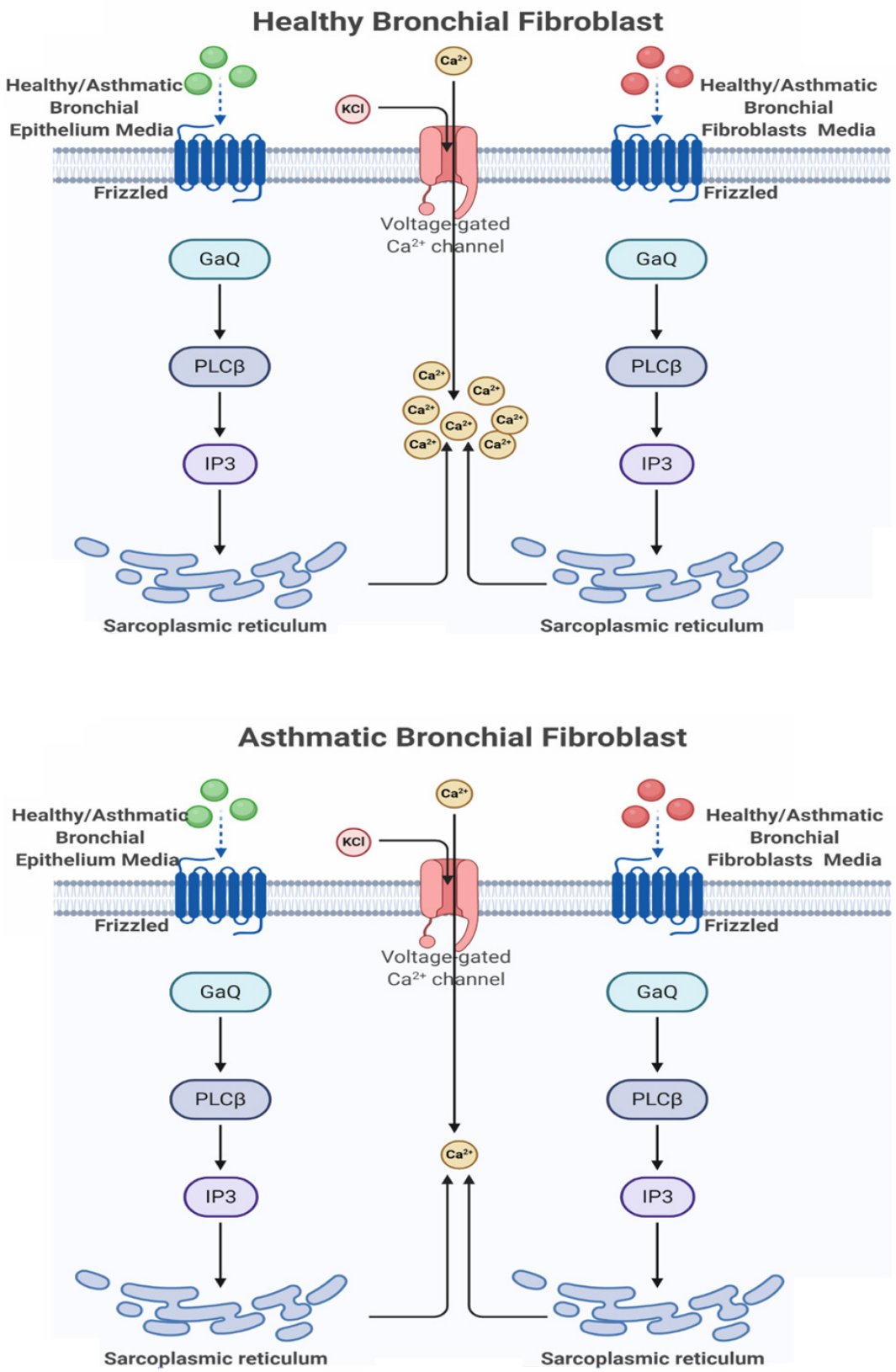


FIGURE 15 | Schematic representation of the effect of healthy and asthmatic bronchial epithelium and fibroblasts supernatant on Ca2+ mobilization in healthy fibroblasts and fibroblasts from asthmatic patients. Created with Biorender.com.

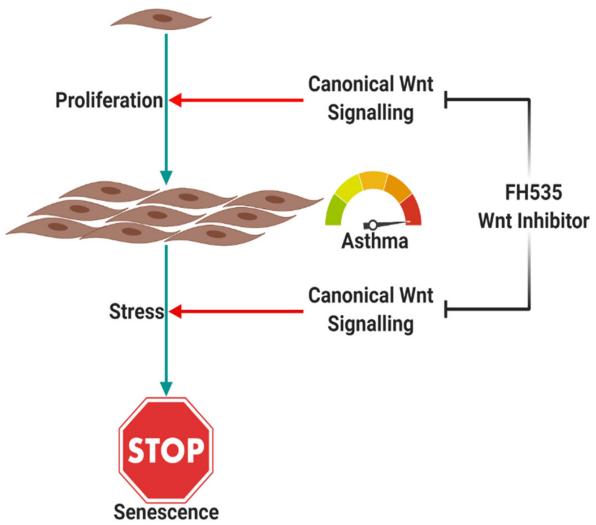


FIGURE 16 | Schematic representation of the possible role of canonical | Wnt signaling on bronchial fibroblast senescence. Canonical Wnt signaling is needed for the proper response of cells to proliferative stimuli, which puts cells under stress. Cells in response to proliferative stress will activate senescence, which is dependent on Wnt signaling too. Created with Biorender.com.

culture conditions as measured by immunofluorescence and immunoblot is illustrated in **Figure 14**.

At the wound edge, cytosolic calcium oscillations are induced in the fibroblasts (Lembong et al., 2017). In human pulmonary fibroblasts, TGF-β stimulates this Ca2+ wave activity, which in turn amplifies extracellular matrix gene expression (Mukherjee et al., 2012). On the other hand, cadherin–cadherin interaction induces Ca2+ transients during cell–cell adhesion (Ko et al., 2001). Uncontrolled Ca2+ oscillations in fibroblasts can lead to pulmonary fibrosis and impairment of lung function (Mukherjee et al., 2015). A schematic representation of the effect of healthy and asthmatic bronchial epithelium and fibroblast supernatant on Ca2+ mobilization in healthy fibroblasts and fibroblasts from asthmatic patients is illustrated in **Figure 15**.

Inhibiting Wnt signaling with FH535 inhibited the growth of healthy fibroblasts with no effect on fibroblasts from asthmatic patients, indicating the deranged signaling pathways in fibroblasts from asthmatic patients. FH535 anti-proliferative effect is mediated by inhibiting the recruitment of β-catenin coactivators (Handeli and Simon, 2008). The known β-catenin coactivators BCL9L/TCF4 might be overproduced in fibroblasts from asthmatic patients that FH535 needs a higher concentration to block them or they have defective PPARD gene needed for the proper action of FH535. Our profiling showed that BCL9 and TCF4 are upregulated in fibroblasts from asthmatic patients, but the difference was statistically not significant. On the other hand, inhibiting Wnt signaling with FH535 significantly decreased the intensity and number of senescent cells compared to Wnt agonist and DMSO, indicating the role of Wnt-CTNNB1 in regulating senescence. This matches the reports that the downregulation of Wnt signaling occurs early during the onset of cell senescence (Ye et al., 2007). Schematic representation of the possible role of Canonical Wnt Signalling on bronchial fibroblasts senescence is shown in **Figure 16**.

CONCLUSION

Our results showed that canonical Wnt signaling is needed for the proper response of cells to proliferative stimuli, which put cells under stress. Cells in response to this proliferative stress will activate senescence mechanisms, which depend on Wnt signaling too. Inhibition of Wnt signaling using FH535 inhibits both proliferation and senescence markers in bronchial fibroblasts compared to DMSO-treated cells. In fibroblasts from asthmatic patients, inhibition of Wnt signaling did not show that effect as Wnt signaling is deranged besides other pathways that might be non-functional. Further understanding of the factors that made fibroblasts from asthmatic patients respond differently will need further exploration to elucidate the interactions between Wnt pathways and other genes that we found to be differentially expressed in asthma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the MUHC Research Ethics Board (2003–1879) and the subjects had provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MH did the conceptualization, data curation, formal analysis, investigation, methodology, validation, software, visualization, and writing—original draft. NE did the data curation, formal analysis, investigation, methodology, validation, and writing—review. RR and KB did the investigation, methodology, and writing—review. IH and SA did the formal analysis, investigation, software, visualization, and writing—review. RO did the resources. QH did the conceptualization, funding acquisition, project administration, resources, supervision, and writing—review and editing. HB did the supervision and writing—review and editing. RH did the conceptualization, funding acquisition, methodology, resources, software, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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WNT Ligand Dependencies in Pancreatic Cancer

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WNT signaling promotes the initiation and progression of pancreatic ductal adenocarcinoma (PDAC) through wide-ranging effects on cellular proliferation, survival, differentiation, stemness, and tumor microenvironment. Of therapeutic interest is a genetically defined subset of PDAC known to have increased WNT/β-catenin transcriptional activity, growth dependency on WNT ligand signaling, and response to pharmacologic inhibitors of the WNT pathway. Here we review mechanisms underlying WNT ligand addiction in pancreatic tumorigenesis, as well as the potential utility of therapeutic approaches that functionally antagonize WNT ligand secretion or frizzled receptor binding.

Keywords: pancreatic ductal adenocarcinoma, intraductal papillary mucinous neoplasms, RNF43, PORCN, R-spondin, WNT/β-catenin signaling, WNT7B, FZD5

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and hard to treat malignancy with an overall 5-year survival of only 10%. It is currently the third leading cause of cancer mortality in the United States (Siegel et al., 2021). PDAC arises from two premalignant histologic precursors—pancreatic intraepithelial neoplasia (PanIN) or macroscopic cystic neoplasia including intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). The molecular hallmark of PDAC is *KRAS* mutation, a near ubiquitous and critical oncogenic driver of tumor initiation and progression. A diverse array of additional signaling pathways and processes further contribute to pancreatic tumorigenesis (Kleeff et al., 2016; Pelosi et al., 2017). Next generation sequencing (NGS) reveals a genetically diverse landscape (averaging >60 mutations/tumor) including four high frequency drivers (*KRAS, CDKN2A, TP53,* and *SMAD4*) and many additional heterogeneous genetic alterations. NGS studies also broadly divide PDAC into (1) classical/epithelial and (2) basal/squamous/quasimesenchymal transcriptional subtypes associated with *GATA6* and *TP63* expression signatures, respectively (Jones et al., 2008; Moffitt et al., 2015; Waddell et al., 2015; Witkiewicz et al., 2015; Bailey et al., 2016; Kleeff et al., 2016; Cancer Genome Atlas Research Network [CGARN], 2017). WNT is a highly enriched molecular mechanism in PDAC based on mutational analysis and expression profiling. Altered expression and activity of upstream or downstream WNT pathway components promote cancer hallmarks linked to pancreatic cancer initiation, progression, dissemination, stemness, and therapeutic resistance (White et al., 2012; Donahue and Dawson, 2016; Makena et al., 2019; Zhong et al., 2020). Of relevance to precision oncology is a subset of PDAC with *ring finger protein 43* (*RNF43)* mutations

conferring growth addiction to WNT ligands. This review briefly summarizes mechanisms of plasma membrane WNT ligand signaling in PDAC and their biological and clinical implications.

REGULATION AND FUNCTION OF WNT LIGAND SIGNALING IN PDAC

Canonical WNT Ligand Signaling in PDAC

Canonical WNT signaling involves oligomerization of frizzled (FZD) receptor and low-density lipoprotein-receptor related protein 5/6 (LRP5/6) by WNT ligand, initiating signaling classically culminating in the stabilization and nuclear translocation of β-catenin (Nusse and Clevers, 2017). Independent of β-catenin, WNT-FZD-LRP5/6 complexes sequester glycogen synthase kinase 3-beta (GSK3β) in multivesicular bodies, preventing its phosphorylation of target substrates. Consequently, canonical WNT signaling inhibits GSK3β phosphorylation-initiated ubiquitin-mediated degradation of numerous target substrates through the WNT stabilization of proteins (WNT-STOP) process. GSK3β sequestration also impinges on other signaling pathways modulated by its phosphorylation, such as mammalian target of rapamycin signaling induced by WNT (WNT-MTOR) (Acebron and Niehrs, 2016; **Figure 1**).

Genetically engineered mouse models (GEMMs), isogenic PDAC cell lines, and patient-derived organoids and xenografts highlight the activity and function of canonical WNT signaling in pancreatic tumorigenesis. Canonical WNT is activated early in PanIN progression and variably across PDAC tumors and cell lines (Pasca di Magliano et al., 2007; White et al., 2012). Genetic or pharmacologic inhibition of WNT ligand signaling or β-catenin itself blocks acinar-to-ductal metaplasia, PanIN, and PDAC in mouse models, including the conditional $Kras^{LSL-G12D}$ (KC) mouse model of PDAC (Zhang et al., 2013). Paradoxically, WNT hyperactivation via *Ctnnb1* stabilizing mutation also blocks PanIN formation and PDAC progression in the KC model (Heiser et al., 2008). Thus, the timing, strength, and manner of WNT activation are critical for pancreatic tumor initiation and progression in the context of oncogenic *KRAS* (MorrisIV., Wang and Hebrok, 2010). In patient samples, hallmark WNT mutations linked to constitutive pathway activation (i.e., *CTNNB1, APC,* etc.) are common primary oncogenic drivers in other non-PDAC pancreatic malignancies (i.e., acinar carcinoma and solid-pseudopapillary neoplasm) (White et al., 2012). By contrast, canonical WNT signaling in PDAC is primarily dysregulated at the plasma membrane level (White et al., 2012; Donahue and Dawson, 2016; Makena et al., 2019; Zhong et al., 2020).

The variable and complex expression patterns of FZD receptors and WNT ligands in PDAC cell lines and tissues raises important questions about functional redundancy or specificity of ligand-receptor combinations. A shared pattern of WNT1 and FZD2 expression correlates with increased total and non-phosphorylated β-catenin in PDAC tissue samples (Zeng et al., 2006). Interestingly, macrophage-derived WNT1 promotes epithelial-mesenchymal transition (EMT) in support of vascular invasion and metastasis in breast cancer (Linde et al., 2018), suggesting a WNT1-FZD2 circuit might be linked to inflammatory cell-mediated paracrine signaling in PDAC. Canonical WNT signaling mediates pancreatic stellate cell (PSC) activation and tumor-stromal crosstalk in PDAC. PSC activation correlates with downregulation of DKK1 and upregulation of WNT2 and β-catenin, while DKK1 antagonizes PSC activation and collagen synthesis (Hu et al., 2014). In organotypic models, activated PSCs secrete WNT2 to drive canonical WNT signaling in PDAC cells, while retinoic acid-induced PSC quiescence reduces WNT activation in PDAC (Froeling et al., 2011; Xu et al., 2015). Thus, paracrine WNT signaling from the tumor microenvironment can drive WNT activation in pancreatic cancer cells.

In relation to autocrine signaling, WNT7B is enriched in PDAC cell lines with high constitutive WNT/β-catenin signaling, is crucial for WNT/β-catenin transcriptional activity, and promotes *in vitro* and *in vivo* tumorigenesis (Arensman et al., 2014). WNT7B, WNT10A, and FZD5 are identified as essentiality genes in a large pooled CRISPR fitness screen of *RNF43*-mutant WNT ligand-addicted PDAC cell lines. FZD5 is therapeutically targetable specifically in *RNF43*-mutant PDAC cell lines and patient-derived xenograft (PDX) using anti-FZD5 antagonistic antibodies with limited FZD8 cross-reactivity (Steinhart et al., 2017). Further highlighting the specificity and potency of specific ligands in autocrine and paracrine signaling, Seino et al. (2018) stratify patient-derived PDAC organoids (PDOs) into three distinct subtypes: (1) growth dependent on WNT ligand provided exogenously or through co-culture with stromal cells; (2) growth sustained by autocrine WNT but sensitive to inhibitors of WNT secretion; or (3) growth independent of WNT. Further phenotypic studies and analysis of patient samples identify epithelial cell-derived (WNT7B and WNT10A) and stromal cell-derived (WNT2 or WNT2A) ligands of functional and clinical significance in PDAC. Interestingly, WNT independent PDOs consistently lack *GATA6* expression but are driven into WNT dependency via exogenous *GATA6* expression (Seino et al., 2018). GATA6 is overexpressed in precursor PanINs and promotes WNT activation and PDAC growth through transcriptional downregulation of the secreted WNT inhibitor DKK1 (Zhong et al., 2011). Thus, WNT ligand dependency is linked to GATA6 expression/function and classical/epithelial transcriptional subtype of PDAC. This transcriptional control of WNT dependency in PDAC is likely highly complex as studies in heart and lung development highlight highly interdependent expression and function of secreted WNT ligands and inhibitors, FZD receptors, and GATA transcription factors in mediating canonical and non-canonical WNT signaling (Afouda et al., 2008; Zhang et al., 2008; Meganathan et al., 2015).

RNF43 and WNT Growth Addiction in PDAC

The ubiquitin E3 ligase RNF43 is a key WNT feedback inhibitor that downregulates canonical signaling by ubiquitinating plasma membrane FZD receptors and LRP5/6 co-receptors, resulting

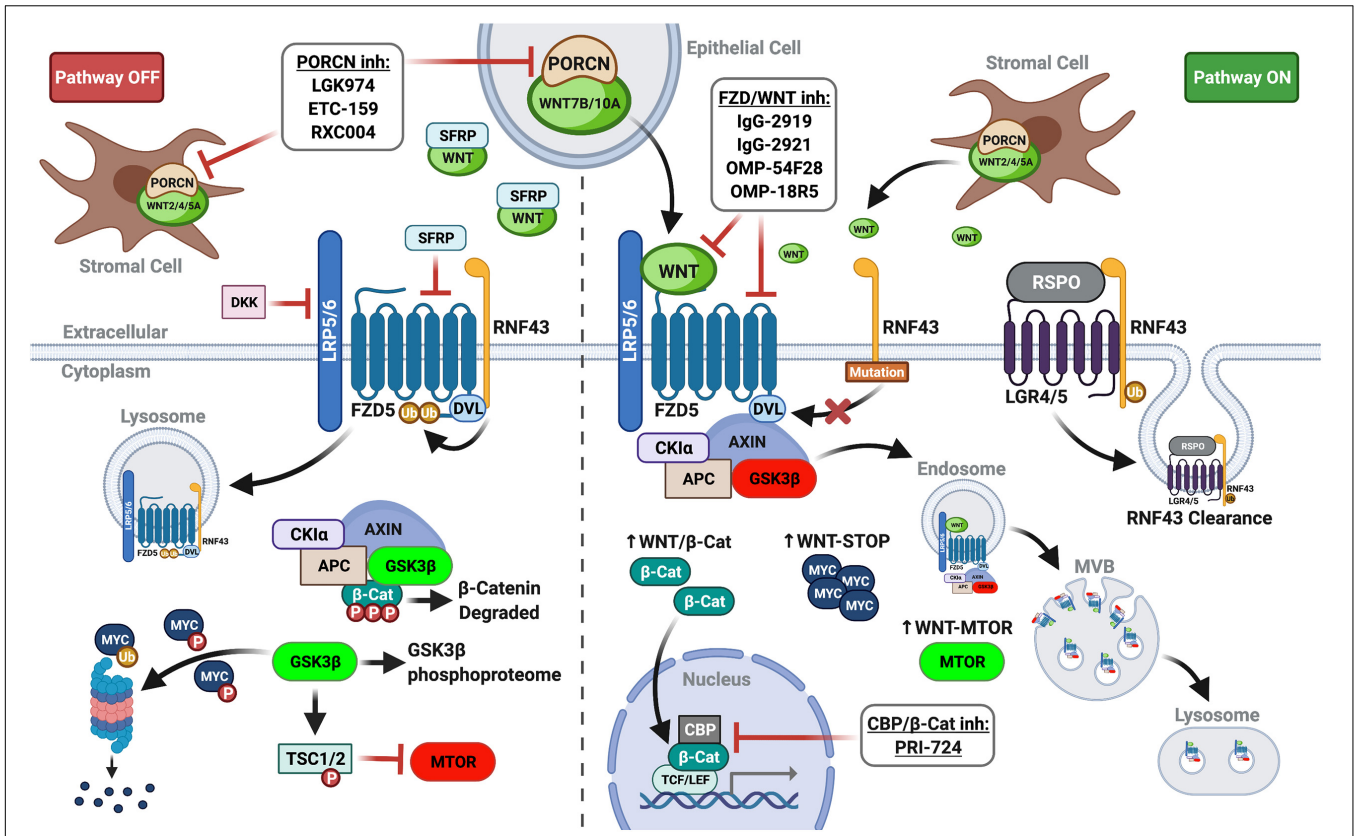

FIGURE 1 | WNT plasma membrane signaling in PDAC. (Left panel) In the absence of FZD-LRP5/6 signaling (lack of WNT ligand and/or inhibition by extracellular DKKs or SFRPs), β-catenin is targeted for degradation. GSK3β activity also impinges other signaling pathways and controls ubiquitin-mediated degradation of proteins. (Right panel) Augmented by *RNF43* mutations or RSPO inhibition of RNF43, WNT ligand signaling re-localizes and inactivates the destruction complex to stabilize β-catenin, increasing its nuclear translocation and co-transcriptional activity. GSK3β sequestration in multivesicular bodies (MVB) prevents substrate phosphorylation, promoting WNT-STOP, WNT-MTOR, and further pathway crosstalk (i.e., de-repression of MAPK/ERK). Thus, upstream WNT pathway inhibitors (PORCN inhibitors, FZD receptor blockers, and FZD decoy receptors) mediate additional actions divergent from downstream WNT pathway inhibitors targeting β-catenin in the nucleus (i.e., PRI-724). Created with BioRender.com.

in their internalization and lysosomal degradation (**Figure 1**). Secreted R-spondin family members (RSPO1-4) inhibit this process by binding leucine-rich repeat-containing G-protein coupled receptor (LGR4/5/6) and RNF43 (Binnerts et al., 2007; Hao et al., 2012) to potentiate WNT ligand signaling. Mutational inactivation of *RNF43* confers growth dependency on autocrine WNT ligand signaling in PDAC lines and predicts response to WNT inhibitors (Jiang et al., 2013). RSPO further regulates cellular hierarchy and cancer stem cell (CSC) phenotypes in PDAC irrespective of *RNF43* mutational status. Subpopulations of PDAC cells with high intrinsic WNT activity express RSPO2, which supports EMT and stemness phenotypes enhancing tumor-initiating and metastatic potential (Ilmer et al., 2015). Thus, PDAC CSC may be further specifically targetable with antagonistic antibodies to RSPO2 or other RSPO members under clinical investigation.

Approximately 5–7% of PDAC and 15–40% of premalignant IPMNs and MCNs harbor mutations in *RNF43*. Comparatively infrequent in PanIN and PanIN-associated PDAC, *RNF43* mutations are primarily linked to the malignant progression in IPMN and MCN (Furukawa et al., 2011; Wu et al., 2011; Jiang et al., 2013; Waddell et al., 2015; Bailey et al., 2016; Cancer Genome Atlas Research Network [CGARN], 2017) and are useful ancillary markers in pancreas cyst fluid diagnostics (Springer et al., 2015). Capture-based whole exome sequencing reveals IPMNs arising as multiple heterogeneous clones with convergent evolution of *RNF43* mutations during dysplastic progression (Fischer et al., 2019). Comprehensive sequencing and functional analysis suggest most *RNF43* non-sense and frameshift mutations and missense mutations in its RING domain and N-terminal region increase WNT activity and predict *in vivo* response to upstream WNT pathway inhibition (Yu et al., 2020). Large genomic deletion of *Rnf43* by inducible CRISPR in KC mice does not lead to cystic neoplasia but does accelerate PDAC progression, implying WNT signaling also facilitates malignant progression of *Kras*-initiated PanIN (Mishra et al., 2020).

Non-canonical WNT Signaling in PDAC

Non-canonical WNT ligand signaling in pancreatic tumorigenesis includes roles in potentiation of drug resistance and metastasis through effects of EMT and cancer stemness (Makena et al., 2019). WNT2 and other WNT ligands are

upregulated in PDAC cells under anchorage-independent conditions. WNT2 suppresses anoikis and potentiates metastasis via non-canonical WNT signaling mechanisms involving fibronectin upregulation and MAP3K7 signaling. WNT2 and WNT5A are enriched in subsets of circulating tumor cells collected from PDAC patients and act as orthogonal drivers of stemness and EMT (Yu et al., 2012; Franses et al., 2020). WNT5A/WNT5B induce EMT and potentiate metastasis across multiple cancer types through FZD2 non-canonical mechanisms involving FYN and STAT3 (Gujral et al., 2014). WNT5A also signals through FZD7 to mediate gemcitabine resistance in PDAC via upregulation of *ABCG2* (Zhang et al., 2021). Upregulated in PanIN and PDAC, WNT5A mediates apoptosis resistance to chemotherapy in PDAC lines through a NFATc2 dependent-mechanism stabilizing β-catenin (Griesmann et al., 2013) highlighting complex and overlapping roles of certain WNT ligands in regulating canonical and non-canonical signaling. Indeed, non-canonical WNT signaling suppresses pancreatic tumorigenesis in certain contexts via its capacity to suppress canonical WNT at different levels. Oncogenic KRAS sequesters calmodulin, which inhibits FZD8 receptor expression to block downstream NFAT and CaMKII-mediated antagonism of β-catenin in PDAC (Wang et al., 2015). This mechanism may explain the requirement for tightly regulated patterns of KRAS and WNT signaling during PDAC initiation and progression (MorrisIV., Wang and Hebrok, 2010).

TARGETING WNT LIGAND DEPENDENCY IN PDAC

Porcupine Inhibitors

Porcupine O-acyltransferase (PORCN) palmitoylates WNT ligands, a critical post-translational modification necessary for proper WNT processing, secretion, and FZD binding (Proffitt and Virshup, 2012). PORCN inhibitors (PORCNi) consistently and potently inhibit WNT/β-catenin transcription and growth of WNT-addicted cancers in both *in vitro* and *in vivo* preclinical models (Jiang et al., 2013; Liu et al., 2013; Arensman et al., 2014; Bailey et al., 2016; Hao et al., 2016; Madan et al., 2018a; Woodcock et al., 2019; Kalantary-Charvadeh et al., 2020). Justifying potential patient selection in their use, LGK974 was the first PORCNi shown to broadly block WNT/β-catenin transcriptional activity while only inhibiting growth of *RNF43*-mutant PDAC lines (Jiang et al., 2013; **Figure 1**). Multiple PORCNi (LGK974, ETC-159, and RXC004) have advanced to phase I or II clinical trials in advanced solid tumors alone and/or in combination with other therapies (Makena et al., 2019). Clinical trials exploring PORCNi in PDAC include NCT01351103, NCT02521844, and NCT03447470. Of note, some of these trials examine PORCNi in combination with immune checkpoint inhibitors given important roles for WNT in cancer immune escape. Targeting WNT will alter key regulators of the tumor immune cycle across tumor cells, antigen presenting cells, and different T cell subsets and has the potential to overcome primary, adaptive, and acquired resistance mechanisms to cancer immunotherapy (Wang et al., 2018).

Porcupine O-acyltransferase inhibitors have been leveraged as tool compounds for in-depth functional studies of WNT ligand signaling in PDAC. In a comprehensive study of *in vitro* and *in vivo* transcriptional dynamics, ETC-159 modulated >20% of expressed genes in *RNF43*-mutant PDAC lines. Altered genes were enriched for targets in cell cycle, nucleic acid metabolism, and ribosomal biogenesis. This transcriptional remodeling involved GSK3β-dependent regulation of β-catenin and MYC, the latter partially mediated via WNT-STOP mechanism independent of β-catenin (Madan et al., 2018a). This provocative finding suggests one critical mechanism of WNT addiction in PDAC may be the stabilization of proteins other than β-catenin (**Figure 1**). An *in vivo* CRISPR loss-of-function screen with ETC-159 identifies PI3K/mTOR pathway as a synthetic vulnerability. ETC-159 combined with pan-PI3K/mTOR inhibitor GDC-0941 more potently suppresses *in vitro* and *in vivo* PDAC tumorigenesis by enhancing cell cycle arrest, cellular senescence, and reduced glucose metabolic flux (Zhong et al., 2019). PORCNi leads to augmented MAPK and JNK activity in PDAC lines. ETC-159 combined with MEK inhibitor Trametinib synergistically inhibits cell cycle progression and *in vivo* tumor growth of *RNF43*-mutant PDAC, leading study authors to propose that WNT may temper excessive and potentially deleterious MAPK/ERK signaling in *RNF43*-mutant PDAC (Zheng et al., 2021).

Anti-FZD Antibodies and Fusion Protein Decoys

The monoclonal antibody OMP-18R5 (Vantictumab) antagonizes WNT signaling by binding multiple FZD receptors (FZDs 1, 2, 5, 7, and 8). OMP-18R5 is effective against PDAC in transgenic and xenograft models alone or synergistically with cytotoxic therapies, including gemcitabine or nab-paclitaxel (Gurney et al., 2012; Zhang et al., 2013; Fischer et al., 2017; Steinhart et al., 2017). Its safety has been evaluated in multiple cancer types, including PDAC (Smith et al., 2013). A phase Ib trial evaluating OMP-18R5 with nab-paclitaxel and gemcitabine in untreated metastatic pancreatic adenocarcinoma (NCT02005315) observed partial disease response in 41.9% and stable disease in 35.5% of patients, a potentially modest improvement over chemotherapy alone. However, definitive conclusions about efficacy were limited by study design and early study termination prior to reaching maximal tolerated dose (Davis et al., 2020).

OMP-54F28 (Ipafricept) (**Figure 1**) is a first-in-class recombinant protein fusing an extracellular portion of human FZD8 receptor to human IgG1 Fc fragment. It acts as a decoy receptor for WNT ligands (Le et al., 2015; Jimeno et al., 2017; Moore et al., 2019). OMP-54F28 alone is more effective than gemcitabine and improves efficacy with paclitaxel in preclinical PDX models (Fischer et al., 2017). A phase Ib trial evaluating OMP-54F28 with nab-paclitaxel and gemcitabine finds an overall response rate (ORR) of 35% and clinical benefit rate of 81%. Although a potentially modest improvement over chemotherapy alone, definitive conclusions regarding the efficacy of

OMP-54F28 were limited by study design and early termination due to concerns surrounding safety and commercial viability (Dotan et al., 2019).

Therapeutic Caveats

On-target effects linked to disruption of WNT and its role in normal homeostasis are concerns for PORCN and FZD inhibitors. Disrupted bone homeostasis is the most serious clinical toxicity observed to date with fragility fractures observed as a significant adverse event with OMP-18R5 (Smith et al., 2013; Tai et al., 2015). OMP-18R5 and OMP-54F28 clinical trials in PDAC were terminated due to concerns surrounding bone complications and commercial viability given an overall lack of therapeutic index across multiple studies (Moore et al., 2019; Davis et al., 2020). Although generally tolerated, PORCNi reduces bone mineral density, strength, and volume in mice by disrupting the balance of adipocytes and osteoblasts arising from mesenchymal stem cells (Funck-Brentano et al., 2018; Madan et al., 2018b). The DNA methylation inhibitor 5-Aza-dC mediates anti-adipogenic and pro-osteoblastogenic phenotypes that are reversible with PORCNi. These phenotypes appear linked to disruption of WNT10A and its regulation of mesenchymal stem cell fate (Chen et al., 2016). As a mitigating strategy, co-administration of the anti-resorptive bisphosphonate alendronate with ETC-159 reverses bone mass loss by rebalancing the activity of osteoclasts and preventing accumulation of bone marrow adipocytes (Funck-Brentano et al., 2018; Madan et al., 2018b). The addition of bone monitoring and bone protective agents with PORCN and FZD inhibitors have been employed in clinical trials although the relative benefit of these mitigating approaches remain uncertain. As an aside, some on-target effects may be clinically desirable. For example, PORCNi ameliorates chemotherapy-induced neuropathic pain via antagonism of canonical WNT ligand signaling in nerve and dorsal root ganglion in rodent models (Resham and Sharma, 2019; Kim et al., 2020) and could benefit patients on chemotherapies with dose-limiting neuropathies.

DISCUSSION

WNT ligand signaling plays key roles in PDAC initiation, progression, dissemination, and therapeutic resistance. Despite exciting results in preclinical models and identification of a subset of PDAC tumors addicted to WNT ligand, the safety and efficacy of upstream WNT inhibitors is uncertain. Adoption of mitigation strategies for dose-limiting side effects and biomarker-driven patient selection could enhance therapeutic index. Additionally, co-administration of highly specific WNT agonists such as next-generation surrogate WNTs that heterodimerize specific FZD isoform-LRP6 combinations (Miao et al., 2020) could facilitate on-target rescue of WNT signaling linked to toxicity while broadly inhibiting WNT systemically with PORCNi or FZD antagonists. Novel approaches such as drug conjugates or functionalized nanoparticles can also be envisioned for targeted delivery of WNT inhibitors specifically to the PDAC tumor microenvironment. Opportunities also exist to leverage known or novel drug combinations targeting tumor cell-specific vulnerabilities elicited by WNT inhibitors, including MEK or MTOR inhibition in combination with PORCNi. Finally, clinically effective and safe WNT inhibitors may ultimately hinge on the development of agents that selectively target PDAC-specific WNT-FZD circuits identified through functional approaches and spatiotemporal analyses of PDAC.

AUTHOR CONTRIBUTIONS

KYA and DWD conceptualized and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Adiponectin as Well as Compressive Forces Regulate *in vitro* β-Catenin Expression on Cementoblasts via Mitogen-Activated Protein Kinase Signaling Activation

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We aimed to investigate the molecular effect that adiponectin exerts on cementoblasts especially in the presence of compressive forces. OCCM-30 cells (M. Somerman, NIH, NIDCR, United States) were used. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) and western blots were employed to verify if the mRNA and protein levels of adiponectin receptors (AdipoRs), mitogen-activated protein kinase (MAPK), and β-catenin signaling were influenced by compressive forces or adiponectin. Moreover, siRNAs targeting *P38*α, *JNK1*, *ERK1*, *ERK2*, and *AdipoRs* as well as pharmacological MAPK inhibition were performed. We found that compressive forces increase the expression of *AdipoRs*. Adiponectin and compression up-regulate *P38*α,*JNK1*, *ERK1*, and *ERK2* as well as β-*catenin* gene expression. Western blots showed that co-stimuli activate the MAPK and β-catenin signaling pathways. MAPK inhibition alters the compression-induced β-catenin activation and the siRNAs targeting *AdipoRs*, *P38*α, and *JNK1*, showing the interaction of single MAPK molecules and β-catenin signaling in response to compression or adiponectin. Silencing by a dominantly negative version of *P38*α and *JNK1* attenuates adiponectin-induced TCF/LEF reporter activation. Together, we found that light compressive forces activate β-catenin and MAPK signaling pathways. Adiponectin regulates β-catenin signaling principally by inactivating the GSK-3β kinase activity. β-Catenin expression was partially inhibited by MAPK blockade, indicating that MAPK plays a crucial role regulating β-catenin during cementogenesis. Moreover, adiponectin modulates GSK-3β and β-catenin mostly through AdipoR1. P38α is a key connector between β-catenin, TCF/LEF transcription, and MAPK signaling pathway.

Keywords: adiponectin, MAPK pathway, β-catenin, cementoblast, compression

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INTRODUCTION

Orthodontically induced inflammatory root resorption (OIIRR) is one of the possible iatrogenic side effects of orthodontic therapy, which has been the subject of many research projects around the world (Yassir et al., 2020). OIIRR is defined as the pathologic removal of cementum and dentin (Kanas and Kanas, 2011). Cementoblasts, the cells comprising the cellular component

of cementum, have a similar gene expression pattern as osteoblasts, including *glycogen synthase kinase-3*β (*GSK-3*β), β-*catenin*, *osteocalcin* (*OCN*), and *osteoprotegerin* (*OPG*) (Saygin et al., 2000; Matthews et al., 2016).

It has been highlighted that β-catenin signaling regulates cementum homeostasis, and a down-regulation of WNT causes root resorptions (Lim et al., 2014). Mechanical compression of the periodontium is one of the numerous conditions occurring during orthodontic tooth movement (Memmert et al., 2019). As part of the periodontium, the cementum is often compressed through orthodontic force application. Thus, cementoblasts become mechanically deformed and start repairing the acellular cementum by replenishing it with cellular mineralized cementum (Diercke et al., 2014). Some research into the biologic regulation of the cementum repairing process have revealed that the regulation of β-catenin signaling is critical for normal cementum remodeling under constant mechanical loading (Lim et al., 2014; Sindhavajiva et al., 2018).

During orthodontic force application, compressive forces also induce human mandibular-derived osteoblast differentiation via WNT/β-catenin signaling (Sindhavajiva et al., 2018). WNT signaling was reported to inhibit cementoblast differentiation and promote their proliferation (Nemoto et al., 2009). Multiple signaling pathways participate in this process (Bikkavilli and Malbon, 2009). Recent advances suggest a possible intersection cross-reacting network: the β-catenin signaling in turn is mediated by the mitogen-activated protein kinase (MAPK) signaling pathway (Bikkavilli et al., 2008; Thornton et al., 2008). The P38 MAPK pathway is known to regulate osteoblast (Thouverey and Caverzasio, 2016) and cementoblast differentiation (Sanchavanakit et al., 2015). Bikkavilli et al. (2008) demonstrated that P38 MAPK inactivates GSK-3β by direct phosphorylation at its C terminus, leading to the activation of β-catenin signaling (Bikkavilli et al., 2008).

Adiponectin, a secreted protein produced mainly by adipocytes (Scherer et al., 1995), binds to its two receptors (AdipoR1 and AdipoR2) (Yamauchi et al., 2003). Adiponectin and its two receptors were reported to be expressed in primary human osteoblasts (Lin et al., 2014) and cementoblasts (Yong et al., 2020), which act as an important signaling link between fat and body weight to bone density (Berner et al., 2004). Unlike other adipokines such as leptin, the concentration of circulating adiponectin in obese human individuals is significantly lower than in non-obese (Naot et al., 2017). Recently, we elucidated the expression of adiponectin receptors on OCCM-30 cells and revealed that adiponectin has a pro-cementogenesis effect on cementoblasts partly through the MAPK signaling pathway (Yong et al., 2020). However, some research reveal that adiponectin has emerged as a key mediator regulating the anti-inflammatory and anti-apoptotic effects of biological activities (Tilg and Wolf, 2005; Sun and Chen, 2010).

It is now of interest how the interaction between the MAPK pathway and β-catenin signaling occurs. Additionally, the mechanism of adiponectin regulation in cementoblasts remains unclear. The aim of the current study is to assess whether β-catenin signaling modulation is regulated via the activation of MAPK signaling. Furthermore, it is aimed to identify the molecular mechanism by which adiponectin-induced MAPK interacts with compression-induced β-catenin.

MATERIALS AND METHODS

Cell Culture

The OCCM-30 cementoblast cell line was kindly provided by Prof. M. Somerman (NIH, NIDCR, Bethesda, Maryland) and maintained in α-MEM (11095-080, Gibco) containing 10% fetal bovine serum (FBS) (10270-106, Gibco) and 1% penicillin/streptomycin (15140-122, Gibco) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. Cells were stimulated using different concentrations of mouse adiponectin/Acrp30/ADIPOQ protein (His Tag) from Sino Biological Inc. (Cat. No.: 113 50636-M08H) with or without compressive force application as described by Kanzaki et al. (2002) and Proff et al. (2014). To achieve this purpose, 33-mm diameter glass cylinders with pulled surfaces were made by Reichmann Feinoptik Inc. (Brokdorf, Germany). Cylinders of different volumes were fabricated in order to reach pressure forces of 1.2, 2.4, and 3.6 gf/cm^2, respectively. The cells were seeded into six-well plates at a density of 3×10^4 cells/well until confluence and covered with the glass cylinders afterward.

To induce cementogenesis, the cell culture medium was supplemented with 10 mM β-glycerophosphate (#35675, Calbiochem) and 50 μg/ml ascorbic acid (6288.1, Roth).

Inhibitors for P38 (SB203580) (#tlrl-sb20, InvivoGen), ERK1/2 (FR180204) (#328007, Calbiochem), and JNK (SP600125) (#tlrl-sp60, InvivoGen) were used.

RNA Silencing

The siRNAs targeting mouse AdipoR1 (SI00890295), AdipoR2 (SI00890323), MAPK1 (SI02672117), MAPK3 (SI01300579), MAPK8 (SI1300691), MAPK14 (SI01300523), negative control (1027280), and cell death control (SI04939025) were purchased from QIAGEN Inc. siRNAs were incubated with 12 μl HiPerFect@ Transfection Reagent (301705, QIAGEN) in 100 μl Opti-MEM medium (31985-062, Gibco) at room temperature for 10 min, and then, each transfection mixture was added into 2.3 ml growth medium in the six-well plate in which OCCM-30 cells were cultured at 60–70% confluence. After siRNA transfection for 24 h, the cells were kept in starvation medium [(α-MEM (11095-080, Gibco) containing 0.5% FBS (10270-106, Gibco) and 1% penicillin/streptomycin (15140-122, Gibco)] for 2 h, and afterward, 100 ng/ml adiponectin (Cat. No.: 50636-M08H, Sino Biological Inc.) was added.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Cells were grown to confluency and kept overnight in starvation medium [α-MEM (11095-080, Gibco) containing 0.5% FBS (10270-106, Gibco) and 1% penicillin/streptomycin (15140-122, Gibco)]. Afterward, cells were either stimulated with adiponectin (100 ng/ml) (Cat. No.: 50636-M08H, Sino Biological Inc.)

or cultivated under compression (1.2, 2.4, and 3.6 gf/cm^2). Total RNA was extracted using NucleoSpin® RNA Kit (740955.50, MACHEREY-NAGEL). RNA concentrations were measured using a spectrophotometer (NanoDrop 2000, Thermo Scientific). Using commercial innuSCRIPT Reverse Transcriptase kit (845-RT-6000100, Analytik Jena), 1.0 μg RNA was transcribed, and 1.0 μl of the resulting cDNA was used at the final reaction volume of 20 μl/well in a CFX96™ Real-Time System Cycler (Bio-Rad). Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) amplification was carried out using the SsoAdvanced™ Universal SYBR@ Green Supermix (1723271, Bio-Rad). The primers for mouse AdipoR1&2 (qMmuCID0023619 and qMmuCID0010157) and MAPK3 (qMmuCED0025043) were purchased from Bio-Rad. The primers for MAPK1, MAPK8, MAPK14, GSK-3β, and β-catenin were purchased from Eurofins Genomics Inc. Primer sets used in the analysis are listed in **Supplementary Table 1**. GAPDH (qMmuCED0027497, Bio-Rad) and β-actin (qMmuCED0027505, Bio-Rad) served as housekeeping genes. Results were analyzed using the Bio-Rad CFX Manager 3.1 software.

Protein Extraction and Western Blot

The OCCM-30 cells were lysed in RIPA buffer (89901, Thermo Scientific) supplied with 3% protease inhibitor (78442, Thermo Scientific). The insoluble material was removed by centrifugation at 14,000 rpm/min for 15 min. Protein concentrations were measured using Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific) on a DR/2000 Spectrophotometer (#4480000, HACH). Then, 20 μg lysate/lane was diluted in a sample loading buffer (#G031, abm) and separated by 10–12% SDS-PAGE gels and then the resolved proteins were transferred electrophoretically to nitrocellulose membranes (1704271, Bio-Rad). Protein loading was verified by Ponceau S staining (6226-79-5, Sigma). Membranes were blocked with 5% non-fat milk (T145.1, ROTH) for 1 h at room temperature and further incubated with the primary antibodies for ERK1/2 (1:1,000, MBS8241746, BIOZOL); phospho-ERK1/2 (p44/42, Thr202/Tyr204) (1:1,000, #4370, Cell Signaling Technology); p54/p56 JNK (1:1,000, #9252, Cell Signaling Technology); phosphor-SAPK/JNK (Thr183/Tyr185) (1:1,000, #4668, Cell Signaling Technology); P38 MAPK (1:1,000, #9212, Cell Signaling Technology); phospho-P38 MAPK alpha (1:1,000, #4511, Cell Signaling Technology); GSK-3β (1:1,000, #12456, Cell Signaling Technology); phospho-GSK-3β (Ser9) (1:1,000, #9323, Cell Signaling Technology); β-catenin (1:1,000, #8480, Cell Signaling Technology); phospho-β-catenin (Ser33/37/Thr41) (1:1,000, #9561, Cell Signaling Technology); and β-actin (1:2,000, ab8227, Abcam) followed by peroxidase-conjugated secondary antibodies including polyclonal goat anti-rabbit (1:2,000, P0448, Dako); rabbit anti-goat (1:2,000, P0160, Dako), and polyclonal goat anti-mouse (1:2,000, P0447, Dako) in 2.5% non-fat milk (T145.1, ROTH) for 1 h at room temperature. The band signal detection was then performed with X-ray Amersham Hyperfilm (28906836, GE Healthcare) utilizing Amersham ECL Western Blotting Detection Reagents (9838243, GE Healthcare) and detected with an OPTIMAX X-Ray Film Processor (11701-9806-3716, PROTEC GmbH) in a dark room.

Immunofluorescence Staining

Cementoblasts were cultured on sterile Falcon™ Chambered Cell Culture Slides (354108, Fisher Scientific) until 50% confluence and afterward were fixed with 4% paraformaldehyde (Cat: 158127, Sigma-Aldrich) dissolved in 1X phosphate-buffered saline (PBS) (1401683, Gibco), adjusted to pH 7.4, for 10 min at room temperature, and permeabilized with 0.5% Triton™ X-100 Surfact-Amps™ Detergent Solution (28313, Thermo-Fisher) for 20 min. Then, cells were kept in a blocking buffer containing 10% goat serum, 0.3 M glycine, 1% BSA (071M8410, Sigma-Aldrich), and 0.1% Tween-20 (P1319, Sigma-Aldrich) for 30 min at room temperature and incubated with primary antibodies for GSK-3β (1:500, #12456, Cell Signaling Technology) and β-catenin (1:500, #8480, Cell Signaling Technology) at 4°C overnight. After washing three times with 1X PBS (1401683, Gibco)–0.1% TRITON X-100 (T-9284, Sigma-Aldrich) for 5 min, the cells were incubated with DyLight 488 goat anti-rabbit polyclonal secondary antibody (1:1,000, ab96899, Abcam), which conjugated to fluorescein isothiocyanate for 1 h. After washing with 1X PBS-Tween-20, DNA was stained using a fluorescent mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (ab104139, Abcam) for 15 min. Staining was analyzed using a high-resolution fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and photographed.

Dual Luciferase (Firefly–Renilla) Assay

OCCM-30 cells were seed into a 96-well plate at a density of 3×10^4 cells per well in 100 μl growth medium [α-MEM (11095-080, Gibco) containing 10% FBS (10270-106, Gibco) and 1% penicillin/streptomycin (15140-122, Gibco)] overnight. The reverse transfection of OCCM-30 cells with silencing RNA was performed with specific siRNAs to knock-down *AdipoR1*, *AdipoR2*, *MAPK1*, *MAPK3*, *MAPK8*, and *MAPK14*. Briefly, cells were incubated with 24.25 μl Opti-MEM antibiotic-free medium (31985-062, Gibco) and 0.75 μl HiPerFect@ Transfection Reagent (301705, QIAGEN) supplemented with 12.5 ng specific siRNA for incubation for 24 h. The cells were simultaneously transfected with the TCF/LEF Reporter Kit (#60500, BPS Bioscience). For control transfection, cells were transfected with DNA mixture by 1 μl of TCF/LEF luciferase reporter vector (#60500, BPS Bioscience) plus negative control siRNA (1027280, QIAGEN); 1 μl of non-inducible luciferase vector (#60500, BPS Bioscience) plus negative control siRNA (1027280, QIAGEN); and 1 μl of non-inducible luciferase vector (#60500, BPS Bioscience) plus specific siRNA in 15 μl Opti-MEM antibiotic-free medium (31985-062, Gibco). For experimental transfection, the DNA mixture of 1 μl TCF/LEF luciferase reporter vector (#60500, BPS Bioscience) plus specific siRNA was incubated in 15 μl Opti-MEM antibiotic-free medium (31985-062, Gibco). All the DNA mixtures were then mixed with 0.35 μl Lipofectamine™ 2000 Transfection Reagent (11668030, Thermo Fisher) in 15 μl Opti-MEM antibiotic-free medium (31985-062, Gibco) at room temperature for 25 min. After 24 h of transfection, the medium was changed to fresh growth medium. Following incubation for

another 23 h, adiponectin (100 ng/ml) was added to stimulate cells for 1 h. We set up each treatment in triplicate.

After 48 h of transfection, the firefly luciferase activities were performed using the BPS Dual Luciferase (Firefly/Renilla) Assay system (#60683-1, BPS Bioscience) using a Mithras LB 940 Luminometer (38099, Berthold Technologies) and analyzed by MikroWin 2000 (Mikrotek Laborsysteme GmbH), which normalized to the Renilla luciferase activities.

Statistical Analysis

Statistical analyses were plotted using the GraphPad Prism 6.0 software (GraphPad Prism Inc., San Diego, CA). Quantitative values are expressed as means ± standard deviation (SD) and analyzed using independent one-way *t*-test followed by Tukey's *post-hoc* test for unpaired samples to determine the statistically significant differences for multiple comparisons. Differences were considered statistically significant at a p-value of < 0.05 and p-values are shown with respect to controls, unless otherwise indicated. Data distribution was analyzed using the Kolmogorov–Smirnov and the Shapiro–Wilk test and visually using QQ plots. All experiments were successfully performed in triplicate.

RESULTS

Adiponectin or Compressions Up-Regulate the Expression of Adiponectin Receptors, MAPKs, and β-Catenin

First, we investigated the expression of adiponectin receptors (AdipoRs) at mRNA and protein levels on OCCM-30 cells. To evaluate the effect that adiponectin as well as compressive forces exert *in vitro* on cementoblasts, cells were additionally stimulated with 100 ng/ml adiponectin. Next, we performed a dynamic analysis of receptor expression, cultivating the cells in the presence of different compressive forces: OCCM-30 cells underwent compressive forces of 1.2, 2.4, and 3.6 gf/cm^2. The RT-PCR analysis revealed that the addition of adiponectin did not significantly influence *AdipoRs* mRNA expression ($p > 0.05$), while the application of compressive forces of 2.4 or 3.6 gf/cm^2 significantly increased its expression ($^*p < 0.05$; **Figure 1A**).

Furthermore, we analyzed the relative mRNA expression of *P38*, *JNK*, and *ERK*. RT-PCR results show that the expression of *P38*α and *JNK1* was strongly up-regulated by adiponectin ($^*p < 0.05$) or compression ($^*p < 0.05$), whereas cells showed a slight up-regulation ($^*p < 0.05$) of *ERK1* and *ERK2* in the presence of adiponectin or compressive forces (**Figure 1C**). Adiponectin exerts a negative effect on the *GSK-3*β mRNA expression ($^*p < 0.05$), while it positively up-regulates β-*catenin* expression ($^{**}p < 0.01$) after 1 h of stimulation (**Figure 1D**). Compressive forces alone decrease the mRNA expression of *GSK-3*β (3.6 gf/cm^2, $^*p < 0.05$) and increase β-*catenin* expression (1.2 gf/cm^2, $^{**}p < 0.01$; 2.4 and 3.6 gf/cm^2, $^*p < 0.05$; **Figure 1D**).

Western blot assays show increased expression of p-P38, p-ERK1/2, and p-JNK as a reaction to adiponectin or compression (**Figures 1A–D**). These observations prompted us to examine the possibility that adiponectin or compression regulates MAP kinase and β-catenin on OCCM-30 cells.

Adiponectin in Combination With Compression Promotes MAPK Signaling Activation

Compressive forces of 2.4 gf/cm^2 cause the phosphorylation of P38 and ERK1/2 after 30 min of cell exposure, whereas the phosphorylation of JNK occurs 1 h after stimulation (**Figures 2A,B**).

The co-cultivation of cementoblasts with adiponectin (100 ng/ml) under compressive forces (2.4 gf/cm^2) resulted in an increased and sustained phosphorylation stage of P38, ERK1/2, and JNK, at different degrees. Western blots (WBs) revealed that the phosphorylation of P38 and ERK1/2 was maintained from 0.5 to 24 h, reaching a peaking during 2–6 h of stimulation. The phosphorylation of JNK occurred after 4 h, reaching a peak at time point 6 h (**Figures 2C,D**).

Furthermore, we found that adiponectin combined with compressive force enhances the protein level of p-P38, p-ERK1/2, and p-JNK on cementoblasts (**Figure 2E**).

The Co-stimuli of Adiponectin With Compressive Forces Enhance β-Catenin Expression on Cementoblasts

Immunocytochemistry staining revealed that adiponectin-mediated GSK-3β expression was almost fully inhibited after 30 min and β-catenin showed an increasing expression 30 min after stimulation (**Figures 3A,B**).

WB analysis shows an attenuated activity of GSK-3β phosphorylation and decreased GSK-3β expression in response to adiponectin (100 ng/ml) over a time period from 30 min to 3 h (**Figures 3C,D**). Adiponectin also enhances β-catenin phosphorylation after 1-h stimulation, but increases β-catenin accumulation concurrently from 5 min to 2 h (**Figures 3C,D**). Surprisingly indeed, we found that adiponectin administration caused a constant increase in the β-catenin expression, the time course of which was coincident with the acute and transient increase of β-catenin phosphorylation in OCCM-30 cells (**Figures 3C,D**).

Next, we performed WBs to verify the effect that compressive forces (2.4 gf/cm^2) alone can have on the cellular accumulation of β-catenin. The time course experiments showed that the activation of β-catenin protein was transiently increased during 10 min to 2 h during compression exposure (**Figures 3E,F**), a fact that indicates that the application of compressive forces initially promotes WNT-independent accumulation of β-catenin in OCCM-30 cell cultures.

Additionally, we observed that adiponectin (100 ng/ml) in combination with compressive forces (2.4 gf/cm^2) upregulates the expression of dephosphorylated β-catenin 10 min after exposure. This effect was sustained over a period of 3 h (**Figures 3G,H**). Furthermore, we found that OCCM-30 cultivated with adiponectin and compression showed a decreasing expression of GSK-3β and increasing expression of β-catenin (**Figure 3I**).

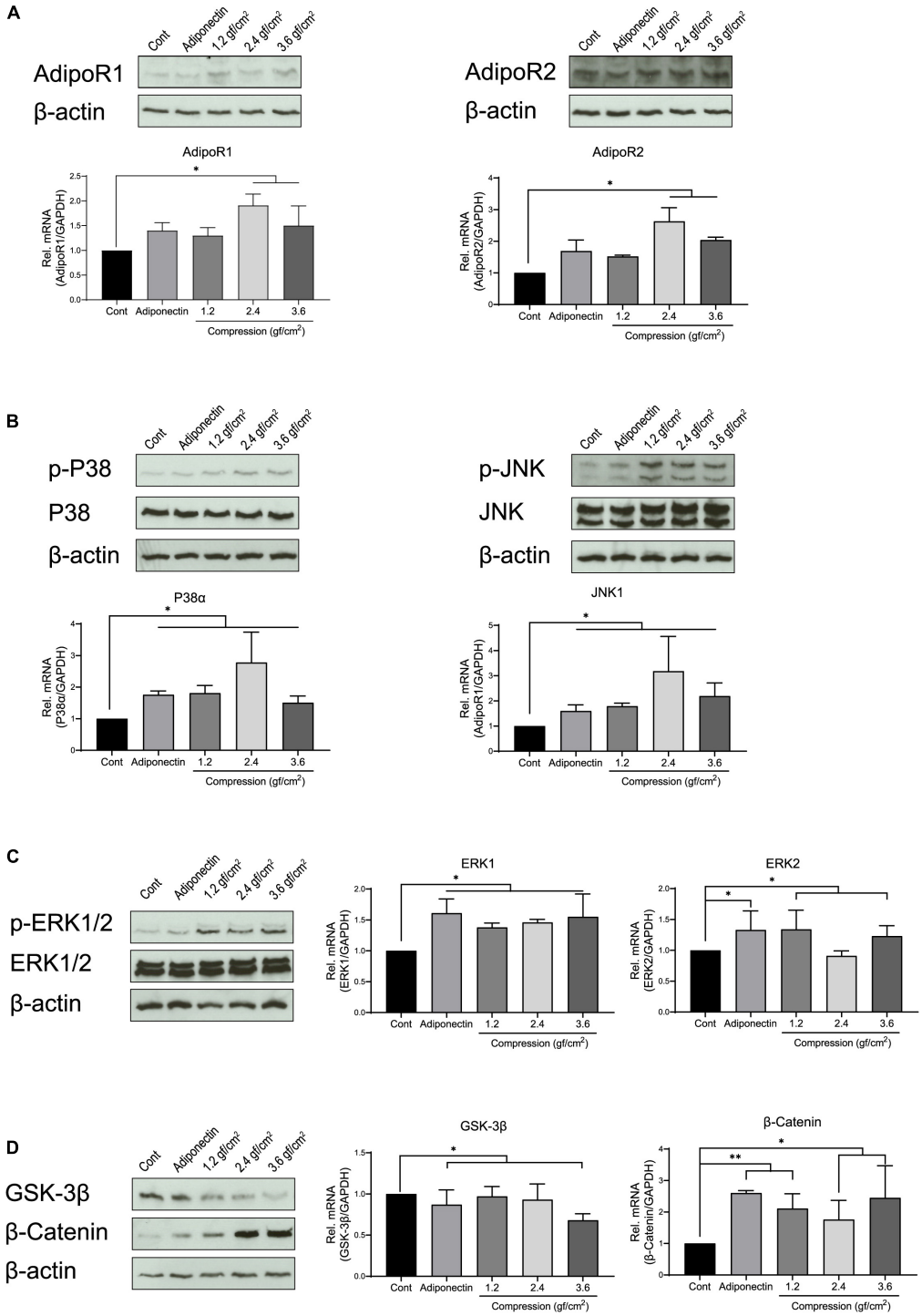


FIGURE 1 | Adiponectin as well as compression regulates the expression of adiponectin receptors (AdipoRs), mitogen-activated protein kinase (MAPK), and β-catenin. **(A)** Representative western blots showing the expression changes of AdipoR1 and AdipoR2 in the presence of adiponectin (100 ng/ml) or compression (1.2, 2.4, and 3.6 gf/cm^2). Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis shows that 2.4 or 3.6 gf/cm^2 compressive forces significantly increase mRNA expression of adiponectin receptors (*AdipoR1* and *AdipoR2*) in mouse OCCM-30 cells (**$p < 0.01$). **(B,C)** Western blots showing the expression of p-P38, p-ERK1/2, and p-JNK induced by adiponectin or compression. **(D)** The OCCM-30 cells show down-regulated mRNA expression of *GSK-3*β when exposed to adiponectin (100 ng/ml) or compression (3.6 gf/cm^2), whereas up-regulated mRNA expression of β-*catenin* (**$p < 0.01$). β-Catenin protein expression was increased after compression stimulation (*$p < 0.05$). Graphics show mRNA expression levels of *P38α*, *JNK1*, *ERK1, ERK2, GSK-3*β, and β-*catenin* after 60-min stimulation with adiponectin or compression. Data were derived from three independent experiments. Data is normalized to 1, and values are expressed as means ± SD. Asterisks indicate significant differences compared to control cells (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$; ns, not significant).

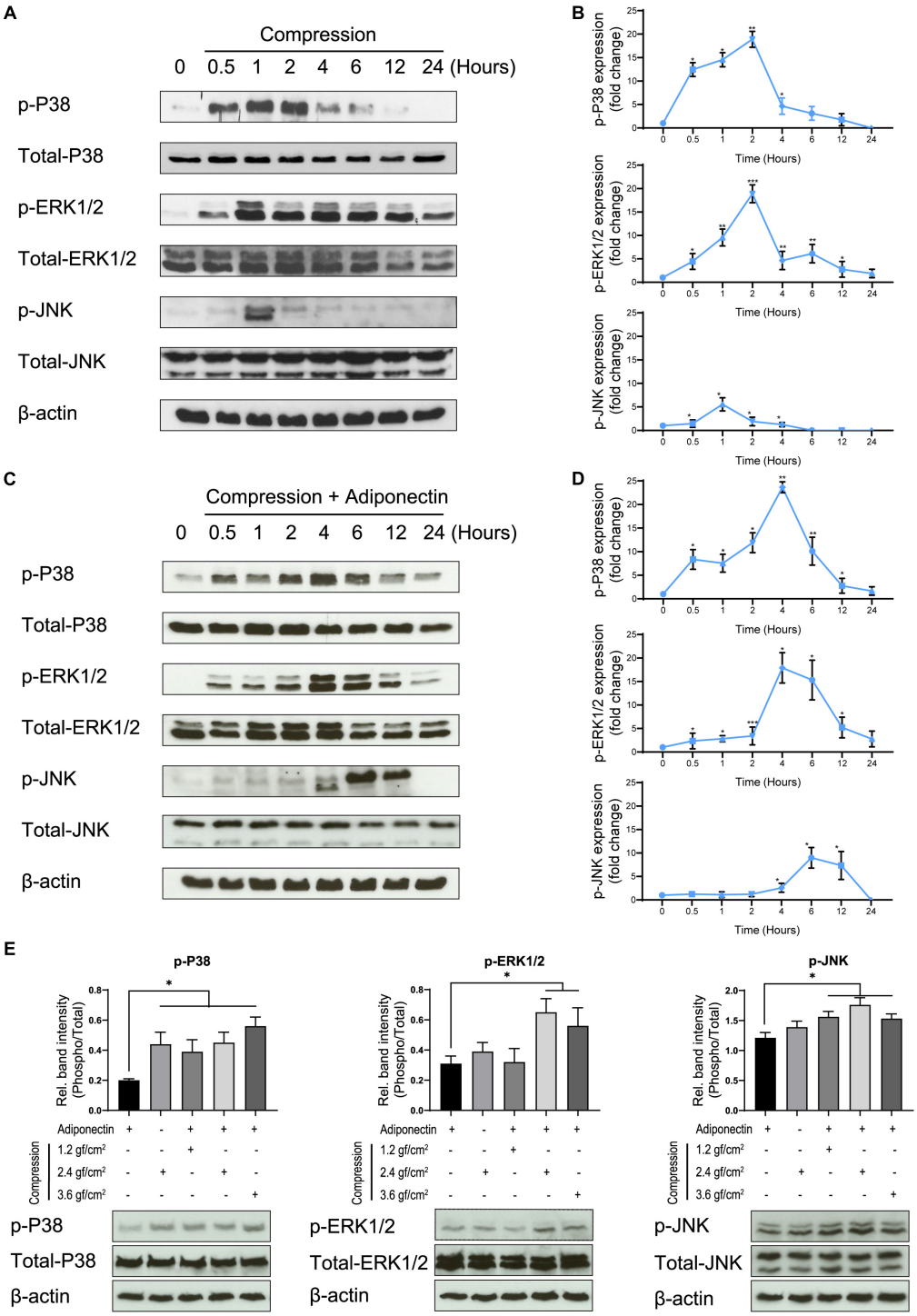


FIGURE 2 | Adiponectin in combination with compression enhances MAPK signaling activation. **(A,B)** Compression promotes P38, ERK1/2, and JNK phosphorylation on OCCM-30 cells. The kinetic protein expression of P38, ERK1/2, and JNK as well as their phosphorylated forms in response to compressive forces of 2.4 gf/cm^2 were analyzed by western blots. β-Actin served as a loading control. **(C,D)** After stimulation with 2.4 gf/cm^2 compression and adiponectin (100 ng/ml), phosphorylated forms of P38, ERK1/2, and JNK were up-regulated at different time points. Graphics represent the relative expression values of p-P38, p-ERK1/2, and p-JNK normalized to control cells at time point 0 as protein fold changes, respectively. **(E)** Western blot showing the expression changes of MAPK protein induced by adiponectin (100 ng/ml), compression (2.4 gf/cm^2), or adiponectin combined with compression (1.2, 2.4, and 3.6 gf/cm^2). The quantification ratio of p-P38, p-ERK1/2, and p-JNK is shown as phosphorylated state unit/total unphosphorylated protein (Phospho/Total). The statistical analysis was based on three independent experiments. Values are shown as the means ± SD. Asterisks indicate significant differences compared to control cells (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$; ns, not significant).

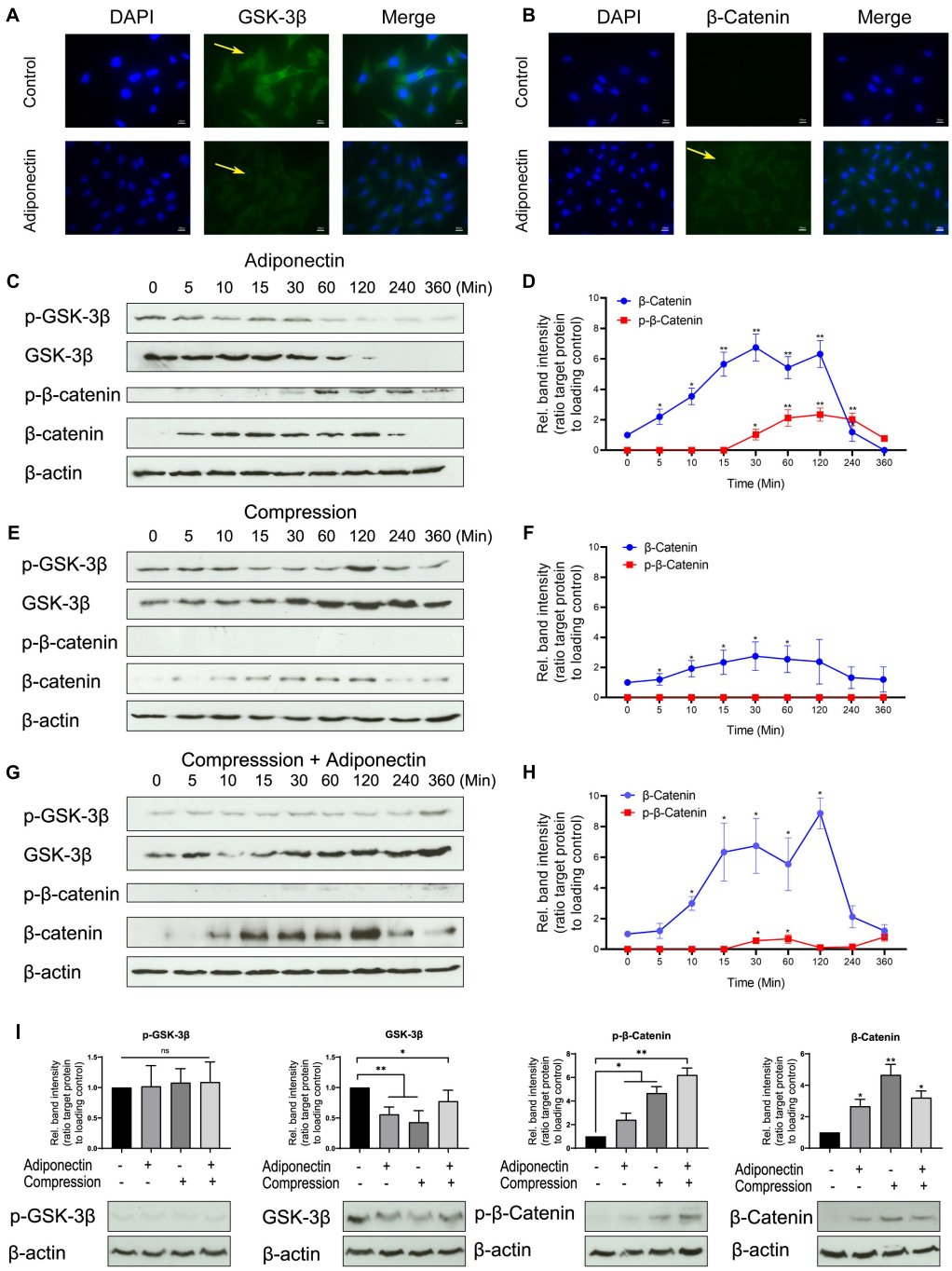


FIGURE 3 | The co-stimuli of adiponectin with compression enhance β-catenin expression on cementoblasts. **(A,B)** Immunofluorescence staining shows that adiponectin (100 ng/ml) decreases the expression of cytoplasmic GSK-3β in OCCM-30 cells and the cellular β-catenin expression was increased after adiponectin (100 ng/ml) addition. **(C,D)** Western blots indicate that after adiponectin (100 ng/ml) addition to OCCM-30 cells, phosphorylated and total GSK-3β protein expression decreased after 1 h, whereas the expression of total β-catenin increased from 5 min to 2 h due to adipokine stimulation. **(E,F)** Compressive forces exert a dual effect on OCCM-30 cells, increasing the expression of GSK-3β as well as the levels of total β-catenin: western blots indicate that the expression of phosphorylated and total GSK-3β as well as total β-catenin is increased after exposure to 2.4 gf/cm^2 compression for 0–6 h. **(G,H)** Compression in combination with adiponectin (100 ng/ml) enhances the expression of β-catenin: the kinetic analysis performed on OCCM-30 cells cultivated under 2.4 gf/cm^2 compression combined with adiponectin (100 ng/ml) shows that total β-catenin was significantly up-regulated after 10 min of stimulation over a period of 4 h. **(I)** Representative western blot showing the expression changes of GSK-3β and β-catenin induced by adiponectin (100 ng/ml) in the presence or absence of compression (2.4 gf/cm^2). Graphic represents the protein intensity, which was quantified as ratio to loading control to show the protein expression of p-GSK-3β, GSK-3β, p-β-catenin, and β-catenin. Data were derived from three independent experiments. Values are expressed as means ± SD. Asterisks indicate significant differences compared to control cells at time point 0 (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$; ns, not significant).

Blockade of MAPKs Alters the Adiponectin and Compression-Induced Activation of β-Catenin on OCCM-30 Cells

Then, we examined whether adiponectin-mediated MAPK signaling activation may cross react with GSK-3β activity. In the absence of exogenous adiponectin, the blockade of ERK1/2 causes the total GSK-3β expression to decrease significantly, whereas by co-stimulation with adiponectin (100 ng/ml), such effect was reversed (**Figure 4A**). In the presence of adiponectin, the blockade of P38 and JNK decreases the total GSK-3β expression, indicating that adiponectin modulates GSK-3β via P38 and JNK signaling.

The expression of total β-catenin was down-regulated in the presence of P38 or ERK1/2 inhibitors, but rescued by adiponectin (100 ng/ml) addition, a fact that suggests that P38, ERK1/2, and JNK modulate GSK-3β and subsequent cellular events such as the cytoplasmic accumulation of β-catenin (**Figures 4A,B**).

In order to investigate whether the MAPK pathway influences β-catenin signaling during compression, the cells were cultivated under compressive forces of 2.4 gf/cm^2 for 60 min. Afterward, we examined by WB the expression of GSK-3β and β-catenin. The results show that the suppression of the MAPK pathway has a significant blockade effect on β-catenin expression. This effect was restored when cells were exposed to compression for 1-h stimulation (**Figures 4C,D**). On the contrary, MAPK inhibition facilitates GSK-3β expression at varied degrees. These effects were counteracted by the application of compressive forces (**Figures 4C,D**).

Furthermore, to determine whether the activation of P38, ERK1/2, and JNK is involved in the stimulation of β-catenin by adiponectin or compression, the cells were preincubated with the pharmacological MAPK inhibitors SB203580 (P38), FR180204 (ERK1/2), and SP600125 (JNK) for 1 h and then cultivated under compressive forces of 2.4 gf/cm^2 co-stimulated with adiponectin (100 ng/ml) for another 1 h.

As a result, we observed that ERK1/2 inhibition as well as JNK inhibition in combination with compression inhibit GSK-3β expression, whereas the total GSK-3β expression was promoted in the group treated with P38 inhibitor in the presence of compression and adiponectin (**Figures 4E,F**). The total expression of β-catenin was slightly reduced in all the groups treated with MAPK inhibitors and compression in the presence or absence of adiponectin in comparison to controls (**Figures 4E,F**), indicating that adiponectin modulates compression-induced GSK-3β and β-catenin expression partly throughout P38 MAPK signaling.

We next tested the effects of knocking down of *AdipoR1*, *AdipoR2*, *P38α*, *ERK1*, *ERK2*, and *JNK1* on adiponectin-stimulated TCF/LEF-sensitive transcription in cementoblasts (**Figure 4G**). Consistent with the effect on β-catenin accumulation, silencing *JNK1* and *P38α* attenuated the TCF/LEF reporter transcription (**Figure 4G**). The effect of silencing RNA targeting *AdipoR1* and *AdipoR2* results in a slight inhibition of TCF/LEF transcription, indicating the involvement of adiponectin receptors in this process (**Figure 4G**).

Adiponectin Modulates GSK-3β and β-Catenin by AdipoR1 Commitment, and P38α and JNK1 Trigger β-Catenin Activation Due to Adiponectin Addition

To define the individual contribution of the adiponectin receptors as well as the MAPK isoforms stimulated with adiponectin on β-catenin regulation, we silenced the mRNA expression of *AdipoR1*, *AdipoR2*, *ERK1* (*MAPK3*), *ERK2* (*MAPK1*), *JNK1* (*MAPK8*), and *P38α* (*MAPK14*) using siRNA transfection. The efficacy of the gene knock-down by siRNA transfections was analyzed by RT-PCR. After a transfection period of 48 h, we could observe effective down-regulation of the target genes (**Figure 5A**).

Next, we stimulated the cells with adiponectin (100 ng/ml), and we observed that the group treated with siRNA against *AdipoR2* showed significant increases of *GSK-3β* ($^*p < 0.05$) and *β-catenin* mRNA levels ($^*p < 0.05$), whereas mock treatment did not alter either *GSK-3β* or *β-catenin* mRNA levels significantly. Conversely, the group treated with siRNA against *AdipoR1* did not show significant differences in the expression of *GSK-3β* or *β-catenin* (**Figure 5B**).

After a 48-h incubation with siRNA against *ERK1*, *ERK2*, *JNK1*, and *P38α* mRNA or mock treatment in the presence or absence of adiponectin, RT-PCR was performed to analyze *GSK-3β* or *β-Catenin* mRNA expression. Adiponectin treatment in *JNK1* and *P38α* siRNA groups has a down-regulation effect on the *GSK-3β* mRNA expression. The silencing of *ERK2* but not that of *ERK1* causes the *GSK-3β* mRNA up-regulation after adiponectin addition ($^{**}p < 0.01$). The single knock-down of *JNK1* or *P38α* results in the reduction of *β-catenin* mRNA expression. This downregulation was rescued after adiponectin addition (100 ng/ml; **Figure 5C**).

After cementogenesis induction, knock-down of *AdipoR1* or *AdipoR2* decreased *osteocalcin* (*OCN*) and *osteoprotegerin* (*OPG*) mRNA expression at varying degrees ($^*p < 0.05$; **Figure 5D**). In the presence of adiponectin, the single silencing of *P38α*, but not that of *ERK1*, *ERK2*, or *JNK1*, significantly decreases *OCN* and *OPG* expression. The showed data indicate that the adiponectin/AdipoR1/P38α cascade is particularly involved in adiponectin-induced cementogenesis ($^{**}p < 0.01$).

DISCUSSION

In the present study, we demonstrated that adiponectin as well as compressive forces activate the MAPK and β-catenin pathways on cementoblasts. Through a cross-link mechanism, compression as well as adiponectin induce β-catenin accumulation on OCCM-30 cells by MAPK pathway commitment principally via AdipoR1. This effect involves multiple mediators including JNK1 and P38α that are key players in this process.

We observed that adiponectin can interact with cementoblasts and influence their biological response to compressive forces promoting the expression of p-P38, p-ERK1/2, and p-JNK. Interestingly, we found a longer up-regulation period of p-P38 and p-ERK1/2 by co-stimulation of adiponectin combined with

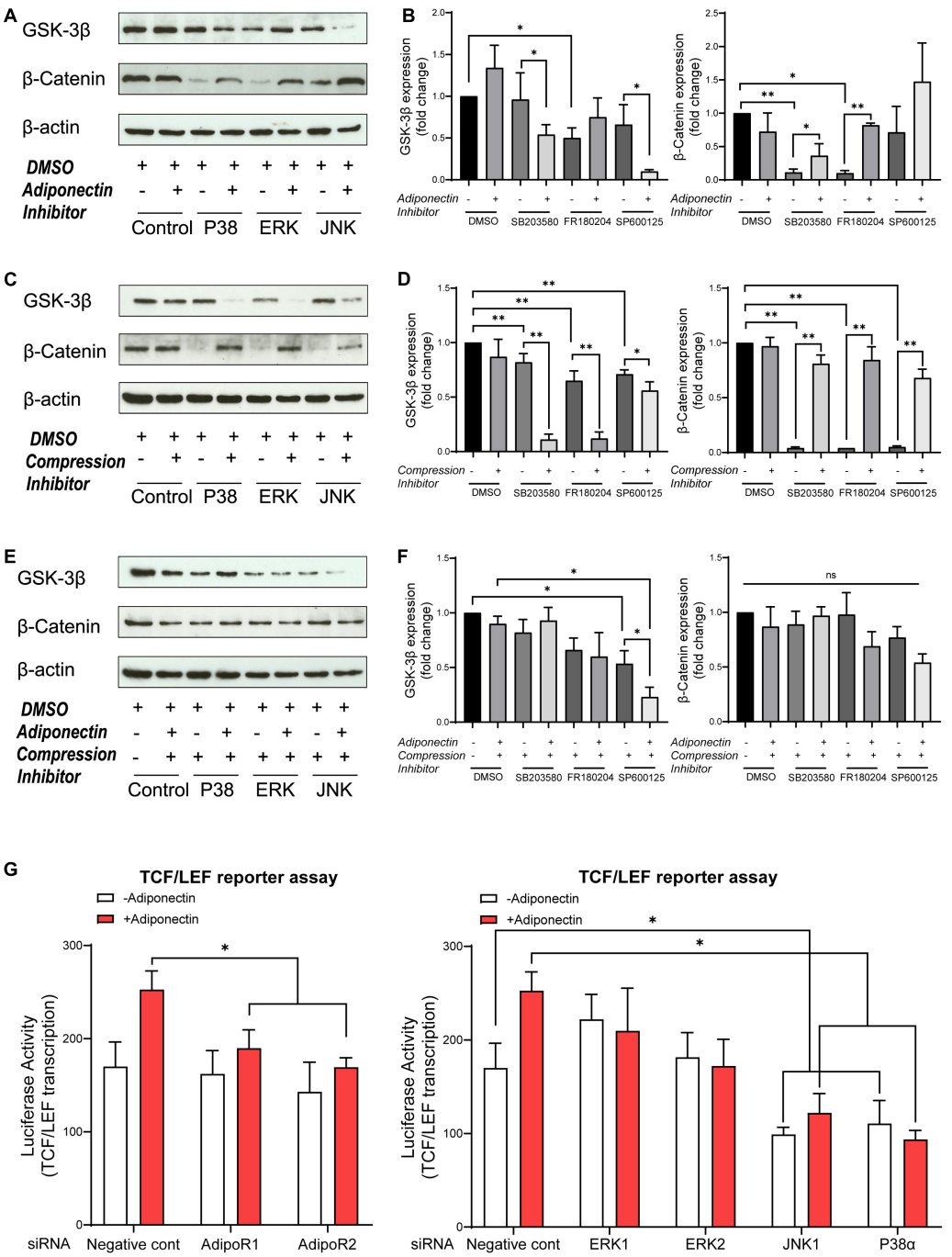


FIGURE 4 | MAPK inhibition blocks β-catenin, whereas adiponectin addition effectively rescues its expression. **(A,B)** Western blots indicate that GSK-3β expression was influenced by MAPK inhibitors 1 h after adiponectin addition: pictures show that addition of SB203580 (P38) and SP600125 (JNK) inhibitors to OCCM-30 cells up-regulate GSK-3β protein expression. The expression of β-catenin was reduced after P38 and ERK1/2 inhibition. The suppressed β-catenin signaling could be rescued by adiponectin (100 ng/ml) in different degrees. **(C,D)** Compressive forces of 2.4 gf/cm^2 decrease the expression of total GSK-3β protein in cells pretreated with SB203580 (P38), SP600125 (JNK), and FR180204 (ERK1/2) inhibitors and increase the expression of cellular β-catenin. Single suppression of P38, ERK, and JNK blocks β-catenin, but its expression was rescued by compression stimulation. **(E,F)** The negative effect that ERK1/2 and JNK inhibition exerts on the expression of total GSK-3β protein on OCCM-30 cells exposed to compression is not altered by adiponectin addition for 1 h of co-stimulation. P38 inhibitor combined with compression decreases the expression of GSK-3β. This effect was enhanced by adiponectin addition. The expression of β-catenin was not significantly altered in the presence of MAPK inhibitors and compression despite adiponectin. Graphics show the variations of GSK-3β and β-catenin protein expression as fold change when cells were exposed to MAPK inhibitors in cells cultivated under compressive forces (2.4 gf/cm^2) and/or adiponectin (100 ng/ml) compared to controls. **(G)** OCCM-30 transfected with siRNA (*AdipoR1*, *AdipoR2*, *P38α*, *JNK1*, *ERK1*, and *ERK2*) as well as TCF/LEF luciferase reporter vector were subsequently treated with adiponectin for 1 h. Asterisks indicate statistical significance (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$; ns, not significant).

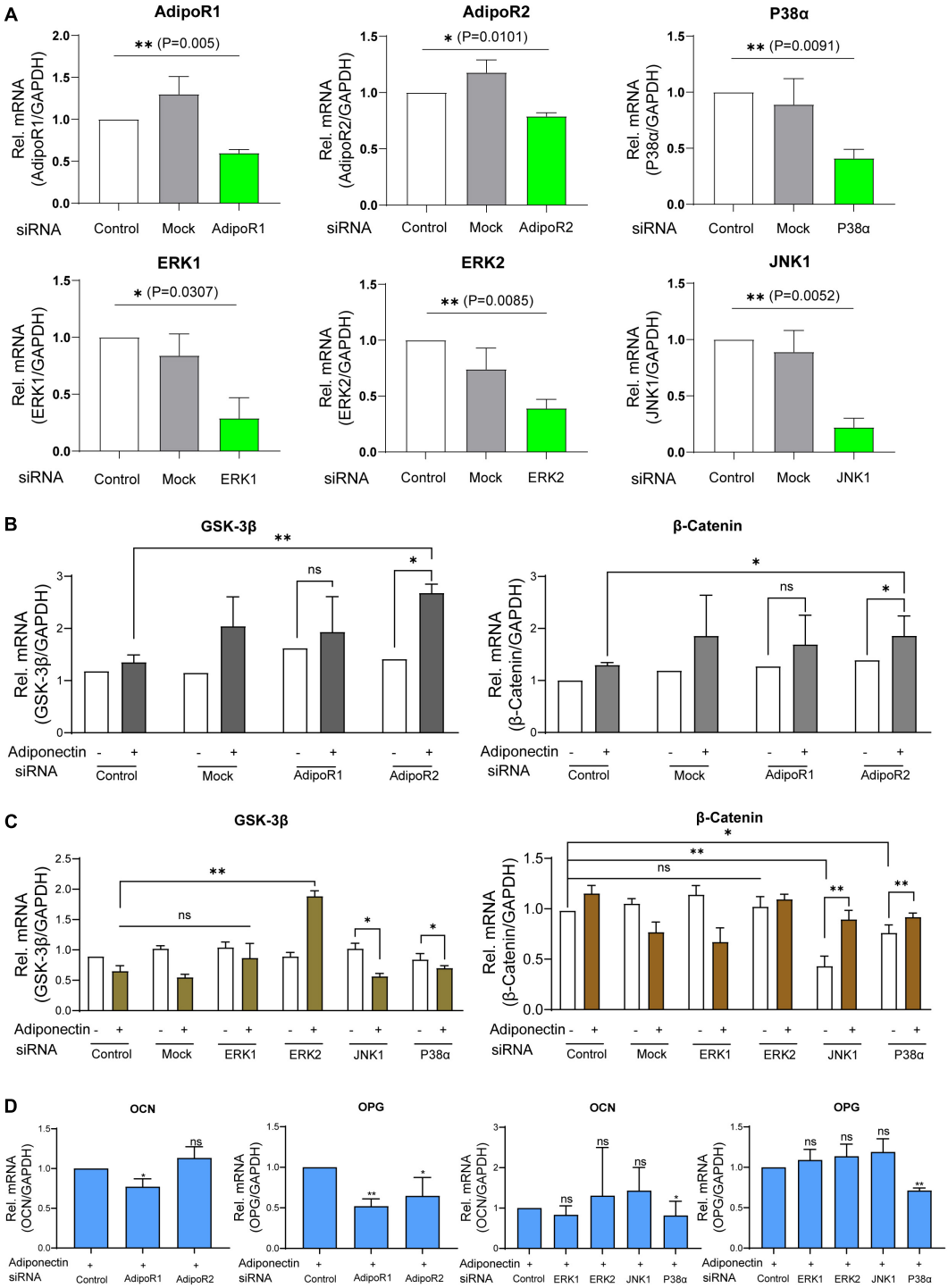


FIGURE 5 | Adiponectin/AdipoR1/P38α cascade is particularly involved in adiponectin-induced cementogenesis. **(A)** The efficacy of siRNA transfections was analyzed by RT-PCR analysis. **(B)** Single knocking down of *AdipoR2* in the presence of adiponectin has a positive effect on *GSK-3*β gene expression and significantly increases β-*catenin* expression (*$p < 0.05$). **(C)** The single silencing of *ERK2* causes increased gene expression of *GSK-3*β after adiponectin addition (**$p < 0.01$). Silencing of *P38*α and *JNK1* slightly activates *GSK-3*β gene expression. After adiponectin addition, its expression was significantly decreased (*$p < 0.05$). Single suppression of *P38*α or *JNK1* significantly decreased β-*catenin* expression (**$p < 0.01$ and *$p < 0.05$, respectively), an effect that was restored after adiponectin addition in both groups. **(D)** Single knocking down of *AdipoR1* or *AdipoR2* has a down-regulating effect on *OCN* (*$p < 0.05$) and *OPG* (**$p < 0.01$) mRNA expression. The single silencing of P38α causes a significant down-regulation of *OCN* (*$p < 0.05$) and *OPG* gene expression in the present of adiponectin (100 ng/ml) (**$p < 0.01$). These effects were not observed by *ERK1*, *ERK2*, and *JNK1* single suppression. Asterisks indicate statistical significance (***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$; ns, not significant).

compression, while the expression levels of p-JNK were altered after co-stimulation and reached an expression peak later. This indicates that adiponectin additionally triggers the MAP kinase pathway. In accordance with our previous data (2020), adiponectin strongly induced the expression of phosphorylated P38, ERK1/2, and JNK on cementoblasts (Yong et al., 2020).

The cooperative interaction between the β-catenin and MAPK signaling pathways has been reported in several studies (Bikkavilli et al., 2008; Thornton et al., 2008; Bikkavilli and Malbon, 2009; Osaki and Gama, 2013). On MC3T3-E1 cells, MEKK2 commitment promotes bone formation by rescuing β-catenin degradation (Greenblatt et al., 2016). Chen et al. (2010) reported that the blockade of P38 phosphorylation eliminates the activation of WNT signaling in preosteoblasts, indicating that osteoblast differentiation triggered by P38 MAPK/β-catenin promotes bone growth in female Sprague Dawley rats (Chen et al., 2010). Decreased phosphorylated P38 and increased ERK protein levels facilitate the β-catenin pathway through enhancing the expression of WNT3 and β-catenin in MC3T3-E1 cells (Guo et al., 2017). TGF-β-activated kinase 1 was reported to promote the phosphorylation of P38, JNK, and β-catenin, and at the same time, it down-regulated GSK-3β expression in mesenchymal stem cells (Yang et al., 2018). In contrast, Wang et al. (2012) showed that pretreatment with a P38 inhibitor (SB203580) on primary osteoblasts did not affect β-catenin protein expression (Wang et al., 2012).

In the present study, we observed that JNK, ERK1/2, as well as P38 chemical inhibition reduce β-catenin expression on OCCM-30 cells time dependently. However, the single suppression of JNK exerted a delayed effect on β-catenin reduction in comparison to cells treated with SB203580 (P38) and FR180204 (ERK1/2) antagonists. Furthermore, the single gene silencing of *ERK1* and *ERK2* as well as *P38*α and *JNK1* showed that adiponectin-induced β-*catenin* activation on OCCM-30 cementoblasts was especially sensitive to *P38*α and *JNK1* knock-down. *JNK1* and *P38*α silencing negatively regulates β-*catenin*, whereas *ERK1* has a significant positive effect on *GSK-3*β expression. Furthermore, β-*catenin* expression was up-regulated after adiponectin addition. In these contexts, our results reveal the role of both molecules triggering adiponectin–MAPK and β-catenin signaling interactions. Our data is consistent with previous studies (Thornton et al., 2008; Sakisaka et al., 2016). Thornton et al. (2008) showed that P38 MAPK inactivates GSK-3β, and this inactivation leads to an accumulation of β-catenin in the brain and thymocytes. Sakisaka et al. (2016) showed that P38 MAPK modulates β-catenin transcriptional activity, but has no effects on the phosphorylated GSK-3β as well as β-catenin expression in dental follicle cells. However, our present results do not clarify the relationship between the expression of GSK-3β and the phosphorylated status of MAPK signaling mediators (P38, JNK, and ERK1/2). Therefore, future studies should be performed to elucidate this mechanism in detail.

In the current study, we verified that compression forces activate β-catenin signaling in OCCM-30 cells. This finding is in accordance with the results of Shuqin et al. (2015) describing that the effect of mechanical strain on OCCM-30 cells regulate RUNX2 and β-catenin expression (Shuqin et al., 2015). Recently, Sindhavajiva et al. (2018) showed that compression induces human mandibular-derived osteoblast differentiation via WNT/β-catenin signaling. However, Korb et al. (2016) demonstrated the compression-induced apoptosis of human primary cementoblasts by up-regulation of the pro-apoptotic gene AXUD1 via a JNK-dependent pathway. Our study shows that the inhibition of β-catenin due to MAPK inhibition was restored by the application of light compressive forces on cementoblasts. Based on this observation, we conclude that MAPK activation is the bridge factor between compression application and β-catenin signaling activation. The proposed schema (**Scheme 1**) depicts that adiponectin activates β-catenin indirectly through the MAPK pathway. Compression forces induced an accumulation of β-catenin, whereas this process could be interacted by MAPK signaling especially through P38α.

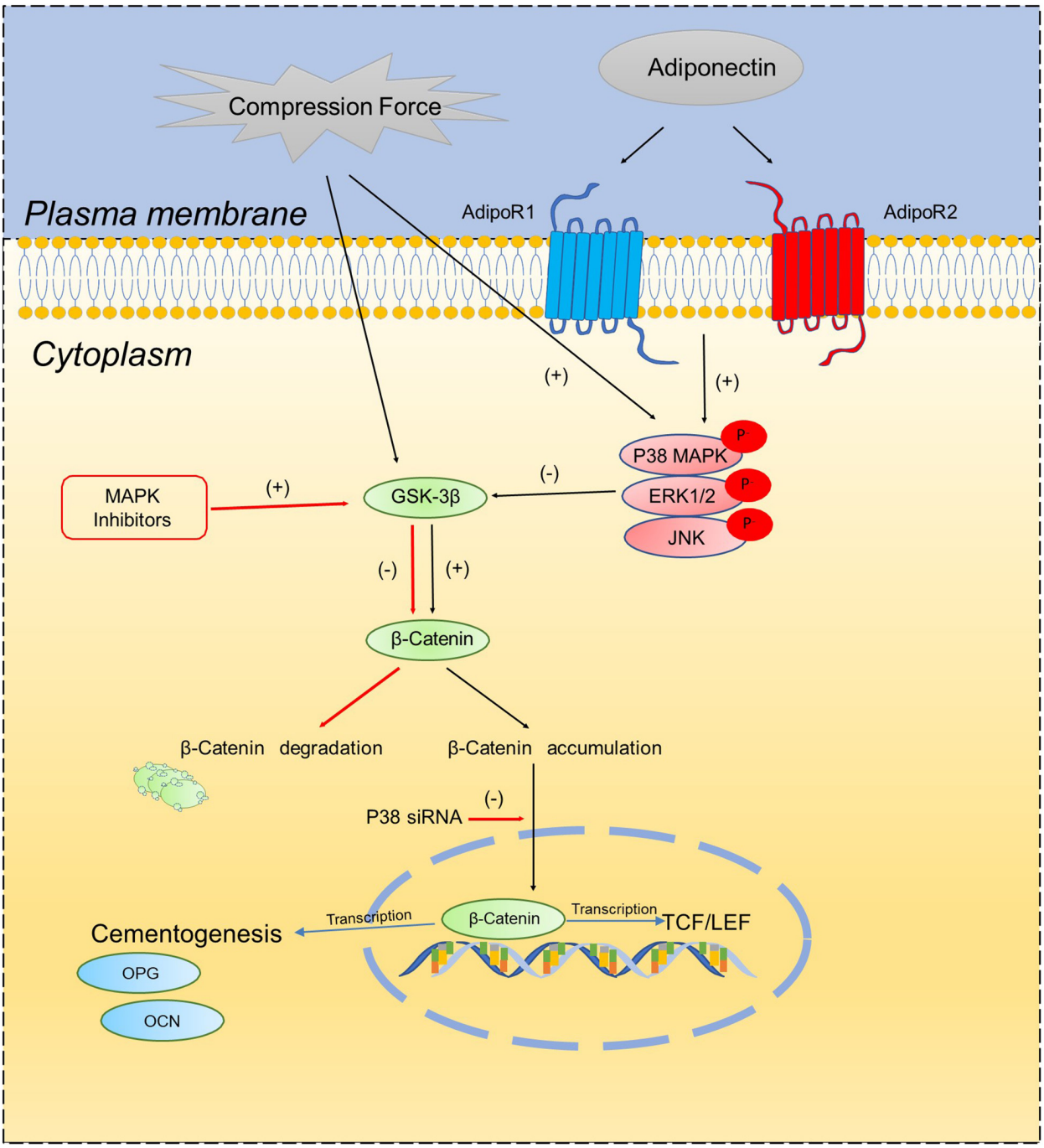
An important observation that warrants discussion is that adiponectin addition combined with compression to OCCM-30 cells did not significantly affect β-catenin expression, even under the inhibition of MAPKs. This fact suggests that adiponectin addition exerts a multistep process in β-catenin modulation that needs to be further investigated. However, all present results imply that MAPK signaling participates in the process of the β-catenin signaling on cementoblasts.

Wang et al. (2017) reported *in vivo* and *in vitro* that adiponectin could facilitate bone mesenchymal stem cell (BMSC) osteogenic differentiation and osteogenesis by the WNT/β-catenin pathway. Nemoto et al. (2009) indicated that the inhibition of GSK-3β on cementoblasts suppresses alkaline phosphatase (ALP) activity and the gene expression of *ALP*, *bone sialoprotein* (*BSP*), and *osteocalcin* (*OCN*) but promotes cell proliferation.

In our experiment, knock-down of *AdipoR2* in OCCM-30 cells induces β-*catenin* up-regulation after adiponectin addition. Furthermore, our findings showing that the up-regulation of GSK-3β mRNA expression occurred after adiponectin addition in AdipoR2-silenced group strongly suggest that the modulation effect that adiponectin exerts on β-*catenin* expression is mostly mediated by AdipoR1. These data raise the possibility that adiponectin indirectly up-regulated β-catenin through the adiponectin/adipoR1 pathway.

OCN and *OPG* mRNA levels, which were identified as cementogenesis biomarkers (d'Apuzzo et al., 2013), were decreased after *P38*α knock-down in the presence of adiponectin. Hence, there is a possibility that *OCN* and *OPG* can be altered by the interplays between MAPK and β-catenin signaling in the cellular responses of cementoblasts under adiponectin and compression forces.

Clinical studies have described that the level of adiponectin is reduced in the serum of obese individuals (Hug et al., 2004). In this context, obese patients undergoing orthodontic treatment may be influenced by these lower levels of circulating adiponectin. As constant or intermittent mechanical compression is present on the stress side of the tooth root surface, we could speculate that less expression of adiponectin–MAPK signaling in this area leads to less activation of β-catenin. The possible clinical implication of this study may be that depressed adiponectin levels in obese subjects during orthodontic tooth



SCHEME 1 | Schematic diagram illustrates the proposed molecular interactions between MAPK signaling pathways and β-catenin on OCCM-30 cementoblasts cultivated under compressive forces and adiponectin.

movement may affect OIIRR in response to mechanical stimulation by decreasing β-catenin, which is capable of inhibiting mediators such as *OPG* and *OCN* that form extracellular matrices in OCCM-30 cells (Qiao et al., 2011). However, further biology response of the cross-talk network into how adiponectin effects such as promoting apoptosis should be identified.

CONCLUSION

In conclusion, we demonstrated that adiponectin as well as compressive forces stimulate both the MAPK and β-catenin signaling pathways on cementoblasts. The inhibition of P38, JNK, and ERK1/2 differently regulates β-catenin expression as well as TCF/LEF transcription. Furthermore, adiponectin regulates the

compression-induced β-catenin signaling via cross-interacting with the MAPK signaling pathway.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JY acquired and analyzed the data. JY, GR-H, and JB interpreted the data and wrote the manuscript. JY, GR-H, and SR conceived, designed, and supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.645005/full#supplementary-material

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REVIEW
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LRP5 and LRP6 in Wnt Signaling: Similarity and Divergence

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The canonical Wnt/β-catenin signaling plays a fundamental role in regulating embryonic development, injury repair and the pathogenesis of human diseases. In vertebrates, low density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6), the single-pass transmembrane proteins, act as coreceptors of Wnt ligands and are indispensable for Wnt signal transduction. LRP5 and LRP6 are highly homologous and widely co-expressed in embryonic and adult tissues, and they share similar function in mediating Wnt signaling. However, they also exhibit distinct characteristics by interacting with different protein partners. As such, each of them possesses its own unique functions. In this review, we systematically discuss the similarity and divergence of LRP5 and LRP6 in mediating Wnt and other signaling in the context of kidney diseases. A better understanding of the precise role of LRP5 and LRP6 may afford us to identify and refine therapeutic targets for the treatment of a variety of human diseases.

Keywords: LRP5, LRP6, Wnt, β-catenin, YAP, kidney fibrosis, chronic kidney disease

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INTRODUCTION

Wnt/β-catenin is an evolutionarily conserved developmental signaling that plays a critical role in cell fate determination, organ development, injury repair and the pathogenesis of human diseases (Clevers, 2006; Tan et al., 2014). The term "Wnt" was derived from a combination of *Drosophila* segmental polarity gene "*Wingless*" and mouse protooncogene "*Int-1*" (Luo et al., 2007; Clevers and Nusse, 2012). Wnt ligands are a family of secreted glycoproteins, consisting of 19 members in mammals (MacDonald et al., 2009; Kawakami et al., 2013). Based on the involvement of key intracellular mediator β-catenin, Wnt signaling is divided into canonical, β-catenin-dependent and non-canonical, β-catenin-independent pathways.

In the quiescent state, β-catenin in the cytoplasm is phosphorylated and degraded by the so-called "destruction complex" in the absence of Wnt ligands. The complex consists of Axin, adenomatous polyposis coli (APC), dishevelled (Dvl), casein kinase 1 (CK1) and glycogen synthase kinase-3 (GSK-3) (Wang et al., 2018; Gajos-Michniewicz and Czyz, 2020). CK1 and GSK3 phosphorylate β-catenin sequentially, thereby tagging β-catenin for ubiquitination/degradation (Polakis, 2002; Tamai et al., 2004). In the Wnt activation state, the binding of Wnt ligands to the seven-pass transmembrane Frizzled (Fzd) receptor and its co-receptors, the low density lipoprotein receptor-related protein -5 or -6 (LRP5/6), leads to dimerization of the two receptors on cell surface and induces conformational changes of these receptors (Nusse and Clevers, 2017). The cytoplasmic tail of LRP5/6 is then phosphorylated by several protein kinases, and subsequently recruits Axin and inhibits the activity of GSK3, resulting in the dissociation of the β-catenin destruction complex

(Wu et al., 2009; Kim et al., 2013). As a result, β-catenin cannot be phosphorylated and degraded. This leads to the stabilization and nuclear translocation of β-catenin, where it binds to transcription factors of the T-cell factor (TCF) and lymphoid enhancer-binding factor (LEF) families to activate the expression of Wnt target genes (van de Wetering et al., 2002; Rao and Kuhl, 2010).

In vertebrates, there are 19 different Wnt ligands and 10 Fzd receptors, but only two coreceptors (LRP5/6) in Wnt signaling (van Amerongen and Nusse, 2009). LRP5/6 are known to play a crucial role in the initiation of Wnt signal transduction, and inhibition of their function blocks Wnt/Fzd signaling (Tamai et al., 2000). While plentiful excellent reviews on Wnt signaling in human diseases, particularly the kidney disorders, have been published (Clevers and Nusse, 2012; Zhou and Liu, 2015; Zuo and Liu, 2018), relatively less is reported on the regulation and divergent functions of LRP5 and LRP6 (Joiner et al., 2013). In this study, we systematically review the structure, regulation and function of LRP5 and LRP6 in the context of kidney diseases, with emphasis on their commonality and uniqueness in mediating Wnt signaling.

COMPONENTS OF WNT RECEPTOR COMPLEX AT THE PLASMA MEMBRANE

There are several structurally unrelated, transmembrane receptor proteins that mediate different Wnt signaling (Grumolato et al., 2010). Both canonical and non-canonical Wnt ligands use common receptors of the Fzd family; however, they employ different co-receptors. LRP5/6 coreceptors are used for canonical Wnt ligands, whereas non-canonical ligands choose other transmembrane proteins (Garcia de Herreros and Dunach, 2019).

Frizzled Receptors

In humans, there are 10 Fzd proteins, named Fzd1-10 (MacDonald and He, 2012). Fzd is a seven-pass transmembrane, atypical G protein-coupled receptor protein. The N-terminus of Fzd contains a conserved 120 amino acid cysteine-rich domain (CRD), which is the main region of Wnt binding (Nusse and Clevers, 2017). The interaction between Wnt and Fzd is promiscuous, in that a single Wnt ligand can bind multiple Fzd and a single Fzd can bind multiple Wnt ligands (Clevers, 2006). It has been shown that the specificity of Wnt signaling depends, at least in part, on the affinities between different Wnt/Fzd pairs (Hsieh, 2004). In addition, the potency of different Fzd proteins to activate canonical Wnt pathway appears not equivalent. Studies have shown that the distinction between canonical Wnt1 and non-canonical Wnt5a lies mainly in the Fzd proteins that interact with them (Holmen et al., 2002). However, it is unclear how the signal initiated by Wnt binding to the CRD of Fzd receptor is transduced across plasma membrane.

The current model for Wnt signaling is that the binding of Wnt to Fzd and LRP5/6 leads to the dimerization or clustering of these two receptors, resulting in the formation of ternary complexes together with different downstream components (Hsieh, 2004; Nusse and Clevers, 2017). Activated Fzd recruits and binds Dvl, enabling Dvl to self-polymerization via its DIX domain (Gammons et al., 2016). The Dvl DIX multimers then further recruit Axin by interacting with the Axin DIX domain (Bienz, 2014), and the locally increased Axin and its associated kinases contribute to the phosphorylation of the cytoplasmic tail of LRP6 (Tamai et al., 2004; Gerlach et al., 2018). Fzd receptors themselves, however, hardly activate the canonical Wnt pathway.

LRP5/6 Co-Receptors

Low-density lipoprotein receptors (LDLR) are involved in a variety of cellular functions. The LDLR family members include LDL receptor, LRP (also named LRP1), megalin (LRP2), VLDL receptor, apoER2 (LRP8), SorLA/LR11, LRP1b, LRP3, MEGF7 (LRP4), and LRP5/6 (Schneider and Nimpf, 2003; Chung and Wasan, 2004). However, each member of the family is expressed in many different tissues and has a wide range of different ligands (Chung and Wasan, 2004; Joiner et al., 2013). Moreover, LRP5 and LRP6 are unique in the number and arrangement of their LDLR repeats compared to other members of the LDLR family (Brown et al., 1998). LRP5 and LRP6, as co-receptors of Wnt ligands and key components of their receptor complex, are necessary for canonical Wnt signaling (Pinson et al., 2000; Tamai et al., 2000), and are the focus of this review.

(Pro)renin Receptor

(Pro)renin receptor (PRR) is a single-pass transmembrane protein at the plasma membrane that transmits renin and prorenin signals (Nguyen et al., 2002). It has been shown that PRR, as a component of Wnt receptor complex, can promote and augment Wnt signaling, although overexpression of PRR itself does not activate Wnt/β-catenin signaling (Cruciat et al., 2010). It is found that phosphorylation of LRP6, which is associated with LRP6 activation, requires vacuolar H^+-adenosine triphosphatase (V-ATPase) activity. (Pro)renin receptor, as a specific adaptor between LRP6 and V-ATPase, is an essential component of the Wnt receptor complex and obligatory for its signal transduction in a (pro)renin-independent manner (Cruciat et al., 2010). Meanwhile, PRR is a direct downstream target of Wnt/β-catenin *in vitro* and *in vivo* (Li et al., 2017). As such, PRR induction and Wnt/β-catenin activation instigate a vicious, self-perpetuating cycle, leading to the amplification of the Wnt/β-catenin signaling (Li et al., 2017; Zuo and Liu, 2018).

Other Receptors

Wnts can also transmit their signal through β-catenin-independent, non-canonical pathway. There are mainly two non-canonical Wnt signaling, the Wnt/planar cell polarity (PCP) pathway and Wnt/Ca^{2+} pathway. The Wnt/PCP signaling can be initiated through the interaction between Wnt and Fzd receptors and their co-receptors, the receptor-like tyrosine kinase (RYK) and receptor tyrosine kinase-like orphan receptor (ROR), to recruit and activate Dvl, which then serves as a scaffold and activator for RhoA and Ras. Activated RhoA and Ras then regulate the activities of Rho-associated kinase (ROCK) and c-Jun N-terminal kinase (JNK), thereby participating in polarized cell orientation and asymmetric

cell movement (Kohn and Moon, 2005; Krishnamurthy and Kurzrock, 2018; VanderVorst et al., 2019). In the Wnt/Ca^{2+} pathway, Wnt binding to Fzd activates Dvl, which leads to Ca^{2+} release from the endoplasmic reticulum and thus activates Ca^{2+}/calmodulin dependent kinase II (CaMKII) and calcineurin. Activated calcineurin via dephosphorylation activates the nuclear factor of activated T cells (NFAT), which translocates to the nucleus and regulates the expression of target genes, thereby regulating cell fate (Krishnamurthy and Kurzrock, 2018; Gajos-Michniewicz and Czyz, 2020).

STRUCTURAL AND FUNCTIONAL SIMILARITY OF LRP5 AND LRP6

The mature protein encoded by human and mouse *Lrp5* cDNAs are 95% identical, indicating that the protein is highly conserved during evolution (Hey et al., 1998). Similarly, the proteins encoded by mouse and human *Lrp6* share 98% identity each other (Brown et al., 1998). Meanwhile, the amino acid sequences of LRP6 and LRP5 proteins have 71% identity (Brown et al., 1998).

Structural Domains of LRP5 and LRP6

LRP5 and LRP6 are single-pass transmembrane proteins with multiple domains (**Figure 1**). The first 24 amino acids at the N-terminus of LRP5 and the first 19 amino acids of LRP6 are predicted to be signal peptides for protein export across the plasma membrane (Brown et al., 1998). The mature proteins of LRP5 and LRP6 are generated by cleavage and removal of the signal peptide. The extracellular domain of LRP5 and LRP6 contains four tandem YWTD-type β-propeller (BP) domains, each followed by an epidermal growth factor (EGF)-like domain, which are named as E1 to E4 from N- to C- terminus. These extracellular domains are responsible for binding Wnt ligands and their inhibitors, such as Dickkopf-related protein 1 (DKK1) and sclerostin (He et al., 2004; Gong et al., 2010; DeBruine et al., 2017). It has been demonstrated that many Wnts such as Wnt1, Wnt2, Wnt2b, Wnt6, Wnt8a, Wnt9a, Wnt9b, and Wnt10b interact with E1E2 domain, while Wnt3 and Wnt3a prefer E3E4 domain (**Figure 1**). Other Wnts including Wnt7a, Wnt7b, and Wnt10a cannot be classified into either group, indicating the possibility that these Wnts may bind to regions other than E1E2 and E3E4 (Gong et al., 2010; MacDonald and He, 2012). These domains are followed by three LDLR type A domains (He et al., 2004). In general, the position and sequence of YWTD motifs are highly conserved in LRP5 and LRP6. However, the LDLR repeats between LRP6 and LRP5 are not highly conserved. Particularly, LDLR3 is only 51% identical between LRP6 and LRP5 (Brown et al., 1998). Mutagenesis studies have shown that LDLR repeats are essential for ligand binding, suggesting that LRP5 and LRP6 may bind related but not highly similar ligands. The cytoplasmic domain of LRP6 consists of 218 amino acid residues, which has 64% identity with LRP5 (Brown et al., 1998). The intracellular domains of LRP5 and LRP6 are rich in proline and serine and contain five reiterated and conserved PPPSPxS motifs, which are the binding sites for Axin and are essential for LRP6 signaling (He et al., 2004; Tamai et al., 2004) (**Figure 1**).

Expression Patterns of LRP5 and LRP6 in Human Tissues

In humans, LRP5 expression level is the highest in liver, while substantial level of expression is also observed in pancreas, prostate, placenta and small intestine. LRP5 expression is detectable in ovary, thymus, skeletal muscle, colon, spleen, kidney, testis, heart, and lung as well, whereas the expression level of this receptor in brain and peripheral leukocytes is very low (Hey et al., 1998). Human LRP6 expression is highest in ovary, with significant levels in the heart, brain, placenta, lung, kidney, pancreas, spleen, and testis. Lower levels of human LRP6 are observed in liver, skeletal muscle, prostate and the mucosal lining of the colon, while the expression of LRP6 is very low in peripheral blood leukocytes, thymus and small intestine (Brown et al., 1998).

It appears that LRP6 is expressed at higher levels in the brain and kidneys than LRP5. Conversely, LRP5 appears to be expressed at higher levels than LRP6 in the liver, thymus, prostate, and small intestine (Brown et al., 1998). In addition, LRP5 is mainly expressed in renal convoluted tubules, but not in the glomeruli or collecting ducts (Figueroa et al., 2000). Both LRP5 and LRP6 are expressed in airway epithelium during lung development, whereas LRP5 but not LRP6 expression is observed in the muscular component of large blood vessels, including the aorta (Wang et al., 2005). These results indicate that LRP5 and LRP6 receptors are expressed differently in various tissues and organs, suggesting that LRP5 and LRP6 may play distinct roles in the pathogenesis of various diseases.

LRP5 and LRP6 in Organ Development

LRP5 and LRP6 are widely co-expressed during embryonic development (Pinson et al., 2000; Houston and Wylie, 2002; He et al., 2004). $Lrp6^{-/-}$ mice die at birth and exhibit a variety of severe developmental abnormalities, including a truncation of the axial skeleton, mid/hindbrain defects, limb defects, microophthalmia, and urogenital malformation (Pinson et al., 2000). These developmental defects are very similar to those of mice carrying Wnt gene mutations, especially *Wnt3a*, *Wnt1*, and *Wnt7a*, but the defects in *Lrp6* mutant embryos are usually less severe than those observed by individual *Wnt* mutants (Pinson et al., 2000). $Lrp5^{+/-}/Lrp6^{-/-}$ and $Lrp5^{-/-}/Lrp6^{-/-}$ embryos arrest prior to mid-gestation, indicating functional redundancy between LRP6 and LRP5 (Kelly et al., 2004). $Lrp5^{-/-}$ mice have a normal morphological appearance and are viable and fertile, but exhibit osteoporosis, subtle defects in eye vasculature (Kato et al., 2002), and metabolic abnormalities (Fujino et al., 2003), suggesting that LRP6 is more important than LRP5 in embryogenesis. Indeed, LRP6 is much more potent in activating Wnt signaling in response to a Wnt ligand in 293T cells (Holmen et al., 2002).

An allelic series of compound mutants reveals the order of progressive loss of Wnt signaling and the severity of developmental abnormalities: $Lrp5^{+/-}$ (normal) $< Lrp6^{+/-} < Lrp5^{-/-} < Lrp5^{+/-}/Lrp6^{+/-} < Lrp5^{-/-}/Lrp6^{+/-} < Lrp6^{-/-} < Lrp5^{+/-}/Lrp6^{-/-} < Lrp5^{-/-}/Lrp6^{-/-}$, indicating that loss of *Lrp6* alleles consistently produces a more

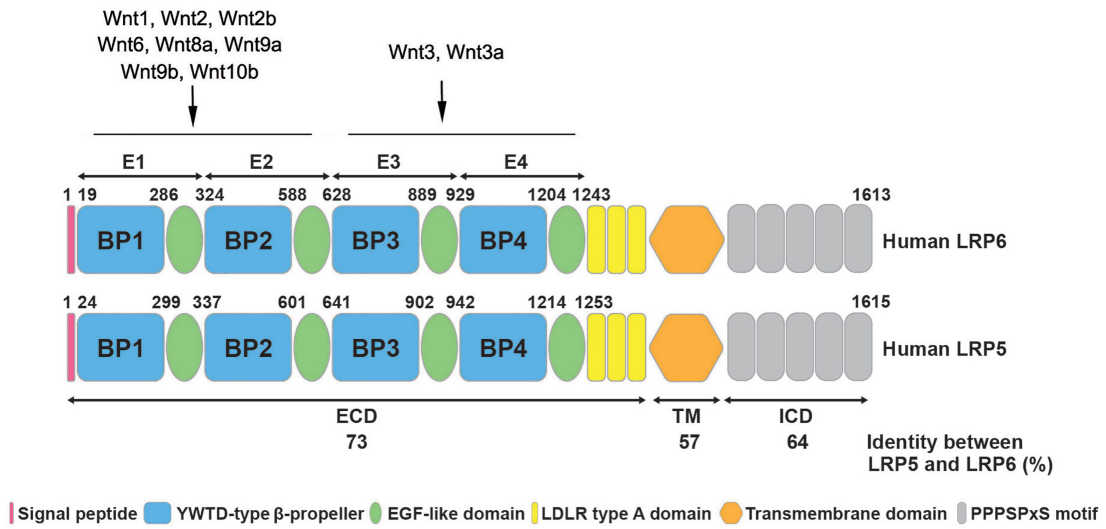

FIGURE 1 | Structural similarity of human LRP5 and LRP6 proteins. The schematic diagram shows the structure of human LRP6 and LRP5 with different colors representing various domains. The numbers listed at the top of the domain represent the boundaries of signal peptides and four EGF-like domains in the full-length protein. The numbers at the bottom shows the amino acid identity between human LRP6 and LRP5. The binding sites of LRP5/6 to different Wnt ligands are indicated. ECD, extracellular domain; TM, transmembrane domain; ICD, intracellular domain.

severe phenotype than loss of *Lrp5*. This difference of LRP5 and LRP6 in organ development may be contributed by their affinity with Wnt ligands or signaling efficacy (He et al., 2004; Kelly et al., 2004). Likewise, the difference in the timing, level and location of embryonic expression of LRP5/6 or their ligands and inhibitors could account for such a separation (Kang and Robling, 2014).

SIGNAL TRANSDUCTION MEDIATED BY LRP5 AND LRP6

When Wnt binds to and activates the Fzd, leading to the recruitment of Dvl and Axin/GSK3 complex to the plasma membrane, triggering GSK3 phosphorylation of LRP5/6 PPPSP motifs (Zeng et al., 2005; Zeng et al., 2008). These series of events eventually result in dephosphorylation of β-catenin and its stabilization (MacDonald and He, 2012).

Kinases That Phosphorylate LRP5/6

A series of amino acid motifs on the intracellular domain of LRP5/6 are phosphorylated following Wnt binding (Tamai et al., 2004), which is essential for Wnt signaling. Up to date, five different kinds of protein kinases are known to phosphorylate LRP5/6, which can be divided into two categories. One is the proline-directed kinases that phosphorylate PPPSPxS motifs, namely GSK3, protein kinase A (PKA), PFTAIRE protein kinase (Pftk) members, and G-protein coupled receptor kinase (GRK5/6). The other is the non-proline directed kinases, namely members of the CK1 family, which phosphorylate the PPPSPxS motifs, the S/T cluster that is the conserved region preceding the first PPPSPxS motif and containing a serine and threonine residues, and other N-terminal sites (Niehrs and Shen, 2010). In addition, on the basis of kinome-wide small interfering RNA (siRNA) screen and confirmative biochemical analysis, a study demonstrates that several proline-directed mitogen-activated protein kinases (MAPKs), such as p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK), are sufficient and necessary for phosphorylation of the PPPSP motif of LRP6 (Cervenka et al., 2011). These studies suggest that cells not only recruit one dedicated LRP6-PPPSP kinase, but also select different kinases based on cell type and the external stimulus (Cervenka et al., 2011).

Proximal Regulatory Events of LRP5/6

The receptor complex is presumed to be a key node in Wnt signal network. However, due to the lack of molecular tools to isolate and analyze endogenous Wnt-binding components, we still lack a comprehensive understanding of the formation, composition and regulation of Wnt signalosomes. There are several proteins known to regulate LRP5/6 activity either positively or negatively. Cripto-1, which is encoded by the Cryptic family 1 gene, is shown to directly bind to LRP5/6, thereby facilitating Wnt3a binding to LRP5/6. Since Cripto-1 is located in lipid rafts, it may also promote caveolin-dependent internalization of LRP5/6, thereby enhancing the canonical Wnt/β-catenin signaling (Nagaoka et al., 2013). Biglycan, a member of the small leucine-rich proteoglycan family, has been reported to enhance canonical Wnt signaling by forming a possible trimeric complex with both Wnt and LRP6 (Berendsen et al., 2011). In addition, other studies have shown that the single-span membrane protein TMEM59 interacts with Fzd and LRP6, which promotes the formation of multimeric Wnt/Fzd complex through the intramembrane interaction, and then the Wnt/Fzd/TMEM59 assemblies merge with LRP6 to form the mature Wnt signalosomes (Gerlach et al., 2018).

In addition to the molecules that positively regulate Wnt/β-catenin signaling, several proteins that negatively regulate this pathway have also been found. The secreted proteins of DKK family, especially DKK1, antagonize Wnt/β-catenin by inhibiting Wnt co-receptor LRP6 (Mao B. et al., 2001; Niehrs, 2006). DKK1 is a high-affinity ligand for LRP6 and inhibits Wnt signaling by blocking the formation of Fzd/LRP6 complex induced by Wnt (Semenov et al., 2001). It is also reported that a secreted protein, Wise, can promote or inhibit Wnt signaling in a context-dependent manner (Itasaki et al., 2003). The Wise protein not only activates the Wnt signal cascade by mimicking some effects of Wnt ligands, but also physically interacts with LRP6 and competes with Wnt8 for bind to LRP6, thus inhibiting the Wnt signaling (Itasaki et al., 2003). Sclerostin, a secreted glycoprotein involved in the regulation of bone metabolism, has been reported to antagonize Wnt signaling by binding to the extracellular domain of LRP5/6 and disrupting Wnt-induced Fzd/LRP5/6 complex formation (Semenov et al., 2005). In addition, Mesd is a specialized molecular chaperone for LRP5/6 and is a universal inhibitor of LRP5/6 ligands (Li et al., 2005; Lu et al., 2010).

Regulation of LRP5/6/β-Catenin Signaling After Wnt Binding

Wnt ligands induce the formation of receptor protein complexes through the successive recruitment of phosphorylation-regulated factors (Vinyoles et al., 2014). It has been proposed that the phosphorylated PPPSPxS motif of LRP5/6 directly inhibits β-catenin phosphorylation by GSK3 in a sequence and phosphorylation-dependent manner, thereby stabilizing β-catenin (Wu et al., 2009). Studies also show that Wnt signaling reduces the cytoplasmic level of GSK3 by sequestering GSK into multivesicular body (MVBS), thereby extending the half-life of β-catenin and stabilizing β-catenin (Taelman et al., 2010; Vinyoles et al., 2014).

The PPPSP motif, reiterated five times in the LRP5/6 intracellular domain, is necessary and sufficient for triggering Wnt/β-catenin signaling. An LRP6 mutant lacking the intracellular domain is defective and in fact blocks Wnt signaling (Tamai et al., 2000, 2004). In contrast, LRP5/6 mutants lacking an extracellular domain, but still being anchored on the cell membrane, seem to have constitutive activity and activate Wnt/β-catenin signaling (Mao B. et al., 2001; Mao J. et al., 2001). In addition, phosphorylated PPPSPxS peptide can sufficiently activate Wnt/β-catenin signal transduction (Wu et al., 2009). Furthermore, the transfer of a single PPPSP motif to LDLR fully activates the Wnt pathway, inducing TCF/β-catenin-responsive transcription in human cells (Tamai et al., 2004).

Several studies have shown that receptor endocytosis is involved in Wnt signaling. For example, Wnt3a and DKK1 induce LRP6 to distinct internalization pathways, thereby activating or inhibiting the β-catenin signaling. Wnt3a induces the caveolin-dependent internalization of LRP6, the phosphorylation of LRP6 and the recruitment of Axin to LRP6 on the cell membrane, leading to stabilizing β-catenin, whereas DKK1 induces clathrin-dependent internalization of LRP6 and inhibits Wnt3a-induced stabilization of β-catenin (Yamamoto et al., 2008). However, the involvement of receptor endocytosis in Wnt signaling is controversial, and contradictory results have been reported regarding the role of clathrin- and caveolin-dependent receptor internalization in Wnt signal transduction (Gagliardi et al., 2008). The pharmacological and molecular tools used to block receptor endocytosis and trafficking are pleiotropic, and sometimes the non-specific effect of these manipulations complicates the interpretations of the studies (MacDonald et al., 2009).

Activation of LRP5/6/β-Catenin Signaling by Other Extracellular Cues

Besides Wnt ligands, LRP5/6 can respond to other extracellular cues, thereby leading to β-catenin activation. The R-Spondin (Rspo) family proteins act as potent activators of Wnt/β-catenin signaling by binding to Fzd8 and LRP6 receptors (Kazanskaya et al., 2004; Nam et al., 2006; Binnerts et al., 2007). It has been shown that human Rspo1 is a high affinity ligand for LRP6, inducing GSK3-dependent phosphorylation and activation of LRP6 (Wei et al., 2007). Interestingly, some studies show that Rspo1 does not directly bind and activate LRP6 but inhibits DKK1-mediated LRP6 internalization through its interaction with Kremen, thereby regulating the Wnt signaling (Binnerts et al., 2007). The different mechanisms may be due to different cell types.

Norrin, a cystine-knot like growth factor that is unrelated to Wnt, has been shown to bind to Fzd4 and LRP5/6 to form a ternary complex, thus activating Wnt/β-catenin signaling (Ke et al., 2013; Chang et al., 2015). In addition, parathyroid hormone (PTH) forms a ternary complex with its receptor PTH1R and co-receptor LRP6, which promotes the rapid phosphorylation of LRP6, leading to the recruitment of Axin to LRP6 and stabilization of β-catenin, and the activation of PKA is crucial for the stabilization of β-catenin induced by PTH (Wan et al., 2008). The extracellular enzyme transglutaminase 2 (TG2) has been reported to bind to LRP5/6 and act as an activating ligand for the LRP5/6, which may activate β-catenin by mediating cross-linking of the LRP5/6 receptors. This finding uncovers a novel activity of TG2 as an agonist of β-catenin signaling (Deasey et al., 2013). Collectively, these studies suggest that LRP5/6 may also act as a co-receptor of other ligands to activate β-catenin signaling.

Some extracellular cues do not bind to LRP6, *per se*, but regulate the activity of kinases used to phosphorylate LRP6, thereby activating β-catenin signaling. For example, hepatocyte growth factor (HGF) can stimulate GSK3-dependent and Wnt-independent LRP6 phosphorylation, thereby stabilizing β-catenin and activating Wnt signaling (Koraishy et al., 2014). In addition, fibroblast growth factor 2 (FGF2) promotes the phosphorylation of LRP6 and accumulation of β-catenin in an ERK1/2-dependent manner (Cervenka et al., 2011). It is also reported that receptor tyrosine kinase (RTK) signaling mediated by FGF receptor 2 (FGFR2) and FGFR3, tropomyosin receptor kinase A (TRKA) and EGF receptor (EGFR) activates Wnt/β-catenin signaling by employing ERK to phosphorylate the PPPSP motif of LRP6 (Krejci et al., 2012).

UNIQUE ACTIONS OF LRP6: BEYOND WNT SIGNALING

Although the overall structure of LRP5 and LRP6 is very similar, it has been shown that their Wnt signal transduction capabilities are not equivalent. For example, in *Xenopus* embryos, LRP6 alone is sufficient to induce axis duplication, while LRP5 is not (Tamai et al., 2000). In addition, overexpression of LRP6 alone, but not LRP5, activates the Wnt/β-catenin signaling in HEK293T cells (MacDonald et al., 2011). On the other hand, LRP5 is essential for mechanotransduction, whereas there is no clear evidence to date that LRP6 is involved in mechanical loading-induced activation of Wnt signaling (Kang and Robling, 2014). Recent studies show that Wnt ligands exhibit a preferential use of LRP5 or LRP6 (Singh et al., 2021). Three groups of Wnt ligands are identified based on their co-receptor specificity: (1) activation of Wnt signaling only through LRP6, (2) through both LRP5 and LRP6, and (3) predominantly through LRP5 (Singh et al., 2021).

Besides divergent roles of LRP5 and LRP6 in Wnt signaling (He et al., 2020; Lim et al., 2021; Singh et al., 2021), increasing evidence suggests that LRP6 displays broad actions that goes beyond Wnt signal transduction. LRP6 can interact with multiple protein partners and acts as a coreceptor for many other extracellular cues.

LRP6 as a Coreceptor of Various Growth Factors

Apart from its well-appreciated role in Wnt signaling, LRP6 is also involved in the regulation of multiple growth factor signaling as a co-receptor. It has been shown that LRP6 interacts closely with platelet-derived growth factor receptor β (PDGFRβ) and transforming growth factor-β (TGF-β) receptor 1 (TβR1) on the cell membrane (Ren et al., 2013). PDGF phosphorylates LRP6 and stimulates the p42/p44 MAPK and JNK to promote pericytes proliferation, while DKK1, an endogenous inhibitor of LRP6, inhibits these responses. Interestingly, pericytes lacking β-catenin also respond to PDGF, confirming that the effect stimulated by PDGF is independent of the Wnt/β-catenin signaling, even though LRP6 is activated (Ren et al., 2013). These investigators also found that both TGF-β and connective tissue growth factor (CTGF) activate LRP6 within minutes, and both stimulate the p42/p44 and JNK pathways, while DKK1 blocks activation of the p42/p44 and JNK and inhibits all migration, activation, and cytoskeletal changes in pericytes in response to TGF-β or CTGF (Ren et al., 2013).

It has also been found that the CTGF domain 4 exhibits low-affinity binding to LRP6, rapidly stimulating LRP6 phosphorylation as well as downstream effectors JNK and p42/p44 activation, which can be inhibited by DKK1 or by silencing LRP6 (Johnson et al., 2017). Moreover, LRP6 binds to several G protein-coupled receptors (GPCRs) (Wo et al., 2016; Kang, 2020). GPCR ligand, such as PTH, can not only promote the interaction between LRP6 and GPCRs and activate β-catenin signaling, but also promote the binding of LRP6 to Gα(s) βγ heterotrimer and activate Gα(s) βγ-coupled GPCR signaling, suggesting that LRP6 may play a role as a general regulator of multiple GPCRs (Wan et al., 2011; Wo et al., 2016). Moreover, another study demonstrates that LRP6 can act as a co-receptor for the PTH receptor to achieve optimal activation of PTH signaling (Pellicelli et al., 2018).

LRP6 Links Wnt to Hippo Signaling

The Hippo pathway is an evolutionarily conserved signaling cascade that regulates organ size and tissue homeostasis by governing cell proliferation and apoptosis (Hong and Guan, 2012; Imajo et al., 2012). There are two key downstream transcriptional co-activators, yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), which mediate the gene regulation and biological functions of the Hippo pathway (Hong and Guan, 2012). Activation of the Hippo pathway phosphorylates YAP/TAZ, rendering them to be sequestrated in the cytoplasm and destructed by ubiquitination-dependent proteasomal degradation (Imajo et al., 2012). Therefore, the Hippo pathway limits the availability and functionality of YAP/TAZ in the nucleus by controlling its distribution and protein levels (Hong and Guan, 2012).

It is reported that in the absence of Wnt, YAP, and TAZ are components of the β-catenin destruction complex, and YAP/TAZ are associated with the destruction complex by binding to Axin (Azzolin et al., 2014). In this regard, the destruction complex is the cytoplasmic sink of YAP/TAZ (Azzolin et al., 2014). Interestingly, the progressive dissociation of YAP/TAZ with Axin parallels with the increased association of Axin with LRP6. In essence, YAP/TAZ and LRP6 apparently compete for binding to the same domain of Axin (Azzolin et al., 2014). When cells are stimulated by Wnt or overexpressed with LRP6, LRP6 releases YAP/TAZ from the destruction complex by replacing them from Axin1, thereby inducing the expression of YAP/TAZ target genes in a YAP/TAZ-dependent manner (Azzolin et al., 2014). This model is supported by another study that DKK3 stabilizes the cell-surface levels of LRP6 by uncoupling LRP6 from the Kremen-mediated internalization machinery, resulting in concomitant activation of β-catenin and YAP/TAZ (Ferrari et al., 2019).

LRP5 mutants lacking the extracellular domain act as constitutive active forms that bind Axin and induce LEF-1 activation by destabilizing Axin and stabilizing β-catenin (Mao J. et al., 2001). Intriguingly, overexpression of full-length LRP5 alone has no effect on the canonical Wnt signaling but acts synergistically with Wnt (Mao J. et al., 2001). Consistent with this result, LRP5 is shown to be associated with Axin, and Wnt3a increases the association of LRP5/Axin (Hay et al., 2009). However, either full-length LRP5 or LRP5 mutants have no effect on YAP/TAZ activity.

Our own data suggest that overexpression of LRP6 alone can activate not only β-catenin but also YAP/TAZ, whereas LRP5 merely activate β-catenin (Ren et al., data unpublished). These results could be explained by two possible mechanisms. One is that overexpression of LRP6 alone leads to homo-oligomerization of LRP6 and conformational changes to form activated forms, thus recruiting Axin and resulting the activation of β-catenin and YAP/TAZ. Alternatively, due to the direct binding of LRP6 to Axin, overexpression of LRP6 alone could compete with

YAP/TAZ to bind Axin, resulting in the simultaneous release of YAP/TAZ and β-catenin from the destruction complex. As shown in **Figure 2**, these studies may well explain how LRP6, but not LRP5, links Wnt signaling to Hippo pathway.

LRP5/6 AND KIDNEY DISEASE

It has been shown that the *Lrp5* gene may play a role in sporadic autosomal dominant polycystic kidney disease (ADPKD) (Cnossen et al., 2016). Earlier studies have identified a total of four different LRP5 variants, which could be pathogenic predicted by *in silico* tools, suggesting that LRP5 variants may contribute to renal cystogenesis. Luciferase assays show that three of the LRP5 variants significantly reduce the activation of Wnt/β-catenin signaling (Cnossen et al., 2016). Further studies, however, are needed to validate these findings. In addition, in *Lrp6* knockout mouse embryo (18.5 days post coitum), macroscopic small cystic kidneys are visible, suggesting a PKD phenotype (Pinson et al., 2000; Wang et al., 2016). These findings indicate that LRP6 plays a pivotal role during early renal development, and that LRP5, while not affecting early renal development, may at least partially contribute to renal cystogenesis after renal maturation by affecting Wnt/β-catenin signaling.

Wnt/β-catenin signaling is reactivated after kidney injury (He et al., 2009; Zhou et al., 2016), and sustained activation of this pathway accelerates acute kidney injury (AKI) to chronic kidney diseases (CKD) progression (Xiao et al., 2016). Although LRP5 and LRP6 are co-receptors in canonical Wnt pathway, their exact role in kidney disease is unclear. Recent studies have shown that the expression of LRP5 is upregulated in renal tubules of type 1 and type 2 diabetes and unilateral ureteral obstruction (UUO) models, and knockout of LRP5 in the kidney of UUO model down-regulates TGF-β/Smad signaling and ameliorates tubulointerstitial fibrosis without changing the Wnt/β-catenin signaling (He et al., 2020). These investigators found that LRP5 could interact with TGF-β receptor I (TβRI) and TβRII, thereby promoting the formation of TβRI/TβRII heterodimers and regulating TGF-β/Smad signaling in human renal tubule epithelial cells (He et al., 2020). Phosphorylated LRP6 has also been reported to co-immunoprecipitate with TβRI. However,

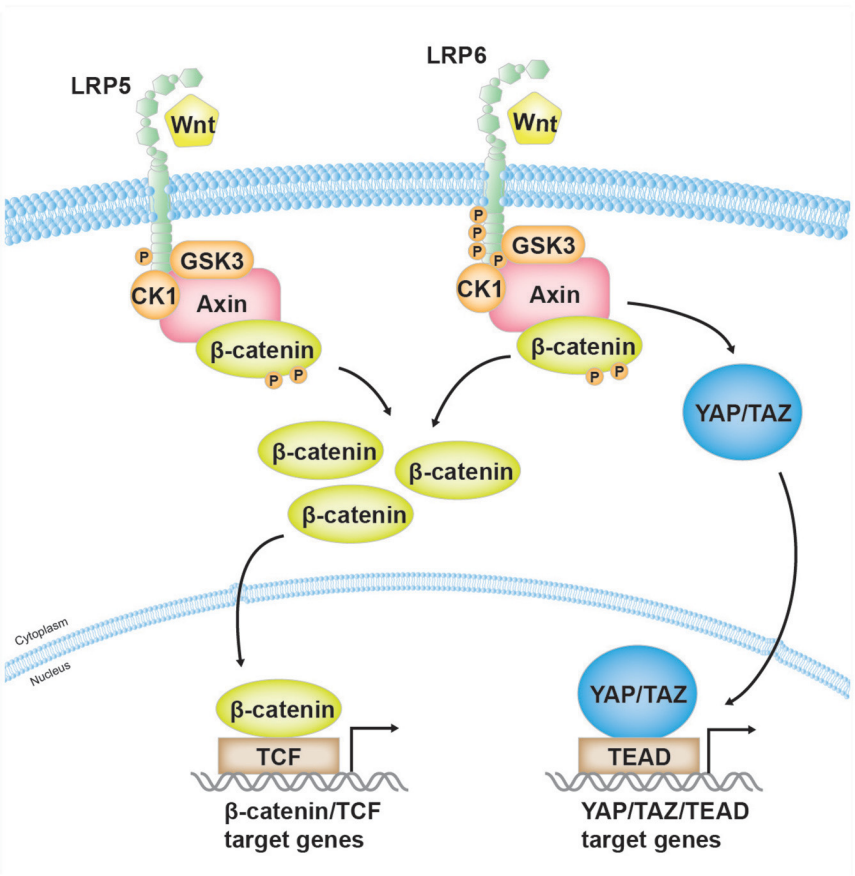

FIGURE 2 | The distinct role of LRP5 and LRP6 in mediating Wnt/β-catenin and YAP/TAZ signaling. Both LRP5 and LRP6, as co-receptors of Wnt ligands, mediate Wnt/β-catenin signaling. However, when Wnt binds to and activates LRP6, activated LRP6, but not LRP5, also competitively binds to the same domain of Axin that is responsible for binding with YAP/TAZ. As a result, this leads to the release of YAP/TAZ from the destruction complex, resulting in their accumulation in the cytoplasm and translocation into the nucleus, where they bind to their cognate transcription factor TEAD and mediate the expression of their target genes.

DKK1 has no effect on canonical TGF-β/Smad signaling in pericytes (Ren et al., 2013). The disparity in these results may be attributable to different cell types. Further studies are needed to fully elucidate the interaction between LRP6 and TβRI.

The expression of LRP6 is also up-regulated in the streptozotocin (STZ)-induced diabetic rat kidneys (Cheng et al., 2016). In another study, 2F1, a functional-blocking monoclonal antibody against the E1E2 domain of LRP6, inhibits Wnt signaling, thereby attenuating renal inflammation, proteinuria and kidney fibrosis in a type 1 diabetes model (Zhou et al., 2012). However, how LRP6 domain-specific ligand interactions mediate different signaling remains poorly understood. In this regard, a study shows that two classes of anti-LRP6 antibodies against E1E2 and E3E4 domains can either inhibit or enhance Wnt/β-catenin signaling (Gong et al., 2010; Ettenberg et al., 2010). Among them, the anti-LRP6 antibody against the E1E2 domain can specifically inhibit Wnt signaling induced by Wnt1, whereas the anti-LRP6 E3E4 domain represses Wnt signaling triggered by Wnt3a. While antibodies that recognize these separate domains may antagonize those Wnts that bind to the same domain of LRP6, antibodies may also enhance signaling mediated by Wnts that bind to different regions, through crosslinking of LRP6 molecules (Gong et al., 2010; Joiner et al., 2013). Similarly, LRP6 antibodies can also potentiate Wnt signal transduction by inhibiting the binding of antagonists, such as DKK1 and sclerostin (Gong et al., 2010). These findings indicate that anti-LRP6 antibodies may be very useful in the treatment of diseases caused by aberrant activation of Wnt/β-catenin signaling. However, one should be careful if the type of Wnt proteins expressed is not known. Because separate binding sites for different subsets of Wnt ligands determine the inhibition or potentiation of Wnt signaling, this complexity can be exploited with antibodies to differentially manipulate Wnt signaling in specific tissues or disease states.

Besides LRP6 antibodies, DKK1, as a natural inhibitor of LRP6, can effectively inhibit pericytes activation, detachment and transition to myofibroblasts *in vivo* in response to kidney injury caused by UUO and unilateral renal ischemia-reperfusion injury (UIRI), thus alleviating renal fibrosis, capillary rarefaction and inflammation (Ren et al., 2013; Johnson et al., 2017). DKK1 also represses β-catenin activation induced by Adriamycin (ADR), TGF-β1 and angiotensin II (AngII), thus alleviating podocyte injury and proteinuria (Dai et al., 2009; Wang et al., 2011; Jiang et al., 2013).

Dysregulation of LRP5/6 not only contributes to kidney diseases but also plays a critical role in the pathogenesis of CKD complications. CKD-mineral and bone disorder (CKD-MBD), characterized by mineral metabolism disorders, vascular calcification (VC), and renal osteodystrophy, is a severe complication in patients with end-stage renal disease (ESRD) (Neven et al., 2018). Mounting clinical evidence has shown that VC is an independent predictor of morbidity and mortality in CKD and ESRD (Blacher et al., 2001; Russo et al., 2011). Elevated parathyroid hormone (PTH) level is one of the major factors associated with progression of VC in hemodialysis patients (Jean et al., 2012). Moreover, PTH is a direct or indirect cause of VC in rats (Neves et al., 2007). As a co-receptor of PTH and Wnts, LRP6 plays a central role in the pathogenesis of CKD-MBD by regulating both PTH and Wnt/β-catenin signaling.

CONCLUSION AND PERSPECTIVES

Over the last two decades, significant progress has been made in our understanding the role of LRP5/6 in mediating Wnt signaling and other signal pathways. It becomes clear that although LRP5 and LRP6 are highly homologous, they are expressed differentially in diverse tissues and organs throughout embryonic and adult stages and possess similar yet divergent actions. In general, LRP6 appears more potent than LRP5 in transmitting Wnt signaling. In addition, LRP6 retains many unique actions by acting as a common coreceptor for numerous extracellular cues and by coupling Wnt with Hippo signaling. In short, LRP6 possesses many unique functions that extend beyond Wnt signaling.

Despite these advances, our current knowledge on the commonality and uniqueness of LRP5 and LRP6 is limited. Up to date, we still know little about the structural basis that accounts for the functional divergence of LRP5 and LRP6. Furthermore, how to target LRP5 and LRP6 by exploiting their commonality and uniqueness *in vivo* for therapeutic intervention remains in its infant stage. Given the complexity of these coreceptors, there will be many obstacles to overcome before any effective remedies can be developed and used in treating various human diseases.

AUTHOR CONTRIBUTIONS

QR reviewed the literature and wrote the manuscript. JC prepared the figures. YL planned the study and revised the manuscript. All authors gave the final approval of the manuscript.

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Wnt3 Is Lipidated at Conserved Cysteine and Serine Residues in Zebrafish Neural Tissue

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Wnt proteins are a family of hydrophobic cysteine-rich secreted glycoproteins that regulate a gamut of physiological processes involved in embryonic development and tissue homeostasis. Wnt ligands are post-translationally lipidated in the endoplasmic reticulum (ER), a step essential for its membrane targeting, association with lipid domains, secretion and interaction with receptors. However, at which residue(s) Wnts are lipidated remains an open question. Initially it was proposed that Wnts are lipid-modified at their conserved cysteine and serine residues (C77 and S209 in mWnt3a), and mutations in either residue impedes its secretion and activity. Conversely, some studies suggested that serine is the only lipidated residue in Wnts, and substitution of serine with alanine leads to retention of Wnts in the ER. In this work, we investigate whether in zebrafish neural tissues Wnt3 is lipidated at one or both conserved residues. To this end, we substitute the homologous cysteine and serine residues of zebrafish Wnt3 with alanine (C80A and S212A) and investigate their influence on Wnt3 membrane organization, secretion, interaction and signaling activity. Collectively, our results indicate that Wnt3 is lipid modified at its C80 and S212 residues. Further, we find that lipid addition at either C80 or S212 is sufficient for its secretion and membrane organization, while the lipid modification at S212 is indispensable for receptor interaction and signaling.

Keywords: Wnt3, zebrafish, fluorescence correlation spectroscopy, fluorescence cross-correlation spectroscopy, selective plane illumination microscopy, FCS diffusion law

INTRODUCTION

Wnts are a class of signaling molecules involved in short- and long-range cell-cell communication that regulates complex tissue patterning during embryogenesis. They coordinate a multitude of developmental processes including cell proliferation, cell polarity induction, lineage specification, cell movement and apoptosis (Clevers and Nusse, 2012; Hikasa and Sokol, 2013). Misregulation of Wnt signaling leads to numerous pathological disorders including cancer (Logan and Nusse, 2004; Azbazdar et al., 2021). To date, 19 wnt genes have been identified in humans and 23 in zebrafish (Miller, 2002; Mikels and Nusse, 2006; Lu et al., 2011). Wnts are ∼ 300-400 amino

acids long (molecular weight of ∼40 kDa) with 22-24 conserved cysteine residues that form crucial intramolecular disulfide bonds which are required for their folding and function (Mikels and Nusse, 2006). After translation, Wnts undergo a series of post-translational lipid and sugar modifications in the endoplasmic reticulum (ER) (Kikuchi et al., 2006; Lorenowicz and Korswagen, 2009; Willert and Nusse, 2012; Torres et al., 2019; Wang et al., 2020). Porcupine (Porc), a membrane bound O-acyl transferase (MBOAT), is known to facilitate the addition of lipid moieties to Wnts which confer a hydrophobic nature to Wnts (Tanaka et al., 2000). Oligosaccharyl transferase (OST) appends N-linked glycans to Wnts (Tanaka et al., 2002; Komekado et al., 2007). The post-translationally modified Wnts are subsequently targeted to the cell membrane and secreted. The secreted hydrophobic Wnts navigate the aqueous extracellular matrix and achieve long-range distribution by either binding to HSPG (Mii et al., 2017; Mii et al., 2020), being packaged inside lipoproteins (Panáková et al., 2005), or being shuttled by carrier proteins such as afamins (Mihara et al., 2016), secreted Frizzled Related Proteins (sFRPs) (Üren et al., 2000; Galli et al., 2006) and Secreted Wg-Interacting Molecule (Swim) (Mulligan et al., 2012). Upon reaching their distal target tissues, they are handed over to their cognate receptors and co-receptors. Wnts are known to interact with a wide range of receptors and co-receptors which play a role in deciding the course of Wnt biological activity, of which the Frizzled (Fzd) receptor super-family is the most extensively studied (Niehrs, 2012). Wnts form a complex with the receptor and co-receptor at the cell membrane and activate the downstream signaling cascade for the transcription of genes that regulate embryonic development and tissue homeostasis (Bilić et al., 2007).

A number of studies have reported the role of lipidation in intracellular trafficking, secretion, transport and function of Wnts (Hausmann et al., 2007; Parchure et al., 2018; Hosseini et al., 2019). Wntless (WIs), a functionally conserved transmembrane protein that shuttles Wnts from the Golgi to the cell membrane, is unable to bind to non-acylated Wnt molecules (Bänziger et al., 2006). As a result, non-acylated Wnts are retained in the Wnt producing cells and hence are not secreted (Coombs et al., 2010; Herr and Basler, 2012). Moreover, lipid adducts also help in the long-range distribution and gradient formation of Wnts. For instance, Mulligan et al., 2012 showed that Swim promotes long-range signaling in Drosophila by interacting with Wingless in a palmitate dependent manner (Mulligan et al., 2012). Similarly, evidence suggests that the lipid modifications in Wnt are central to the interaction of Wnts with afamin (Naschberger et al., 2017) and sFRP (Janda and Garcia, 2015). Recently, it was demonstrated how glypicans change conformation to accommodate the lipid tails to facilitate paracrine signaling of Wnts (McGough et al., 2020). In addition to secretion and transport, lipid modifications in Wnts also mediate their interaction with receptors and co-receptors to activate Wnt signaling. The crystal structure of Xenopus Wnt8 (xWnt8) in complex with mouse Fzd8 (mFzd8) revealed that the interaction occurs by xWnt8 projecting its lipid tail into the hydrophobic groove of the mFzd8 cysteine rich domain (CRD) (Janda et al., 2012). The importance of lipid modification on Wnts, from translation to signaling activity, thus initiated detailed investigations on the type and sites of lipid modifications in different Wnts.

Nusse et al. first performed a mass-spectrometric analysis on the proteolytic fragments of purified mouse Wnt3a (mWnt3a) and Drosophila Wnt8 (dWnt8) to identify the type and position of lipidation. They demonstrated that these proteins were modified at their conserved cysteine residue (C77 in mWnt3a and C51 in dWnt8) with a thioester-linked palmitic acid, and mutating this cysteine residue decreased their hydrophobic nature and activity (Willert et al., 2003). Another subsequent mass-spectrometric study by Kurayoshi et al. (2007) further confirmed the addition of palmitate at the homologous C104 residue in mouse Wnt5a. In addition to the palmitoylation at cysteine, Takada et al. reported that mWnt3a is lipidated at a conserved serine residue (S209) with a oxyester-linked palmitoleic acid (Takada et al., 2006). However, a later published 3.25 Å resolution xWnt8-Fzd8 crystal structure identified serine (S187 in xWnt8) as the only lipid addition site, while the corresponding cysteine residue (C55 in xWnt8) was found to be engaged in a conserved disulfide bond making it conformationally unfavorable to serve as a lipid modification site (Janda et al., 2012).

Although the crystal structure recognized serine as the only consensus lipidation site across all Wnts, mutations in the conserved cysteine residue diminishes the hydrophobicity, membrane localization, secretion, and activity in several Wnts. For instance, C93A Wg is retained inside the cells without being secreted and does not activate Wnt signaling in *Drosophila* imaginal disks (Franch-Marro et al., 2008), while C104 mWnt5a and C77A mWnt3a are secreted similar to wildtype but do not interact with Fzd and fail to regulate Wnt pathways in L-cells (Willert et al., 2003; Kurayoshi et al., 2007). MacDonald et al. clarified that a mutation at the conserved cysteine residue results in hydrophilic partitioning and inactive Wnt signaling, due to the aggregation of Wnts by ectopic intermolecular disulfide bonds which buries the lipid adduct in the oligomerized mutant, and not due to the lack of an acyl group at the cysteine residue. However, this does not explain how several Wnt proteins mutated at their homologous serine residues are localized on the cell membrane, are secreted, and remain functional (Franch-Marro et al., 2008; Galli and Burrus, 2011; Tang et al., 2012; Azbazdar et al., 2019; Speer et al., 2019). Given the major structural and functional roles linked with Wnt lipidation, the possibility of an additional lipidation site in at least some Wnt molecules cannot be excluded. To further confound the situation, the impact of inhibiting acylation in Wnts differs with different Wnt ligands and depends on the cellular context. For example, it was recently documented that non-acylated Wnt1 and Wnt5a were unable to initiate Wnt signaling, whereas non-acylated Wnt8 and Wnt3a retained their receptor binding capacity and signaling activity in Xenopus embryos. Furthermore, these results varied when their secretion and activity were examined *in vitro* (Speer et al., 2019). Therefore, it is imperative to characterize lipid modifications for each Wnt ligand in a relevant biological context to comprehend their structure and function.

Wnt3, a member of the Wnt family, is engaged in a number of developmental processes such as primitive streak formation,

neurogenesis, vasculogenesis and limb development to name a few (Bulfone et al., 1993; Liu et al., 1999; Garriock et al., 2007; Anne et al., 2013). In zebrafish, Wnt3 is expressed in embryonic neural tissues (Clements et al., 2009; Teh et al., 2015), and our group mapped the expression, dynamics and interactions of zebrafish Wnt3 in vivo to understand its distribution and influence on brain development (Veerapathiran et al., 2020). We had earlier shown in vivo that Wnt3 associates with cholesterol rich domains on the cell membrane and blocking the action of Porc by C59 lowers, but does not abolish, its confinement in the domains in a concentration-dependent manner and results in defective brain development (Teh et al., 2015; Ng et al., 2016). Recently, Azbazdar et al. reported that lipidation of Wnt3 at S212 is dispensable for secretion and binding to Fzd8 receptor in HEK293T cells (Azbazdar et al., 2019). Therefore, it remains unclear whether zebrafish Wnt3 is only lipidated at serine, and how lipidation influences its membrane organization, secretion, receptor binding and activation of Wnt signaling *in vivo*.

To address these questions, we created Wnt3-EGFP constructs mutated at their putative lipidation sites, either cysteine 80 (Wnt3C80A-EGFP) or serine 212 (Wnt3S212A-EGFP) or at both sites (Wnt3C80AS212A-EGFP). These constructs were compared and analyzed for *in vivo* dynamics using confocal fluorescence correlation spectroscopy (FCS), membrane organization using single plane illumination microscopy-FCS (SPIM-FCS), receptor interaction using fluorescence cross-correlation spectroscopy (FCCS) and signaling activity of the lipidation site mutants with Wnt3-EGFP. FCS is a single molecule sensitive technique that measures the concentration and diffusion dynamics of fluorescently labeled molecules in a small observation volume based on the fluorescence fluctuations they create during their transit (Elson and Magde, 1974; Krichevsky and Bonnet, 2002; Enderlein et al., 2004). SPIM-FCS is a multiplexed modality of FCS that records fluctuations from the entire illumination plane in a sample using fast array detectors and generates spatial maps of diffusion coefficients and concentrations in a single measurement which provides information on the membrane organization of a probe (Wohland et al., 2010; Singh et al., 2013; Xue Wen et al., 2017). The ability of the camera-based FCS to bin pixels post-acquisition to create multiple observation areas allows us to easily adapt the FCS diffusion law, a plot of diffusion time against the observation area, to SPIM-FCS (Wawrezinieck et al., 2005; Ng et al., 2015; Veerapathiran and Wohland, 2018). On the other hand, in FCCS, the fluorescence fluctuations of two bound molecules tagged with spectrally different fluorophores are correlated as they transit the observation volume to measure their molecular interaction (Bacia and Schwille, 2007; Ries et al., 2009; Shi et al., 2009; Foo et al., 2012; Schwille and Rigler, 2013). Our results demonstrate that while Wnt3C80A-EGFP and Wnt3S212A-EGFP localize on the cell membrane, albeit with reduced localization to the ordered membrane domains as compared to Wnt3-EGFP, Wnt3C80AS212A-EGFP fails to localize to the cell membrane. While Wnt3C80A-EGFP and Wnt3S212A-EGFP were secreted and were found in the brain ventricle, Wnt3C80AS212A-EGFP was not detected in the extracellular spaces. Finally, we found that Wnt3C80A-EGFP interacts with Fzd1, although with a lower binding affinity as compared to Wnt3EGFP, whereas Wnt3S212A-EGFP did not interact with receptors. However, both mutants fail to activate the Wnt-signaling pathway. Overall, our work suggests that Wnt3 in zebrafish has two lipid modifications at its C80 and S212 residues which play different functional roles. While either residue is sufficient for Wnt3 secretion and membrane localization, the lipidation at S212 seems necessary for receptor interaction and activation.

MATERIALS AND METHODS

Confocal Microscope Setup

An Olympus FV1200 laser scanning confocal microscope (IX83; Olympus, Japan) was used for this study. The samples were illuminated using a 488 nm laser for the EGFP tagged constructs and a 543 nm laser for the mApple tagged constructs. The emitted signal passes through a 120 μm (1 airy unit) pinhole and is filtered by a 510/23 emission filter (Semrock, United States) for EGFP and a 605/55 emission filter (Semrock, United States) for mApple before it reaches the PMT detector for imaging. For FCS and FCCS measurements, emission filters (Semrock, United States) of 510/20 for EGFP and 615DF45 for mApple were used and the emission was detected using a single photon sensitive avalanche photodiode (SPAD; SPCM-AQR-14, PerkinElmer, United States). The signal was recorded and processed using SymPhoTime 64 (PicoQuant, Germany) to calculate the correlation functions. For Quasi PIE-FCCS measurements, the sample was illuminated simultaneously using a 485 nm pulsed laser (LDH-D-C-488; PicoQuant) at 20 MHz repetition rate and the 543 nm continuous wave laser of the confocal microscope. The emission was separated using a dichroic mirror 560 DCLP and was directed towards a 510/23 (Semrock, United States) or a 615DF45 filter (Semrock, United States) for the different detection channels. The auto- and cross-correlation functions were calculated using a custom written program in Igor Pro (Wavemetrics, United States[1].

Confocal Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy extracts information such as the concentration and dynamics of fluorescent molecules from the fluorescence intensity fluctuations in a small observation volume. The fluctuations are generated from processes such as translational diffusion, rotational diffusion, flow, chemical reactions, and fluorophore blinking. The intensity fluctuations are temporally autocorrelated to extract the information embedded in them. The autocorrelation function (ACF) is given by

$$G(\tau) = \frac{\langle F(t) \cdot F(t+\tau) \rangle}{\langle F(t) \rangle \cdot \langle F(t+\tau) \rangle} \quad (1)$$

where F(t) is the intensity at time t and τ is the lag time. The actual form of the ACF can be derived for different processes generating

[1]https://www.dbs.nus.edu.sg/lab/BFL/index.html

the fluctuations. For a 3D free diffusion of a single species (3D – 1 particle – 1 triplet model) with a triplet state, the theoretical ACF is given by

$$G(\tau)_{3D,1p,1t} = \frac{1}{N}\left(1+\frac{\tau}{\tau_d}\right)^{-1}\left(1+\frac{\tau}{K^2\tau_d}\right)^{-\frac{1}{2}} f_{trip}(\tau) + G_\infty, \quad (2)$$

where N is the number of molecules in the observation volume, τ_d is the diffusion time of the molecule, G_∞ is the convergence at long lag times, K is the structure factor which determines the shape of the confocal volume and f_{trip} (τ) is the triplet state function given by

$$f_{\text{trip}}(\tau) = 1 + \frac{F_{\text{trip}}}{1-F_{\text{trip}}} e^{-\frac{\tau}{\tau_{\text{trip}}}}. \quad (3)$$

where, F_{trip} is the fraction of particles in the triplet state and τ_{trip} is the triplet relaxation time.

In case of two diffusion components, the correlation function for a two-component 3D diffusion process is

$$G(\tau)_{3D,2p,1t} = \frac{1}{N}\left[(1-F_2)\left(1+\frac{\tau}{\tau_{d1}}\right)^{-1}\left(1+\frac{\tau}{K^2\tau_{d1}}\right)^{-\frac{1}{2}} + F_2\left(1+\frac{\tau}{\tau_{d2}}\right)^{-1}\left(1+\frac{\tau}{K^2\tau_{d2}}\right)^{-\frac{1}{2}}\right] f_{\text{trip}}(\tau) + G_\infty \quad (4)$$

where F_2 is the fraction of the second component (3D – 2 particle – 1 triplet model). For two-dimensional diffusion, such as on the cell membrane, equations 2 and 4 become

$$G(\tau)_{2D,1p,1t} = \frac{1}{N}\left(1+\frac{\tau}{\tau_{d1}}\right)^{-1} \times f_{\text{trip}}(\tau) + G_\infty \quad (5)$$

and

$$G(\tau)_{2D,2p,1t} = \frac{1}{N}\left[(1-F_2)\left(1+\frac{\tau}{\tau_{d1}}\right)^{-1} + F_2\left(1+\frac{\tau}{\tau_{d2}}\right)^{-1}\right] f_{\text{trip}} + G_\infty \quad (6)$$

and are referred to as 2D – 1 particle – 1 triplet model and 2D – 2 particle – 1 triplet model, respectively. The system was first calibrated with 5 nM Atto 488 dye (ATTO-TEC GmbH, Germany) for the 488 and 485 nm lasers and 5 nM Atto 565 dye (ATTO-TEC GmbH, Germany) for the 543 nm laser line. The ACF for the calibration dyes (D of 400 $\mu m^2/s$) was fit using equation 2 and the free fit parameters were N, τ_d, τ_{trip}, F_{trip}, and G_∞. All measurements were taken at room temperature with an acquisition time for each measurement of 60 s. For FCS measurements, the fitting models were chosen by Bayes inference-based model selection which determines the most suitable model given the data and noise (Sun et al., 2015). The fit models selected for Wnt3S212A-EGFP, Wnt3C80A-EGFP and Wnt3-EGFP was a 2D – 2 particle – 1 triplet model for measurements in cell membranes, while a 3D – 2 particle – 1 triplet model was used for measurements in the BV. For secEGFP measurements, a 3D – 1 particle – 1 triplet model was used. The laser power used for all FCS measurements was 8.3 μW of 488 nm continuous wave laser.

Quasi PIE Fluorescence Cross-Correlation Spectroscopy

Fluorescence cross-correlation spectroscopy is useful in studying the interaction between molecules in live samples. When two interacting molecules tagged with spectrally different fluorophores pass through the confocal volume, the fluorescence intensity fluctuations from the two channels can be cross-correlated to obtain the cross-correlation function (CCF), G_x (τ), given by

$$G_x(\tau) = \frac{\langle F_g(t)\cdot F_r(t+\tau)\rangle}{\langle F_g(t)\rangle\cdot\langle F_r(t)\rangle} \quad (7)$$

where F_g and F_r represents the fluorescence intensity in the green and red channels, respectively. The ACFs were fitted using 2D-2particle-1triplet model as described in equation 6.

In order to determine interaction of Wnt3S212A-EGFP and Wnt3C80A-EGFP with Fzd1, a pulsed 485 nm laser line and a continuous 543 nm laser line were simultaneously used to excite EGFP and mApple, respectively. The intensity in each channel were autocorrelated to obtain their respective ACF and cross-correlated to obtain the CCF. Fluorescence lifetime correlation spectroscopy (FLCS) based statistical filtering was applied to eliminate spectral cross talk (Padilla-Parra et al., 2011; Kapusta et al., 2012). The apparent dissociation constant (K_d) was calculated as detailed in Veerapathiran et al. (2020). Laser power used for FCCS measurements were 8.8 μW for 485 nm pulsed laser and 8.5 μW for 543nm continuous wave laser.

Sample Preparation for Diffusion Law Analysis on SPIM

Early-stage zebrafish embryos expressing Wnt3S212A-EGFP, Wnt3C80A-EGFP and Wnt3-EGFP were taken at 2-3 days-post-fertilization (dpf) and anesthetized in MS-222 (Merck) solution. These embryos were placed in low melting 1% agarose (UltraPureTM Low Melting Point Agarose, 16520100, Thermofisher Scientific, United States) mixed with MS-222 solution and mounted into a Fluorinated Ethylene Propylene (FEP) tube of 1.1 × 1.5 mm^2 cross-section (FT 1.1 × 1.5, Adtech Polymer Engineering, England, United Kingdom) with the help of a plastic wire in such a way that their dorsal hind brain region faced one of the walls of the FEP tube. In such a position, the light sheet illuminated the coronal plane of the embryo and the detection objective faced and thus imaged the dorsal region of the embryo.

SPIM Setup

A home-built SPIM described in Singh et al. (2013) and Krieger et al. (2015) was used to measure the membrane organization and dynamics of Wnt3 and its lipidation site mutants. The system contains an illumination arm that creates a Gaussian light sheet

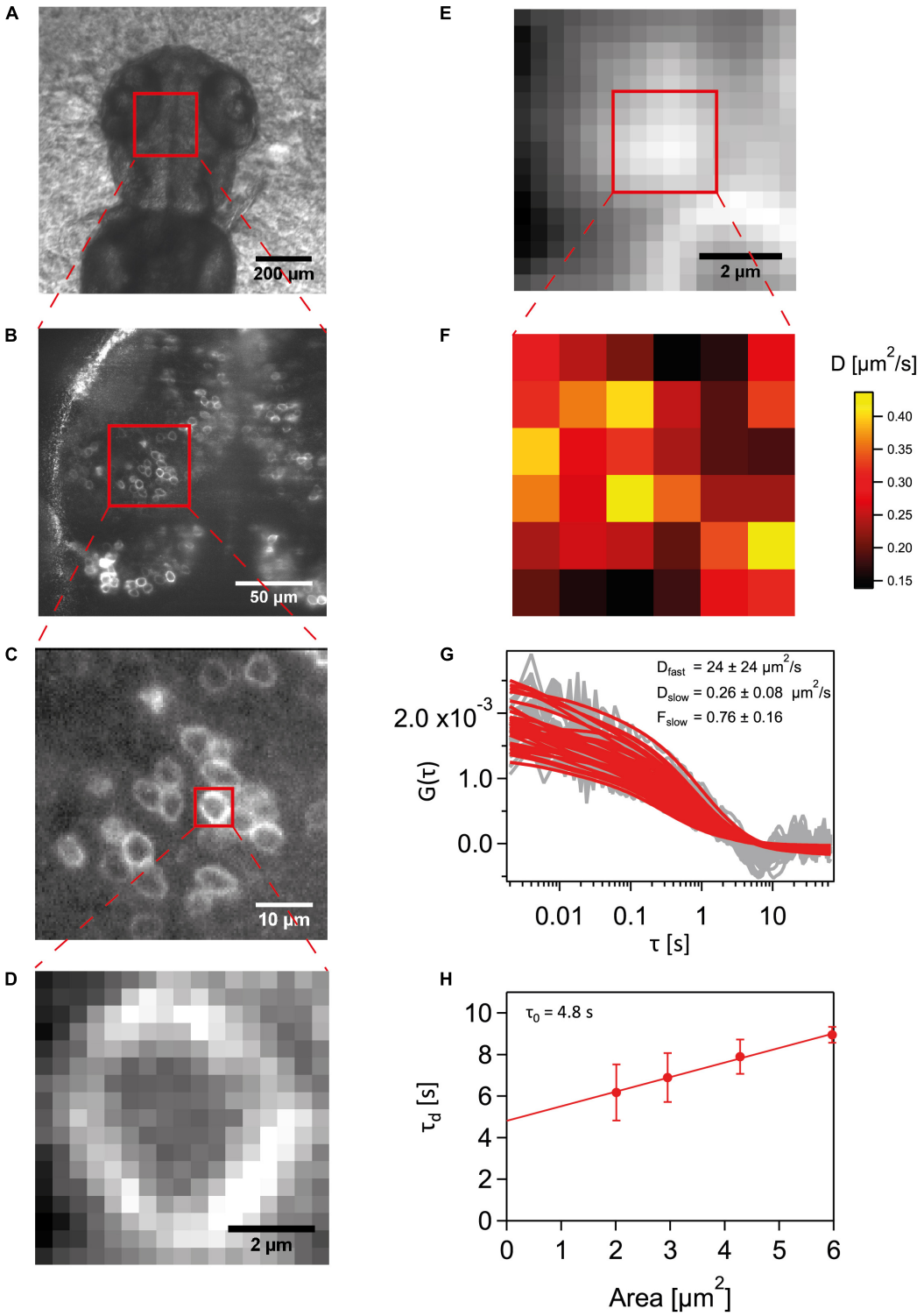

FIGURE 1 | Schematic for a SPIM-FCS experiment. **(A)** A stereomicroscope image of the dorsal region of a 2-3 dpf Wnt3-EGFP expressing embryo. **(B)** sCMOS camera image capturing a large field of view of Wnt3-EGFP expressing cells at the midbrain-anterior hind brain region. **(C)** EMCCD camera image of a smaller subregion expressing Wnt3-EGFP fluorescent cells. **(D)** Cropped detector region of the imaging area with only a single cell in view. **(E)** A summed-up intensity projection of the 100,000 frames imaging the apical membrane of the cell imaged in **(D)**. **(F)** A 6 6 subregion's slow component diffusion coefficient map obtained from the fit values of the ACFs. **(G)** The raw autocorrelation data in gray and their corresponding fits in red. **(H)** Diffusion law plot for the 6 6 region to obtain the τ_0 intercept value.

to illuminate the sample in the sample chamber, and a detection arm with the detection objective, tube lens and a camera to image the embryo. A 488 nm diode laser line (Cobolt 06-MLD 488nm 0488-06-01-0100-100, Cobolt AB,Sweden) was used for all measurements. This was directed through an optical fiber (kineFLEXP-3-S-405..640-1.0-4.0-P2, Qioptiq, United States). The beam from this fiber was reflected onto the illumination arm with a 45-degree mirror and passed through an achromatic cylindrical lens of 75 mm focal length (ACY254-075-A; Thorlabs Inc., United States) and an illumination objective (SLMPLN 20 × /0.25; Olympus, Japan) to form the light sheet. The light sheet thickness obtained had a $1/e^2$ radius of approximately 1.1 μm. The thinnest section of the light sheet was aligned to match the focal plane of the detection objective (LUMPLFLN 60 × /1.0, Olympus, Japan).

The embryo was mounted in the FEP tube that was held by a self-closing forceps and was mounted on a motorized stage with three linear positioning systems (Q-545 Q-MotionR Precision Linear Stage; Physik Instruments, Germany) with piezo motors for the three axis and one rotation stage (DT-34 Miniature Rotation Stage; Physik Instruments, Germany). The sample chamber was a 3 cm × 3 cm × 3 cm cube with an opening on top for mounting the sample and an opening on the side for the detection objective. The sample chamber was filled with water and the embryos were lowered into the chamber from the top with the motorized stage. The detection objective was placed in a mounting hole on one side of a custom-made sample chamber and mounted on a piezo flexure objective scanner (P-721 PIFOC; Physik Instruments, Germany) for controlling the position of the detection objective with respect to the light sheet. The emission obtained by the detection objective was passed through a filter (FF03-525/50-25, Semrock, United States) and was projected onto an EMCCD camera (Andor iXon3 860, 128 x 128 pixels; Andor Technology, United Kingdom) by a tube lens (LU074700, f = 180 mm, Olympus, Tokyo, Japan). A flip mirror was used to alternatively project the image onto a sCMOS camera (OCRA-Flash4.0 V2; C11440, Japan) with a large field of view to visualize the global position of the embryo and to decide on the region of interest.

Data Acquisition and Fitting for SPIM-FCS

An illustration for a general sequence of steps involved in SPIM-FCS and diffusion law measurements is given in **Figure 1**. Fluorescent cells in the cerebellum and midbrain of 2–3 dpf embryos were chosen to perform SPIM-FCS measurements. The embryo samples experienced a laser intensity of ∼60 W/cm^2, which was used on the majority of the samples except for nine cells expressing Wnt3S212A-EGFP wherein intensity of 120 W/cm^2 was used. The global region of interest was identified by using a sCMOS camera allowing for a large field of view. Cells expressing the fluorescent molecules of interest were chosen and then imaged on the EMCCD camera to perform Imaging FCS to take advantage of the signal amplification from the EM gain. The acquisition field was cropped to image only the cell of interest and the sample mounting stage was moved in steps of 300 nm to image a region on the apical membrane. 100,000 frames were recorded with an exposure time of 2 ms. For analysis, the image stacks were summed up to obtain an intensity projection to find the region containing only the membrane and then the image stacks were accordingly cropped to 6 × 6 pixels. The intensity traces of each pixel were corrected for bleaching and were autocorrelated using the ImageJ plugin Imaging FCS 1.52[2]. Data from cells drifting during the measurement or having significant traces of mechanical vibrations in their autocorrelations were excluded.

These ACFs were fit with a 2-component fitting model. Here, the fast component was captured by a 3D free diffusion fitting model to account for the dynamics of molecules in the intracellular and extracellular space. Diffusion on the membrane was accounted for by using a 2D free diffusion fitting model for the slow component (Sankaran et al., 2009). The fitting function is given below.

$$G(\tau)_{SPIM} = \frac{1}{N}\left[(1-f_2)\,p(D_1,\tau)\left(1+\frac{4D_1\tau}{\omega_z^2}\right)^{-\frac{1}{2}} + f_2 p(D_2,\tau)\right] + G_\infty \quad (8)$$

where

$$p(D,\tau) = \left[\frac{\sqrt{4D\tau+\omega_{xy}^2}}{a\sqrt{\pi}}\left(e^{-\left(\frac{a}{\sqrt{4D\tau+\omega_{xy}^2}}\right)^2}-1\right) + erf\left(\frac{a}{\sqrt{4D\tau+\omega_{xy}^2}}\right)\right]^2 \quad (9)$$

Here, N is a fitting parameter that represents the total number of particles in the observation volume, D_1 and D_2 are the 3D fast component and the 2D slow component's diffusion coefficient fitting parameters respectively, f_2 is the fraction of the slow component fitting parameter and G_∞ is the fitting parameter for obtaining the convergence value at infinite lag times. ω_{xy} is the PSF of the objective (644 nm) and ω_z is the $1/e^2$ thickness of the light sheet (∼1.1 μm). The pixel size of the EMCCD was 24 μm and with a magnification of 60 × each pixel effectively imaged 0.4 μm in the sample (represented as 'a' in equation 9). As the acquisition time of 2 ms was much too slow to detect triplet fluctuations, that component of dynamics was not included in the fitting function unlike the confocal FCS fitting model.

The bleach correction model typically used to linearize a decaying trace is a polynomial function. However, the polynomial function cannot correct for infrequently observed clusters which cause transient intensity bursts. These clusters change the shape of the ACF curve and results in an apparent low diffusion coefficient. As we observed clusters in our samples, we used a line segment-based bleach correction that divides the intensity trace into equal segments, linearizes each segment and concatenates the linearized intensity trace. This mostly abolishes the influence

[2]https://github.com/ImagingFCS/Imaging_FCS_1_52

of the aggregates on the correlation functions. We chose a line segment size of 5000 data points as this removes the influence of the rare clusters and other slow fluctuations but leaves the fast and slow diffusion coefficients unchanged. If line segments much smaller are chosen, the line segment correction overcorrects resulting in diffusion coefficient overestimations.

SPIM-FCS Diffusion Law

The theory of the FCS diffusion law was initially developed by Lenne and colleagues (Lenne et al., 2006) and later adapted for camera-based FCS modalities by our group (Ng et al., 2015, 2016; Bag et al., 2016; Veerapathiran and Wohland, 2018). Briefly, it characterizes a molecule's mode of diffusion as free diffusion or transiently trapped domain confined diffusion or cytoskeleton meshwork hindered hop-diffusion based on the dependence of the probe's diffusion coefficient. The FCS diffusion law is a plot of diffusion time (τ_d) versus effective observation area (A_{eff}) (equation 10). In camera-based FCS, A_{eff} is the convolution of the PSF with the detection area, and a wide range of A_{eff} is obtained from a single FCS measurement by software binning of the pixels after image acquisition. The τ_d for each observation area is plotted and by analyzing the *y*-intercept value (τ_0) we can classify the mode of diffusion as free diffusion ($\tau_0 = 0$), domain confined diffusion ($\tau_0 > 0$) or cytoskeleton influenced hop-diffusion ($\tau_0 < 0$).

$$\tau\,(A_{\text{eff}}) = \tau_0 + \frac{A_{\text{eff}}}{D_{\text{eff}}} \tag{10}$$

Larger bin sizes have sufficient area to capture the fluorescence fluctuations coming from the movement of molecules in and out of an observation volume. As 1x1 region exhibited larger variability, the diffusion coefficient from that point was not included for the diffusion law. Pixel binning from 2×2 to 5×5 was used instead to obtain different A_{eff} and their corresponding τ_d values. The slower diffusion component pertaining to that of the membrane was used as D_{eff} to obtain the τ_d values which were then plotted against A_{eff} values. The plots were fit to a straight line to obtain the τ_0 intercept value. Diffusion law measurements with negative slopes or that resulted in highly nonlinear convex or concave curves resulted in either extremely high or low diffusion law intercepts. This happened in less than 25% of all measurements and these were excluded as they were judged to result from membrane curvature and thus a non-flat geometry of the membrane within the light sheet.

Generation of Transgenic Lines and Constructs

A stable wnt3 promoter-driven line was generated as described (Teh et al., 2015) by PCR amplification of Wnt3-EGFP and pEGFPN2 fusion with HindIII and KpnI flanked primers: HindIII_Wnt3F, 5′-aagcttATGGATTTGTACCTGGTTGGAT-3′ and KpnI_Wnt3R, 5′ggtaccaaTTTACATGTATGTACGTCGTAGA3′. From this, Wnt3-EGFP SV40 polyA fragment was excised to prepare the 4-kbWnt3EGFP-miniTol2 recombinant plasmid.

SecEGFP sequence was also obtained in a similar way by replacing Wnt3EGFP ORF with secEGFP into the 4-kbWnt3EGFP-miniTol2 recombinant plasmid which resulted in a 4-kbsecEGFP-miniTol2 recombinant plasmid which was co-injected with Tol2 transposase at 1–2 cell stage for 4 kb wnt3 promoter-driven somatic expression of SecEGFP.

The constructs for the mutants (Wnt3C80A-EGFP and Wnt3S212A-EGFP) were created using by means of site directed mutagenesis targeting two base pairs of TGT (cytosine) into GCT (alanine) of wnt3 for Wnt3C80A-EGFP and targeting one base pair of **T**CA (serine) into **G**CA (alanine) of Wnt3 gene of Wnt3S212A-EGFP in the recombinant DNA vector (pminiTol2 containing 4 kb upstream zebrafish Wnt3 promoter driving the expression of Wnt3-EGFP).

Wnt3S212A-EGFP was generated by site directed mutagenesis at wt Wnt3-EGFP by targeting 2 basepairs, TGT (cytosine) into **GC**T (alanine), of Wnt3 gene. The following primers were used: forward: 5′CCATGGGCTGgCAGGCAGCTG3′ and reverse: 5′CATTTACAGCGCAGGTGCATGTTC3′. The PCR product was ligated into pGFP-N2-zfWnt3-S212A vector. Wnt3C80A-EGFP was generated by side directed mutagenesis at wt Wnt3-EGFP by targeting 1 basepair of **T**CA (Serine) into **G**CA (alanine) of Wnt3 gene of Wnt3S212A-EGFP in the recombinant DNA vector (pminiTol2 containing 4 kb upstream zebrafish Wnt3 promoter driving the expression of Wnt3-EGFP). The following primers were used: forward: 5′TATCCAGGAGgcTCAGCACCAGTTCC3′ and reverse: CCCAGTTTGACTCCTTCC. The PCR product was ligated into pGFP-N2-zfWnt3-C80A.

For Wnt3C80AS212A-EGFP, mutated gene of both C80A and S212A were amplified as inserts using following primers from pGFP-N2-zfWnt3-C80A and pGFP-N2-zfWnt3-S212A, respectively: Insert-C80A(mTol2) forward primer: ATGGATTTGTACCTGGTTGG and Insert-C80A (mTol2) reverse primer: GCAAGAAAGAAAACTAGAGATTC TTGTTTgcggccgctctagagtcgcggccgctttact. Pminitol2-4kbwn t3pro plasmid was amplified with respective primers for both C80A and S212 A separately using following primers: Vector_tol2_reverse primer: TCCAACCAGGTACAAATCCA TggtggcgaccggtggatcG Vector_Tol2_forward primer: agcg gccgcAAACAAGAATCT using Gibson assembly.

Microinjection and Zebrafish Sample Preparation

All wild type zebrafish (*Danio rerio*) were maintained at 28°C and embryos were obtained by allowing them to mate naturally according to the University's animal care protocol (BR18-1023). 40 ng of required plasmid was injected into the zebrafish embryos at one-cell stage and the embryos were incubated at 28°C. All the embryos were screened for fluorescence before measurement at 48pf and embryos older than 24 hpf were treated with 1-phenyl-2-thiourea to prevent melanin formation and subsequent pigmentation. Healthy embryos, dechorionated and anesthetized with MS-222 (Merck), were laterally mounted on a glass bottom dishes (MATEK dish) in 1% low gelling agarose (Merck).

Capped Sense mRNA Synthesis and Whole-Mount *in situ* Hybridization (WMISH)

Capped sense RNAs of wt Wnt3-EGFP, Wnt3C80A-EGFP and Wnt3S212A-EGFP were synthesized with mMessage mMachine Kit (Thermo Fisher Scientific, Waltham, MA, United States). For phenotypic observation and WMISH, 100 pg of mRNA was injected into one-cell transgenic Tg(7xTcf-Xla.Siam:nlsm-Cherry^{ia}) zebrafish embryos (Moro et al., 2012). The Wnt antagonist IWR-1 was added to the embryo water at 8 h-post-fertilization (hpf) and kept until 24 hpf. Embryos were initially photographed at 24 hpf under a Zeiss Stemi 508 stereo microscope (Carl Zeiss AG, Jena, Germany). Next, embryos were fixed at 24 hpf in 4% paraformaldehyde (PFA) dissolved in PBS overnight. WMISH was performed with mCherry antisense RNA probe as described previously (Jowett and Lettice, 1994).

RESULTS

C80A and S212A Mutations Do Not Affect Wnt3 Secretion *in vivo*

To study the influence of lipidation on the action of zebrafish Wnt3 *in vivo*, we first examined whether a single point mutation at the putative lipidation sites perturbs Wnt3 secretion *in vivo*. We injected one-cell stage wildtype (WT) embryos with either Wnt3C80A-EGFP or Wnt3S212A-EGFP plasmids and determined via confocal FCS if they are secreted in the fourth brain ventricle (BV). The BVs are cerebrospinal fluid filled cavities enriched with several extracellularly secreted signaling factors, including Wnt (Ochoa et al., 2015; Kaiser et al., 2019; Kaiser and Bryja, 2020). We had earlier demonstrated that functional Wnt3-EGFP, driven by the 4 kb wnt3 promoter in the transgenic line Tg (-4.0wnt3:Wnt3EGFP), is secreted extracellularly and diffuses in the BV (Teh et al., 2015; Veerapathiran et al., 2020) as two fractions: a fast component with a diffusion coefficient D_{fast} of 56.9 ± 11.9 $\mu m^2/s$ and a slow fraction with a D_{slow} of 3.6 ± 2.6 $\mu m^2/s$ (fraction of slow component F_{slow} = 0.1 ± 0.1) (Teh et al., 2015). Therefore, we performed FCS in the BV to detect if the mutants are secreted similar to Wnt3-EGFP. We obtained autocorrelation functions (ACF) for both Wnt3C80A-EGFP and Wnt3S212A-EGFP, suggesting that the mutants are secreted into the BV (**Figure 2**). The correlation functions for the mutant constructs were fitted with a 3D-2particle-1triplet model (see Materials and Methods), and the D for both components were computed (see **Table 1**). We observed a fast diffusing component (D_{fast} of 52.5 ± 10.3 $\mu m^2/s$ for Wnt3C80A-EGFP; 54.4 ± 10.4 $\mu m^2/s$ for Wnt3S212A-EGFP) and a slow diffusing component (D_{slow} = 6.8 ± 6 $\mu m^2/s$ and F_{slow} = 0.3 ± 0.2 for Wnt3C80A-EGFP; D_{slow} = 4.6 ± 2.0 $\mu m^2/s$ and F_{slow} = 0.3 ± 0.1 for Wnt3S212A-EGFP). However, when we injected the double mutant Wnt3C80AS212A-EGFP construct at the one-cell stage and performed FCS measurements in the BV, we observed correlation functions characteristic for autofluorescence with a very fast diffusion coefficient (∼200 $\mu m^2/s$ in Wnt3C80AS212A-EGFP injected embryos, compared to 225 ± 56.1 $\mu m^2/s$ for wild type un-injected embryos) that are unphysical for proteins of a size of Wnt3 (**Figure 2D**).

The low intensity and high D value in the BV of unlabeled WT embryos indicates diffusion of auto-fluorescent molecules in the BV (**Figure 3**). When we injected embryos with the secretory peptide of Fibroblast growth factor 8a (Fgf8a) tagged with EGFP (secEGFP) as a positive control, a comparatively high molecular brightness (∼2000-2200 counts per particle per second (cps)) was observed in the BV compared to unlabeled WT embryos, and FCS analysis yielded a D of 72.90 ± 9.4 $\mu m^2/s$, consistent with the free diffusion coefficient of EGFP (**Figure 3**). As a negative control, when we injected a plasma membrane targeting peptide tagged with mEGFP (PMT-mEGFP), strong signal was detected on the cell membrane whereas the molecular brightness was low (∼700–800 cps. The ACF in the BV of embryos expressing PMT-mEGFP yielded a D of 201.30 ± 42.10 $\mu m^2/s$; similar to what was observed in the unlabeled WT embryos (**Figure 3**).

C80A and S212A Mutants of Wnt3 Are Associated With Ordered Plasma Membrane Domains

Next, we investigated if C80A and S212A mutations alter the membrane association of Wnt3 in vivo. The addition of lipid groups to Wnts improves their affinity to the cell membrane and helps in their partitioning to ordered membrane domains (Gao et al., 2011; Nile and Hannoush, 2016; Azbazdar et al., 2019). This prompted us to characterize the influence of lipidation on the membrane dynamics and organization of Wnt3 *in vivo*. We first measured the dynamics of Wnt3-EGFP, Wnt3C80A-EGFP, Wnt3S212A-EGFP and Wnt3C80AS212A-EGFP in cell membranes of cells within the zebrafish brain using confocal FCS (**Figure 4**). As previously published, confocal FCS measurements of Wnt3-EGFP result in two fractions, a fast one (D_{fast} of 31.1 ± 8.5 $\mu m^2/s$), representing non-membrane bound proteins either in the cytosol or extracellular space, and a slow fraction (D_{slow} of 0.6 ± 0.2 $\mu m^2/s$; F_{slow} = 0.6 ± 0.05) that is characteristic for membrane diffusion (Veerapathiran et al., 2020). Therefore, we performed FCS on the cell membrane in the cerebellum to determine whether the mutants localize on the cell membrane similar to Wnt3-EGFP.

Wnt3C80A-EGFP and Wnt3S212A-EGFP mutant constructs were both found on the cell membrane similar to Wnt3-EGFP. The correlation functions for the mutant constructs were fit with a 2D-2particle-1triplet model (see Materials and Methods), and the D for both components were computed (see **Table 1**). We observed a fast diffusing component (D_{fast} of 27.2 ± 7.8 $\mu m^2/s$ for Wnt3C80A-EGFP; 28.1 ± 4.9 $\mu m^2/s$ for Wnt3S212A-EGFP) and a slow diffusing component (D_{slow} = 0.6 ± 0.2 $\mu m^2/s$ and F_{slow} = 0.6 ± 0.04 for Wnt3C80A-EGFP; D_{slow} = 0.7 ± 0.3 $\mu m^2/s$ and F_{slow} = 0.5 ± 0.06 for Wnt3S212A-EGFP) (**Table 1**). However, Wnt3C80AS212A-EGFP is not found on the plasma membrane and we did not obtain any ACFs on the cell membrane (**Figure 4**). In order to locate expression of the mutants in the

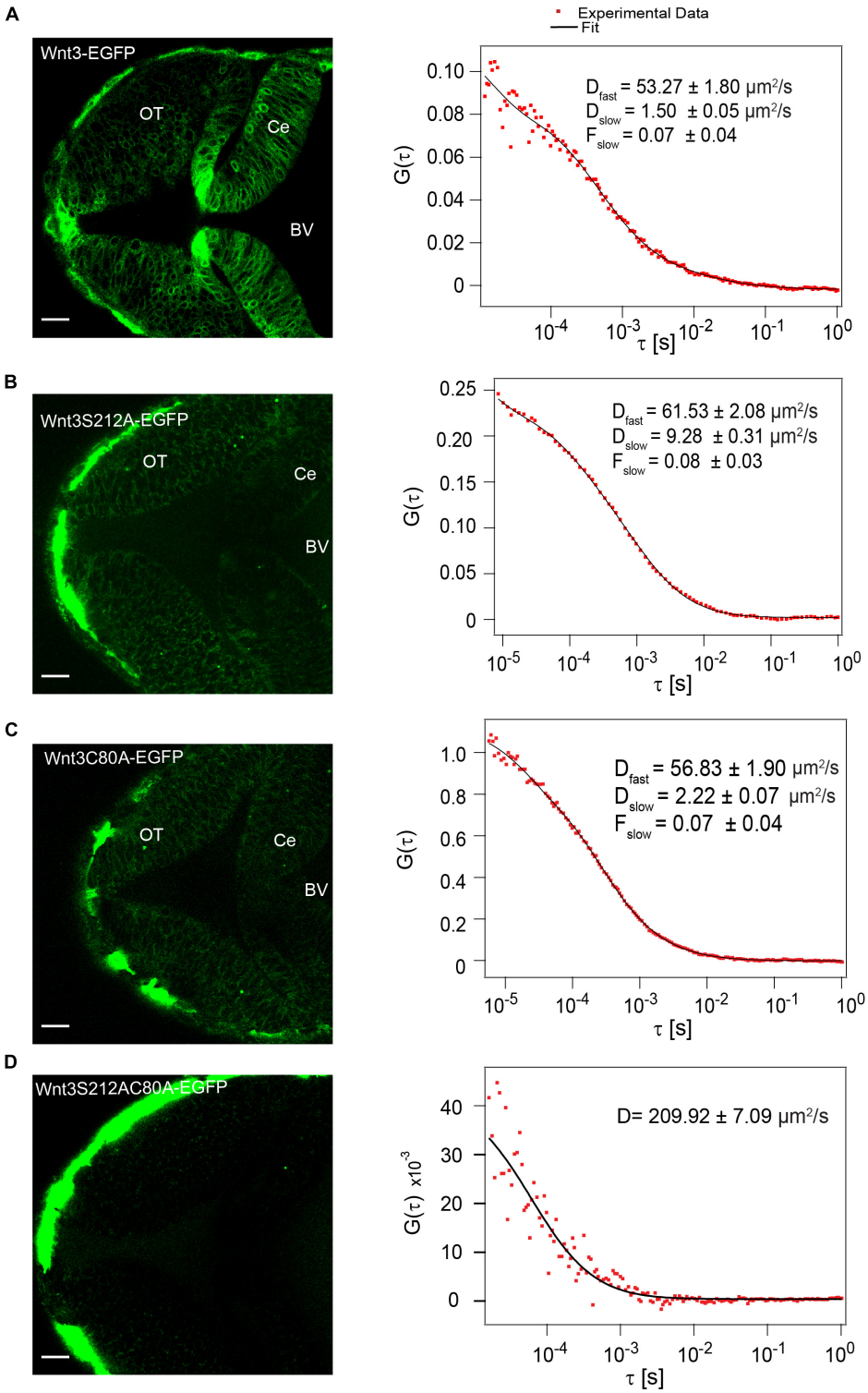

FIGURE 2 | Influence of C80 and S212 lipidation on secretion *in vivo*. **(A)** Expression of Wnt3-EGFP in the zebrafish brain at ∼48 hpf (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a Wnt3-EGFP FCS measurement in the BV (right). **(B)** Expression of Wnt3S212A-EGFP in the zebrafish brain at ∼48 hpf (left) and a representative autocorrelation function (ACF; dots) and fit (line) of Wnt3S212A-EGFP FCS measurement in BV (right). **(C)** Expression of Wnt3C80A-EGFP in the zebrafish brain at ∼48 hpf (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a Wnt3C80A-EGFP FCS measurement in the BV (right). **(D)** Expression of Wnt3S212AC80A-EGFP in the zebrafish brain at ∼48 hpf (left) and a representative autocorrelation function (ACF; dots) and fit (line) of Wnt3S212AC80A-EGFP FCS measurement in BV (right). The FCS curves were fitted using 3D-2particle-1triplet model. BV, fourth brain ventricle; Ce, cerebellum; OT, optic tectum. Orientation: anterior to the left. Scale bar 20 µm.

TABLE 1 | Diffusion coefficients of Wnt3-EGFP, Wnt3C80A-EGFP and Wnt3S212A-EGFP in cell membrane and fourth brain ventricle as measure by confocal FCS.

Region	Sample	D_{fast}	D_{slow}	F_{slow}	No. of measurements
Cell Membrane	Wnt3-EGFP	31 ± 9	0.60 ± 0.2	0.60 ± 0.05	14 (3)
	Wnt3S212A-EGFP	28 ± 5	0.7 ± 0.3	0.50 ± 0.06	17 (4)
	Wnt3C80A-EGFP	27 ± 8	0.6 ± 0.2	0.60 ± 0.04	24 (4)
Brain Ventricle	Wnt3-EGFP	57 ± 12	3.6 ± 2.6	0.1 ± 0.1	16 (3)
	Wnt3S212A-EGFP	54 ± 10	4.6 ± 2.0	0.3 ± 0.1	13 (4)
	Wnt3C80A-EGFP	53 ± 10	6.8 ± 6.0	0.3 ± 0.2	11 (4)

Data are mean ± S.D. Number for zebrafish embryos used for each case is mentioned in bracket.

source and target cells, we co expressed the mutant constructs with PMT-mApple, a protein tethered to the inner leaflet of the cell membrane, which is expressed under a 4 kb Wnt3 promoter, marking source cells of Wnt3 expression. While PMT-mApple localizes to the cell membrane, Wnt3C80AS212A-EGFP is cytosolic. In case of Wnt3C80A-EGFP and Wnt3S212A-EGFP, the mutants at least partially co-localize with PMT-mApple on the cell membrane of source cells but were also found away from the source region (**Figure 5**).

While we measured the mobility of the membrane marker PMT-mEGFP in the cell membrane of the zebrafish brain as a control, we obtained a D_{fast} of 41.8 ± 5.0 $\mu m^2/s$ and a D_{slow} of 1.30 ± 0.40 $\mu m^2/s$, although here the D_{fast} corresponds solely to the cytosolic fraction, as PMT-mEGFP is not secreted extracellularly (**Figure 6**). Overall, as the D_{slow} for the two mutants are in the same order of magnitude as Wnt3-EGFP and the membrane marker, we can infer that both Wnt3C80A-EGFP and Wnt3S212A-EGFP are localized on the membrane in zebrafish embryos. The difference in fluorescence intensities for Wnt3C80-EGFP and Wnt3-S212A-EGFP as compared to Wnt3-EGFP is due to different expression levels as the mutant plasmid constructs were injected in zebrafish embryo at 1-2 cell stage while Wnt3-EGFP is a transgenic line driven by a 4 kb Wnt3 promoter. Therefore, membrane localization for Wnt3C80-EGFP and Wnt3S212A-EGFP was confirmed based on the slow diffusion component and fraction of the slow diffusion component.

We have previously shown that Wnt3 partitions into ordered plasma membrane environments *in vivo* (Ng et al., 2016; Sezgin et al., 2017; Azbazdar et al., 2019). On establishing that Wnt3C80A-EGFP and Wnt3S212A-EGFP are localized on the membrane, we next used the SPIM-FCS diffusion law to understand the role of lipidation in its domain confinement (**Figure 1**). SPIM-FCS is a multiplexed FCS modality that integrates array detectors and SPIM to simultaneously quantify the in vivo dynamics of proteins at a large number of contiguous points across a large area of the sample in a single measurement (Wohland et al., 2010; Singh et al., 2013). The FCS diffusion law utilizes the scale dependence of diffusion to provide nanoscopic information on the membrane organization of the probe such as molecular trapping and meshwork compartmentalization (Lenne et al., 2006). In SPIM-FCS, this is adapted as the SPIM-FCS diffusion law by measuring the diffusion time (τ_d) as a function of the effective observation area, A_{eff}, which is adjusted by pixel binning of the image stack.

Diffusion of molecules on membranes is typically hindered through its interactions with the cytoskeletal meshwork or raft domains. As these interactions are happening at a spatial scale below the diffraction limit, an indirect way to understand the nature of hindrances is to measure the extent of deviation from free diffusion. By plotting the diffusion time (y-axis) vs the area a molecule transvers in space (x-axis) and extrapolating the plot to 0 on the y-axis, we obtain a y-intercept value (τ_0). We can use this value to infer the type of diffusion exhibited by the molecule on the membrane. In free diffusion, as the time a molecule takes to transverse a given area scales linearly with time, the τ_0-value is close to zero ($-0.2 \leq \tau_0 \leq 0.2$) But in cases of transient domain confinement, molecules initially take a very long time to transverse a short area, and thus the τ_0 intercept value becomes positive ($\tau_0 > 0.2$). With increasing domain confinement, there is an increase in τ_0 and conversely, reduced domain confinement leads to low τ_0-values. A negative intercept ($\tau_0 < -0.2$) corresponds to cytoskeleton influenced hop-diffusion coming from initial fast dynamics within the meshwork compartments and subsequent slow down over larger areas beyond the compartment spaces (Wawrezinieck et al., 2005; Bag et al., 2016) (see section "Materials and Methods").

As the thickness of the light sheet used is much larger than the thickness of a cell membrane, fluorescent molecules in the intracellular and extracellular spaces diffusing in 3D space also get excited. These molecules contribute to the fast component of the autocorrelation function in SPIM-FCS. The dynamics of the membrane-bound molecules diffusing on a 2D cell membrane are captured by the slow component. Thus, a two-component model, with the fast component's fitting function using a 3D free diffusion model and the slow component's function using a 2D free diffusion model was used to fit the ACFs. While the slow acquisition rate of the camera (∼ 1 ms) is sufficient to capture the diffusion dynamics on the membrane, it is not fast enough to accurately capture the diffusion coefficient of the fast-diffusing molecules. Nevertheless, we were able to obtain diffusion coefficient values for the fast and slow component in the same range as that of confocal FCS measurements. However, we had to set an upper bound for the diffusion coefficient of 100 $\mu m^2/s$ for the fast component to exclude erroneous fits and non-physical fit values. As we were interested in the

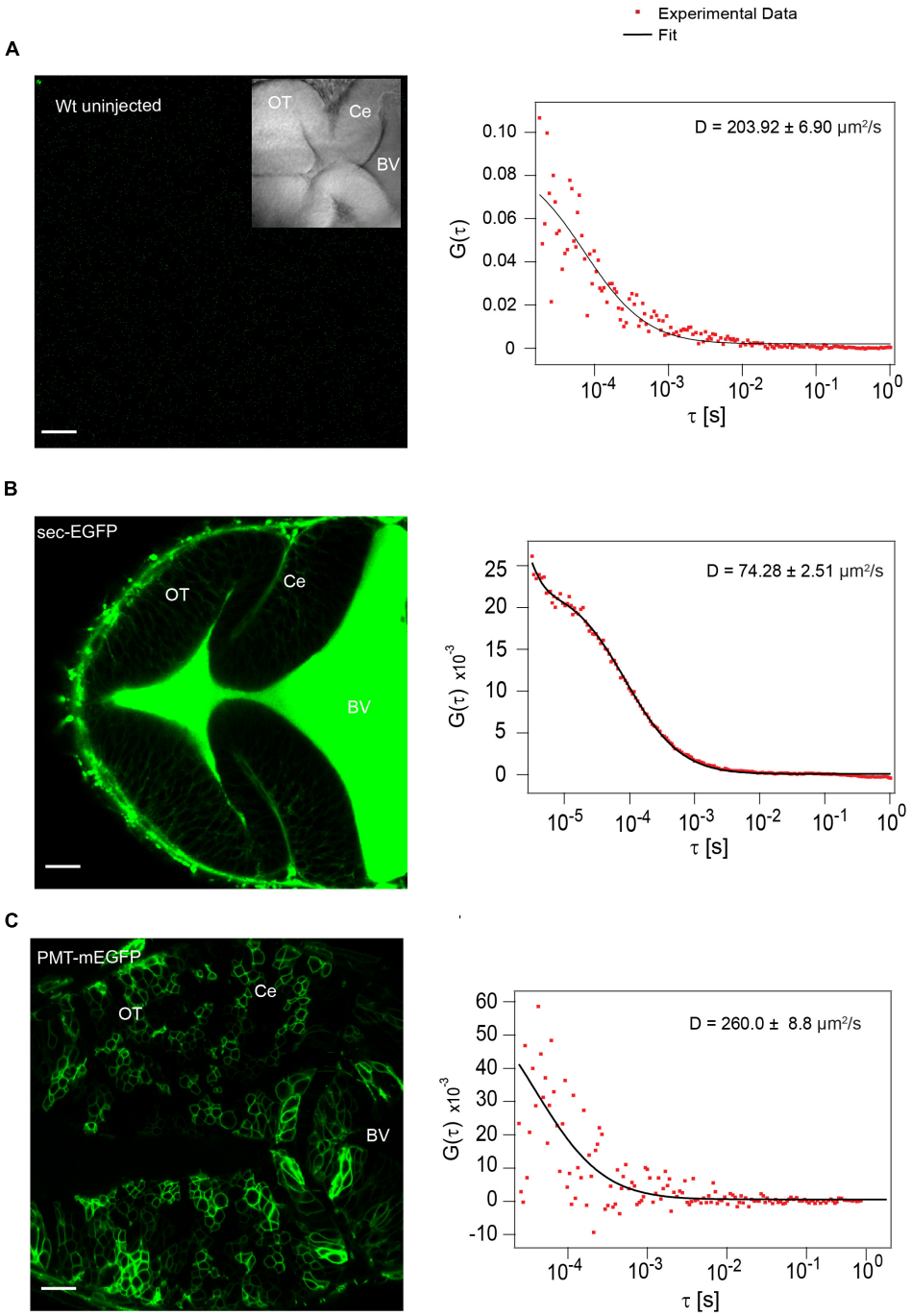

FIGURE 3 | Secretion of positive and negative control in the BV **(A)** Auto fluorescence signal from wild type (Wt) unlabeled zebrafish embryo and representative autocorrelation function (ACF; dots) and fit (line) of a FCS measurement in the BV at ∼48 hpf.). **(B)** Expression of sec-EGFP in the zebrafish brain at ∼48hpf (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a FCS measurement in the BV (right). **(C)** Expression of PMT-mEGFP in the zebrafish brain at ∼48 hpf (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a FCS measurement in the BV of zebrafish (right). The FCS curves were fitted using 3D-2particle-1triplet model. BV, fourth brain ventricle; Ce, cerebellum; OT, optic tectum. Orientation: anterior to the left. Scale bar 20 µm.

membrane dynamics, of Wnt3 and its mutants, diffusion law analysis was performed only for the slow component. Our SPIM-FCS diffusion law analysis revealed that both Wnt3-EGFP and mutants Wnt3C80A-EGFP and Wnt3S212A-EGFP undergo domain-confined diffusion with a τ_0 of Wnt3-EGFP (3.9 ± 1.0 s) higher than those of the mutants Wnt3C80A-EGFP (2.5 ± 0.6 s) and Wnt3S212A-EGFP (3.1 ± 0.7 s). Lower τ_0-values for the mutants imply that they are less confined to ordered

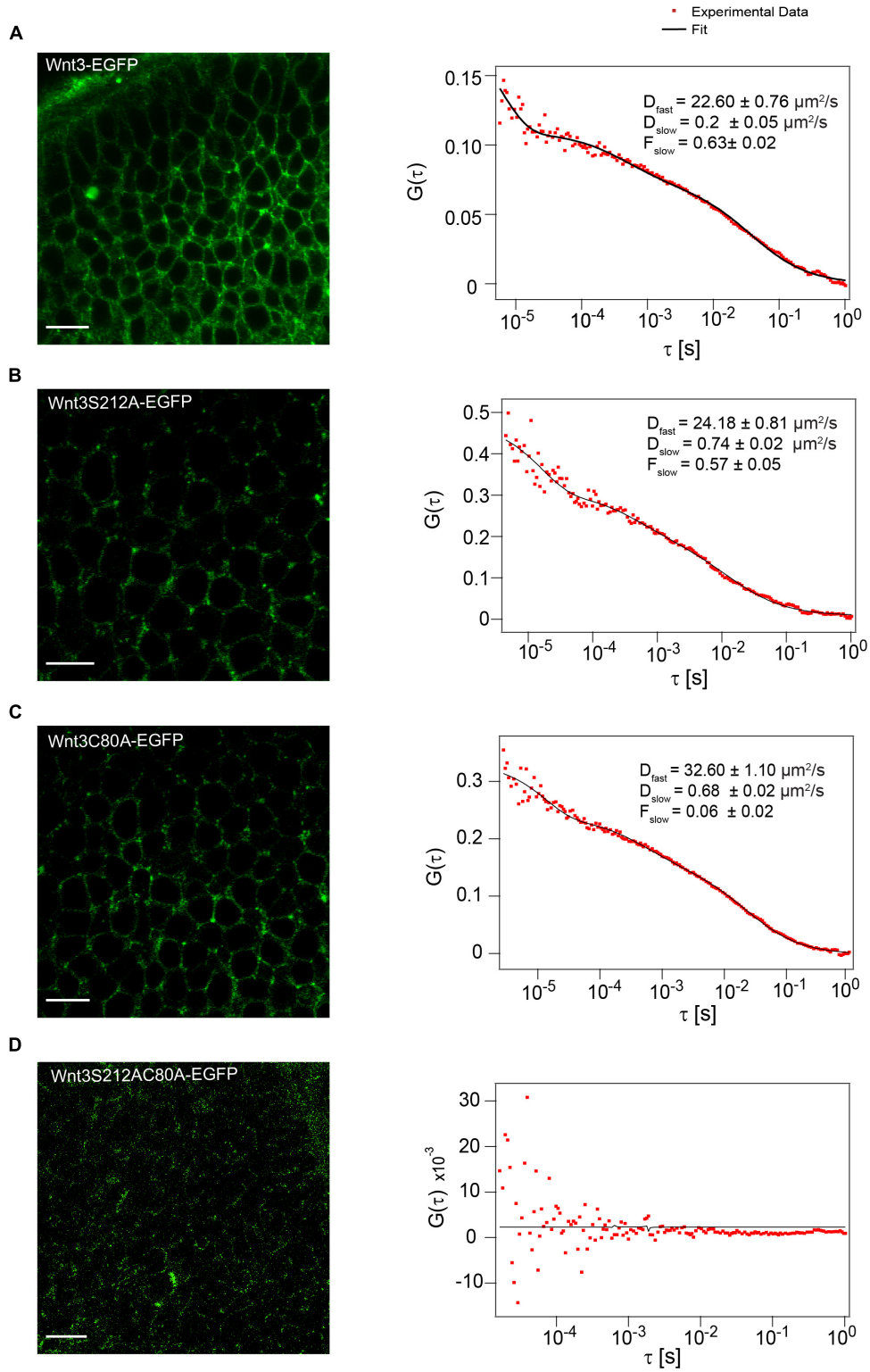

FIGURE 4 | Influence of C80 and S212 lipidation on membrane localization *in vivo*. **(A)** Expression of Wnt3-EGFP on the cell membrane (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a Wnt3-EGFP FCS measurement at a cell membrane (right). **(B)** Expression of Wnt3S212A-EGFP on the cell membrane (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a Wnt3S212A-EGFP FCS measurement at a cell boundary (right). **(C)** Expression of Wnt3C80-EGFP on the cell membrane (left) and a representative autocorrelation function (ACF; dots) and fit (line) of a Wnt3C80A-EGFP FCS measurement at a cell membrane (right). **(D)** Expression of Wnt3S212AC80-EGFP on the cell membrane (left) (Image brightness was increased for clear visualization) and a representative autocorrelation function (ACF; dots) and fit (line) of a Wnt3S212AC80A-EGFP FCS measurement at a cell membrane (right). The FCS curves were fitted using 2D-2particle-1triplet model. BV, fourth brain ventricle; Ce, cerebellum; OT, optic tectum. Orientation: anterior to the left. Scale bar 10 µm.

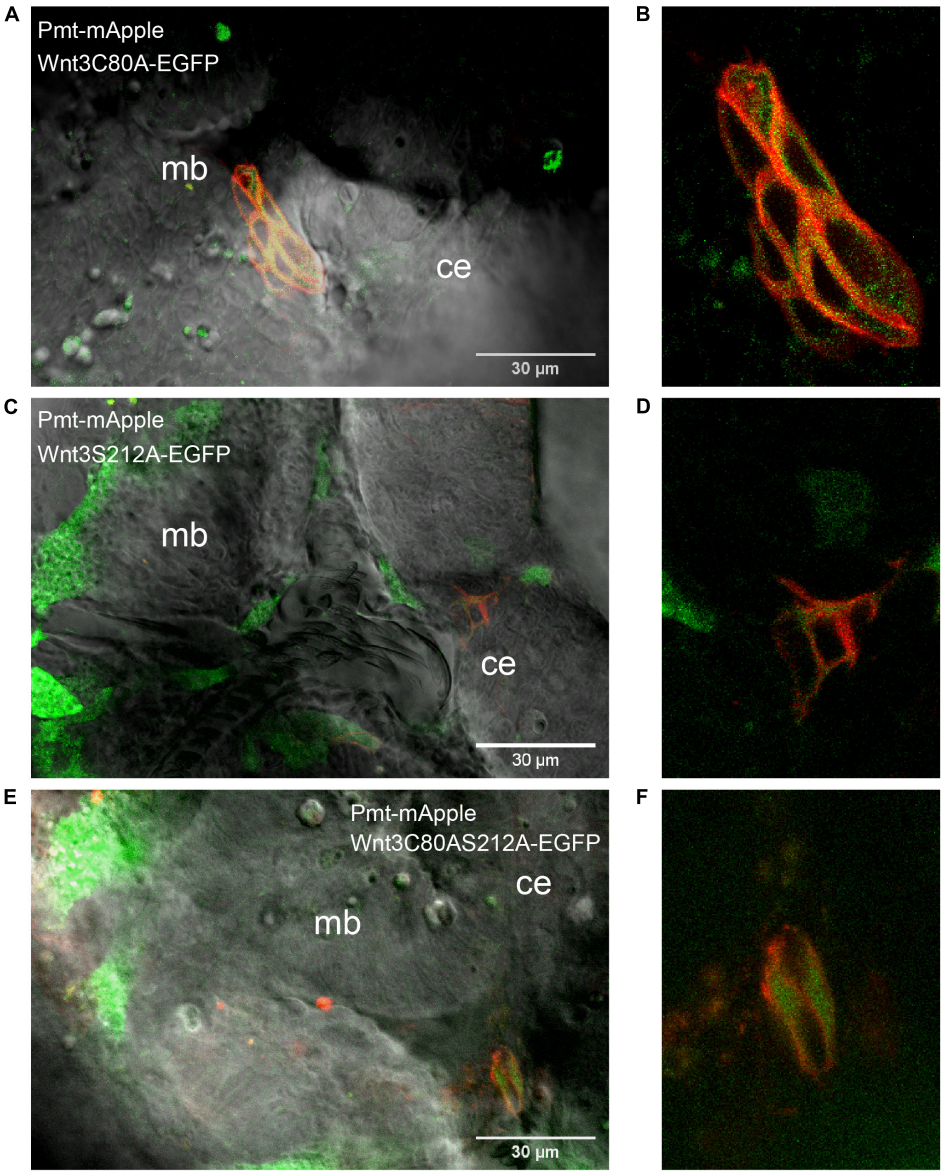

FIGURE 5 | Expression profile of the mutants co expressed with PMT-mApple. Co-expression of mutants (Wnt3C80A-EGFP, Wnt3S212A-EGFP, and Wnt3S212AC80A-EGFP) and PMT-mApple in the zebrafish brain at ∼48 hpf. PMT-mApple is a protein located on the inner leaflet of the cell membrane. It is expressed under a 4kb Wnt3 promoter and serves to mark the source cells of Wnt3 expression. **(A)** Expression profile of Wnt3C80A-EGFP co-expressed with PMT-mApple in zebrafish brain. **(B)** Expression of Wnt3C80A-EGFP co-expressed with PMT-mApple in the midbrain. **(C)** Expression profile of Wnt3S212A-EGFP co-expressed with PMT-mApple in zebrafish brain. **(D)** Expression of Wnt3S212A-EGFP co-expressed with PMT-mApple in the midbrain. **(E)** Expression profile of Wnt3C80AS212A-EGFP co-expressed with PMT-mApple in zebrafish brain. **(F)** Expression of Wnt3C80AS212A-EGFP co-expressed with PMT-mApple in the midbrain.

membrane domains as compared to Wnt3-EGFP (**Table 2** and **Figure 7**).

C80 but Not S212 Residue Is Dispensable for the Interaction of Wnt3 With Fzd1 *in vivo*

The high-resolution crystal structures of Wnt-Fzd complexes revealed the presence of a deep hydrophobic pocket in the CRD of Fzd in which Wnts insert their lipid tail indicating a critical role of lipidation in receptor interactions (Janda et al., 2012; Hirai et al., 2019). As we had earlier established that Wnt3 strongly interacts with Fzd1 in the dorsal cerebellum and midbrain hindbrain boundary of live zebrafish embryos with an apparent dissociation constant (K_d) of ∼ 115 nM (Veerapathiran et al., 2020), we aimed to monitor the interactions of Wnt3C80A-EGFP and Wnt3S212A-EGFP with Fzd1-mApple in the same regions. We used quasi-PIE FCCS, a modality of

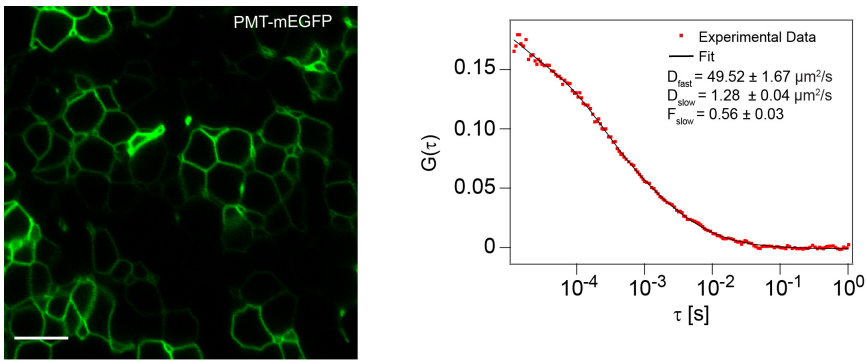

FIGURE 6 | Membrane localization and dynamics of PMT-mEGFP Cell membrane localization of PMT-mEGFP (left) and a representative autocorrelation function (ACF; dots) and fit (line) of FCS measurement at the cell membrane (right). The FCS curves were fitted using 2D-2particle-1triplet model. Scale bar 10 µm.

FCCS that uses a pulsed laser line along with a continuous wave laser line to illuminate the sample. This approach facilitates the statistical filtering of background, spectral bleed through and detector after-pulsing (Padilla-Parra et al., 2011; Yavas et al., 2016). Embryos co-expressing Wnt3C80A-EGFP Fzd1-mApple yielded positive cross-correlations suggesting that the Wnt3C80A-EGFP retains its interaction with Fzd1 receptor in vivo. The apparent dissociation constant (K_d) was calculated to be 313 ± 42 nM (**Figure 8**), signifying that C80A mutant binds with a lower affinity to Fzd1 receptor as compared to Wnt3-EGFP. Interestingly, mutation at the S212 residue abolished the Wnt3-Fzd1 interaction as no cross-correlations were observed in embryos co-expressing Wnt3S212A-EGFP and Fzd1-mApple (**Figure 8**). As positive control, we injected PMT-mApple-mEGFP at the one-cell stage which showed a higher cross-correlation amplitude compared to the sample, while as negative control, we co-expressed PMT-mApple and PMT-mEGFP which showed no cross-correlation (**Figure 8**). These results clearly suggest that the lipidation at S212 residue is vital for its in vivo interaction with Fzd1.

C80 and S212 Lipidations Are Essential for Wnt3 to Activate β-Catenin Signaling

Lastly, to test whether lipidation of Wnt3 at the conserved cysteine and serine affects Wnt/β-catenin signaling activity, we overexpressed Wnt3-EGFP, Wnt3C80A-EGFP or Wnt3S212A-EGFP in zebrafish embryos by injecting their capped sense RNAs at the one-cell stage. We next treated the embryos with the Wnt antagonist IWR-1 for 16 h until 24 hpf. IWR treatment caused a reduction in tail elongation while Wnt3-EGFP effectively suppressed eye formation at 24 hpf, a distinctive phenotype caused by enhanced Wnt/ß-catenin signaling (Özhan et al., 2013). IWR could significantly restore the eye phenotype caused by Wnt3-EGFP. Neither Wnt3C80A-EGFP nor Wnt3S212A-EGFP overexpression exhibited any phenotypic alteration as compared to the control (**Figure 9**).

To examine the direct influence of the Wnt3EGFP and Wnt3 lipidation site mutant constructs on Wnt/β-catenin signaling, we took advantage of a transgenic zebrafish reporter of Wnt/ß-catenin signaling *Tg(7xTcf-Xla.Siam:nlsm-Cherry^{ia})* (Moro et al., 2012). IWR robustly inhibited reporter activity in all domains of expression. In contrast to strong reporter activation by Wnt3-EGFP in the posterior site of embryos at 24 hpf, Wnt3C80A-EGFP or Wnt3S212A-EGFP overexpression had no detectable effect on the reporter activity as shown by whole mount in situ hybridization (WMISH) (**Figure 9**).

During gastrulation, canonical β-catenin signaling mediates specification of the ventrolateral mesoderm by repressing the dorsal organizer (Lekven et al., 2001). To test whether Wnt3 lipidation affects mesodermal patterning of the embryo, we injected mRNAs of Wnt3 constructs into 1-cell embryos and examined development of the organizer at early development. While Wt Wnt3 caused a significant restriction of the dorsal organizer region marked by *goosecoid* (*gsc*) expression (Yao and Kessler, 2001) at early gastrula (shield, 6 hpf) stage, neither Wnt3C80A-EGFP nor Wnt3S212A-EGFP altered the size of organizer region as compared to control (**Figure 10**). Taken together, these data indicate that lipid modifications of Wnt3 at both C80 and S212 are necessary for downstream activation of Wnt/β-catenin signaling.

These results show that point mutations at either S212 or C80 residue of Wnt3 do not impede its membrane localization and secretion, however, localization to the ordered membrane domains is compromised. There are two possible explanations for this: (i) Wnt3 is dually lipidated at its C80 and S212 position, and the lipid tail at one residue compensates for the absence of the lipid moiety at the mutated residue (ii) Wnt3 is lipidated only at S212, but its membrane localization and secretion is independent of its lipidated.

DISCUSSION

Post-translational lipidation involves the covalent modification of proteins with lipids in the cytoplasm or in the lumen of the organelles involved in the secretory pathway. Among the secreted signaling molecules regulating development, Wnts and Hedgehog are the most commonly reported lipid modified morphogens with diverse functions (Parchure et al., 2018).

TABLE 2 | SPIM-FCS and SPIM diffusion law results for Wnt3-EGFP, Wnt3C80A-EGFP and Wnt3S212A-EGFP.

Sample	D_{Fast} [$\mu m^2/s$](N_{ACFs})	D_{Slow} [$\mu m^2/s$](N_{ACFs})	F_{Slow}	τ_0 [s]	Cells (embryos)
Wnt3-EGFP	29 ± 22 (515)	0.40 ± 0.17 (635)	0.5 ± 0.2	3.9 ± 1.0	18 (4)
Wnt3C80A-EGFP	22 ± 18 (317)	0.55 ± 0.27 (374)	0.5 ± 0.2	2.5 ± 0.6	11 (4)
Wnt3S212A-EGFP	27 ± 18 (478)	0.48 ± 0.20 (561)	0.5 ± 0.2	3.1 ± 0.7	16 (4)

Data are mean ± S.D.

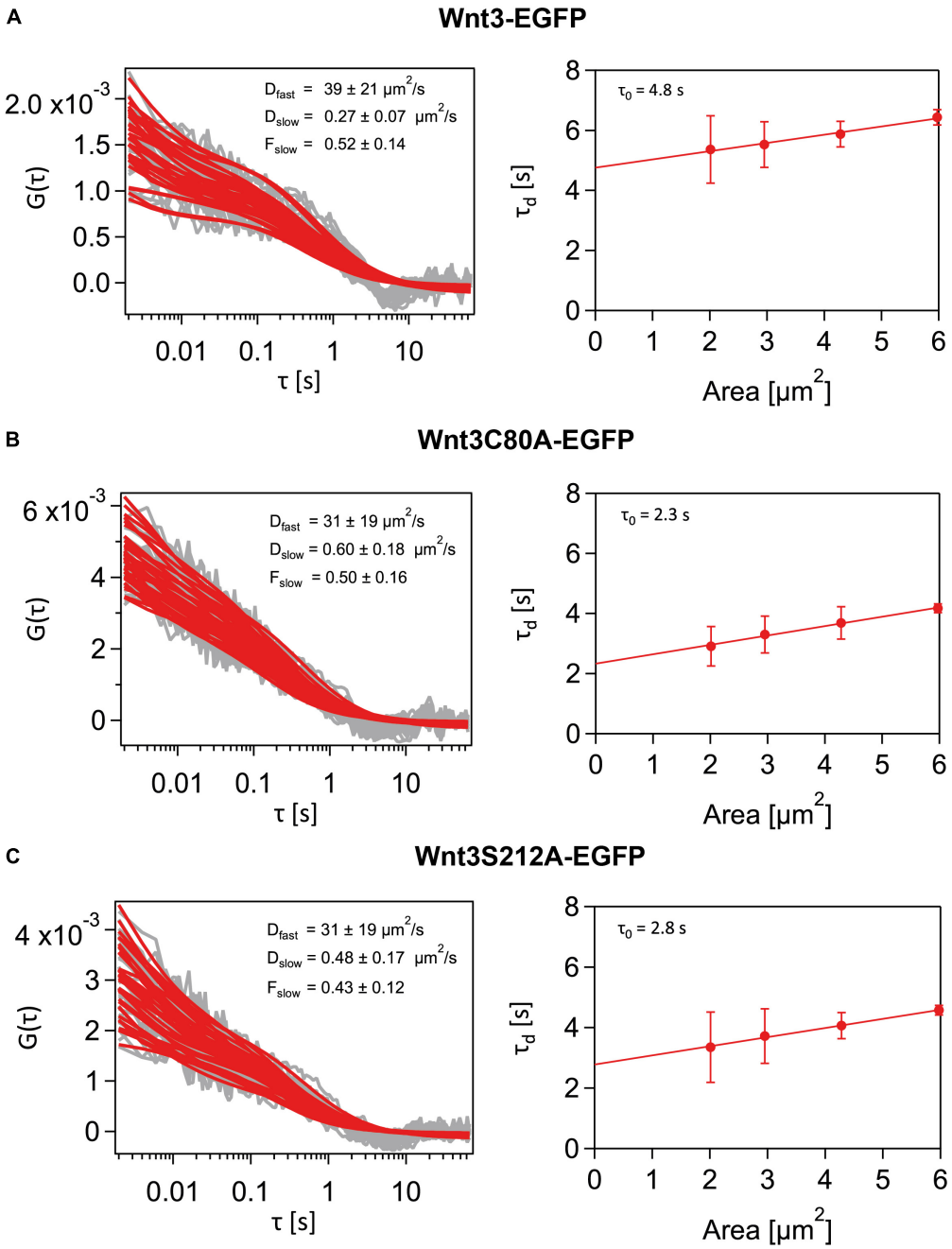

FIGURE 7 | Representative SPIM-FCS and Diffusion law data from a cell. **(A)** Autocorrelation functions (left) and Diffusion law plot (right) for a Wnt3-EGFP expressing cell *in vivo*. Wnt3-EGFP expressing cells have higher τ_0 value than the mutants. **(B)** Autocorrelation functions (left) and Diffusion law plot (right) for a Wnt3C80A-EGFP mutant-expressing cell *in vivo*. **(C)** Autocorrelation functions (left) and Diffusion law plot (right) for a Wnt3S212-EGFP mutant expressing cell *in vivo*. All autocorrelation function plots have the raw correlation data in gray and their corresponding fit in red.

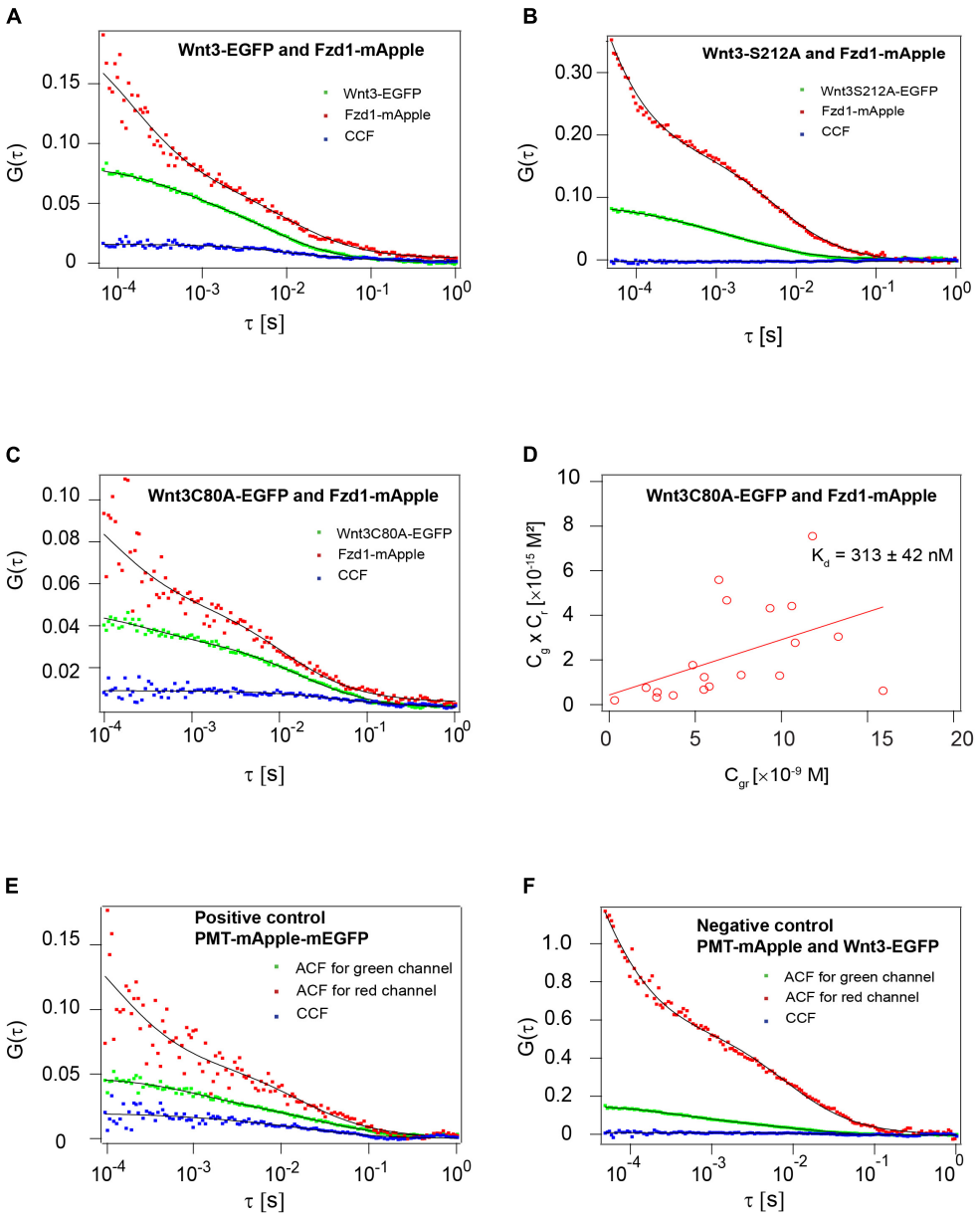

FIGURE 8 | Influence of C80 and S212 lipidation on the interaction of Wnt3 with Fzd1 receptor. **(A)** Representative auto- and cross-correlation functions (dots) and fits (lines) of a Wnt3-EGFP and Fzd1mApple FCCS measurement. The positive cross-correlation function indicates Wnt3EGFP interacts with Fzd1mApple *in vivo*. **(B)** Representative auto- and cross-correlation functions (dots) and fits (lines) of a Wnt3S212A-EGFP and Fzd1mApple FCCS measurement. No cross-correlation function indicates Wnt3S212A-EGFP does not interact with Fzd1mApple *in vivo*. **(C)** Representative auto- and cross-correlation functions (dots) and fits (lines) of Wnt3C80A-EGFP and Fzd1mApple. The positive cross-correlation function indicates Wnt3C80-EGFP interacts with Fzd1mApple *in vivo*. **(D)** Determination of apparent dissociation constant (K_d) for Wnt3C80A-EGFP and Fzd1mApple interaction *in vivo*. Cg, Cr, and Cgr represents the concentration of unbound Wnt3C80A-EGFP, unbound Fzd1mApple, and bound Wnt3C80A-EGFP and Fzd1mApple molecules, respectively. The estimated apparent K_d ($K_d = C_g \times C_r / C_r C_{gr}\ C_{gr}$) for Wnt3C80A-EGFP and Fzd1mApple in vivo is 313 ± 42 nM. **(E)** Representative auto- and cross-correlation functions for PMT-mApple-mEGFP which is a positive control showing a clear cross-correlation. **(F)** Representative auto- and cross-correlation functions for embryos co-expressing PMT-mApple and PMT-mEGFP as a negative control.

Almost all Wnts, except WntD in *Drosophila*, are post-translationally lipidated, and this lipid modification has a regulatory role in mediating membrane-protein interaction, secretion and signaling activity (Nadolski and Linder, 2007; Jiang et al., 2018). Initial mass spectrometry studies marked cysteine and serine as putative lipid addition sites (Willert et al., 2003; Takada et al., 2006). Later however, the crystal structure of XWnt8 (Janda et al., 2012), along with biochemical studies using metabolic labeling and click chemistry (Gao and Hannoush, 2014; Miranda et al., 2014), identified serine as the solitary lipidation

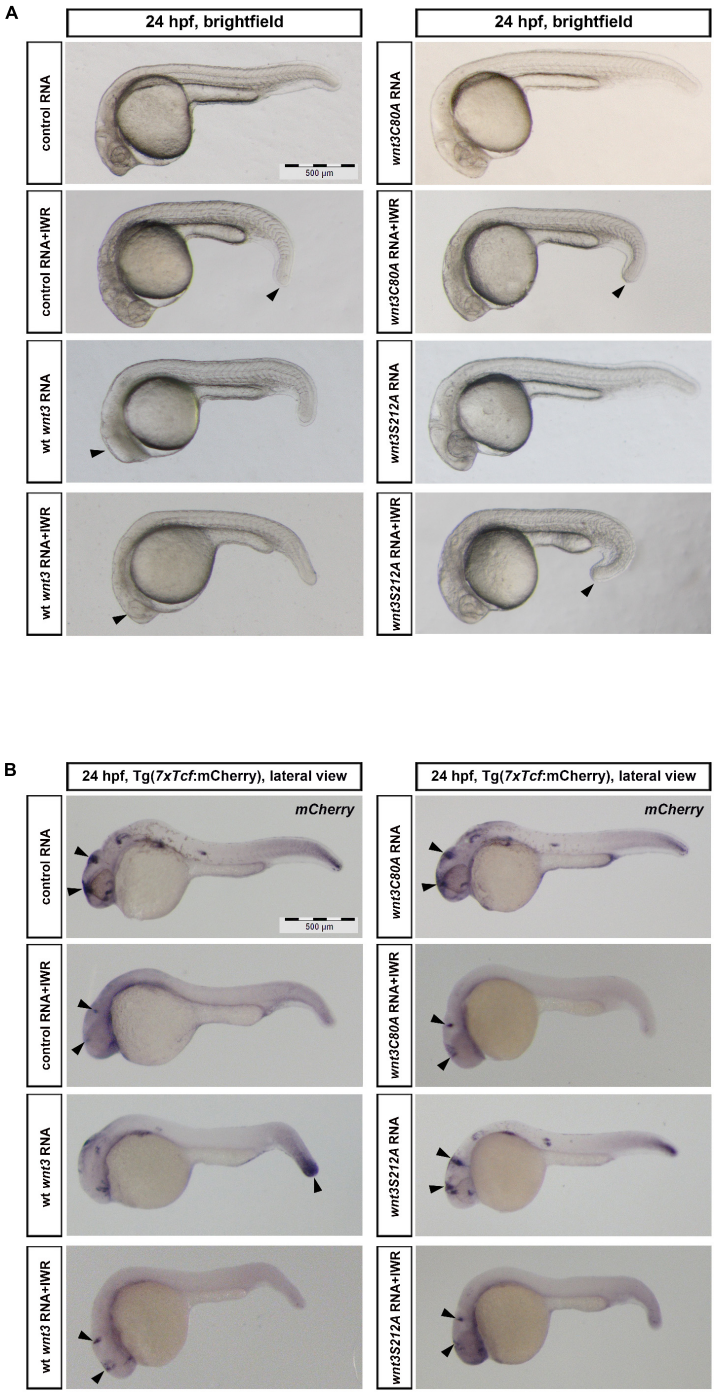

FIGURE 9 | Influence of C80 and S212 residues on Wnt signaling activity **(A)** Morphological phenotypes at 24 hpf of embryos injected/treated with capped sense RNAs of control (EGFP 100 pg, 37/37 embryos), control +IWR (42/42 embryos, arrowhead: reduction in tail elongation), wt Wnt3 (100 pg, 47/53 embryos, arrowhead: loss of eye), Wnt3-EGFP + IWR (36/43 embryos, arrowhead: restoration of eye), Wnt3C80A-EGFP (100 pg, 41/43 embryos), Wnt3C80A-EGFP + IWR (41/44 embryos, arrowhead: reduction in tail elongation), Wnt3S212A-EGFP (100 pg, 52/54 embryos) or Wnt3S121A-EGFP + IWR (43/46 embryos, arrowhead: reduction in tail elongation). IWR was used at a concentration of 10 µM and DMSO was used in groups that were not treated with IWR. **(B)** Whole mount in situ hybridization (WMISH) in the transgenic Tg(*7xTcf-Xla.Siam:nlsm-Cherry*^{ja}) canonical Wnt/ß-catenin reporter embryos showing the effects of control (EGFP 100 pg, 33/33 embryos, arrowheads: anterior Wnt expression domains), control + IWR (45/45 embryos, arrowheads: reduction in anterior Wnt expression domains), Wnt3-EGFP (100 pg, 41/49 embryos, arrowheads: increase in posterior Wnt expression domain), Wnt3-EGFP + IWR (40/45 embryos, arrowheads: restoration of anterior Wnt expression domain), Wnt3C80A-EGFP (100 pg, 45/48 embryos, arrowheads: no effect on anterior Wnt expression domains), Wnt3C80A-EGFP + IWR (38/39 embryos, arrowheads: reduction in anterior Wnt expression domains), Wnt3S212A-EGFP (100 pg, 44/46 embryos, arrowheads: no effect on anterior Wnt expression domains) or Wnt3S121A-EGFP + IWR (50/53 embryos, arrowheads: reduction in anterior Wnt expression domains) on canonical Wnt signaling. IWR was used at a concentration of 10 µM and DMSO was used in groups that were not treated with IWR1. *mCherry* WMISH shows upregulation of signaling in Wnt/ß-catenin reporter embryos by Wnt3-EGFP but not by Wnt3C80A-EGFP or Wnt3S212A-EGFP.

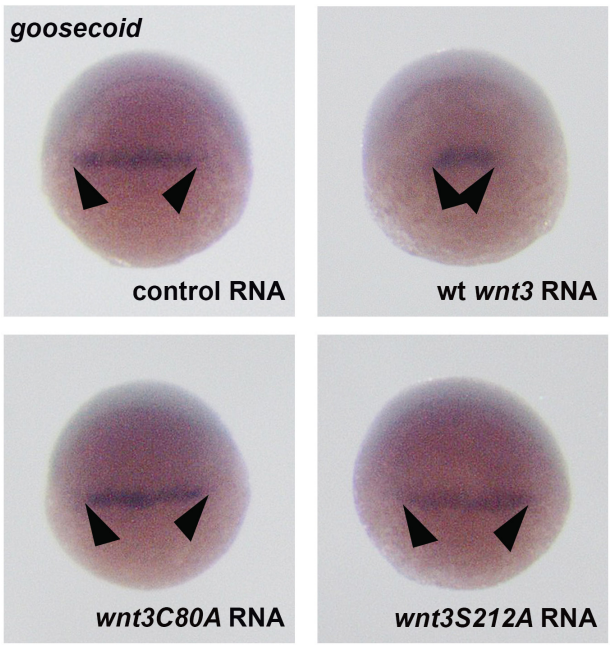

FIGURE 10 | Whole mount *in situ* hybridization for the dorsal organizer marker gene *goosecoid* (*gsc*) in wt embryos at the shield stage, showing the effects of control (EGFP 100 pg, 35/35 embryos, arrowheads: normal *gsc* expression domain), Wnt3-EGFP (100 pg, 31/35 embryos, arrowheads: reduction in *gsc* expression domain), Wnt3C80A EGFP (100 pg, 38/41 embryos, arrowheads: no effect on *gsc* expression domain), and Wnt3S212A-EGFP (100 pg, 32/34 embryos, arrowheads: no effect on *gsc* expression domain).

site across all Wnts. This was further supported by functional studies where the Wnt3a S209A mutant expressed in L-cells was not secreted into the media (Takada et al., 2006) and the S239A Wg mutant diminished downstream Wnt signaling in Drosophila imaginal disks (Franch-Marro et al., 2008). However, in a few Wnt proteins, mutating the conserved serine residue did not perturb their in vivo expression, secretion, or function, which cannot be explained by a single lipid addition site. Additionally, the conformation of Wnts could also differ in its free and bound state (Janda and Garcia, 2015). Therefore, in this work, we investigated the possibility of lipid modifications at the conserved serine and cysteine residues and the importance of lipidation in Wnt trafficking and activity.

The addition of hydrophobic groups to soluble proteins increases their affinity to membranes, thereby influencing their membrane organization and function. Furthermore, these lipid modifications also drive their compartmentalization in organized membrane domains which serve as portals for protein-protein interactions, oligomerization, and signaling among others (Levental et al., 2010a,b; Lingwood and Simons, 2010; Resh, 2013). As zebrafish Wnt3 is known to partition into cholesterol rich membrane domains and inhibition of Porc by C59 is known to decrease its association with these domains, here we monitored the membrane localization and domain compartmentalization of C80A and S212A Wnt3EGFP mutants in the zebrafish brain. From our diffusion law results, we observed that both Wnt3C80A-EGFP and Wnt3S212A-EGFP mutants are found on the membrane albeit, with a lower degree of domain confinement as compared to Wnt3-EGFP embryos. However, the double mutant Wnt3C80AS212A-EGFP construct, was not detected on the membrane.

Our results demonstrate that disrupting either the conserved cysteine or conserved serine does not abolish membrane localization for Wnt3 in the zebrafish brain. However, its association with ordered domains is reduced as shown by the lower τ_0-values for the mutants (Wnt3S212A-EGFP and Wnt3C80A-EGFP) as compared to Wnt3-EGFP. This suggests that lipidation at either residue is sufficient for membrane binding of Wnt3 in zebrafish but lipidation at both residues is required for its correct partitioning into ordered membrane domains. This can be seen by comparing τ_0-values for the mutants (Wnt3C80A-EGFP and Wnt3S212A-EGFP) with Wnt3-EGFP, which are lower and thus indicate reduced confinement to ordered membrane domains.

The BV serves as a route for signaling molecules to achieve long-range transport in the central nervous system (Kaiser et al., 2019). In the zebrafish brain, Wnt3 is secreted into the BV as it is adjacent to the Wnt3-expressing cells of the cerebellar rhombic lip. So next, we studied how mutations in Wnt3 C80 and S212 residues affect their secretion into the BV. Similar to our results on membrane organization, we observed that Wnt3C80A-EGFP and Wnt3S212A-EGFP mutants are secreted into the BV, while mutating both residues simultaneously renders Wnt3 incapable of secretion. This further substantiates the possibility that Wnt3 in zebrafish is lipidated at both its C80 and S212 position. These results are consistent with another study in which Tang et al. (2012) observed membrane localization and secretion of single Wg C93A and S239A mutants but not of Wg double mutants.

Indeed, lipid modifications are believed to mediate the interaction of Wnt with Wls, the intracellular Wnt chaperone protein that transports Wnts to the membrane and facilitates their secretion (Bartscherer et al., 2006; Herr and Basler, 2012). Blocking the interaction of Wnt with Wls or mutating Wls leads to intracellular accumulation of Wnt proteins and degradation (Bänziger et al., 2006). As we note in this study that Wnt3C80A-EGFP and Wnt3S212A-EGFP single mutants are targeted to the membrane and secreted into the BV, while Wnt3C80AS212A-EGFP is not, we can assume that a single lipid modification in the Wnt3 single mutants maintains their interaction with Wls, while the lack of lipidation at both residues diminishes Wnt3-Wls interaction. However, how mutations at C80 and S212 residue modulate the interaction of Wnt3 with Wls *in vivo* requires further investigation.

Overall, disruption of Wnt lipidation at C80 or S212 does not abolish membrane binding as shown by the slow diffusion coefficient for Wnt3C80A-EGFP and Wnt3S212A-EGFP mutant which is of the same order as the slow diffusion coefficient for Wnt3-EGFP. However, neither mutant can activate downstream signaling. This lack of downstream signaling might be a result of reduced localization of mutants to the ordered lipid membrane domains which is demonstrated by the lower τ_0 values for the mutants as compared to Wnt3-EGFP. The importance of membrane organization of proteins has been discussed by

Azbazdar et al. (2019) who have proposed that Wnt3 acylation is necessary for its localization to ordered domains in the membrane and subsequently to downstream signaling. It is possible that partitioning into the ordered membrane domains helps in Wnt complex signalosome formation which leads to the activation of the signaling pathway. However, this needs further investigation.

Finally, when secreted Wnt3 reaches its target tissues, how do covalent lipid modifications of Wnt3 regulate receptor binding and signaling activity? To answer this question, we measured how the mutations at C80 and S212 residue impact Wnt3 interaction with Fzd1 using quasi-PIE FCCS. We found that Wnt3C80A-EGFP maintains its interaction with Fzd1-mApple, albeit with an almost three times lower binding affinity when compared to Wnt3-EGFP. In contrast, Wnt3-EGFP is unable to interact with Fzd1-mApple *in vivo* when the S212 residue is substituted with alanine. Our results are in line with the description of the serine residue positioning its lipid adduct into the hydrophobic pocket of Fzd as determined from the crystal structure of xWnt8-mFzd8 complex. Interestingly, we observed that despite Wnt3C80A-EGFP binding to Fzd1 receptor, it does not activate downstream signaling. Although Azbazdar et al. also reported that non-acylated Wnt3 interacts with Fzd8 but fails to activate downstream β-catenin signaling in HEK293 cells (Azbazdar et al., 2019), the reason still remains elusive. However, note that these observations are tissue-specific and context dependent and results might vary if the same Wnt is being studied in a different tissue/region in zebrafish or in a different organism. Finally, we analyzed the effect of C80A and S212A mutation on the downstream signaling pathway and found that Wnt3C80A-EGFP and Wnt3S212A-EGFP failed to activate the β-catenin signaling pathway as demonstrated by whole mount in situ hybridization for the *goosecoid* (*gsc*)

Collectively, the *in vivo* functional studies in this work indicate that zebrafish Wnt3 experiences lipid modifications at its conserved cysteine and serine residues, which regulate its secretion and activity in neural cells. However, additional investigations are required to further substantiate our results. First, a detailed mass-spectrometric characterization of Wnt3 purified from zebrafish neural cells is required to precisely identify the type of lipids and the sites of lipidation in zebrafish Wnt3. Moreover, palmitoylation, the most commonly reported form of lipid modification in Wnts, has a unique characteristic of being reversible (Ganesan and Levental, 2015; Azbazdar et al., 2019). Hence, it is critical to check whether Wnt3 undergoes a series of palmitoylation and depalmitoylation events and how that affects its distribution and function *in vivo*. Lastly, the signaling range of Wnts in vivo could also be lipidation dependent, as shown by Baena-Lopez et al. (2009) for Wg in *Drosophila*. Therefore, it is also essential to examine how changes in lipidation modulates the range and gradient kinetics of Wnt3 during vertebrate brain development.

In conclusion, we infer that zebrafish Wnt3 is dually lipidated at its C80 and S212 residue. We find that single mutants at C80 or S212 are both binding to membranes, albeit with reduced domain localization, and are secreted. But only Wnt3C80A-EGFP binds to its receptor Fzd1, however without activating signaling in zebrafish neural cells. The double mutant Wnt3C80AS212A-EGFP is neither found on the membrane nor is it secreted. Overall, this study sets the framework for a detailed understanding of the molecular basis of Wnt lipidation during mammalian brain development and developing novel drugs for diseases that therapeutically target the Wnt signaling network.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by NUS Institutional Animal Care and Use Committee (IACUC) breeding protocol BR18-1023.

AUTHOR CONTRIBUTIONS

TW, SV, and GO designed the experiments. SV and DD performed the FCS and FCCS experiments. AN performed and analyzed the SPIM-FCS measurements. YA performed the phenotype characterization and reporter line assay for downstream Wnt β-catenin signaling. CT prepared the constructs and transgenic zebrafish lines. SV, DD, GO, and TW wrote the manuscript. All authors contributed to the discussion. All authors contributed to the article and approved the submitted version.

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REVIEW
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WNT5B in Physiology and Disease

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WNT5B, a member of the WNT family of proteins that is closely related to WNT5A, is required for cell migration, cell proliferation, or cell differentiation in many cell types. WNT5B signals through the non-canonical β-catenin-independent signaling pathway and often functions as an antagonist of canonical WNT signaling. Although WNT5B has a high amino acid identity with WNT5A and is often assumed to have similar activities, WNT5B often exhibits unique expression patterns and functions. Here, we describe the distinct effects and mechanisms of WNT5B on development, bone, adipose tissue, cardiac tissue, the nervous system, the mammary gland, the lung and hematopoietic cells, compared to WNT5A. We also highlight aberrances in non-canonical WNT5B signaling contributing to diseases such as osteoarthritis, osteoporosis, obesity, type 2 diabetes mellitus, neuropathology, and chronic diseases associated with aging, as well as various cancers.

Keywords: WNT5B, WNT signaling, development, cancer, WNT5A

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INTRODUCTION

Wingless-related integration site (*Wnt*) genes are evolutionarily conserved, secreted proteins that are essential for developmental and biological processes. In 1982 the first *Int-1* gene was discovered and this proto-oncogene was activated by integration of the mouse mammary tumor virus to induce breast tumors (Nusse and Varmus, 1982), suggesting the importance of *Int* genes in cancer. The mutation of the *wingless* (*wg*) gene in Drosophila created the wingless phenotype (Sharma and Chopra, 1976) by interrupted segment polarity in fly larva (Rijsewijk et al., 1987), indicating its role in development. Over 30 years ago, the *wg* gene was shown to be a homolog with the mouse *Int-1* gene, leading to a combination of these two names to WNT (Rijsewijk et al., 1987).

The WNT family now contains 19 WNT genes, falling into 12 WNT subfamilies in mammalian genomes. All WNT genes encode proteins around 40 kDa in size and contain highly conserved cysteines (Miller, 2002; Clevers and Nusse, 2012). Mammalian WNT proteins are palmitoylated at conserved serine residues by a special palmitoyl transferase, Porcupine (PORCN), in the endoplasmic reticulum (Takada et al., 2006; Galli et al., 2007; Rios-Esteves et al., 2014). Zebrafish WNT3 is lipidated at both cysteine and serine residues (Dhasmana et al., 2021). The activity of PORCN is essential for the secretion of WNT ligands. Then, the seven-transmembrane protein Wntless/Evi (Wls) in the endoplasmic reticulum escorts mature hydrophobic WNT proteins to be secreted at the plasma membrane or released in exosomes, leading to both autocrine and paracrine effects (Banziger et al., 2006; Routledge and Scholpp, 2019).

The WNT signaling pathway is divided into two main branches: the non-canonical (β-catenin-independent) signaling pathway and the canonical (β-catenin-dependent) signaling pathway (**Figure 1**). WNT ligands bind to various receptors and co-receptors. There are 10 members of the Frizzled (FZD) protein family, which serve as receptors for both the canonical and non-canonical signaling pathways. The pairing between specific FZDs and another receptor, such as low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), receptor Tyr kinase-like orphan receptor 1 or 2 (ROR1/2) and receptor Tyr kinase (RYK), directs the downstream signaling pathway (Azbazdar et al., 2021). Canonical WNT signaling is initiated by binding of WNT ligands (e.g., WNT3A and WNT10B) to a heterodimeric receptor complex formed by a Frizzled (FZD) and LRP5/6. The signaling output of the canonical WNT pathway is determined by the level of cytosolic β-catenin, which is under the strict control of the destruction complex. The destruction complex is composed of APC (Adenomatous Polyposis Coli), AXIN1, and two constitutively active kinases [glycogen synthase kinase (i.e., GSK3β) and casein kinase (i.e., CK1α)], which associate with β-catenin and promote its polyubiquitination by phosphorylating the degron motif of β-catenin (Stamos and Weis, 2013). Subsequently, phosphorylated β-catenin can be recognized by the F-box/WD-repeat protein β-TrCP within the SCF ubiquitin ligase complex, which facilitates the targeting of cytosolic β-catenin, leading to its proteasome-dependent degradation (Hart et al., 1999; Kitagawa et al., 1999). Binding of WNT ligands with FZD/LRP receptors induces stoichiometric sequestration of the destruction complex components AXIN1/CK1α/GSK3 onto the receptor complex and phosphorylation of LRP5/6, which are enhanced by Disheveled family members (DVL1-3). Receptor engagement leads to the accumulation of cytoplasmic β-catenin, which translocates into the nucleus, where it binds to members of the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family to drive transcription of WNT/β-catenin target genes such as *AXIN2* and *MYC* (amongst others) (Lecarpentier et al., 2019).

Some WNT ligands (e.g., WNT5A and WNT5B) can activate non-canonical WNT pathways, which are independent of β-catenin stabilization (Xiao et al., 2017). Non-canonical WNT signaling pathways are subdivided into the WNT-planar cell polarity (WNT-PCP) signaling pathway and the WNT-calcium (WNT-Ca^{2+}) signaling pathway. Although these two pathways show overlapping receptors such as ROR and RYK, they utilize different downstream effectors (Chen et al., 2021). In the WNT-PCP signaling pathway, Disheveled (DVL) forms a complex with DVL-associated activator of morphogenesis 1 (DAMM1) to induce the small GTPase Ras homology family member (Rho) activation (Habas et al., 2001). Then Rho activates downstream kinases such as Rho-associated protein kinase (ROCK) (Winter et al., 2001) or JUN-N-terminal kinase (JNK) (Strutt et al., 1997). Alternatively, DVL activates RAC to trigger JNK activity, which controls gene expression via JNK-dependent transcription factors (Fanto et al., 2000). The activation of WNT-PCP signaling results in cell polarity, cell migration and convergent extension [Convergent extension (CE) is a critical process by which the tissue of an embryo is restructured to converge (narrow) along one axis and extend (elongate) along a perpendicular axis by cellular movement] (Niehrs, 2012). The WNT-Ca^{2+} signaling pathway activates heterotrimeric G proteins to trigger phospholipase C (PLC) activity, which stimulates diacylglycerol (DAG) and inositol-1,4,5-triphosphate, type 3 (IP3) production leading to intracellular calcium flux (Slusarski et al., 1997), as well as calcineurin, calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) activation (Kuhl et al., 2000; Wang et al., 2010). In addition, the transcriptional regulator nuclear factor associated with T cells (NFAT) is stimulated via the YES/CDC42/CK1α pathway and is translocated to the nucleus to control transcription of genes involved in morphogenesis, mesenchymal to epithelial transition and metastasis (Dejmek et al., 2006; Burn et al., 2011).

Herein, the review will focus on non-canonical WNT5B signaling and its role in both normal physiology and disease, especially in comparison to WNT5A.

WNT5B ACTIVITY AND SIGNALING

The WNT5 subfamily of WNT proteins consists of WNT5A and WNT5B. The Wnt5 gene is present in Cnidarians (diploblastic animals including freshwater polyps and hydroids, sea anemones and corals, and jellyfish) (Guder et al., 2006). Duplication can be observed in Xenopus, but not in all chordates, such as chickens. *WNT5B* is the genetic paralog of *WNT5A* (Bergstein et al., 1997). They have 87 and 80.5% amino acid identity in mice and humans, respectively (Gavin et al., 1990; Saitoh and Katoh, 2001). Both WNT5A and WNT5B show similar expression patterns and functions in some cell types, such as cardiomyocytes (Mazzotta et al., 2016; Albanese et al., 2017) and lung fibroblasts (van Dijk et al., 2016; Wu et al., 2019). However, WNT5A and WNT5B are expressed in non-overlapping patterns during mouse development (Gavin et al., 1990; Lickert et al., 2001; Church et al., 2002), suggesting that WNT5A and WNT5B activity are distinctly different during embryogenesis. Moreover, distinct effects of WNT5A and WNT5B are found in chondrocytes (Church et al., 2002; Yang et al., 2003), bone (Maeda et al., 2012; Brommage et al., 2014), adipocytes (Kanazawa et al., 2005; Maeda et al., 2012), mammary epithelial cells (Kessenbrock et al., 2017), and myeloid cells (de Rezende et al., 2020; see **Table 1** and specific tissues below).

Wnt5b was first identified in 1990 and was found to be expressed during embryogenic development (Gavin et al., 1990). Mouse *Wnt5b* is located on chromosome 6 (Church et al., 2009) and encodes a 3.2 kb single RNA species (Gavin et al., 1990) and in turn, 49 and 46.5 kDa proteins can be visualized by immunoblotting after post-translational modification (Smolich et al., 1993). Although mouse and human WNT5B show 87.74% gene similarity and 94% amino acid identity[1], the rodent *Wnt5b* promoter and human *WNT5B* promoter are significantly divergent (Katoh and Katoh, 2005). Human *WNT5B*, located at chromosome 12p13.33 (Saitoh and Katoh, 2001), has 4 exons and alternative promotors that

[1] www.genecards.org

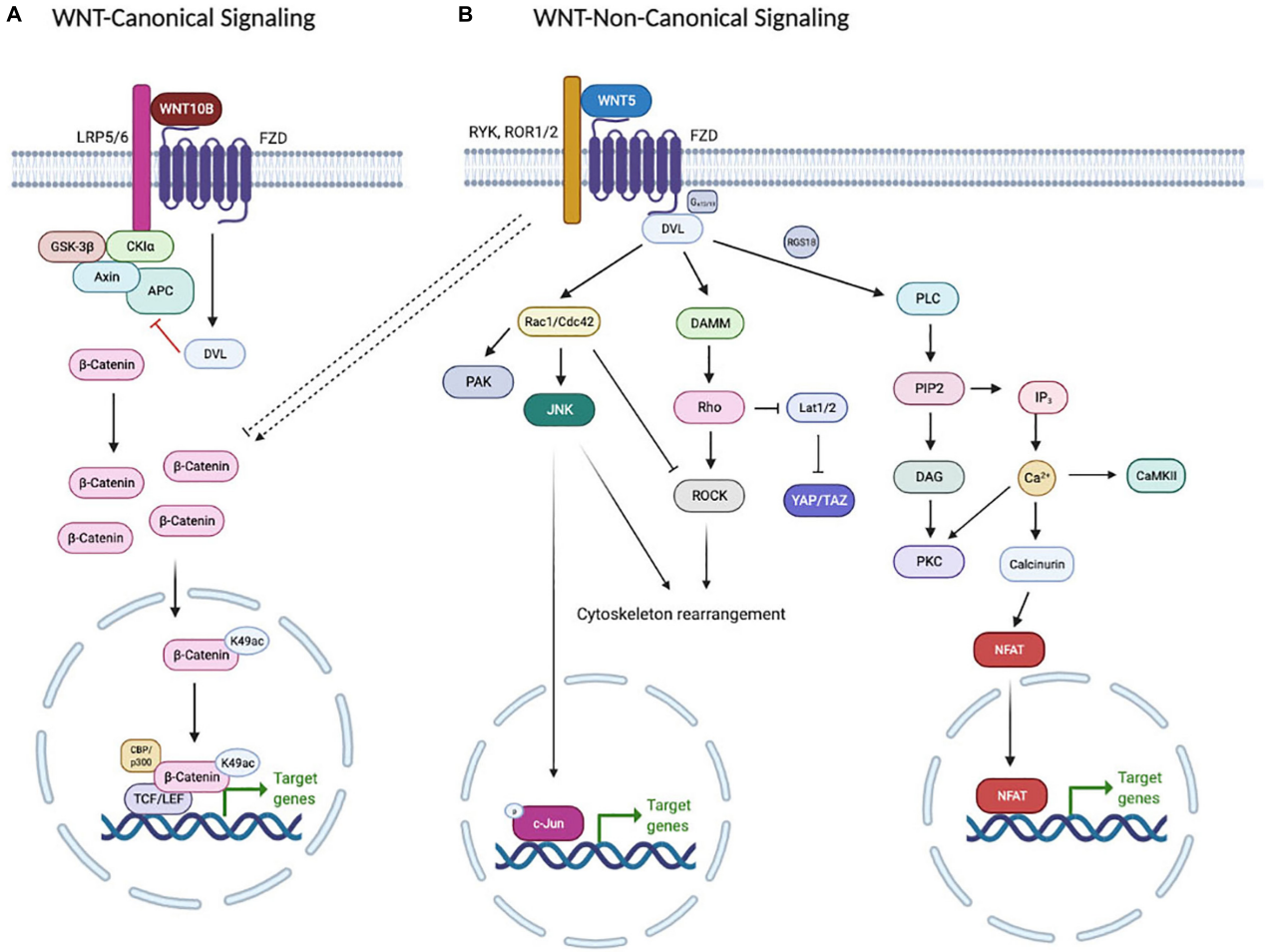

FIGURE 1 | WNTs can signal through **(A)** β-catenin-dependent (or canonical) WNT signaling, **(B)** WNT-PCP signaling or WNT-Ca^{2+} signaling. See the text for details.

encode *WNT5B* into two isoforms differing at the first exon: isoform 1 (NM_032642.2) consists of exon 1A and isoform 2 (NM_030775.2) consists of exon 1B. Exons 1A and 1B correspond to the 5′-UTR, while exon 2 contains the starting methionine (Katoh and Katoh, 2005). The consequence of the differing exon usage is unknown.

WNT5B functions through the non-canonical WNT signaling pathway (Lin et al., 2010; Bradley and Drissi, 2011; Park et al., 2015; van Dijk et al., 2016; Mattes et al., 2018). While it is called β-catenin-independent signaling, WNT5B is often an antagonist to β-catenin signaling (Santiago et al., 2012; Mattes et al., 2018; Wu et al., 2019; **Figure 1**). The receptors and coreceptors [FZD protein(s)] to which WNT5B binds is still controversial. A structure-based prediction shows that the cystine-rich domain of FZD6 binds with WNT5B into a palm-shaped opening structure (Dahiya et al., 2019) and then activates a small G_0 protein (Katanaev and Buestorf, 2009). In contrast, fluorescence recovery after photobleaching (FRAP) revealed that WNT5B did not affect FZD6 mobility, suggesting a lack of WNT5B-FZD6 interactions, at least in the HEK293T model system (Kilander et al., 2014). *In silico* analysis predicts that FZD8 has the highest affinity (Agostino et al., 2017; Dahiya et al., 2019), while *in vitro* and *in vivo* studies show that WNT5B can bind to FZD1 (Park et al., 2015), FZD2 (van Dijk et al., 2016), FZD4 (Mazzotta et al., 2016; Sarin et al., 2018), FZD5 (Sarin et al., 2018), FZD6 (Katanaev and Buestorf, 2009; Mazzotta et al., 2016), and FZD7 (Yin et al., 2020).

ROLE OF WNT5B IN DEVELOPMENT

WNT signaling is crucial for embryonic development in all animal species and the topic has been well-reviewed by others (van Amerongen and Nusse, 2009; Steinhart and Angers, 2018). The role of WNT5B signaling in embryonic development is less clear. In general, WNT signaling in embryonic development is context-dependent in both invertebrates and vertebrates. In embryos, WNT signaling has definitive roles for ventral-dorsal and anterior-posterior organization during various stages of early development. The organization of cells requires different WNT inputs to mediate planar cell polarity (PCP) during developmental processes such as gastrulation and

TABLE 1 | Distinct effects of WNT5A and WNT5B.

Organ/Tissue	Cell type	WNT5A	References	WNT5B	References
Embryo		Controls convergent extension (z)	Ye et al., 2013	Controls convergent extension (z)	Ye et al., 2013
Palate		Knockouts had a short palate (z)	He et al., 2008	Knockouts had a short palate (z)	Rochard et al., 2016
Bone		*Wnt5a*$^{-/-}$ showed skeletal defects in the mouse fetus (m) *Wnt5a*$^{\pm}$ exhibited low bone mass (m)	Yamaguchi et al., 1999 Maeda et al., 2012	High-throughput *Wnt5b*$^{-/-}$ slightly increased bone mass (m)	Brommage et al., 2014
	Chondrocyte	Expressed at joint and perichondrium (c) Activated transition from proliferative chondrocyte zone to pre-hypertrophy zone by repressing *Ccnd1*, *Sox9,* and *Col2a1*, while increasing *p130* (m)	Church et al., 2002 Yang et al., 2003	Expressed at pre-hypertrophic zone (c) Promoted proliferative chondrocyte zone but suppressed the transition to pre-hypertrophy zone by elevating *Ccnd1*, *Sox9,* and *Col2a1*, while suppressed *p130* (m)	Church et al., 2002 Yang et al., 2003
	Osteoblast	Promoted osteoblastogenesis by upregulating *Lrp5/6* expression and activating β-catenin-dependent signaling pathway (m)	Okamoto et al., 2014	No report of the mechanism of action	
	Osteoclast	Increased osteoclastogenesis by upregulating RANK through ROR2/JNK signaling pathway (m)	Maeda et al., 2012	Activated osteoclast differentiation via RYK receptor (murine RAW264.7 monocytic cells)	Santiago et al., 2012
	Synovial mesenchymal stem cell	Aggravated joint degeneration by inducing senescence and inflammatory cytokines (h)	Huang et al., 2020	Promoted joint degeneration by inhibiting chondrocyte differentiation and ECM secretion (h)	Huang et al., 2020
Adipose tissue	Adipocyte	Suppressed adipogenesis (r)	Tang et al., 2018	Promoted adipogenesis and increased the level of *PPARγ*, *C/EBP-α*, *ADIPOQ* and *LEP* (mouse 3T3-L1 cell line) Inhibited nuclear translocation of β-catenin (mouse 3T3-L1 cell line)	Kanazawa et al., 2004 Kanazawa et al., 2005
Pancreas	β-cell	Less expressed and colocalized with insulin- and glucagon-immunoreactive cells (m) No effect on NKX6.1 production in human iPSC (h)	Heller et al., 2002 Vethe et al., 2019	Strongly expressed and localized with insulin- and glucagon-immunoreactive cells (m) Increased the level of NKX6.1 in human iPSC (h)	Heller et al., 2002 Vethe et al., 2019
Cardiac tissue	Cardiomyocyte	No effect on pacemaker differentiation (z/h)	Ren et al., 2019	Induced pacemaker differentiation through activating β-catenin-dependent signaling pathway (z/h)	Ren et al., 2019
Nervous system	Differentiated neural stem cell	Slightly increased in differentiated cells (m)	Choi et al., 2011	Highly elevated in differentiated cells (m)	Choi et al., 2011
	Dorsal ganglia root and spinal cord dorsal horn	Decreased after morphine treatment and morphine withdrawal (m)	Wu et al., 2020	Strongly increased after morphine treatment and morphine withdrawal (m)	Wu et al., 2020
	Hippocampal dentate gyrus	Downregulated in ictal zone and peri-ictal zone after seizure (m)	Gupta and Schnell, 2019	Elevated in ictal zone but reduced in peri-ictal zone after seizure (m)	Gupta and Schnell, 2019
	Mechano-sensory Cell	No effect on cilia-mediated mechanosensory cells (z)	Louwette et al., 2012	Induced ciliogenesis in inner ear and migration of neuromast (z)	Louwette et al., 2012
Mammary gland	Mammary epithelial cell	Endogenously expressed and unable to transform C57MG cell morphology Increased mammosphere formation through RYK receptor (m) Inhibited mammary gland branching via ROR2 receptor (m)	Wong et al., 1994 Kessenbrock et al., 2017	Exogenously expressed and induced C57MG morphological transformation Suppressed mammosphere formation through ROR2 and RYK-independent receptors (m) No effect on mammary gland branching (m)	Wong et al., 1994 Kessenbrock et al., 2017
Lung	Alveolar epithelial progenitors (AEPs)	Represses the growth of lung organoid co-cultures of fibroblasts and epithelial progenitors (m) Highly expressed in COPD (h)	Wu et al., 2019 van Dijk et al., 2016	Represses the growth of lung organoid co-cultures of fibroblasts and epithelial progenitors (m) Represses the growth and differentiation of AEPs (m) Highly expressed in COPD (h)	Wu et al., 2019 van Dijk et al., 2016

(Continued)

TABLE 1 | Continued

Organ/Tissue	Cell type	WNT5A	References	WNT5B	References
Immune system	Myeloid cells	No effect on IL-3 and GM-CSF-induced myeloid differentiation (m) Knockdown *Wnt5a* did not affect thrombocyte production (z)	de Rezende et al., 2020 Louwette et al., 2012	Exhibited divergent effects on IL-3 and GM-CSF-induced myeloid differentiation (m) Knockdown *Wnt5b* showed severe thrombocytopenia (z)	de Rezende et al., 2020 Louwette et al., 2012
	T cells	Not expressed in thymocytes, peripheral T cells and epithelial cells (m)	Balciunaite et al., 2002	Expressed in thymocytes, peripheral T cells and epithelial cells (m) Highly expressed in $CD4^+$ $CD8^+$ double positive thymocytes and decreases by 75-95% during later stages of T cell development (m)	Balciunaite et al., 2002
	Whole blood	No change in expression in septic shock (m/h)	Gatica-Andrades et al., 2017	Increased expression in septic shock and correlated with IL-6, IL-10, and TNF (m/h)	Gatica-Andrades et al., 2017
	Lymphatic system	Increased migration of lymphatic endothelial cells, leading to elongated tube formation. (m)	Lutze et al., 2019	Necessary and sufficient for lymphatic endothelial specification (z/h) Enhances the lymph vessel formation, permeability and migration of lymphatic endothelial cell (h)	Nicenboim et al., 2015 Wang et al., 2017
Cancer	Breast cancer	Controversial: Correlated with better prognosis and induced tumorigenicity (h)	Zeng et al., 2016	Correlated with a worse prognosis in BLBC (h)	Yang et al., 2014; Jiang et al., 2019
	Colorectal cancer	Promotes migration and invasion (h) Inhibits cell proliferation and EMT (h)	Bakker et al., 2013 Cheng et al., 2014	Promoted cell proliferation, migration and invasion through the JNK signaling pathway (h)	Zhang et al., 2016
	Pancreatic cancer	Leads to pancreatic cancer progression and chemo-resistance (h)	Wei et al., 2014; Bo et al., 2016; Ram Makena et al., 2019	Not studied	
	Lung cancer	Overexpressed in NSCLC compared to normal tissue (h) Overexpression is correlated with poor overall survival (h)	Huang et al., 2005; Zhang et al., 2020	Overexpressed in NSCLC compared to normal tissue (h) Overexpression is correlated with poor overall survival (h)	Huang et al., 2005; Zhang et al., 2020
	Oral squamous cell carcinoma	TGF-β1 stimulated Slug which decreased WNT5A expression (h)	Hino et al., 2016	TGF-β1 stimulated Slug which increased WNT5B expression (h)	Hino et al., 2016
	Osteosarcoma	Correlated with advanced surgical stage and tumor metastasis (h) Increased migration and invasion of osteosarcoma cell lines (h)	Lu et al., 2012. Enomoto et al., 2009; Wang et al., 2018	Similarly expressed compared to the expression of *ROR2* (h) No study on the function in osteosarcoma	Morioka et al., 2009
	Ovarian cancer	Suppresses growth of epithelial ovarian cancer (h)	Bitler et al., 2011	Increases stemness (h)	Raghavan et al., 2019
	Brain cancer	Correlated with overall worse survival (h)	Xu et al., 2020	Correlated with better overall survival (h)	Xu et al., 2020
	Hepato-cellular carcinoma	No significant difference in WNT5A expression in tumor and adjacent-normal tissue (h)	Dong et al., 2019	High expression in tumor compared to adjacent-normal tissue (h)	Dong et al., 2019
	Leukemia	Highly expressed in CLL and correlates with CLL aggressiveness (h) Expression is lost in AML (h)	Janovska et al., 2016 Martin et al., 2010	Highly expressed in CLL and AML (h)	Janovska et al., 2016 Zheng et al., 2017

z, zebrafish; c, chicken; m, mouse; r, rat; and h, human.

neural tube closure. The polarization of node cells along the anterior–posterior axis of a mouse embryo was mostly unknown until the discovery of the asymmetric expression of *Wnt5a* and *Wnt5b* in the posterior side, which coincided with an anterior expression of WNT inhibitor SFRP (Minegishi et al., 2017). The opposing distribution of *Wnt5a*/*Wnt5b* gradients with that of SFRP guides intercellular signaling via PCP to polarize the cells along the anterior-posterior axis and to make a break of the left–right symmetry of the cells. The data suggest that WNT5A/5B have a redundant role in governing the break of left-right symmetry in cells, but it is unknown exactly which receptor/pathway this action is mediated through.

Vertebrate gastrulation is critical to establishing the germ layer and to coordinate the body axes, which are guided by cell movements. During gastrulation in zebrafish development, silencing of focal adhesion kinase (FAK1A) utilizing morpholinos led to the loss of convergent extension, impaired epiboly (cellular movement of squamous epithelium) and hypoblast cell migration. FAK1A combined with WNT5B rescued convergent extensions mediated by the FAK1A phenotype (Hung et al., 2020). Conversely, an injection of subthreshold levels of *Wnt5b* antisense morpholino oligonucleotides led to enhanced loss of convergent extension in the null FAK1A zebrafish. The results suggest that gradient increases of *Wnt5b* expression change or hamper or complement the loss of FAK1A activity on CE. Furthermore, CE was mediated by RAC1 and CDC42 actin dynamics. WNT5A also regulates convergent extensions in the zebrafish embryo (Ye et al., 2013).

Palate morphogenesis in the zebrafish at E4.5 is also formed by CE (Rochard et al., 2016). *Wnt5b* knockout zebrafish had a short palate. The ablation of *Wnt5b* affected the columnar organization but not the single layer stacking during CE. These results demonstrate that WNT5B controls the anterior-posterior axis in palate formation. WNT5A also regulates the anterior-posterior axis of the palate, similar to WNT5B (He et al., 2008). Interestingly, a translocation in the WNT5A locus has been implicated in human cleft palates (Blanton et al., 2004).

Cilia and basal bodies on the cell help control convergent extension. Basal body proteins [encoded by *bbs1*, *bbs4,* and *bbs6* (*mkks)*] were found to interact with WNT11 and WNT5B and signal through the WNT-PCP pathway (Gerdes et al., 2007). Knockout of BBS4 produced the strongest phenotype, as determined by defective tail extension. The authors suggest that WNT11 and WNT5B may have partially redundant roles, as mutants fail to extend axial tissues in zebrafish. Moreover, BBS4 was also shown to affect canonical WNT signaling in a mammalian cell system (using HEK293T cells) to measure ciliary function. Upon BBS4 suppression, β-CATENIN accumulated and increased nuclear Disheveled 3 (DVL3) localization, with increases in corresponding WNT reporter activity. Mechanistically, the authors link the stability of β-CATENIN protein degradation, in the absence of BBS4 expression, to BBS4's ability to physically interact with the proteasomal subunit RPN10. These results demonstrate a link between WNT/β-catenin signaling and non-canonical signaling (WNT-PCP) in convergent extension.

ROLE OF WNT5B IN BONE

WNT5B is involved in many aspects of bone physiology, including bone formation, bone maintenance and bone degradation. *Wnt5b* is expressed in mouse limb development; meanwhile, *Wnt5a* was not detected (Witte et al., 2009). In total long bone extracts, *Wnt5b* has the highest expression in young mouse bone (6 weeks old), decreases by adulthood (6 months old) and remains constant in aged mice (18 months old). *Wnt5a* shows the same pattern of expression, but its expression level is fourfold lower compared to *Wnt5b* at each timepoint (Rauner et al., 2008).

WNT5B is expressed in mesenchymal stem cells (MSCs) (Fazzi et al., 2011; Charoenpanich et al., 2014) and the lineage cells, including chondrocytes (Church et al., 2002; Bradley and Drissi, 2011; Duesterdieck-Zellmer et al., 2015) and osteoblasts (Rauner et al., 2008). WNT5B is also expressed in osteoclasts (Santiago et al., 2012). WNT5A also has high expression in MSCs, as does WNT5B (Fazzi et al., 2011), but the level of WNT5A is higher than WNT5B in osteoblasts (Maeda et al., 2012; Kemp et al., 2014), adipose tissues (Akoumianakis et al., 2019), as well as osteoclast precursor bone marrow-derived macrophages (Maeda et al., 2012). High-throughput knockout of *Wnt5b* revealed an increase in bone mineral density (BMD) (Brommage et al., 2014), indicating that WNT5B affects bone negatively, but this has not been investigated beyond analyzing BMD. In comparison, knockout of *Wnt5a* results in skeletal defects in the mouse fetus and perinatal lethality (Yamaguchi et al., 1999). *Wnt5a*$^{\pm}$ mice exhibit low bone mass with decreases in both osteoblastogenesis and osteoclastogenesis (Maeda et al., 2012), in contrast to the high bone mass in *Wnt5b* knockout mice. The skeletal phenotype of the WNT5A knockout mouse is also due to its proliferative and PCP effects in mesenchyme (Andre et al., 2015). The role of WNT5B in specific bone cells (chondrocytes, osteoblasts and osteoclasts) (**Figure 2**) and bone diseases (osteoarthritis and osteoporosis) is discussed below.

Chondrocytes

Endochondral ossification is the major process for long bone formation in which mesenchymal cells must first condense and differentiate into chondrocytes to establish a cartilaginous structure before gradually being replaced by bone cells. During this process, chondrocyte regions can be divided into two zones, Zone I (articular and resting chondrocytes) and Zone II (proliferative chondrocytes), depending on cell morphologies, developmental functions and fates. Zone I corresponds to the epiphysis and contains two cell types: articular chondrocytes which will become permanent articular cartilage to maintain normal joint function throughout life and resting chondrocytes, which contain stem cell-like activity and embed into the growth plate (Abad et al., 2002). Meanwhile, Zone II maintains highly proliferative chondrocytes in the growth plate, which will undergo hypertrophy. The proliferative activity of Zone II is dependent on Zone I and requires tight regulation from systemic and local factors (Hunziker, 1994; Karp et al., 2000).

WNT5B is highly expressed in cartilage canal chondrocytes during neonatal development (Duesterdieck-Zellmer et al., 2015). In long bones, WNT5B is restricted to the pre-hypertrophic zone (Zone II) (Church et al., 2002), and acts as a local factor to activate proliferation of chondrocytes in Zone I by downregulation of *p130* (an Rb family transcription factor) and upregulation of *Ccnd1, Sox9, and Col2a1*, leading to the promotion of Zone II formation (Yang et al., 2003; **Figure 2**). Overexpression of *Wnt5b* delays terminal chondrocyte differentiation and hypertrophy by suppressing *Col10a1* expression, and results in an open skull, shortened long bones and reduced ossification in the embryo (Yang et al., 2003).

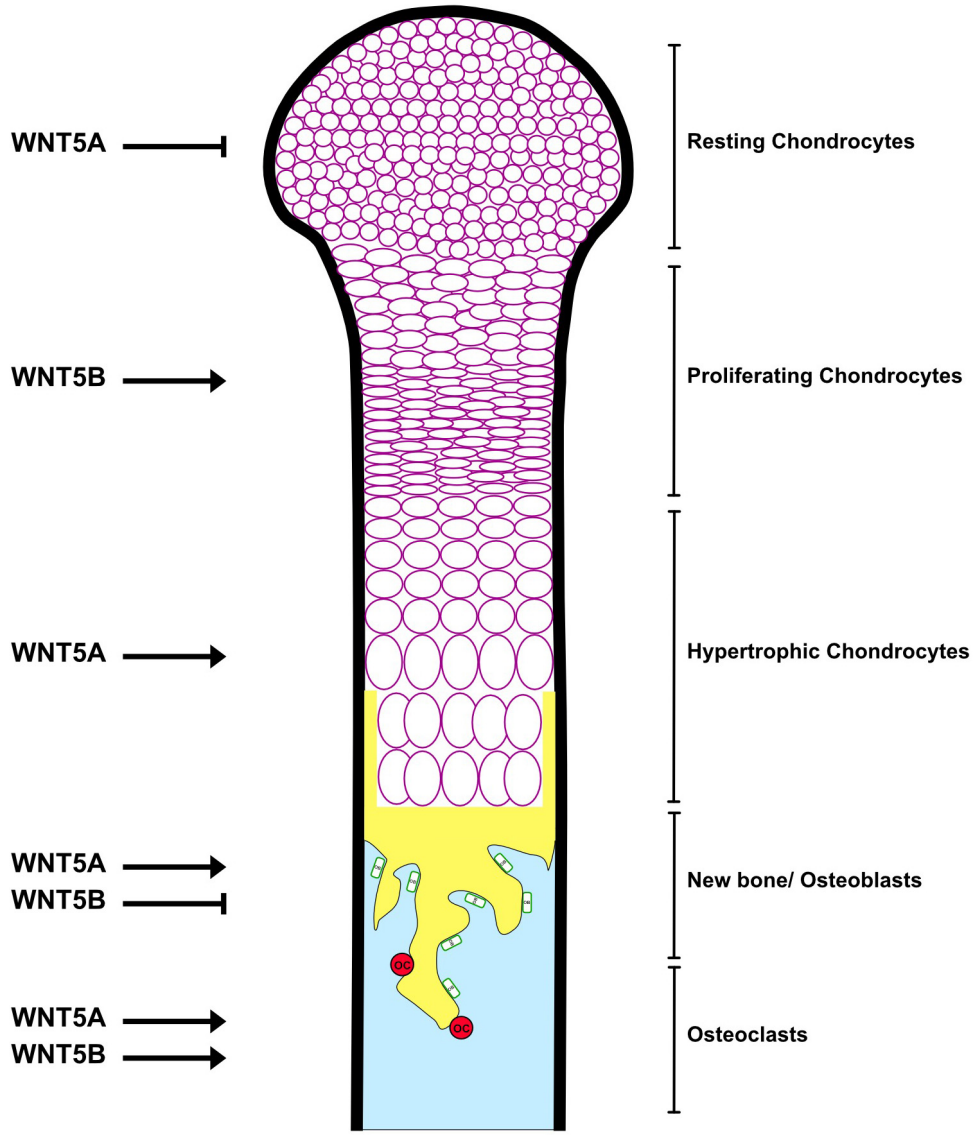

FIGURE 2 | The roles of WNT5A and WNT5B in the bone. See the text for details.

Interestingly, misexpression of *Wnt5b* causes shortened limbs (Church et al., 2002), suggesting that precise conditions of WNT5B are important to regulate endochondral ossification.

WNT5B also controls other processes involved in cartilage development. WNT5B is required for craniofacial cartilage development in zebrafish. WNT5B regulates chondrocyte stacking in a Wnt/PCP-independent signaling pathway (Sisson et al., 2015) and increases chondrogenic cell proliferation via Wls and Fgf3 (Wu et al., 2015). WNT5B activates mesenchymal chondroprogenitor cell migration in a JNK-dependent manner (WNT-PCP signaling pathway). In addition, WNT5B decreases cell aggregation and prevents chondrocyte hypertrophy by reducing cadherin expression and destabilizing cadherin receptors via Src-mediated phosphorylation of membrane-bound β-catenin. Notably, phosphorylation of β-catenin by WNT5B results in decreased membrane-associated β-catenin and increased nuclear β-catenin, thereby activating β-catenin's transcriptional activity (Bradley and Drissi, 2011), in contrast to inhibition of β-catenin signaling by WNT5B observed in osteoblasts, adipocytes, lung and other tissues (see below).

Although WNT5A and WNT5B both delay chondrocyte differentiation (Yang et al., 2003), their roles are distinct. WNT5A is expressed in joints and in the perichondrium, which is considered Zone I (Church et al., 2002). WNT5A inhibits proliferation and the transition from Zone I to Zone II by repressing *Ccnd1*, *Sox9* and *Col2a1* and elevating *p130*. Meanwhile, WNT5A promotes the transition from Zone II to the pre-hypertrophic zone, which will develop into the hypertrophic zone (Yang et al., 2003; **Figure 2**). Misexpression of *Wnt5a* results in truncated limbs and fused joints (Church et al., 2002),

supporting the positive effect of WNT5A on hypertrophy and long bone formation.

Osteoblasts

In osteoblast lineage cells, WNT5A and WNT5B exhibit redundant effects in mesodermal progenitor cells (MPCs), which have mesenchymal and endothelial differentiation potential (Petrini et al., 2009). WNT5A and WNT5B activate MPCs from quiescence and induce mesenchymal differentiation to MSCs through the FZD1/Calmodulin signaling pathway. The levels of WNT5A and WNT5B are high when the proliferation rate is low (early MSCs) and then decrease during exponential growth (late MSCs) (Fazzi et al., 2011). Then WNT5A and WNT5B expression increases during osteoblast differentiation (Hurson et al., 2007; Rauner et al., 2008; Okamoto et al., 2014), but the effects of WNT5A and WNT5B are divergent when MSCs commit to being osteoblasts. WNT5A promotes osteoblastogenesis by upregulation of *Lrp5/6* expression and results in enhanced β-catenin dependent signaling (Okamoto et al., 2014). Osteoblast lineage-specific *Wnt5a* conditional knockout mice show impaired osteoblastogenesis and decreased trabecular bone volume (Maeda et al., 2012). In contrast, there is some preliminary indication that WNT5B suppresses osteoblast differentiation (**Figure 2**). High expression of WNT5B, due to the regulation of circular RNA CDR1as, reduces β-catenin levels leading to decreased osteoblastogenesis in bone marrow mesenchymal stem cells (BMSCs) from steroid-induced osteonecrosis (Chen et al., 2020). However, the direct effects of WNT5B on normal osteoblast differentiation have not been studied.

Mechanical loading regulates bone remodeling by stimulating bone formation directly. Osteocytes, which contain sensors of strain energy, secrete sclerostin to inhibit WNT/β-catenin-dependent signaling, resulting in suppressed osteoblastogenesis in mechanical unloading conditions (Robling and Turner, 2009). The effects of mechanical loading on WNT5A and WNT5B were shown in osteoblast progenitors but have never been tested in osteocytes. Cyclic tension strain elevated WNT5A and WNT5B in BMSCs and MSCs from osteoporotic donors, respectively (Charoenpanich et al., 2014; Gu et al., 2018). In rat tendon-derived stem cells, uniaxial mechanical tension (UMT) activates osteoblast differentiation and upregulates WNT5A, WNT5B, ROR2 and RAC1, then WNT5A and WNT5B promote UMT-induced osteoblastogenesis via the activation of JNK (Liu X. et al., 2015). WNT5A also induces osteoblast differentiation from BMSCs through FZD4/JNK signaling under mechanical stimulation (Gu et al., 2018). Notably, the distinct actions of WNT5B, but not WNT5A, between normal and mechanical loading-induced osteoblast differentiation demonstrate that WNT5B can act differently in varied conditions.

Bone and teeth both have a mineralized extracellular matrix and have similar properties of differentiation. Ten WNT ligands are expressed in the dental epithelium and mesenchyme during mouse tooth development. *Wnt5b* is specifically expressed in the papilla mesenchyme of mouse incisors at E16 and E18 (Suomalainen and Thesleff, 2010). Mechanical forces (hydrostatic pressure) upregulate expression of *WNT5B* in human exfoliated deciduous teeth stem cells and activate odontoblast differentiation (Miyazaki et al., 2019), similar to what is observed after mechanical forces in rat tendon-derived stem cells (Liu X. et al., 2015).

Bone fracture healing requires several steps and signaling pathways to regenerate new bone (Marsell and Einhorn, 2011). Indirect suppression of WNT5B and β-catenin via miRNAs that target parathyroid hormone (Yao et al., 2018) and PANX3 (Jia and Zhou, 2018) results in inhibition of fracture healing. Moreover, knockout of Toll-like receptor 4 (TLR4), which inhibits the WNT/β-catenin signaling pathway, promotes fracture healing and increases β-catenin, WNT4, WNT5B, PCNA, and BMP-2 (Zhao et al., 2020), suggesting that β-catenin and WNT5B play similar roles in bone fracture healing. As both WNT/β-catenin-dependent and independent signaling pathways are involved in specific generative patterns of bone repair (Heilmann et al., 2013; Houschyar et al., 2018), WNT5B might regulate β-catenin activities to generate new bone. Nevertheless, the mechanisms that link WNT5B and β-catenin in this process are not understood.

Osteoclasts

WNT5A and WNT5B are expressed in the osteoclast lineage and promote bone resorption by enhancing osteoclast differentiation (**Figure 2**). Nevertheless, their mechanisms of action are dissimilar. WNT5B increases TRAP activity through the RYK receptor and suppresses β-catenin signaling in osteoclasts (Santiago et al., 2012). Meanwhile, WNT5A is highly expressed, secreted from osteoblast lineage cells and increases osteoclastogenesis by upregulating receptor activator of nuclear factor-kB (RANK) in bone marrow macrophages (BMMs), which are osteoclast precursors, via the ROR2/JNK non-canonical signaling pathway (Maeda et al., 2012). Notably, the expression of the receptors RYK and ROR2 in osteoclast lineages differs between the Maeda et al. (2012) and Santiago et al. (2012) studies. RT-PCR shows that RYK is highly expressed in both undifferentiated and differentiated murine osteoclast precursor monocytic cells (RAW264.7), while ROR2 is less expressed, and knockdown of the RYK receptor abolishes the effect of WNT5B (Santiago et al., 2012), supporting the argument that WNT5B signals through RYK signaling. In contrast, Maeda et al. (2012) found that ROR2 is in BMMs and conditional knockout of *Ror2* in RANK-expressing cells prevents WNT5A-induced osteoclastogenesis. The two contrasting results may be due to using an *in vitro* vs. an *in vivo* system. Thus, further work will be required to determine the role of WNT5B in the differentiation and activity of osteoclasts.

Bone Diseases

Osteoarthritis (OA) is a common age-dependent degenerative joint disease found in women more frequently than men. It is characterized by progressive degeneration of the components of extracellular matrix (ECM) of the articular cartilage and subchondral bone associated with secondary inflammatory factors (Dijkgraaf et al., 1995). Loss of the canonical WNT signaling pathway can be observed in OA pathogenesis. The specific knockout of β-catenin (*Ctnnb1)* at the superficial zone

promotes OA development (Xuan et al., 2019) and β-catenin is decreased in OA, while p-JNK, p-CaMKII and p-PKC are increased (Huang et al., 2020). WNT5B is upregulated in OA patients (Hopwood et al., 2007; Shi and Ren, 2020) and has higher expression in trabecular bone from women compared to men (Hopwood et al., 2007), indicating that it is a candidate to stimulate OA progression. WNT5B activates synovial MSC (SMSC) proliferation and migration via the Hippo-YAP signaling pathway (Tao S.C. et al., 2017). However, WNT5B inhibits chondrocyte differentiation and ECM secretion as well as promotes fibrosis by increasing collagen type I secretion (Tao S.C. et al., 2017; Huang et al., 2020), leading to joint degeneration. For inflammatory-induced OA, lipopolysaccharide activates WNT5B expression in human chondrocyte cells (CHON-001 cells) (Shi and Ren, 2020). WNT5B expression correlates with TGF-β1, a key player in OA pathology, in osteoblasts from OA patients (Kumarasinghe et al., 2012). WNT5B also increased MMP13 expression in SMSCs, which may lead to joint degradation (Huang et al., 2020).

WNT5A is also elevated in OA patients (Martineau et al., 2017) and shows similar effects on SMSC proliferation and migration as WNT5B (Tao S.C. et al., 2017). Nevertheless, the inhibition of WNT5A on SMSC differentiation to chondrocytes is different from WNT5B. WNT5A aggravates joint degeneration by inducing senescence and inflammatory cytokines such as IL-1β and IL-6 in SMSCs, whereas WNT5B does not (Huang et al., 2020). Taken together, WNT5A and WNT5B are involved in OA pathogenesis but the mechanisms of action are different.

Osteoporosis is a bone metabolic disorder in which there is a loss of BMD due to an imbalance between bone formation and bone resorption, leading to an increased risk of bone fractures (Imel et al., 2014). The effect of WNT5B on osteoporosis is still unclear due to the lack of research on WNT5B in normal bone cells. However, several genetic studies link single nucleotide polymorphisms (SNPs) near WNT5B to osteoporosis. In 2012, a genome-wide meta-analysis first reported that WNT5B might affect BMD through SNP rs2887571 (**Figure 3**; Estrada et al., 2012). rs2887571 is correlated with BMD in children, and children carrying the risk allele at rs2887571 benefited from physical activity (Mitchell et al., 2016). However, since rs2887571 is located in a non-coding intergenic region, the true target gene or consequence of the polymorphism is still unknown. Other SNPs in the *WNT5B* gene (rs2240506, rs7308793, rs4765830, and rs735890) are also associated with BMD (Zheng et al., 2016). Interestingly, SNP rs735890 in WNT5B disrupts mRNA secondary structure and dictates the binding of miRNA, resulting in less translation of WNT5B and increased BMD (Amjadi-Moheb et al., 2018). The molecular mechanisms of how WNT5B is influenced by these SNPs should be further investigated.

ROLE OF WNT5B IN ADIPOSE TISSUE

WNT5B has been shown to both transiently increase (Kanazawa et al., 2005) and decrease in early adipogenesis. However, both studies demonstrated that WNT5B activates adipocyte differentiation by elevating the level of adipocyte markers, such as peroxisome proliferator-activated receptor-γ (PPARγ), C/EBPα, AP2, Adiponectin and Leptin (Kanazawa et al., 2004; van Tienen et al., 2009). Furthermore, WNT5B abolishes the effect of WNT3A-suppressed adipogenesis (Kanazawa et al., 2005) by inhibiting β-catenin nuclear translocation and suppressing canonical WNT targeted genes such as *IGF-1*, *WISP-1,* and *VEGF-C* (Kanazawa et al., 2005; van Tienen et al., 2009). Conversely, WNT5A inhibits adipogenesis. $Wnt5a^{-/-}$ mice have an increased number of adipocytes (Maeda et al., 2012) and WNT5A suppresses adipocyte differentiation from rat adipose-derived stem cells *in vitro* (Tang et al., 2018). This demonstrates the opposite effects of WNT5A and WNT5B in adipocytes, as well as in chondrocytes and osteoblasts, all of which originate from MSCs.

ROLE OF WNT5B IN THE PANCREAS

WNT5B and WNT5A are expressed in embryonic mesenchyme where β-cells differentiate during mouse pancreatic development. WNT5B is strongly upregulated and localizes around insulin- and glucagon-immunoreactive cells; WNT5A, in contrast, has less expression and low localization with these cells (Heller et al., 2002). WNT5A increases ROR2 on the cell membrane, while WNT5B elevates the level of c-JUN, the downstream signaling of JNK (Vethe et al., 2019), suggesting that WNT5A and WNT5B act through the β-catenin-independent signaling pathway in pancreatic β-cells. In this study, the receptor for WNT5B was not analyzed, nor was the receptor for WNT5A, beyond the observation of increased ROR2 on the cell membrane. WNT5A and WNT5B together were shown to increase differentiation of β-like cells from induced pluripotent stem cells. In addition, WNT5A together with WNT5B or WNT5B alone, but not WNT5A alone, increase the level of NKX6.1, a transcription factor that controls β-cells fate, identity and function (Schaffer et al., 2013; Taylor et al., 2013), in human induced pluripotent stem cells differentiated to β-like cells (Vethe et al., 2019).

ROLE OF WNT5B IN TYPE 2 DIABETES MELLITUS

A case-control association study showed that SNP rs2270031, located in a WNT5B intron (**Figure 3**), is strongly associated with type 2 diabetes mellitus (T2DM) in a Japanese population (Kanazawa et al., 2004). In addition, a study in a UK population combined rs2270031 with rs7903146 (located in an intron of *TCF7L2*, a transcription factor for *glucagon-like peptide-1),* to show a risk effect for T2DM (Salpea et al., 2009). The necessity to combine the two genes to show an effect might be due to the fact that Caucasian subjects rarely have the risk allele at rs2270031. WNT5B regulates both adipocytes and pancreatic cells, two main cell types affected in diabetes, and mis-regulation of WNT5B via this SNP or otherwise in these cell types may lead to disease. Therefore, the effects of WNT5B in T2DM need further investigation.

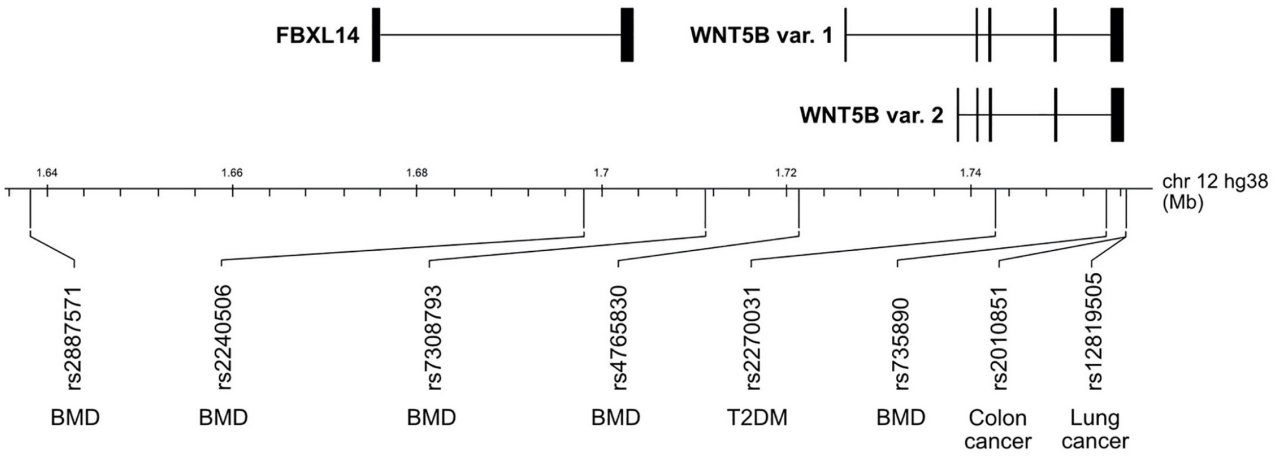

FIGURE 3 | WNT5B single nucleotide polymorphisms (SNPs) in 12p13.33 implicated in disease. SNP rs2887571, rs2240506, rs7308793, rs4765830, and rs735890 are associated with variations in BMD. SNP rs2270031 is linked to Type 2 diabetes mellitus (T2DM). SNPs rs2010851 and rs12819505 are associated with colon cancer and lung cancer, respectively.

ROLE OF WNT5B IN CARDIAC TISSUE

WNT5A and WNT5B are expressed at various stages of heart development. They direct the commitment of mesoderm to cardiogenic mesoderm by elevating *MESP1*, the earliest cardiogenic marker, via the ROR2/JNK WNT signaling pathway (Mazzotta et al., 2016). WNT5A and WNT5B also stimulate the differentiation of cardiomyocytes via FZD4/6/Ca^{2+} WNT signaling pathway in late development (Mazzotta et al., 2016). However, only WNT5B promotes pacemaker cardiomyocytes, which are essential for the initiation and maintenance of proper heart rhythm through the activation of the canonical β-catenin/WNT signaling pathway (Ren et al., 2019). Interestingly, *activation* of the canonical pathway by WNT5B is observed here, as it is in chondrocytes, but in contrast to *inhibition* of β-catenin signaling by WNT5B observed in osteoblasts, adipocytes, lung and other tissues.

Aortic valve calcification causes aortic valve stenosis, which induces heart failure. WNT5A and WNT5B show the same effects on calcification development. Their expression is significantly, positively correlated with pathological calcified valves. WNT5A and WNT5B reduce proliferation and induce apoptosis of human aortic valve interstitial cells, increase osteogenic gene expression and increase calcium deposition and formation in calcified nodules *via* the JNK/Akt1/MAPK38 signaling pathway (Albanese et al., 2017). Fetuin A, a calcification inhibitor, reduces the expression of *WNT5A* and *WNT5B* (Khan et al., 2020).

ROLE OF WNT5B IN THE NERVOUS SYSTEM

WNT5B is moderately expressed in the human and zebrafish fetal brain, especially in midbrain-hindbrain boundary constriction in zebrafish and is required for basal constriction via activation of focal adhesion kinase (FAK) (Gutzman et al., 2018). Then its expression decreases in adulthood (Saitoh and Katoh, 2001) similar to WNT5A (Gavin et al., 1990). The effects of WNT5A and WNT5B are both different and redundant in the nervous system, depending on cell types and contexts. Neural stem cells (NSCs) have the capacity to differentiate into either neurons, astrocytes or oligodendrocytes in the central nervous system (CNS). *Wnt5b* is increased more than twofold, while *Wnt5a* is only slightly elevated, in differentiated cells compared to NSCs (Choi et al., 2011). The median motor column (MMC) contains a set of motor neurons arranged in a longitudinal array and innervates axial muscles. WNT5B and WNT5A redundantly control MMC identity and connectivity by establishing a ventral^{high} to dorsal^{low} signaling gradient, subsequently promoting the persistent expression of LIM Homeobox protein 3 and 4, the transcription factors for cell specification (Agalliu et al., 2009).

WNT5B is also required for the mechanosensory system in mammalian and lower vertebrates. In zebrafish (*Danio rerio*), WNT5B regulates ciliogenesis in the inner ear and migration of neuromasts to the lateral line, a sensory organ of fishes, through the RGS18-mediated WNT-Ca^{2+} signaling pathway, whereas WNT5A does not have any effect on cilia-mediated mechanosensory cells (Louwette et al., 2012). In the juvenile rat cochlea, WNT5B is expressed in non-sensory supporting cells, and more strongly expressed in fibrocytes of the spiral limbus and spiral ligament (Daudet et al., 2002). This suggests a function of WNT5B in late cochlea differentiation, as well as auditory function, although no functional experiments have been performed to date.

WNT5B and WNT5A have the same effects on the development and degradation of retinal and optic nerves. Both proteins regulate retinal neuropil formation in the outer retina. WNT5A and WNT5B are selectively secreted by rod bipolar cells to rod photoreceptors, and then control the patterning of the outer plexiform layer via the RYK/FZD4/FZD5/DVL

signaling pathway (Sarin et al., 2018). WNT5A and WNT5B are also elevated in a mouse model of retinal degradation (Yi et al., 2007), suggesting that they function during photoreceptor injury. However, the mechanism of action requires further investigation. Moreover, WNT5A and WNT5B are expressed in the neural retina, but not in retinal pigment epithelium (RPE) in the mouse embryo (Liu et al., 2003). A study in human embryonic stem cell-derived RPE cells supported that WNT5B and its downstream signaling have to be suppressed, while high expression of BMP7 is required in order to successfully go through epithelialization of the RPE cells (Choudhary et al., 2015).

WNT5B also controls optic nerve regeneration in lower vertebrates. Axon growth in zebrafish requires the activation of small G-proteins, such as CDC42 and Rac1, whereas RhoA has to be suppressed. *Wnt5b* is increased, while *Wnt10a* is decreased and *Wnt5a* has no change, after optic nerve transection. WNT5B stimulates the CDC42/Rac1/JNK/PAK signaling pathway and represses RhoA via CDC42, together with the reduction of β-catenin level in the nucleus due to the decrease of WNT10A. Both of these actions lead to axon extension (Matsukawa et al., 2018).

The expression of WNT5B is affected in several nerve injuries and neuropathology. Morphine treatment induces WNT5B accumulation in the dorsal ganglia root (DRG) and it is exported to the central terminus of the spinal cord dorsal horn (DH) after morphine withdrawal. Translocated WNT5B at the DH then increases Ca^{2+} influx via RYK, resulting in behavioral and neurochemical alteration of morphine withdrawal. Meanwhile, the level of *Wnt5a* expression is decreased after morphine treatment and morphine withdrawal (Wu et al., 2020). Chronic constriction injury-induced neuropathic pain and complete Freund's adjuvant-induced chronic inflammation also elevate the level of *Wnt5b* expression in the DRG. Notably, the expression level of *Ryk* is raised in the same pattern as *Wnt5b* and a RYK antagonist abolishes the effects of the WNT/RYK/CaMKII/NR2B signaling pathway in neurons (Liu S. et al., 2015; Wu et al., 2020). However, there is no direct investigation of the effect of WNT5B on nerve injury. In addition, epilepsy is neural network remodeling in the hippocampus dentate gyrus, which associates with the alteration of WNT signaling pathways. WNT5B is significantly dysregulated between the ictal zone and peri-ictal zone, the center and remodeling network region after seizure induction, respectively. In contrast, *Wnt5a* is downregulated in both regions (Gupta and Schnell, 2019). Nevertheless, the mechanisms of action of WNT5B in epilepsy are poorly understood. Lastly, an epigenome-wide association study revealed that alteration in DNA methylation at the first intron of the longest isoform of *WNT5B* is associated with Alzheimer's disease progression (Smith et al., 2019). However, the consequences of the methylation and different isoform functions remain unknown.

Together, these data suggest that WNT5B has varied expression in both the CNS and the peripheral nervous system, and exhibits several roles during embryonic development and pathogenesis, such as in neuron identity and patterning, neuron regeneration, and the mechanosensory system.

ROLE OF WNT5B IN THE MAMMARY GLAND

Wnt5a and *Wnt5b* are expressed in the more differentiated luminal epithelial cells of the mammary gland (Ji et al., 2011). *Wnt5b* is also expressed in the mammary gland during mid-pregnancy and disappears in lactation, while *Wnt5a* is expressed in early pregnancy (Gavin and McMahon, 1992). In the mammary gland WNT5B signals through ROR2 and RYK-independent receptors. WNT5B inhibits mammosphere formation by downregulating proliferation genes, such as *Mcm2* and *Ki67*, as well as luminal differentiation genes, such as *Gata3*. WNT5A, in contrast, increases the number and size of mammospheres via the RYK receptor, and suppresses mammary gland branching via the ROR2 receptor (Kessenbrock et al., 2017), whereas WNT5B does not have any effect on mammary gland branching. These data, again, suggest distinct roles of WNT5A and WNT5B. Further work on the role of WNT5B in the mammary gland should be performed, as there are numerous reports of WNT5B in breast cancer (see below).

ROLE OF WNT5B IN THE LUNG

Both WNT5A and WNT5B are expressed in the lung and have been implicated in lung pathogenesis, including COPD and lung cancer. WNT5A and WNT5B are secreted from lung fibroblasts in a paracrine manner to inhibit alveolar epithelial progenitors, while inducing epithelial differentiation. WNT5B, but not WNT5A, induces expression of Aquaporin 5 (Aqp5, a marker for alveolar type I cells) and Surfactant protein C (Sftpc, a marker for alveolar epithelial type II cells). WNT5B also shows a stronger repressive effect on alveolar organoid formation compared to WNT5A. However, canonical WNT/β-catenin signaling is repressed by both WNT5A and WNT5B in these cells. WNT5A and WNT5B seem to have similar functions, but are not identical (Wu et al., 2019).

Chronic obstructive pulmonary disease (COPD) is caused by defective epithelial lung repair associated with an abnormal inflammatory response. Alteration of fibroblast functions also plays an important role in COPD pathogenesis. WNT5B has higher expression in COPD patients (Heijink et al., 2016), suggesting a role in its pathogenesis. Furthermore, TGF-β1, a key player in fibroblast activation to repair lung injury, increases *WNT5B* expression and then WNT5B stimulates ECM production (fibronectin and α-smooth-muscle actin) and myofibroblast differentiation via FZD8 (Spanjer et al., 2016). WNT5B induces an inflammatory response in MRC-5 human fibroblasts and primary human lung fibroblasts by increasing IL-6 and CXCL8 secretion via the FZD2/Tak1/JNK signaling pathway (van Dijk et al., 2016). WNT5A is also highly expressed and impairs lung repair in COPD patients through the WNT/β-catenin-independent signaling pathway (Baarsma et al., 2017). These studies indicate that non-canonical WNT5A and WNT5B have similar roles in the lung and activating the development of COPD.

ROLE OF WNT5B IN HEMATOPOIESIS

Hematopoietic stem cells (HSCs) generate the balance between lymphoid and myeloid cell types which impact immunity efficiency and tissue homeostasis. WNT5B is expressed in HSCs (de Rezende et al., 2020) and has been reported in the development of myeloid and lymphoid lineage cells.

Acceleration of myelopoiesis in the elderly contributes to the development of chronic diseases associated with aging. Primitive cells such as HSCs and multipotent progenitors are more sensitive to IL-3, which supports stem cell maintenance, while committed myeloid progenitors are more sensitive to Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF), which drives differentiation and proliferation. WNT5B exhibits divergent effects on IL-3 and GM-CSF-induced myeloid differentiation, which relate to aging-associated myeloid imbalance. WNT5B enhances HSCs and progenitors by suppressing IL-3-mediated myeloid differentiation and non-canonical WNT signaling genes such as *c-Fos*, *c-Jun,* and *Cdc42*. Meanwhile, WNT5B accelerates GM-CSF-driven myelopoiesis and upregulates non-canonical WNT signaling genes, leading to progenitor cell exhaustion. Notably, osteoclasts are derived from the monocyte/macrophage lineage and WNT5B may play a role in osteoclast progenitor cells to affect bone mass. Furthermore, WNT5B controls megakaryocyte differentiation through RGS18 as depletion of *Wnt5b* in zebrafish embryo shows severe thrombopenia (Louwette et al., 2012). Interestingly, *Wnt5a* is expressed in primitive HSCs and niche cells, as is *Wnt5b*, but WNT5A does not affect IL-3- and GM-CSF-induced HSC differentiation (de Rezende et al., 2020).

The primary lymphoid organ for T-cell development is the thymus. The thymus functions to ensure that T-cells are selected to prevent autoimmunity. The T-cell section for non-autoimmune T-cells is mediated by both distinct thymic stromal cells and cortical and medullary epithelial cells (Anderson and Jenkinson, 2001). The full maturation of thymic epithelial cells (TECs) occurs in the third pharyngeal pouch (ppIII) of the thymus. Growth and development of TECs (Anderson and Jenkinson, 2001) are dependent on Forkhead Box N1 (FoxN1) expression, and in its absence, epithelial morphogenesis in the thymus is aberrant. These epithelial cells lack the ability to attract lymphoid precursors to the thymus primordium. *Wnt5b* expression is found in thymocytes, peripheral T cells and epithelial cells. Meanwhile, *Wnt5a* has no expression in these cells. Interestingly, *Wnt5b* is highly expressed in $CD4^+$ $CD8^+$ double-positive thymocytes and decreases by 75–95% during later stages of T cell development. Moreover, the transduction of cortical TEC 1-2 cells with *Wnt5b* upregulates FoxN1 and WNT5B is strictly localized with FoxN1 in ppIII. This suggests that WNT5B is involved in lymphocyte differentiation by regulating FoxN1 (Balciunaite et al., 2002), but the mechanism of WNT5B on T cells is still poorly understood. In addition, WNT5B also contributes to a dysregulated immune response and the production of inflammatory cytokines to the infection. WNT5B, but not WNT5A, increases in whole blood from patients with septic shock and directly correlates with IL-6, IL-10, and TNF (Gatica-Andrades et al., 2017).

ROLE OF WNT5B IN THE LYMPHATIC SYSTEM

The lymphatic system origins have had two opposing models for close to 100 years, one suggests a venous origin for lymphatic endothelial cells, and the other a more random coalescence of discontinuous, independent lymphatic vessels arising from mesenchymal-derived cells. A study in zebrafish demonstrated that lymphatic progenitor cells, which arise from a specialized angioblast within the cardinal vein that can also give rise to arterial veins, are governed by WNT5B activity (Nicenboim et al., 2015). WNT5B is necessary and sufficient for lymphatic endothelial specification. WNT5B promoted the angioblast to lymphatic transition in both zebrafish and human embryonic stem cells, suggesting this function by WNT5B is evolutionarily conserved. WNT5B has also been shown to be secreted from tumor cells to induce tumor lymphangiogenesis (Wang et al., 2017). WNT5B enhanced the lymph vessel formation, permeability and migration of lymphatic endothelial cells. These findings can potentially lead to the development of regeneration of lymphatic vessels for human usage or inhibition of lymphangiogenesis in cancer.

WNT5A also increased migration of lymphatic endothelial cells, leading to elongated tube formation. This was demonstrated in mice, using the dermal lymphatic vascular system, and not compared to WNT5B in the same system (Lutze et al., 2019).

ROLE OF WNT5B IN CANCER

Breast Cancer

WNT1 was originally identified due to its ability to transform mammary gland cells. WNT2, WNT3A, WNT7A, WNT7B, WNT10B, and WNT5B are also highly transforming of mouse mammary cells. In contrast, WNT5A (and WNT4 and WNT6) failed to induce transformation (Wong et al., 1994).

WNT5B is overexpressed in the majority of Triple Negative (TNBC)/Basal-Like Breast Cancer (BLBC) cell lines and primary patient samples and is correlated with a worse prognosis (Yang et al., 2014; Jiang et al., 2019). In addition, WNT5B was found to be amplified in 3.2% of breast cancers, although the type of breast cancer with WNT5B amplification was not described (Jiang et al., 2019). Knockdown of *WNT5B* inhibited the proliferation, migration and invasion of Bcap-37 and MDA-MB-231 cells *in vitro* and reduced the expression of basal-like markers (Yang et al., 2014; Jiang et al., 2019). Mechanistically, WNT5B was shown to promote TAZ activation by promoting the transcription of SLUG, which is a master regulator of epithelial-mesenchymal transition (EMT) as well as a regulator of TAZ nuclear localization and subsequent activation (Samanta et al., 2018). In addition, knockdown of *WNT5B* in MDA-MB-231 and Bcap-37 cells showed reduced tumor growth in BALB/c-nu mice compared with control cells. Targeting WNT5B (and all WNTs) through the addition of WNT inhibitors pyrvinium pamoate (a CK1α activator) or LGK-974 (a Porcupine inhibitor) also reduced tumor growth and proliferation *in vivo* (Jiang et al., 2019).

The regulation of WNT5B expression in breast cancer (or other cell types) is not well-studied. However, it was shown that WNT5B is suppressed by miR145-5p in three basal-like breast cancer cell lines. Mechanistically, insulin-like growth factor-2 mRNA-binding protein 3 (IMP3), which is correlated with TNBC, stabilizes *WNT5B* by suppressing miR145-5p.

WNT5A and WNT5B also have different roles in mammary gland development and TNBC. In contrast to WNT5B, many publications show WNT5A as a tumor suppressor in breast cancer, correlating its loss with a worse prognosis (Zeng et al., 2016). However, the opposite has also been shown: WNT5A is higher in breast carcinomas and induces tumorigenicity in breast cancer (Iozzo et al., 1995; Zeng et al., 2016). Therefore, the roles of WNT5A and WNT5B in breast cancer need further research.

Pancreatic Cancer

Genomic analysis of pancreatic ductal carcinoma revealed four major subtypes (Bailey et al., 2016) and the WNT signaling pathway was found to be enriched in the squamous subtype, which is the most aggressive subtype. Specifically, overexpression of canonical WNT ligands such as *WNT2* and *WNT7A* has been implicated in pancreatic progression via persistent activation of β-catenin signaling. In addition, the non-canonical WNT ligand WNT5A has been shown to lead to pancreatic cancer progression and chemoresistance (Wei et al., 2014; Bo et al., 2016; Ram Makena et al., 2019).

Only a few studies have suggested roles for WNT5B in normal or cancerous pancreatic cells. Although WNT5B has not been studied in pancreatic cancer patient samples, *WNT5B* mRNA is highly expressed in the pancreatic cancer cell line PANC-1 (Harada et al., 2017). Panc-1 cancer cells (and the lung adenocarcinoma cell line A549) can induce the invasive ability of neighboring epithelial cells both *in vitro and in vivo*. WNT3 and WNT5B are secreted from mesenchymal-transitioned cancer cells to induce the metastatic potential of neighboring epithelial cells (Kato et al., 2014). Knockout of *WNT5B* in Panc-1 cells reduced migration and invasion, but not proliferation. In control PANC-1 cells, addition of TGF-β increased the expression of *Snail* and *Vimentin* mRNA and decreased *E-cadherin*. However, in *WNT5B* knockout cells, TGF-β-dependent increases of *Snail* and *Vimentin* mRNA were reduced and the expression of *E-cadherin* mRNA decreased. These results suggest that WNT5B may be involved in TGF-β-induced EMT (Harada et al., 2017).

WNT5B has also been shown to be secreted in exosomes from PANC-1 cells. WNT5B-associated exosomes promoted cancer progression in a paracrine manner, as seen in their ability to induce proliferation and migration on A549 lung cancer cells (Harada et al., 2017). The contribution of WNT5B in exosomes vs. secretion needs to be explored in both pancreatic cancer and other cell types.

Lung Cancer

Many WNT ligands, both canonical and non-canonical, and WNT signaling proteins are misregulated in lung cancer. Specifically, both WNT5A and WNT5B are overexpressed in non-small cell lung cancers (NSCLC) compared to normal tissue and their overexpression is correlated with poor overall survival (Huang et al., 2005; Zhang et al., 2020). Knockdown of *WNT5B* reduced the proliferation and growth rate of lung adenocarcinoma both *in vitro* and *in vivo* by inducing cell-cycle arrest at the G1/S phase. Mechanistically, WNT5B was shown to regulate the metabolism of the lung cancer cells, including the amino acid transporter LAT1. In addition, miR-5587-3p was shown to repress WNT5B in these cells (Zhang et al., 2020).

In a group of non-small cell lung carcinomas, variants for rs12819505 (**Figure 3**) were significantly associated with worse prognosis (Stewart et al., 2014). The function of this SNP, which is a few hundred base pairs downstream of the WNT5B coding sequence is unknown.

Colorectal Cancer

WNT signaling is highly important in colorectal cancer (CRC) through the regulation of proliferation, differentiation, and cell fate. WNT signaling is activated in normal intestinal crypts, which is crucial for stem cell maintenance and tissue homeostasis. The canonical WNT/β-catenin pathway is constitutively activated in more than 90% of colorectal cancers. APC suppresses β-catenin-mediated WNT signaling and is thus a tumor suppressor gene. Germline mutations in APC lead to familial adenomatous polyposis (FAP) and account for about 1–2% of CRC. Somatic mutations in APC have also been found in a majority of sporadic CRC (Aoki and Taketo, 2007).

In contrast to β-catenin-dependent signaling, very little is known about WNT5B in CRC. At least five CRC cell lines express WNT5B and the addition of WNT5B to COLO 205 cells promoted cell proliferation, migration and invasion through the JNK signaling pathway (Zhang et al., 2016). Examination of patient samples for mRNA and/or protein levels could determine if there is a role for WNT5B in CRC.

The polymorphism rs2010851 in the 3′ UTR of *WNT5B* (**Figure 3**) was shown to predict tumor recurrence in stage II colon cancer. This specific *WNT5B* SNP is significant because it shows for the first time that a WNT polymorphism can predict early tumor recurrence in colorectal cancer (Paez et al., 2014), and suggests that more research needs to be done to understand the consequences of both the SNP and WNT5B in CRC.

Similarly, the role of WNT5A in CRC is not clear, as WNT5A has both been shown to promote migration and invasion (Bakker et al., 2013) and to inhibit cell proliferation and EMT (Cheng et al., 2014). WNT5A is down-regulated in a majority of colon cancers and suppressed during colon cancer metastasis (Li and Chen, 2012; Tao J. et al., 2017). The contradictory roles of WNT5A in CRC could be due to two different *WNT5A* splice forms, with each form having opposing functions (Huang et al., 2017).

Oral Squamous Cell Carcinoma

Expression profiles from 40 patients with oral squamous cell carcinoma (OSCC) revealed a small (1.5-fold) increase in *WNT5B* mRNA in 40% of cases. In addition, WNT5B mRNA and protein were higher in patients with nodal metastases (Wang et al., 2017). As in many other cancer types, knockdown of WNT5B decreased OSCC cell line proliferation and migration (Takeshita et al., 2014; Wang et al., 2017). WNT5B was further

shown to activate CDC42 and RhoA, which are involved in cell migration (Takeshita et al., 2014). In the HSC-4 OSCC cell line, TGF-β1 stimulated Slug which increased WNT5B expression, while decreasing WNT5A expression. In turn, WNT5B increased the expression of MMP-10, a matrix metalloproteinase that induces tumor progression and invasion (Hino et al., 2016).

Osteosarcoma

Osteosarcoma is a cancer that starts in the bone, and affects mostly children, especially during puberty. In osteosarcoma, it has been identified that the interaction of WNT5B and ROR2 can enhance cell migration, which supports a potential role of ROR2 and WNT5B in the metastatic pathway of osteosarcoma cells. *ROR2* was overexpressed in 56% of patient samples and the expression pattern of *WNT5B* expression is concurrent with that for *ROR2*. Knockdown of *ROR2* suppressed proliferation and invasion of osteosarcoma cells, suggesting that ROR2 and WNT5B could be promising therapeutic targets for osteosarcoma patients (Morioka et al., 2009).

While only one paper exists on the significance of *WNT5B* in osteosarcoma, several highlight the importance of *WNT5A*. WNT5A was overexpressed in 81% of 42 osteosarcoma immunohistochemistry samples and was significantly correlated with advanced surgical stage and tumor metastasis (Lu et al., 2012). Specifically, WNT5A increased migration and invasion of osteosarcoma cell lines (Enomoto et al., 2009; Wang et al., 2018). As in CRC, the different isoforms of WNT5A have different roles in osteosarcoma cell lines (Vaidya et al., 2016). WNT5B also has two different promoters and splice forms, suggesting the need to also investigate WNT5B isoforms in osteosarcoma.

Ovarian Cancer

WNT5B is important in ovarian cancer, particularly in ovarian cancer stem cells (OCSC). Immune cells, including macrophages, are enriched in ovarian cancer ascites. Raghavan et al. (2019) demonstrated that WNT5B from macrophages induces expression of the stem cell marker *ALDH* in the OCSC in a paracrine manner. They also showed a 50% decrease in β-catenin protein expression after WNT5B knockdown, indicating cross-talk with canonical WNT signaling (Raghavan et al., 2019). Zong et al. (2020) also demonstrated the importance of WNT5B in OCSC and demonstrated that WNT5B induces ALDH expression in OCSC. Their research proposed that the tumor suppressor Disabled homolog 2-interacting protein (DAP2IB) down-regulates WNT5B in OCSC and proposed the pathway in which EZH2 inhibits DAP2IB, which inhibits WNT5B signaling. Addition of recombinant WNT5B to the ovarian cancer cell line OVCAR3 induced JNK and c-Jun phosphorylation and downstream stemness related genes (Zong et al., 2020). They further suggested that combining an EZH2 inhibitor, or other epigenetic therapy, with a non-canonical WNT pathway inhibitor for ovarian cancer would be optimal.

While not much is known about the clinical implications of *WNT5B*, high expression of *WNT5A* has been significantly correlated with ovarian cancer stage, poorer overall survival and poorer progression-free survival (Peng et al., 2011). WNT5A has been shown to regulate ovarian cancer invasion and migration. In addition, it has been shown that WNT5A may mediate vasculogenic mimicry and EMT in ovarian cancer cells via PKC-α signaling (Qi et al., 2014). In contrast, Bitler et al. (2011) propose that loss of *WNT5A* expression predicts worse outcomes in patients with Epithelial Ovarian Cancer (EOC) and that *WNT5A* expression suppresses the growth of EOC cells by initiating cellular senescence. While both WNT5B and WNT5A have been shown to be expressed in ovarian cancer, their functions are distinct.

Brain Cancer

There are more than 120 types of brain and central nervous system tumors. The presence and/or biology of WNT5B in these tumors are mostly unknown. The WNT5B locus has been shown to be amplified in astrocytomas (Schiffman et al., 2010), but this has not been further described. The expression of *WNT5B* has been shown in gliomas and atypical teratoid rhabdoid tumors (ATRT), but studies beyond gene expression have not been performed. *WNT5B* expression was correlated with better overall survival, in contrast to patients with elevated *WNT5A* expression that had overall worse survival (Xu et al., 2020). In a set of ATRT samples, *WNT5B* was upregulated in 19 of the 20 samples. Furthermore, WNT inhibitors were shown to decrease proliferation in these cells. WNT5B was shown to bind to FZD1 but the functional role of WNT5B in ATRT patients is still unknown (Chakravadhanula et al., 2015).

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) can be divided into well-differentiated (epithelial, conserved hepatocyte morphology) and poorly differentiated (mesenchymal, more motile and invasive) tumors. A study of HCC cell lines revealed that canonical WNT ligands (WNT3, WNT8B, and WNT10B) are expressed in well-differentiated tumors, while non-canonical WNT ligands such as WNT5B and WNT5A are correlated with poorly differentiated tumors. This study further proposes a potential crosstalk mechanism by which non-canonical WNT5A inhibits canonical WNT signaling (Yuzugullu et al., 2009).

The expression of WNT ligands was also evaluated in 360 HCC tumor tissues and 50 adjacent non-tumor tissues by RNA-sequencing. The expression of five WNT genes (*WNT2B*, *WNT3A*, *WNT6*, *WNT8B,* and *WNT10B*) was higher in tumor tissues than adjacent normal cells. Six WNTs, including WNT5B, showed the opposite pattern of expression, suggesting that WNT5B may have anti-tumor activity in HCC. Meanwhile, there was no significant difference in WNT5A expression in tumor and adjacent normal tissue (Dong et al., 2019).

Leukemias

Several groups have shown that WNT5B is significantly higher in chronic lymphocytic leukemia (CLL) than normal B cells. Specifically, WNT5B is higher in patients without IgV mutations (Lu et al., 2004; Memarian et al., 2009; Janovska et al., 2016). This higher expression of WNT5B correlates with CLL aggressiveness in patients. WNT5A also is highly expressed in CLL and correlates with CLL aggressiveness. However, the signaling induced by WNT5A and WNT5B is not identical, as

only WNT5A induced DVL3 phosphorylation and only WNT5A correlated with basal migration (Janovska et al., 2016).

WNT5B was also shown to be highly expressed in EpCAM^{+} cells (leukemia stem cells) in acute myeloid leukemia (AML) and to associate with the resistance of EpCAM^{high} myeloid leukemia cells to cytotoxic chemotherapy (Zheng et al., 2017). In contrast, WNT5A expression is epigenetically silenced in a majority of patients with AML (Martin et al., 2010).

CONCLUSION

WNT5B is often assumed to have a similar function as WNT5A because they belong to the WNT5 subfamily and share a high amino acid identity. They have similar roles in convergent extension during development, lymphatic development, MMC identity in the nervous system, retinal development, and aortic valve calcification (see **Table 1**). However, WNT5B exhibits unique, often opposing, effects on development and homeostasis in many tissue types including bone, adipose tissue, mammary gland, and myeloid cells. Non-canonical WNT5B functions through both WNT-PCP and WNT-Ca^{2+} signaling pathways, which are responsible for not only cell movement, but also cell proliferation, and cell differentiation. Disrupted WNT5B signaling leads to the progression of diseases such as osteoarthritis, osteoporosis, obesity, type 2 diabetes mellitus and chronic diseases associated with aging, as well as cancers. Understanding the mechanistic effects of WNT5B, which are not well known, could be translated to the development of potential therapies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CORRECTION
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Corrigendum: WNT5B in Physiology and Disease

Sarocha Suthon[1], *Rachel S. Perkins*[1], *Vitezslav Bryja*[2,3], *Gustavo A. Miranda-Carboni*[4,5] *and Susan A. Krum*[1,5]*

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Keywords: Wnt5B, Wnt signaling, development, cancer, Wnt5a

A Corrigendum on

WNT5B in Physiology and Disease

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In the original article, there were two errors.

(1) Our information on Wnt modification and secretion was out of date. Mouse Wnts are not palmitoleated on cysteines—that was an error in mutational analysis by Karl Willert. All the cysteines in Wnt are engaged in disulfide bonds (DOI 10.1126/science.1222879, 10.1074/jbc.m114.575027). However, a new reference describes WNT palmitoylation in zebrafish WNT3A (Dhasmana et al., 2021).

(2) The WLS protein binds to Wnt in the ER, not the Golgi. The Golgi localization of WLS was also an error due to the use of an epitope tag on the c-terminus (10.1016/j.devcel.2014.03.016, 10.1016/j.cell.2020.11.038).

A correction has been made to ***the introduction, paragraph number*** 2

The WNT family now contains 19 WNT genes, falling into 12 WNT subfamilies in mammalian genomes. All WNT genes encode proteins around 40 kDa in size and contain highly conserved cysteines (Miller, 2002; Clevers and Nusse, 2012). Mammalian WNT proteins are palmitoylated at conserved serine residues by a special palmitoyl transferase, Porcupine (PORCN), in the endoplasmic reticulum (Takada et al., 2006; Galli et al., 2007; Rios-Esteves et al., 2014). Zebrafish WNT3 is lipidated at both cysteine and serine residues (Dhasmana et al., 2021). The activity of PORCN is essential for the secretion of WNT ligands. Then, the seven-transmembrane protein Wntless/Evi (Wls) in the endoplasmic reticulum escorts mature hydrophobic WNT proteins to be secreted at the plasma membrane or released in exosomes, leading to both autocrine and paracrine effects (Banziger et al., 2006; Routledge and Scholpp, 2019).

Accordingly, the following reference has been added to the original article:

Dhasmana, D., Veerapathiran, S., Azbazdar, Y., Nelanuthala, A. V. S., Teh, C., Ozhan, G., et al. (2021). Wnt3 is lipidated at conserved cysteine and serine residues in zebrafish neural tissue. *Front. Cell Dev. Biol.* 9:671218. doi: 10.3389/fcell.2021.671218

And the following reference has been removed from the original article:

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The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has 5 been updated.

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REVIEW
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A Role for Frizzled and Their Post-Translational Modifications in the Mammalian Central Nervous System

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The Wnt pathway is a key signalling cascade that regulates the formation and function of neuronal circuits. The main receptors for Wnts are Frizzled (Fzd) that mediate diverse functions such as neurogenesis, axon guidance, dendritogenesis, synapse formation, and synaptic plasticity. These processes are crucial for the assembly of functional neuronal circuits required for diverse functions ranging from sensory and motor tasks to cognitive performance. Indeed, aberrant Wnt–Fzd signalling has been associated with synaptic defects during development and in neurodegenerative conditions such as Alzheimer's disease. New studies suggest that the localisation and stability of Fzd receptors play a crucial role in determining Wnt function. Post-translational modifications (PTMs) of Fzd are emerging as an important mechanism that regulates these Wnt receptors. However, only phosphorylation and glycosylation have been described to modulate Fzd function in the central nervous system (CNS). In this review, we discuss the function of Fzd in neuronal circuit connectivity and how PTMs contribute to their function. We also discuss other PTMs, not yet described in the CNS, and how they might modulate the function of Fzd in neuronal connectivity. PTMs could modulate Fzd function by affecting Fzd localisation and stability at the plasma membrane resulting in local effects of Wnt signalling, a feature particularly important in polarised cells such as neurons. Our review highlights the importance of further studies into the role of PTMs on Fzd receptors in the context of neuronal connectivity.

Keywords: post-translational modification, Frizzled receptors, Wnt signalling, CNS connectivity, trafficking

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INTRODUCTION

Wnt secreted proteins are key regulators of neuronal circuit formation and function. Wnts bind to several receptors resulting in the activation of different Wnt signalling pathways. The most common receptors are Frizzled receptors (Fzd), which often interact with co-receptors to initiate different Wnt signalling cascades. Through these receptors, four Wnt signalling cascades can be activated: the canonical Wnt/β-catenin pathway, the Wnt/divergent canonical/transcription-independent pathway, the planar cell polarity (PCP), and the Wnt/calcium pathway (**Figure 1**) promoting different cellular functions, all of which have been extensively reviewed (Kohn and Moon, 2005; Clevers, 2006; Salinas, 2007; De, 2011; Gray et al., 2011; Devenport, 2014; Acebron and Niehrs, 2016; Nusse and Clevers, 2017).

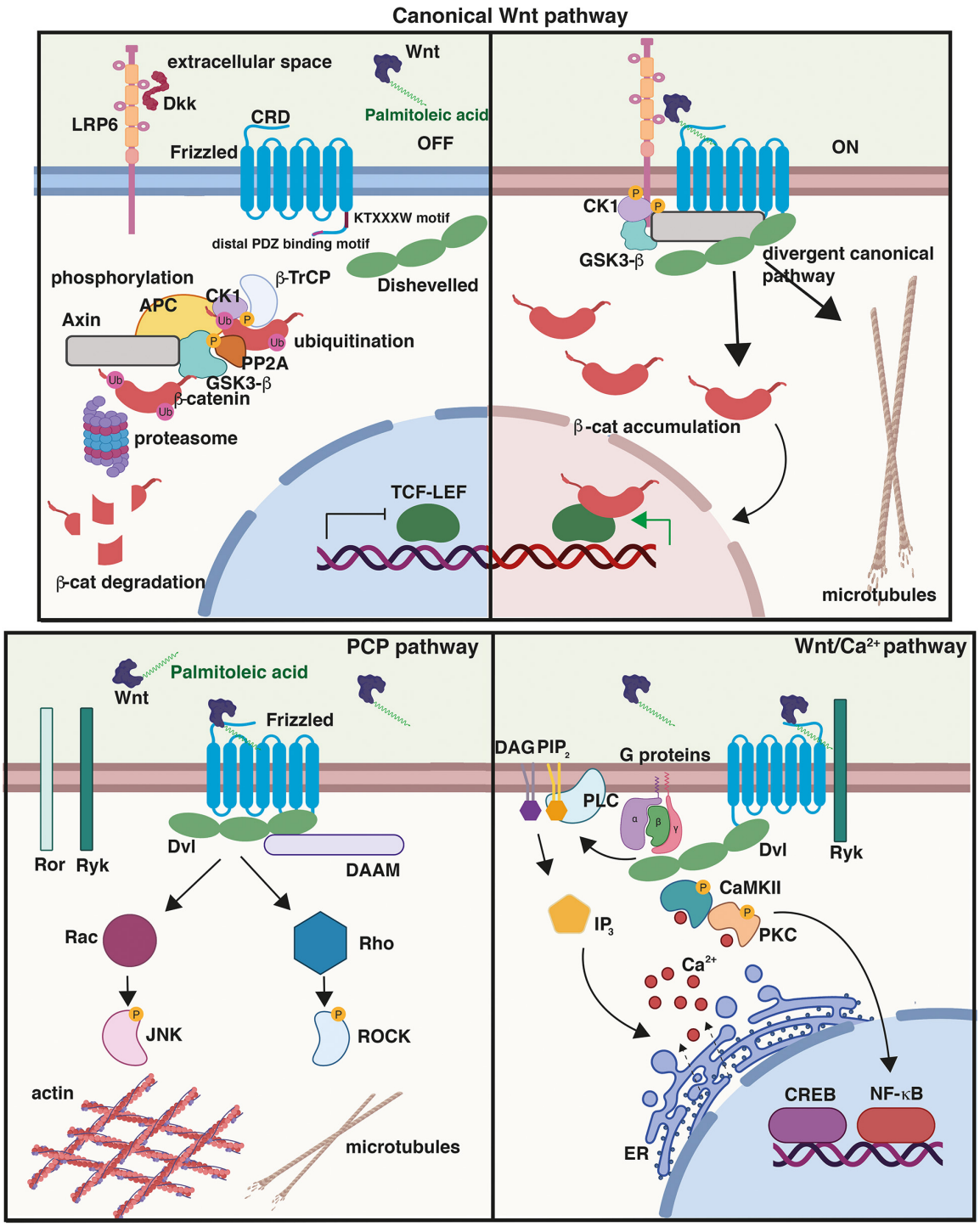

FIGURE 1 | Wnt signalling pathways. (Top) Canonical or Wnt/β-catenin signalling cascade. In the absence of Wnt ligands or in the presence of secreted antagonists like Dkk1, the destruction complex composed of Axin, APC, GSK3β, and β-catenin promotes the ubiquitination and phosphorylation of β-catenin. In the presence of Wnts, Frizzled (Fzd) and co-receptor LRP5/6 dimerise triggering the recruitment of Dvl to the PM and the assembly of signalosome complex; β-catenin is stabilised and then translocates to the nucleus driving the transcription of Wnt target genes through its interaction with TCF/LEF transcription factors. Activation of the divergent canonical/transcription-independent pathway downstream of GSK3β and independent of β-catenin promotes microtubule remodeling which includes axonal remodeling in neurons, cell growth and mitosis in dividing cells, and the maturation of germ cells. Cysteine-rich domain (CRD), conserved KTXXXW Dvl-binding motif, and distal PDZ-binding motifs on Fzd are shown. In the planar cell polarity (PCP) pathway, Wnt binding to Fzd results in the activation of the GTPases Rho and Rac and their effectors ROCK and JNK, respectively, leading to cytoskeletal reorganisation. Activation of the Wnt/Ca^{2+}, another β-catenin-independent pathway, results in the release of Ca^{2+} from the endoplasmic reticulum (ER), which activates the protein kinases CAMKII and PKC. This pathway triggers downstream events that include the activation of transcription factors NFκB and CREB and the transcription of downstream regulator genes. Created with BioRender.com.

Frizzled receptors were originally identified in *Drosophila* (Gubb and García-Bellido, 1982), but their role as Wnt receptors was not recognised until 1996 (Bhanot et al., 1996). Functionally, Fzd receptors are critical for the establishment of tissue and cell polarity, embryonic development, regulation of cell proliferation among other processes, and patterning of the central nervous system (CNS) (Huang and Klein, 2004; Wang et al., 2016; Zeng et al., 2018). During postnatal CNS development, Fzd receptors, like their Wnt ligands, play diverse roles including axon guidance, dendritogenesis, and synapse formation (Wang et al., 2016). In the adult nervous system, Wnt–Fzd signalling is required for synaptic plasticity and for synapse maintenance (Inestrosa and Arenas, 2010; Dickins and Salinas, 2013; Oliva et al., 2013a; Purro et al., 2014; Marzo et al., 2016; Buechler and Salinas, 2018; Ferrari et al., 2018; McLeod et al., 2018).

Frizzled receptors belong to the "Frizzled class" within the superfamily of G-protein coupled receptors (GPCRs) (Foord et al., 2005; Schulte and Bryja, 2007; Dijksterhuis et al., 2014) of which 10 Fzd receptors have been identified in mammals, Fzd1–10 (Schulte, 2010). Fzd receptors are 500 to 700 amino acids long and exhibit some characteristics typical of GPCRs: an extracellular N-terminus domain that contains multiple glycosylation sites, followed by seven transmembrane (TM) domains, and an intracellular C-terminus domain, which is subject to post-translational modifications (PTMs) and that interacts with different G proteins ($G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ proteins) (Bhanot et al., 1996; Shulman et al., 1998; Schulte and Bryja, 2007; Nichols et al., 2013). The N-terminus of Fzd receptors contains a fairly conserved cysteine-rich domain (CRD) (Huang and Klein, 2004; Wang Y. et al., 2006), characterised by a hydrophobic cavity required for binding to the palmitoleate moiety present on Wnt ligands (**Figure 1**; Janda et al., 2012; DeBruine et al., 2017; Nile and Hannoush, 2018). In contrast, the intracellular C-terminus is highly variable between different Fzd receptors, except for a highly conserved KTXXXW motif required for binding to the scaffold protein Dishevelled (Dvl), a critical component of all Wnt cascades (Wang H. et al., 2006; Sharma et al., 2018). These findings suggest that although the C-terminus and PTMs of Fzd receptors may vary, these receptors require Dvl to activate Wnt signalling.

Frizzled–Wnt signalling is important for maintaining a healthy nervous system. Indeed, deficiency in Fzd receptors leads to neurodevelopmental defects (Wang et al., 2016). For example, Fzd9 deficiency is responsible for some of the aspects of the multisystem developmental disorder Williams–Beuren (Ranheim et al., 2005; Zhao et al., 2005). Furthermore, impaired Wnt signalling has been linked to neurodegenerative diseases such as Alzheimer's disease (Purro et al., 2012; Sellers et al., 2018), and downregulation of *FZD2* and *FZD3* has been observed in the aging brain (Folke et al., 2019). This raises the important question: how is Fzd function regulated in health and disease? Fzd receptors are essential for Wnt signalling activation and their function is modulated by PTMs such as glycosylation, phosphorylation, and ubiquitination, all of which can affect trafficking, localisation, and their ability to signal. However, these PTMs are largely understudied in the nervous system. Here, we review the current understanding of Fzd PTMs and how those described in the CNS affect Fzd function during postnatal development of the mammalian CNS. For PTMs described outside the CNS, we discuss how they could play a role in Fzd function in neuronal connectivity.

FUNCTION OF FRIZZLED RECEPTORS IN THE MAMMALIAN CNS

Frizzled receptors are crucial Wnt receptors that mediate diverse functions in neurons including neurogenesis, axon guidance, dendritogenesis, synapse formation, and synaptic plasticity (Sahores et al., 2010; Budnik and Salinas, 2011; Oliva et al., 2013b; Ferrari et al., 2018; McLeod et al., 2018). Fzd receptors are enriched at specific subcellular compartments (Wu et al., 2004), suggesting that specific subcellular localisation of Fzd could allow the activation of the Wnt pathway in specific compartments and not the entire cell. This feature would be crucial in polarised cells such as neurons, during axon guidance and synapse assembly. Indeed, local activation of Wnt signalling *via* CAMKII is critical to regulate spine growth and synaptic strength at dendritic spines (Ciani et al., 2011). Here, we focus on Fzd receptors involved in CNS connectivity.

Axon guidance is a crucial process that permits the navigation of axons to their appropriate targets during development (Salinas, 2012; Stoeckli, 2018). This process is followed by the terminal remodeling of axons and growth cones that allow the subsequent assembly of the synapses (Salinas, 2012). Two receptors, Fzd5 and Fzd9, are present on axonal growth cones (Shah et al., 2009; Slater et al., 2013). Fzd9 is selectively expressed in the developing and adult hippocampus, where it localises to dendrites and efferent axons postnatally (**Table 1**; Zhao and Pleasure, 2004). In contrast, Fzd5 is expressed at the peak of synaptogenesis in the mouse hippocampus, with expression increasing postnatally (Sahores et al., 2010). In cultured rat hippocampal neurons, Fzd9 is present along the axon and axonal growth cones during the early stages of their development (Shah et al., 2009; Varela-Nallar et al., 2012), whereas Fzd5 is distributed exclusively on axonal growth cones during development up to the stage of dendritic outgrowth (Varela-Nallar et al., 2012; Slater et al., 2013), but in both axons and dendrites from neuronal maturation stage (Sahores et al., 2010; **Figure 2A**). This suggests that Fzd5 could play a role in the initial establishment of neuronal polarity and in the morphogenesis of neuronal processes and that its function may differ in dendrites versus axons (**Table 1**). Indeed, loss and gain-of-function experiments showed that Fzd5 is required for neuronal polarity and neurite growth (Slater et al., 2013). Studies on another Fzd, Fzd3, demonstrated that this receptor is required *in vivo* for axon growth and guidance in the forebrain, cranial and spinal motor neurons, sensory neurons, and the sympathetic nervous system, mainly through activation of the PCP signalling pathway (Wang et al., 2002, 2016; Lyuksyutova et al., 2003; Fenstermaker et al., 2010; Armstrong et al., 2011; Hua et al., 2013, 2014; Schafer et al., 2015; Feng et al., 2016; Ghimire and Deans, 2019). However, its role in postnatal development is less known. Overall, these findings show that specific enrichment of Fzd5, Fzd9, and

TABLE 1 | Frizzled (Fzd) receptors in the nervous system: localisation, function, and post-translational modifications (PTMs).

Fzd	Localisation	Function	PTMs (CNS)	PTMs (outside CNS)	Major interacting partners	References
Fzd1	Presynaptic	Presynaptic differentiation			Wnt3a	Varela-Nallar et al. (2009)
Fzd3	Soma, dendrites, axons, and synapses	*In vivo* for axon growth and guidance, synaptogenesis in dendrites, presynaptic differentiation	Phosphorylation: • Hyperphosphorylation required for PCP signalling and growth cone guidance *in vivo* • Hyperphosphorylation in presynaptic fraction Glycosylation: • N42 in CRD domain and N356 in second extracellular loop • Shisa2 inhibits glycosylation of Fzd3, reducing surface levels		PCP signalling components: Celsr3, Vangl2	Wang et al. (2002, 2016), Lyuksyutova et al. (2003); Fenstermaker et al. (2010), Armstrong et al. (2011); Shafer et al. (2011), Hua et al. (2013, 2014), Onishi et al. (2013); Schafer et al. (2015), Feng et al. (2016); Thakar et al. (2017), Ghimire and Deans (2019)
Fzd4	Dendrites	Activity-independent dendrite morphogenesis			Wnt5a	Bian et al. (2015)
Fzd5	Axons, dendritic shaft	Initial establishment of axonal polarity, presynaptic differentiation		• Ubiquitination by ZNRF3 and RNF43 which results in receptor endocytosis and degradation • Deubiquitination by USP6, maintains receptor at PM	Wnt7a	Sahores et al. (2010); Hao et al. (2012), Koo et al. (2012); Varela-Nallar et al. (2012), Slater et al. (2013); Madan et al. (2016), McLeod et al. (2018)
Fzd7	Dendritic growth cones, postsynapse	Dendritogenesis, postsynaptic differentiation		Inhibition of N-linked glycosylation by Shisa in the ER suppresses maturation and trafficking of Fzd to PM in HEK293	Wnt7a	Yamamoto et al. (2005); Varela-Nallar et al. (2012), Ferrari et al. (2018); McLeod et al. (2018)
Fzd9	Axon, dendrites	Axon guidance, postsynaptic assembly			Wnt5a, non-canonical Wnt signalling pathway	Shah et al. (2009); Varela-Nallar et al. (2012), Ramírez et al. (2016)

Fzd3 in growth cones is required for axonal outgrowth in different brain regions.

Dendritic morphogenesis is essential for the formation of functional neuronal networks (Prigge and Kay, 2018; Lefebvre, 2021). Fzd4 is localised in dendrites where it mediates activity-independent dendrite morphogenesis downstream of Wnt5a during postnatal development, as demonstrated by loss and gain-of-function experiments in cultured hippocampal neurons (**Table 1**; Bian et al., 2015). Fzd4 mRNA expression levels increase during the first two postnatal weeks in the mouse hippocampus and cerebral cortex, concomitantly with *Wnt5a* expression (Bian et al., 2015). Interestingly, signalling *via* Fzd4 is through its less conserved distal PDZ-binding motif rather than *via* the conserved motif to which Dvl1 binds (Wong et al., 2003; Bian et al., 2015). Although the function of Dvl1 has not been ruled out completely, the data suggest a primarily Dvl1-independent mechanism (Bian et al., 2015). This is of particular importance as Dvl is crucial for integrating and coordinating the activation of all Wnt signalling cascades (Sharma et al., 2018). In addition, Fzd4 is important for other brain functions. *In vivo* studies suggest that Fzd4 is important for the maintenance of blood–brain barrier function and plasticity in the mature CNS vascular structure (Wang et al., 2012).

Fzd7 is another receptor that plays an important role in dendritogenesis (**Table 1** and **Figure 2A**). In the hippocampus, Fzd7 expression increases from birth, reaching its peak in the adult (Ferrari et al., 2018). Endogenous Fzd7 localises along the neurite shaft and at dendritic growth cones (Ferrari et al., 2018). Loss and gain-of-function studies in cultured hippocampal neurons and loss-of-function *in vivo* showed that Fzd7 functions as a receptor for Wnt7b-mediated dendritic growth and complexity *via* Dvl1, CAMKII, and JNK (Ferrari et al., 2018). Therefore, Fzd7 promotes dendritogenesis *via* two non-canonical Wnt pathways (Ferrari et al., 2018).

Synapse formation is a complex process that requires the coordinated assembly of thousands of proteins at both sides of the synapse. Fzd receptors such as Fzd1, Fzd3, Fzd5, Fzd7, and Fzd9 play an important role in synaptogenesis (Varela-Nallar et al., 2009; Sahores et al., 2010; Schafer et al., 2015; Ramírez et al., 2016; McLeod et al., 2018). In particular, Fzd1, Fzd3, and Fzd5 regulate presynaptic differentiation, whereas Fzd7 and Fzd9 are important for postsynaptic assembly (**Table 1** and

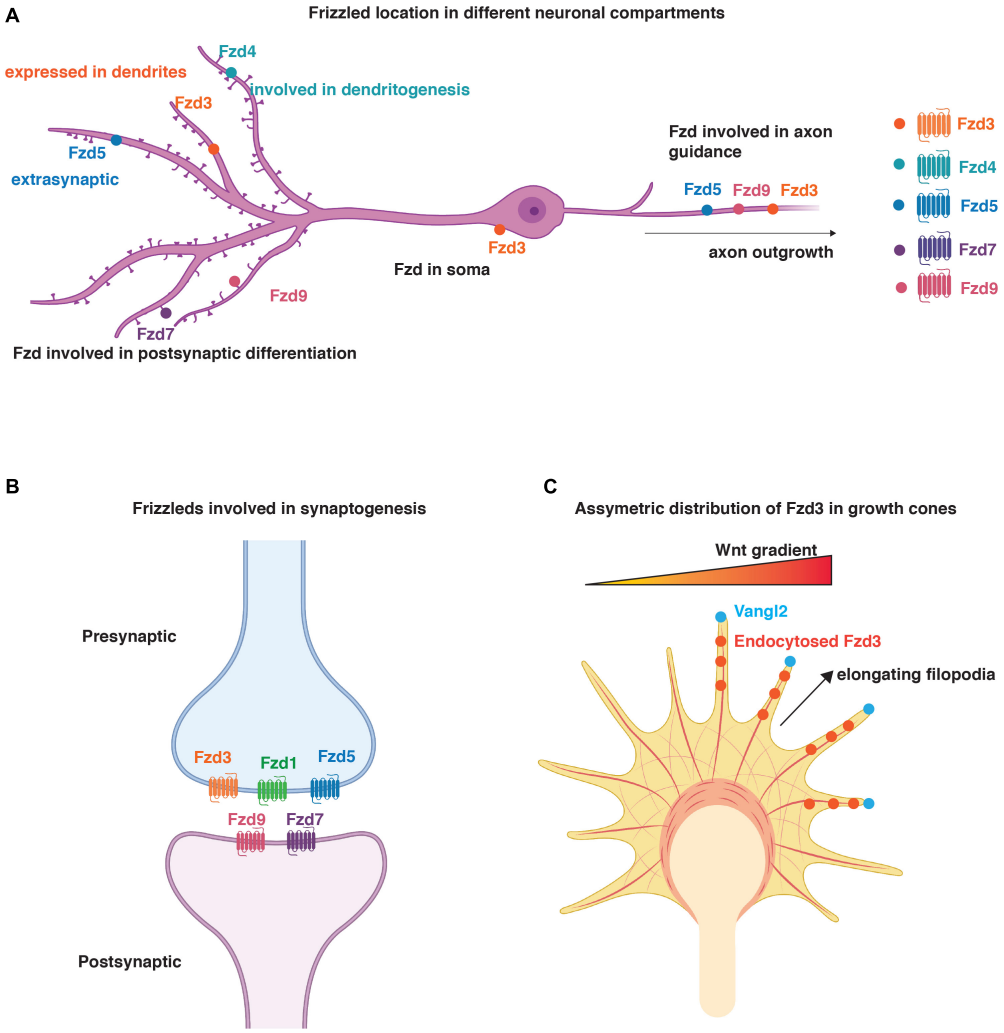

FIGURE 2 | Distribution and function of Fzd in neurons. **(A)** Schematic representation of localisation of Fzd receptors to different neuronal compartments. Fzd3 is localised to the soma, axons, and dendrites. Fzd4, Fzd7, and Fzd9 are present in dendrites and promote dendritogenesis and/or synaptogenesis. Fzd3, Fzd5, and Fzd9 play a role in axon outgrowth. Fzd4 is also involved in dendritogenesis. Fzd5 is present in axons where it regulates synapse formation. It is also present in dendrites but its function in this neuronal compartment is unknown. **(B)** Schematic representation of Fzd involved in synaptogenesis. Fzd3, Fzd1, and Fzd5 are presynaptically localised, whereas Fzd9 and Fzd7 are present postsynaptically. **(C)** Representation of asymmetric distribution of Fzd3 in growth cones due to the asymmetric localisation of Vangl2 at elongating filopodia. Here, Vangl2 promotes Fzd3 endocytosis and PCP signalling in certain filopodia to ensure asymmetric signalling for growth cone steering in response to Wnt gradients (Onishi et al., 2014). **(A–C)** Created with BioRender.com.

Figure 2B). These findings highlight the idea of local signalling, where enrichment of different Fzd receptors in distinct cellular compartments results in specific outcomes such as pre- or postsynaptic development.

Fzd1 is highly expressed in the hippocampus during postnatal development (Shimogori et al., 2004; Lein et al., 2007; Varela-Nallar et al., 2009; Mardones et al., 2016). Wnt3a ligand through Fzd1 regulates presynaptic differentiation and function as both the overexpression of Fzd1 and treatment with Wnt3a increase the number of bassoon puncta, a function that is blocked by exposure to a peptide containing the CRD domain of Fzd1. Notably, Fzd1 is present in synaptosome fractions from adult rat brains, suggesting a role in mature synaptic function (Varela-Nallar et al., 2009), but this has not been demonstrated.

In cultured hippocampal neurons, Fzd3 is expressed in the cell bodies, dendrites, and axons (**Figure 2A**) and co-localises with the presynaptic marker Vglut1 (Davis et al., 2008). Consistently, *in vivo* studies demonstrate that Fzd3 is localised presynaptically at glutamatergic synapses where it interacts with Cadherin EGF LAG seven-pass G-type receptor 3 (Celsr3), a key component of the PCP pathway, to promote synapse formation in the postnatal hippocampus (Thakar et al., 2017). These results suggest that Fzd3 interacts with PCP signalling components at the presynaptic side to promote glutamatergic synapse formation.

In addition to its role in neuronal polarity and neurite outgrowth (Varela-Nallar et al., 2012; Slater et al., 2013), Fzd5 also plays a role in presynaptic assembly (**Table 1**). Studies using loss and gain-of-function studies in cultured hippocampal

neurons demonstrated that Fzd5 is a presynaptic receptor for Wnt7a (Sahores et al., 2010). Fzd5 KD by expression of shRNAs or by acute Fzd5 loss-of-function achieved by using the soluble Fzd5-CRD domain that binds Wnts blocks Wnt7a-mediated synaptogenesis in cultured hippocampal neurons. In contrast, Wnt7a treatment or gain-of-function of Fzd5 induces presynaptic assembly (Sahores et al., 2010). Notably, high-frequency stimulation (HFS), a well-established paradigm that induces long-term potentiation and the formation of synapses (Bolshakov et al., 1997; Bozdagi et al., 2000), increases surface levels of Fzd5. In contrast, blockade of Fzd5 function with CRD domain during HFS prevents the localisation of Fzd5 to cell surface and to synapses. Importantly, the CRD domain of Fzd5 fully blocks HFS-induced synaptogenesis (Sahores et al., 2010). The results demonstrate that Fzd5 is required for activity-mediated synapse formation.

Fzd5 is also present along the dendritic shaft but is not enriched at dendritic spines (McLeod et al., 2018; **Figure 2A**). This finding raises the question of the role of Fzd5 in dendrites and how Fzd receptors are trafficked to those specific cellular locations. Interestingly, *Fzd5* is also expressed in the retina, the hypothalamus, and the parafascicular nucleus (PFN) of the thalamus (Shimogori et al., 2004; Liu et al., 2008), and studies of Fzd5-deficient mice revealed that Fzd5 is required for the survival of adult PFN neurons but not for their development (Liu et al., 2008). In summary, Fzd5 plays diverse roles in the postnatal CNS, but its potential role in dendrites requires further studies.

Two other Fzd receptors (Fzd7 and Fzd9) are expressed in the hippocampus. Their expression increases from birth, reaching its peak in the adult (Shimogori et al., 2004; Varela-Nallar et al., 2012; Ferrari et al., 2018). In mature neurons, Fzd7 is enriched in the postsynaptic fraction and localises to dendritic spines (**Table 1**; McLeod et al., 2018). Loss and gain-of-function studies of Fzd7 as well as acute blockade of endogenous Wnts in cultured hippocampal neurons showed that Wnt7a–Fzd7 postsynaptic signalling mediates LTP-dependent spine plasticity by promoting synaptic AMPA receptor localisation *via* CAMKII, PKA, and ERK cascades (McLeod et al., 2018). Thus, Fzd7 is required for Wnt7a/b-mediated dendritic development and for structural and functional plasticity of synapses (McLeod et al., 2018).

Fzd9 is highly enriched in postsynaptic synaptosome preparations from adult rat brains. Importantly, loss-of-function studies demonstrate that Fzd9 is required for Wnt5a-mediated increase in dendritic spine density in cultured hippocampal neurons (**Table 1**; Ramírez et al., 2016). Mechanistically, Fzd9 interacts with heterotrimeric G proteins resulting in the activation of non-canonical Wnt signalling pathways including CAMKII, JNK, and protein kinase C (PKC) (Ramírez et al., 2016). However, the *in vivo* role for Wnt5a/Fzd9 signalling has not been examined.

HOW PTMs DETERMINE THE FUNCTION OF FRIZZLED RECEPTORS

Post-translational modifications are biochemical modifications that are incorporated onto one or more amino acids after protein synthesis. PTMs vary greatly, but they all modulate the biochemical properties of proteins beyond that conferred by amino acids alone. PTMs are master regulators of protein trafficking, subcellular localisation, and function. There are different types of PTMs ranging from the covalent attachment of proteins or functional groups to their proteolytic cleavage (Mann and Jensen, 2003; Kannicht and Fuchs, 2008; Khoury et al., 2011; Vidal, 2011; Millar et al., 2019). The most common forms of PTM are N-linked glycosylation and phosphorylation (Khoury et al., 2011).

Wnts are post-translationally modified proteins. As secreted proteins, they are glycosylated but have a unique PTM as they are palmitoyleolated by a membrane-bound O-acyltransferase called Porcupine in the ER, which adds palmitoleic acid to serine residues on the Wnt protein (Hausmann et al., 2007; Nile and Hannoush, 2018). This modification regulates Wnt secretion and their interaction with Frizzled receptors (Kakugawa et al., 2015). Palmitoyleolation is a reversible PTM as Wnts can be de-palmitoyleolated by the de-acylase protein Notum in the extracellular space, resulting in the inhibition of Wnt signalling (Kakugawa et al., 2015). In turn, Fzd receptors are also PTM by glycosylation, ubiquitination, and phosphorylation that modify their localisation and function. To date, phosphorylation is the most described PTM on Fzd receptors in the CNS, followed by glycosylation.

The distribution of Fzd at the PM confers spatio-temporal dynamics of signalling activation (Huang and Klein, 2004). For example, Fzd5 is enriched presynaptically (Sahores et al., 2010), whereas Fzd9 is enriched postsynaptically (Ramírez et al., 2016), raising the question: what controls the trafficking and retention of Fzd receptors at these specific PM locations? A possible mechanism could be through PTMs. Indeed, Fzd receptors are phosphorylated, an important modification that regulates their function during axon guidance (Shafer et al., 2011; Onishi et al., 2013). Fzd receptors have canonical motifs for phosphorylation by protein kinase A (PKA) and PKC and casein kinase II on their C-terminus (Wang and Malbon, 2004). As Fzd receptors have highly variable C-terminal domains, it is possible that not all Fzd receptors will be modified by phosphorylation and could be regulated in different ways. For example, in mammalian systems, Fzd6 has been reported to be phosphorylated on pSer-648 by casein kinase I d in epithelial cells (Strakova et al., 2018), whereas Dvl-dependent phosphorylation of Fzd3 requires Ser-576 (Yanfeng et al., 2006). Additional sites for the phosphorylation of Fzd3 were also identified: Ser-508, Thr-541, Thr-562, Ser-587, Ser-624, and Ser-636, all of which reduced Dvl-induced phosphorylation when mutated to alanine (Yanfeng et al., 2006).

A particular example of the regulation of Fzd phosphorylation is through Celsr3, an essential component of the PCP pathway, which regulates Fzd3 phosphorylation. Mice lacking Celsr exhibit impaired PCP signalling and hyperphosphorylated Fzd3 (Wang and Nathans, 2007; Onishi et al., 2013). In addition, a recent study showed that both loss-of-function of leucine-rich repeat kinase 2 (LRRK2) and gain-of-function of mutated LRRK2 lacking kinase activity showed anterior–posterior guidance errors after midline crossing *in vivo* (Onishi et al., 2020). Furthermore, they identified

LRRK2 as a new protein which both directly phosphorylates Fzd3 on threonine 598 (T598) and indirectly promotes Dvl1-induced Fzd3 hyperphosphorylation by acting as a scaffold and recruiting other kinases (Onishi et al., 2020). Overall, hypo- or non-phosphorylated Fzd3 is required for PCP signalling and for growth cone guidance *in vivo* (**Table 1**; Shafer et al., 2011; Onishi et al., 2013, 2020).

Studies also showed a correlation between the level of Fzd3 phosphorylation and endocytosis. Activation of the PCP pathway requires Fzd receptor endocytosis (Yu et al., 2007; Sato et al., 2010). Consistent with this, overexpression of Dvl1 and a mutant of Fzd3, whose phosphorylation cannot be induced by Dvl1, promotes Fzd3 internalisation and the consequent increase in PCP signalling in dissociated commissural neurons (Shafer et al., 2011; Onishi et al., 2013). In addition, stimulation with recombinant Wnt5a results in Fzd3 endocytosis at growth cones of dissociated commissural neurons, where Dvl2 and atypical PKC (aPKC) inhibit Dvl1-induced hyperphosphorylation of Fzd3. This process leads to Fzd3 endocytosis and the consequent increase in PCP signalling (Onishi et al., 2013). Together, these data show that Dvl1-mediated hyperphosphorylated Fzd3 is maintained at the PM and inhibits PCP signalling, whereas hypo- or non-phosphorylated Fzd3 is internalised resulting in the activation of the PCP cascade (**Table 1**). Thus, phosphorylation is an important PTM that regulates the localisation of Fzd3 at the PM and its ability to signal. In contrast to Fzd3, the role of phosphorylation on other Fzd remains to be determined.

Loss and gain-of-function experiments led to the conclusion that another core PCP pathway component Vangl2 (Van Gogh2), a four pass transmembrane protein, also inhibits Dvl1-mediated Fzd3 hyperphosphorylation and promotes its endocytosis resulting in PCP signalling (Shafer et al., 2011). Vangl2 is localised predominantly at the PM and is enriched at the tips of filopodia and sites where filopodia emerge in commissural axon growth cones. In addition, when Dvl1 and Fzd3 are co-expressed, they target each other to the plasma membrane of growth cones (Shafer et al., 2011). The antagonistic actions of Dvl1 and Vangl2 on Fzd3 phosphorylation allow the sharpening of PCP signalling to occur locally on tips of filopodia to sense directional cues by Wnts causing the turning of growth cones (Shafer et al., 2011).

Another interesting feature is the asymmetric localisation of Wnt components in neurons. Vangl2 is localised asymmetrically to the tip of elongating filopodia as opposed to those that are shrinking, suggesting that Vangl2 promotes Fzd3 endocytosis and PCP signalling in some filopodia to ensure asymmetric response to Wnts and, consequently, signalling for growth cone steering (Onishi et al., 2014; **Figure 2C**). Whether the asymmetric localisation of Fzd3 is due to changes in PTM remains to be determined.

Vangl2 also antagonises Celsr3 during glutamatergic synapse formation as Vangl2 inhibits synapse assembly, whereas Celsr3 promotes synapse assembly (Thakar et al., 2017). Analysis of the presence of PCP components at synaptic membrane fractions (SMF) and postsynaptic density (PSD) demonstrates that Celsr3 and Celsr2 are present in both fractions. In addition, Dvl1 is enriched in SMF, whereas Dvl2 and Vangl2 are enriched in PSD fraction (Thakar et al., 2017). Interestingly, the hyperphosphorylated form of Fzd3 is more abundant in the SMF than the PSD, and the unphosphorylated form of this receptor is enriched in the PSD fraction. These findings are consistent with the data showing that Fzd3 is hyperphosphorylated in a Dvl1-mediated manner, a process which is inhibited by Dvl2 and Vangl2 (Shafer et al., 2011; Onishi et al., 2013). Overall, these data suggest that PCP components are asymmetrically localised in glutamatergic synapses (Thakar et al., 2017) to mediate local effects on PCP signalling as observed by hyperphosphorylated and unphosphorylated forms of Fzd3.

Asymmetric distribution of Fzd3, which is closely related to Fzd6 (Schulte, 2010), has been observed outside the CNS. In epithelial cells of the fallopian tube, phosphorylated Fzd6 (pSer-648 by casein kinase I d) is predominantly localised to the apical side compared with total Fzd6, which is evenly distributed on both the apical and basal plasma membrane of these epithelial cells (Strakova et al., 2018). This could suggest that asymmetric phosphorylation rather than asymmetric distribution of Fzd6 leads to polarised signalling (Strakova et al., 2018). Overall, these studies demonstrate that phosphorylation is an important PTM, which affects the function of Fzd both in epithelial cells and the CNS and is responsible for polarised and, therefore, local signalling.

Although phosphorylation is the best understood PTM for Fzd in the CNS, glycosylation of Fzd has also been described in rodent commissural axon growth cones (Onishi and Zou, 2017). Fzd receptors were first identified to be glycosylated at the N-terminus in the ER in HEK293 cells suggesting that this PTM might be important for Fzd maturation and trafficking to the PM (Yamamoto et al., 2005; **Figure 3**). Shisa, an ER-resident protein, interacts with immature Fzd protein in the ER, preventing further processing of N-linked glycosylation and suppressing its maturation and trafficking to the cell surface in HEK293 (Yamamoto et al., 2005). Therefore, Shisa inhibits Wnt signalling. The role of Shisa has been demonstrated for Fzd7 and Fzd8; however, all Fzd receptors are predicted to be N-glycosylated at a conserved YNTxT motif (**Table 1**; MacDonald and He, 2012). Indeed, Shisa2 was later on shown to inhibit Fzd3 glycosylation at two sites (N42 in the CRD domain and N356 in the second extracellular loop) and, consequently, Fzd3 cell surface presentation in HEK293 (Onishi and Zou, 2017). Shisa2 knockdown in commissural neurons was also shown to increase Fzd3 protein on the surface of growth cones, resulting in precocious anterior turning of commissural axons before or during midline crossing (**Table 1**; Onishi and Zou, 2017). In addition, Shh-Smoothened signalling activates PCP signalling in commissural neurons by inhibiting Shisa2 and, therefore, resulting in Fzd3 trafficking to the membrane and activation of PCP signalling (Onishi and Zou, 2017). Further studies are required to establish the precise role of Shisa on other Fzd receptors in different cellular contexts in the CNS. For example, at the synapse, Shisa9, the first Shisa to be identified in mice, plays an important role in AMPA receptor (AMPAR) desensitisation (von Engelhardt et al., 2010). Another isoform, Shisa7, is associated with AMPAR and regulates synaptic function (Schmitz et al., 2017). In

contrast, Shisa6 traps AMPAR at postsynaptic sites (Klaassen et al., 2016). The presence of different Shisa proteins in the brain raises the question: which Shisa isoforms regulate Fzd localisation and function in the mammalian CNS? Is Shisa2 specific to Fzd3 in axonal growth cones? What is the relationship of Shisa with Fzd at the synapse and other neuronal compartments?

Another PTM that regulates Fzd receptor function, but has not been described in the CNS, is ubiquitination, where cycles of ubiquitination/deubiquitination control the degradation of Fzd receptors and their recycling to the PM (Hao et al., 2012; Koo et al., 2012; Moffat et al., 2014). Fzd can be multi-monoubiquitinated on lysine residues, located in their cytoplasmic loops between different transmembrane domains, by the highly related and membrane-localised RING E3 ligases Zinc and acid finger protein 3 (ZNRF3) and ring finger protein 43 (RNF43) (Hao et al., 2012; Koo et al., 2012). Studies in HEK293T cells show that a Fzd5 mutant, which cannot be ubiquitinated as all cytosolic residues are mutated to arginine, is resistant to RNF43-mediated internalisation, suggesting that ubiquitination plays an important role in Fzd internalisation and, therefore, has the ability to detect Wnt ligands (**Table 1**; Koo et al., 2012). Indeed, ubiquitination of Fzd reduces their levels at the PM resulting in the downregulation of Wnt signalling by promoting Fzd endocytosis and lysosomal degradation (Hao et al., 2012; Koo et al., 2012; Moffat et al., 2014; **Figure 3**). Interestingly, ubiquitination by ZNRF3 and RNF43 has also been shown to regulate surface levels of the Wnt co-receptor LRP5/6 in the same way as Fzd (Hao et al., 2012; Koo et al., 2012). Wnt binding to Fzd and co-receptor LRP5/6 induces the dimerisation of these two receptors activating signalling *via* the β-catenin canonical pathway (Bilic et al., 2007; Komiya and Habas, 2008; Feng and Gao, 2015; Nusse and Clevers, 2017). Therefore, this raises the question of whether Fzd and co-receptors are regulated in the same manner and whether PTMs on Fzd influence their interaction with co-receptors. Future research will shed new light into this.

R-spondins are secreted glycoproteins and are effective Wnt agonists that bind to ZNRF3 forming a trimeric complex between R-spondin, the R-spondin receptor LGR4, and ZNRF3, which results in the membrane clearance of ZNRF3 (Hao et al., 2012). Therefore, R-spondins inhibit ZNRF3-dependent ubiquitination of Fzd receptors, thus potentiating Wnt signalling (Hao et al., 2012). Double knockout of ZNFR3 and RNF43 induces intestinal adenoma, a Wnt-dependent cancer, likely as a result of increased Fzd receptors at the cell surface (Koo et al., 2012). Indeed, loss-of-function mutations of ZNFR3/RNF42 have been observed in many other types of cancers (Koo et al., 2012; Hao et al., 2016; Katoh and Katoh, 2017). In contrast, Fzd receptors are deubiquitinated by UBPY/ubiquitin-specific protease 6 and 8 (USP6 and USP8). Gain and loss-of-function studies showed that USP6 stabilises the membrane pool of Fzd5 (**Table 1**; Madan et al., 2016) and USP8 promotes the recycling of Fzd receptors to the PM, increasing their cell surface localisation and therefore enhancing Wnt signalling both in mammalian cells and *Drosophila* wing (Mukai et al., 2010). USP6 has also been shown to regulate cell surface abundance of LRP6 as expression of wild-type USP6 increases surface levels of LRP6. However, it is unclear whether it directly deubiquitylates LRP6 (Madan et al., 2016). Interestingly, the Wnt antagonist Dickkopf-1 (DKK1) or LRP5/6 siRNA blocked USP6-induced Wnt signalling, suggesting that a Wnt-ligand receptor complex is required for USP6 function (Madan et al., 2016). These data support the view that ubiquitination/deubiquitination cycles are crucial in regulating surface levels of Fzd and co-receptors LRP5/6 and, therefore, Wnt signalling.

DISCUSSION

Increasing evidence suggests that PTMs of Frizzled receptors play important roles in their function and cellular localisation. However, only phosphorylation and glycosylation have been described to modulate Fzd function in the central nervous system (CNS). Here, we have focussed our attention on the specific cellular localisations of Fzd in the mammalian CNS and how PTMs contribute to their function in the CNS. The exact mechanisms for these modifications remain poorly understood.

Currently, phosphorylation is the most understood PTM of Fzd in the CNS. This PTM regulates Fzd3 localisation at the PM during growth cone guidance and glutamatergic synapse formation, where asymmetric distribution of unphosphorylated/phosphorylated Fzd3 has been observed alongside asymmetric distribution of components regulating Fzd3 phosphorylation (Shafer et al., 2011; Onishi et al., 2013; Onishi and Zou, 2017; Thakar et al., 2017). Thus, asymmetric distribution or asymmetric phosphorylation of Fzd is important for local signalling.

Glycosylation has also been shown to be important in controlling surface levels of Fzd both in HEK293 cells (Fzd7 and Fzd8) and in the CNS (Fzd3). Inhibition of glycosylation by Shisa proteins in both systems decreased Fzd receptors at the PM and, consequently, dampened Wnt signalling (Yamamoto et al., 2005; Onishi et al., 2020). As many key proteins involved in synaptic transmission are N-glycosylated, this PTM on Fz receptors could be an important regulator of neurotransmitter release, excitability and synaptic potentiation (Scott and Panin, 2014). Further studies are required to establish the precise role of different Shisa proteins on Fzd receptors in the context of neuronal connectivity.

On the other hand, ubiquitination of Fzd has only been described in cell lines. The role of ubiquitination/deubiquitination cycles on Fzd is better understood as these processes control the degradation of Fzd receptors and their recycling to the PM in cell lines (Hao et al., 2012; Koo et al., 2012; Moffat et al., 2014). Given the data in heterologous cells and how important glycosylation and ubiquitination/deubiquitination regulate surface localisation of receptors such as AMPAR and GABAR in the CNS (von Engelhardt et al., 2010; Klaassen et al., 2016; Schmitz et al., 2017), it is likely that these PTMs regulate the trafficking, localisation, and function of Fzd in the CNS.

By modulating the stability of Fzd at the PM, PTMs modulate their ability to signal. For example, PTMs could

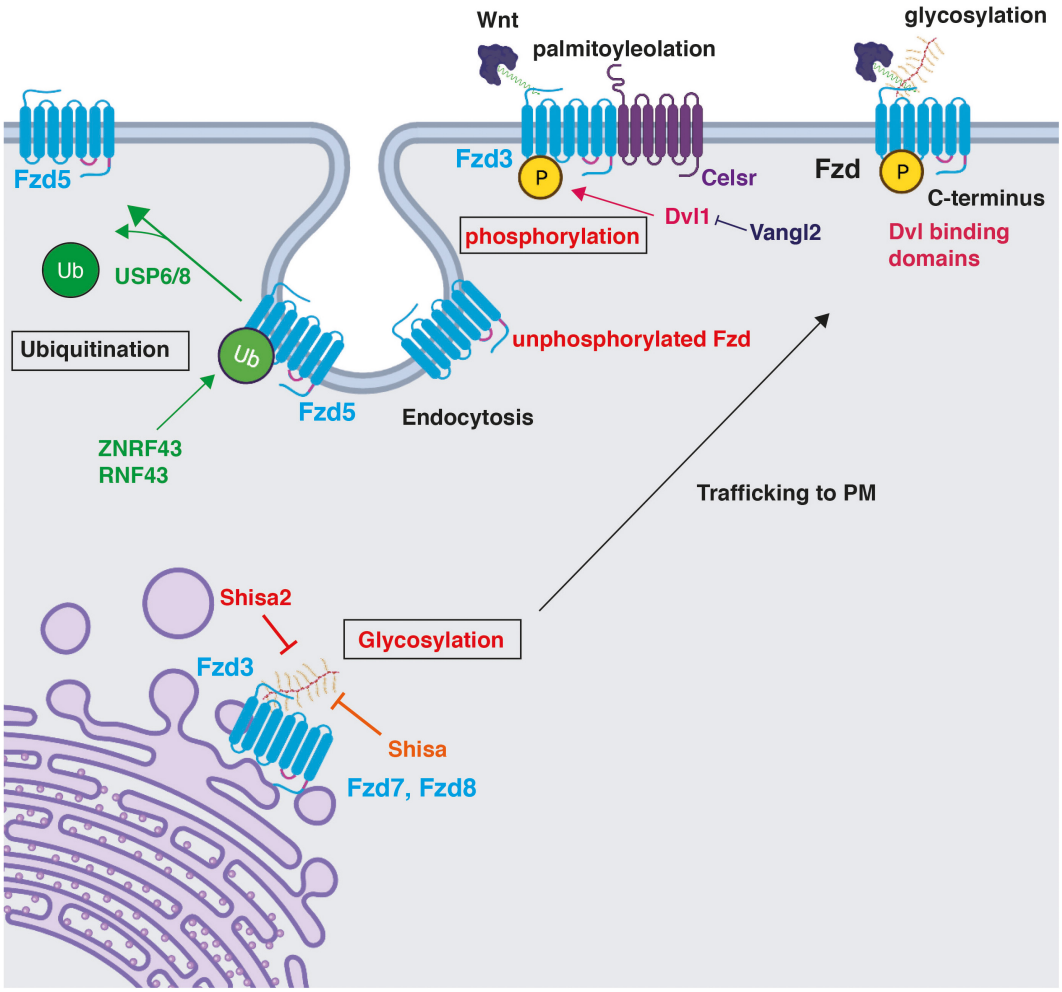

FIGURE 3 | Post-translational modifications on Frizzled receptors and their impact on Fzd function. Fzd receptors are post-translationally modified. Phosphorylation and glycosylation are shown in red as described in the CNS. Phosphorylation maintains Fzd at the PM (Shafer et al., 2011; Onishi et al., 2013). A particular example of the regulation of Fzd phosphorylation is through Celsr3, an essential component of the PCP pathway. Dvl1 promotes Fzd3 hyperphosphorylation, which is inhibited by Vangl2 (Shafer et al., 2011). Glycosylation occurs on the N-terminus in the ER and promotes trafficking of Fzd7 and Fzd8 to the plasma membrane, a process that is inhibited by the ER-resident protein Shisa in HEK293 (Yamamoto et al., 2005), and by Shisa2 in rat commissural neurons (Onishi and Zou, 2017). Ubiquitination by ZNRF3 and RNF43 occurs on multiple lysine residues in cytoplasmic loops and results in Fzd5 internalisation and degradation (Hao et al., 2012; Koo et al., 2012; Moffat et al., 2014). USP6/8 deubiquitinate Fzd5, stabilising the membrane pool of Fzd5 and promoting its recycling to the PM (Madan et al., 2016). Created with BioRender.com.

influence the interaction of Fzd with their co-receptors such as LRP5/6 thereby affecting canonical Wnt signalling or other pathways. Interestingly, LRP5/6 surface levels are modulated by ubiquitination in the same was as Fzd (Koo et al., 2012; Hao et al., 2016), and deubiquitination by USP6 requires a Wnt–ligand-engaged Fzd–LRP5/6 receptor complex (Madan et al., 2016). Similarly, PTM could regulate the interaction between Fzd and another co-receptor such as Ror or Ryk, influencing the PCP pathway. This raises the question of whether PTM of Fzd receptors and their co-receptors are coordinated and whether certain PTMs depend on Wnt ligand interaction.

In summary, Fzd are post-translationally modified by phosphorylation, glycosylation, and ubiquitination/deubiquitination. In the CNS, however, although phosphorylation of Fzd is the best understood PTM, it has only been described for Fzd3. Indeed, phosphorylation plays a role in the asymmetric distribution of Fzd3 receptors during axon guidance and at the synapse. However, whether other Fzd receptors are regulated by phosphorylation and how this PTM will affect their function remains to be determined. As phosphorylation occurs on the highly variable C-terminal of Fzd receptors, this PTM is likely to vary between Fzd receptors. In contrast, although glycosylation has been demonstrated to be important only for Fzd3 at the cell surface during axon guidance so far, given that it has been shown to regulate Fzd7 and Fzd8 in the same manner in HEK293 cells, combined with the fact that Fzd N-termini are highly conserved suggests that it is likely

that glycosylation regulates all Fzd receptors in a similar manner. Nevertheless, this remains to be determined and other PTM modifications in the CNS remain to be identified. Given the important role of other PTMs in regulating the localisation and function of Fzd receptors in other tissues, future studies will shed light into the molecular mechanisms that control the localisation of these receptors to specific plasma membrane domains and how local activation of Wnt signalling in specific neuronal/cellular compartments is achieved and how they influence neuronal connectivity in the nervous system.

AUTHOR CONTRIBUTIONS

PP-V and PS designed the outline of the review and wrote the manuscript. PP-V created the figures. Both authors contributed to the article and approved the submitted version.

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Changes in Wnt and TGF-β Signaling Mediate the Development of Regorafenib Resistance in Hepatocellular Carcinoma Cell Line HuH7

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Hepatocellular carcinoma (HCC) is an aggressive, chemo resistant neoplasm with poor prognosis and limited treatment options. Exploring activated pathways upon drug treatment can be used to discover more effective anticancer agents to overcome therapy resistance and enhance therapeutic outcomes for patients with advanced HCC. Human tumor-derived cell lines recapitulate HCC diversity and are widely used for studying mechanisms that drive drug resistance in HCC. In this study, we show that regorafenib treatment activates Wnt/β-catenin signaling only in hepatoblast-like HCC cell lines and induces enrichment of markers associated with hepatic stem/progenitor cells. Moreover, activation of Wnt/β-catenin signaling *via* Wnt3a/R-Spo1 treatment protects these cells from regorafenib induced apoptosis. On the other hand, regorafenib resistant cells established by long-term regorafenib treatment demonstrate diminished Wnt/β-catenin signaling activity while TGF-β signaling activity of these cells is significantly enhanced. Regorafenib resistant cells (RRCs) also show increased expression of several mesenchymal genes along with an induction of CD24 and CD133 cancer stem cell markers. Moreover, regorafenib resistant cells also exhibit significantly augmented *in vitro* and *in vivo* migration capacity which could be reversed by TGF-β type 1 receptor (TGFb -R1) inhibition. When combined with regorafenib treatment, TGFβ-R1 inhibition also significantly decreased colony formation ability and augmented cell death in resistant spheroids. Importantly, when we knocked down TGFβ-R1 using a lentiviral plasmid, regorafenib resistant cells entered senescence indicating that this pathway is important for their survival. Treatment of RRCs with TGFβ-R1 inhibitor and regorafenib significantly abolished pSTAT3, pSMAD2 and pERK (44/42) expression suggesting the involvement of both canonical and non-canonical pathways. In conclusion, our data suggest that HCC tumors with aberrant activation in the Wnt/β-catenin pathway, might have higher intrinsic regorafenib resistance and the inhibition of this pathway along with regorafenib administration might increase regorafenib-induced cell death in

combinational therapies. However, to resolve acquired regorafenib resistance developed in HCC patients, the combined use of TGF-β pathway inhibitors and Regorafenib constitute a promising approach that can increase regorafenib sensitization and prevent tumor recurrence.

Keywords: hepatocellular carcinoma, regorafenib, Wnt/β-catenin, TGFβ, resistance

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the third leading cause of cancer-related deaths worldwide (Bertuccio et al., 2017; Savitha et al., 2017; Yang et al., 2019; Pérez et al., 2020). HCC is an aggressive, chemo resistant neoplasm with complex etiology and high metastatic potential. The majority of diagnosis is done at an advanced stage where HCC patients are not suitable for potentially curative therapies including liver transplantation or surgical resection. Therefore, systemic therapy still is the main therapeutic option for advanced HCC patients (Ohri et al., 2016).

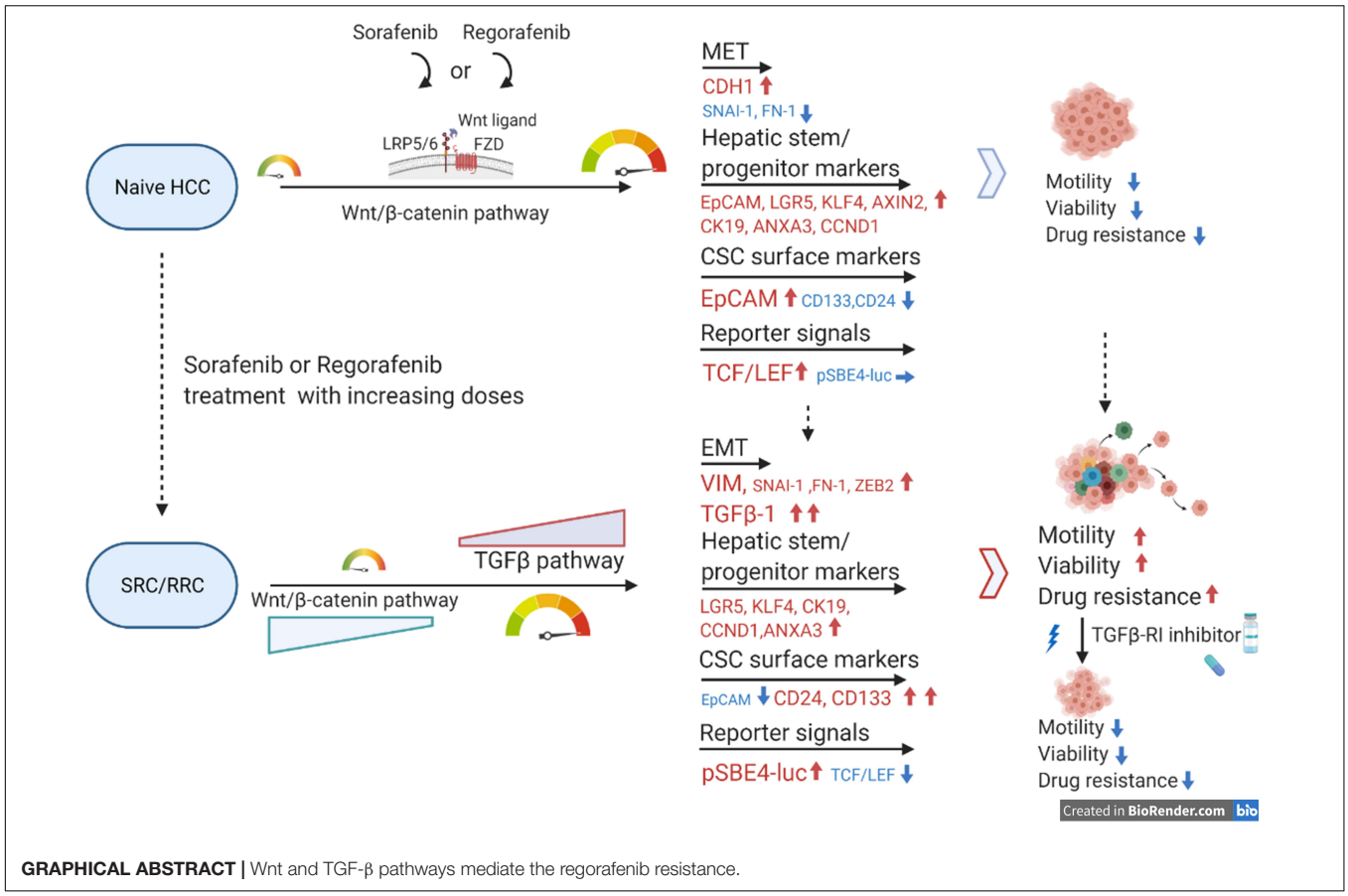
Multi-kinase inhibitors Sorafenib and Lenvatinib, are the two systemic therapies approved first-line for unresectable HCC (Kudo et al., 2018; Xie et al., 2018; Personeni et al., 2019). Moreover, the combination of programmed cell death ligand 1 (PD-L1) inhibitor atezolizumab and VEGFR inhibitor bevacizumab was also recently approved for first-line treatment for advanced HCC. However, since majority of Phase 3 trials are done after sorafenib failure, sorafenib still remains the globally accepted first-line treatment for advanced HCC despite its poor therapeutic response and high rates of resistance (Kudo et al., 2018; Personeni et al., 2019; Kim et al., 2020). Regorafenib, the fluoro analog of sorafenib, is one of the second-line treatments in patients who failed sorafenib therapy. Despite the fact that regorafenib increases the overall survival of patients who progress on sorafenib, the efficacy of this drug is also limited by primary or acquired therapy resistance and high interindividual variability (Bruix et al., 2017; Rimassa et al., 2017; Ettrich and Seufferlein, 2018; Finn et al., 2018; Tovoli et al., 2018). High intra and inter tumor heterogeneity in HCC influences disease progression, prognosis, and variable tumor response of patients to treatment (Ueshima et al., 2017; Iavarone et al., 2019; Hacioglu et al., 2020; Lee M.J. et al., 2020; Lee Y. et al., 2020; Ogasawara et al., 2020). Unfortunately, there are currently no molecular markers for currently used systemic treatments (sorafenib, lenvatinib, regorafenib, and others) in HCC which could be used in therapeutic decision-making to improve patient outcomes. Therefore, it is essential to investigate the molecular mechanisms underlying the variable response to drug treatment and contribute to the development of drug resistance in order to enhance treatment efficacy in HCC.

Human tumor-derived cell lines recapitulate HCC diversity and are widely used for studying mechanisms that drive drug resistance and sensitivity in HCC. Recent characterization of human liver cancer cell lines (LCCLs) identified three subgroups of LCCLs according to the differentiation state and transcriptome (Caruso et al., 2019). The first group of LCCLs include Huh7, HepG2 and Hep3B which express hepato-specific genes and fetal/progenitor markers with active Wnt signaling. Among these cell lines, HepG2 carries a deletion in β-catenin gene (Exon 3–4) resulting in its constitutive activation. In contrast, Hep3B harbors mutations in AXIN1, a major member of β-catenin degradation machinery, leading to stabilization and accumulation of β-catenin protein. Finally, although Huh7 contains wild type β-catenin gene, due to mutations in p53 gene and amplification of FGF19 gene, β-catenin activity is increased in this cell (Cagatay and Ozturk, 2002; Ding et al., 2017).

On the other hand, the other two subgroups of LCCLs are less differentiated with an activation of the TGF-β and noncanonical β-catenin pathways (Caruso et al., 2019; Rebouissou and Nault, 2020). The goal of this study was to analyze the various responses of HCC cell lines to regorafenib treatment and identify molecular pathways that could be used as new potential drug targets for combinational treatment regimens in HCC and/or serve as molecular markers for predicting regorafenib sensitivity.

In this study, we show that regorafenib treatment activates Wnt/β-catenin signaling only in hepatoblast-like HCC cell lines and induces enrichment of markers associated with hepatic stem/progenitor cells. Moreover, Wnt/β-catenin signaling activation by Wnt3a/R-Spo1 treatment protects these cells from regorafenib induced apoptosis. However, regorafenib resistant cells established by long-term regorafenib treatment demonstrate diminished Wnt/β-catenin signaling activity. On the other hand, the TGF-β signaling activity of these cells is significantly enhanced. Regorafenib resistant cells also have increased mesenchymal gene expression along with an induction of CD24 and CD133 cancer stem cell markers. Moreover, TGF-β type1 receptor (TGFβ-R1) inhibition could augment regorafenib induced cell death and reverse increased migration capacity of regorafenib resistant cells. In addition, knocked down of TGFβ-R1 in regorafenib resistant cells induced senescence indicating that this pathway is important for the growth and survival of cells. Treatment of resistant cells with TGFβ-R1 inhibitor and regorafenib significantly abolished pSTAT3, pSMAD2 and pERK (44/42) expression suggesting the involvement of both canonical and non-canonical pathways.

These results suggest that although acute Regorafenib treatment of hepatoblast-like cells initially creates a Wnt/β-catenin pathway-mediated increase in epithelial and stemness related markers, long-term regorafenib treatment of these cells leads to TGF-β pathway activation and the induction of mesenchymal cancer stem cell markers accompanied with an increased *in vivo* metastatic ability mediated by TGF-β pathway. Thus, our data suggest that for HCC tumors with aberrant Wnt/β-catenin activation, the inhibition of this pathway along with regorafenib administration might increase regorafenib-induced cell death and thus ameliorate treatment outcome.



GRAPHICAL ABSTRACT | Wnt and TGF-β pathways mediate the regorafenib resistance.

However, for acquired regorafenib resistance developed in HCC patients, the combined use of TGF-β pathway inhibitors and Regorafenib constitute a promising approach for regorafenib sensitization and to prevent tumor recurrence.

MATERIALS AND METHODS

Cell Culture

The previously authenticated cell lines were used in this study (Karagonlar et al., 2020). All cells were grown at 37°C, 5% CO2 in RPMI Medium 1640 supplemented with % 2–10 FBS, 1% NEAA, 2 mmol·L^{-1} Glutamax, 1% pen/strep. Sorafenib and Regorafenib resistant cells were created by treating parental cell lines with increased doses of drugs starting with their IC50 values. For a period of 8–12 months, at each passage, the drug concentrations were increased between 0.2 and 0.5 μM according to cell viability and proliferation rate of the cells. MTT analysis was performed to confirm the resistance. Established cell lines are maintained under the following drug concentrations: Sorafenib: 7.2 μM and Regorafenib: 8.4 μM.

Reporter Assays

The plasmids used in TCF/LEF reporter assay were a gift from Dr. Hans Clevers at Hubrecht Institute. Transfections were performed as previously described (Karabicici et al., 2021) using Lipofectamine 2000. For TCF/LEF reporter assays, 24 h after transfection, cells were treated with Regorafenib (5 or 10 μM), Wnt3a/R-Spo1, IWR-1(10 μM) or a combination of these molecules. For TGF-β reporter assays, 24 h after transfection, cells were treated with 5 ng/mL TGF-β1 (Peprotech) (Cat#100-21) or 5 μM TGF-β type 1 receptor (ALK5) inhibitor (Selleckchem) (Cat: #SB525334) for an additional 24 h.

Fluorescent Staining

Immunofluorescence stainings were performed as previously described (Karabicici et al., 2021), Beta-catenin (BD, 610153)(1:50), p-Beta-catenin (S675)(4176)(1:50), Cytokeratin 19 (sc-6278)(1:50), E-Cadherin (sc8426)(1:50), EpCAM (2929S)(1:50), Vimentin (sc-373717)(1:250), alpha-SMA (ab21027)(1:50), p-Smad2 (S255)(ab188334)(1:50), t-Smad2 (ab40855)(1:50) and Phalloidin-iFluor 488 (ab176753) antibodies were used for the stainings. Cells were visualized using the Confocal LSM 800 microscope.

Apoptosis Assay

For the detection of apoptosis, cells were stained using AnnexinV Apoptosis Detection Kit (Biolegend; Cat #640922) as described in the manufacturer's instructions. Annexin V and PI stainings were analyzed using BD LSR Fortessa flow cytometer.

Real-Time q-PCR

Real-time q-PCR experiments were performed as previously described (Karabicici et al., 2021). using7500 Fast RT PCR System (Applied Biosystems). The relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. The primers are given in **Supplementary Table 1**.

Flow Cytometry

Flow cytometry experiments were performed as previously described (Karabicici et al., 2021). For all cell lines, 2×10^6 cells were seeded into a 10 cm dish 1 day before the experiment. Cells were treated with Regorafenib, Wnt3a/R-spondin or their combination for 24 h and then were stained with EpCAM-FITC (1:50), CD133-APC (1:50) and CD24-APC (1:50) antibodies. Cells were analyzed using the BD LSR Fortessa flow cytometer.

MTT

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was performed as previously described (Karagonlar et al., 2020). Briefly, cells were seeded in 96-well plates (5×10^3 cell/well) 1 day before the experiment, then treated with increasing concentrations of Sorafenib or Regorafenib (0, 2, 4, 8, 16, 32 μM) for 48 h.

Colony Formation

For all colony formation assay cells were seeded six well plates at 500–1,000 cells per well. One day later cells treated with 5 μM Regorafenib or 5 μM Sorafenib. The cells were kept in culture for about 7–10 days so that they could form a colony of minimum 50 cells. During this time, fresh medium was added first after 3–4 days and then every 2 days.

At the end of the period, the medium on the cells was removed and cells were washed with 1 × PBS, then fixed with cold methanol for 20 min. After fixation, colonies were stained with crystal violet for 20 min and then washed by immersing them in a container kept under running water to remove excess dye. Plates were dried, then imaged using a camera. Colonies were analyzed with the Fiji cell counter tool of Image J and colony numbers were graphed using GraphPad Prism.

Spheroid Formation

The hanging drop method was used for the formation of spheroids. Briefly, 1×10^3 cells/30 μl were prepared for all treated or transfected cell lines. Then 30 μl droplets were pipetted on the interior of a 10 cm plate lid. Then the lid was inverted and the plate was incubated for 3 days to start the formation of the spheroids. After 3 days of incubation, plate lids were inverted again and the spheroid medium was collected without disturbing the spheroids. Then the treatment medium was added to the spheroids. After 2 and 4 days in the hanging cell culture, cells were imaged under the stereo microscope using 5× zoom. Then spheroid areas were calculated using threshold calculating methods in the "Adjust" section in ImageJ. Results and statistical analyses were performed with GraphPad Prism.

Western Blot

Cells were seeded onto 6 cm dishes ($1–1.5 \times 10^6$ cells/plate) 1 day before treatments. TGFβ-R1 inhibitor (5 μM), Regorafenib (5 μM), Sorafenib (5 μM) or combined treatments were performed for 48 h for all conditions. After treatment, cell media were removed and washed with 1× PBS containing 0.1 mM NaF and 0.1 mM Na3VO4. Then cells were scraped on ice and collected into eppendorf tubes. Cell pellets were lysed using RIPA buffer (150 mM NaCl, %1 NP-40, %0.5 sodium deoxycholate, % 0.1 SDS, 50 mM Tris(pH:8),with 10 mM NaF, 10 mM Na3VO4, 1× phosphatase inhibitor (Pierce,Thermo,A32957,United States) and protease inhibitor (Pierce, Thermo 88666,United States) freshly added. Cell lysates were kept on ice for 30 min and were vortex for every 5 min. Then the cell lysates were sonicated (Diagenode SA Picoruptor 163007 E.C.) for 30 s and centrifuged at max speed for 20 min at 4°C. Supernatants were collected and protein concentrations were calculated using BCA Protein assay kit (Pierce, Thermo, 23225, United States). For all experiments 50 or 100°μg total protein was loaded to jels and incubated overnight with the following antibodies: Akt (p-Ser473)(4060S), Akt (9272),p-Smad3 (Ser423/425)(9520), p-Smad2 (S255)(ab188334),Smad2/3 (D7G7) (8685),Smad4 (sc-7966),p-B catenin (S675)(4176), Beta Catenin (BD, 610153), p-GSK-3α/β (Ser21/9) (9331), GSK-3α/β (sc-7291), p-p38 MAPK (Thr180/Tyr182) (D3F9) (4511),p38 MAPK (9212), p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (4370), p44/42 MAPK (Erk1/2) (137F5) (4695), p-Stat3 (Tyr705) (D3A7) (9145),Stat3 (124H6) (9139). Blot images were taken using the Licor (CLX-1137 United States) detection system and analyzed using ImageJ "analyze"-"gel"-"plot lines" tools.

β-Galactosidase Assay

β-galactosidase assay was performed as previously described (Karabicici et al., 2021). Briefly, cells were seeded on the six well plates (7×10^4) or 12 well plates (2×10^4) 1 day before treatment. Then cells were treated with 5 ng/ml TGF-β1, 5 μM TGFβ -R1 inhibitor or transfected using shTGFβ -R1. 100 nM Doxorubicin treatment for 2 days was used as a positive control. After 6 days, the cell media were removed, cells were washed with 1 × PBS and then were stained with Biovision senescence detection kit as described in the manual. The cells were then imaged using a light microscope and results were analyzed using GraphPad Prism Software.

Scratch Assay

$3–3.5 \times 10^5$ cells were seeded on the 12 well plates 1 day before the experiment. Next day, a straight line was drawn using a yellow 200 μl pipette tip from top to down in the center of the cell monolayer. Residual cells were washed and cell media were replaced with control or treatment media. Cells were imaged at day 0, day 1, day 2, and day 3. From the images, wound area was calculated using Image J MRI wound healing tool with a range of 50–100 threshold parameter. Statistical analysis and wound closure percentages were calculated using GraphPad Prism software.

SubG1 Assay

For the Sub G1 assay all mediums and cells were collected in 50 ml sterile tubes after the treatments (Regorafenib, Wnt3a/R CM (condition medium) or combination) for 48 h. Then the cell pellet was fixed with dropwise addition of cold %70 ethanol to the pellet while vortexing. Cells were kept on ice for 2 h then were centrifuged at 400 × *g* for 5 min. Then the pellet was washed two times with Phosphate-citrate buffer (192 parts of 0.2 M Na2HPO4 and eight parts of 0.1 M Citric acid; pH:7.8). To eliminate RNA, cells were treated with 50 μl 100 μg/ml Ribonuclease A solution for 15 min. After that 450 μl of 50 μg/ml PI was added and cells were incubated for an extra 15 min. Then cells were analyzed at the low flow rate under 400 events/seconds in BD LSR Fortessa flow cytometer and results were analyzed using FlowJo software (Becton Dickinson, Heidelberg, Germany).

Lentiviral Transfection

Viral plasmids were produced in Hek293T cells as previously described (Karagonlar et al., 2020). 2×10^5 cells/well were plated in six well plates 1 day before the experiment. Next day, cells were transfected with shPLKO.1 empty control plasmid or shTGFB-RI plasmid (TRCN221535, Broad Institute) targeting TGFB-RI transcript. Cells were then with 6 μg/ml puromycin (A1113803, Gibco) for 3 days. After that, puromycin was removed and cells were maintained in their standard growing media.

Nucleofection

Nucleofection protocol was performed according to Lonza P3 Primary Cell 4D-Nucleofector™ X Kit L. Briefly, 5 μg TCF/LEF 5 μg Renilla and shB-cateninC4 or empty pSUPER plasmids were mixed with 100 μl of nucleofection solution, which contains 5×10^5 cells. Transfection was done using the CA-137 program. Cells were incubated for 10 min in RT after nucleofection and then were seeded in 12 well plates. After 48 h, nucleofection efficiency was measured by Dual-luciferase reporter assay.

Whole-Mount *in situ* Hybridization

Tg(7xTCF-Xla.Siam: nlsmCherry)^{ia5}(designated *TCFsiam*) zebrafish embryos were crossed with WT embryos. At 8 h postfertilization (hpf), embryos were dechorionated with Pronase enzyme and incubated with 5 μM Regorafenib containing E3 medium for 24 h and 48 h. At the end of the 24 and 48 h incubation times, embryos were fixed in 4% PFA in PBS overnight. mCherry probe synthesis and whole-mount *in situ* hybridizations were performed as described previously (Moro et al., 2012).

Zebrafish Xenograft

Untreated HuH7 cells (CTRL), HuH7 cells treated with 5 μM Regorafenib (Reg5), Regorafenib resistant cells (RRC), and Sorafenib resistant cells (SRC) were labeled with 2 mg/ml DiI (V2288, Molecular Probes) before injection. The cells were then resuspended to a final density of 40,000 cells/μl in 10%FBS in PBS and injected into the yolk of 2 days old dechorionated embryos (∼250 cells/embryo). The injected embryos were incubated at 34°C in E3 media until 5 dpi.

Statistics

Statistical analyses were done using GraphPad Prism 7(GraphPad Software, Inc., California, United States) software. Two-tailed unpaired student *t*-test was used to determine statistical significance between 2 experimental groups. Differences between groups were considered as "$>$ 0.05" (n.s.), "$\leq$ 0.05" (*), "$\leq$ 0.01" (**), "$\leq$ 0.001" (***) "$\leq$ 0.0001" (****).

RESULTS

Regorafenib Increases TCF/LEF Reporter Activity Both *in vitro* and *in vivo*

We first performed MTT analyses to determine IC50 values of drugs on hepatoblast-like (HuH-7, Hep3B, and HepG2), mesenchymal (SNU387, SNU449) cell lines and acquired drug resistant clones of HuH-7 (SRC and RRC). MTT analyses demonstrated that IC50 values of mesenchymal cell lines for regorafenib were significantly higher than hepatoblast-like cells (**Figure 1A**). Regorafenib treatment also significantly reduced migration, 3-D growth and colony formation of hepatoblast-like cells (**Supplementary Figure 1**). Of note, we showed that the sorafenib resistant clones also had acquired regorafenib resistance while regorafenib resistant clones had become sorafenib resistant (**Figure 1A**).

It has previously been shown that hepatoblast-like cell lines exhibit higher basal levels of Wnt/β-catenin pathway activity while mesenchymal-like Snu387 and Snu449 cell lines have repressed Wnt/β-catenin pathway activity (Yuzugullu et al., 2009). So, we investigated if the difference on drug resistance of HCC lines can be explained *via* the characteristics of Wnt/β-catenin signaling. We first analyzed the effect of sorafenib and regorafenib treatment on TCF/LEF reporter activity of HCC cell lines under basal culture conditions as well as with ligand induction. Here we demonstrated that sorafenib and regorafenib treatments alone significantly increase TCF/LEF reporter transcriptional activity of hepatoblast-like cell lines while in mesenchymal-like cell lines Snu387 and Snu449, drug treatments have no significant effect on TCF/LEF reporter activity (**Figure 1B**). Moreover, although treatment with canonical Wnt pathway ligands Wnt3a/R-Spo1 was able to induce TCF/LEF activity of both hepatoblast like and mesenchymal-like cell lines, additional enhancement of Wnt3a/R-Spo1 induced TCF/LEF reporter activity upon regorafenib treatment was only detected in hepatoblast-like HuH7, HepG2, and Hep3B cell lines (**Figure 1C**). Notably, regorafenib treatment alone or in combination with Wnt3a/R CM significantly increased TCF/LEF activity and β-catenin phosphorylation in HuH7 cells (**Figure 1D**).

Moreover, to detect if regorafenib activates Wnt/β-catenin signaling *in vivo*, we utilized *Tg(7xTCF-Xla.Siam)* Wnt/β-catenin reporter fish (Moro et al., 2012). The transgenic fishes were crossed with WT fishes and at 8 hpf, embryos were treated with 5 μM regorafenib for 24 and 48 h. At indicated time points, Wnt/β-catenin reporter activity was detected by *in situ* hybridization. For both time points, TCF/LEF activity was greater

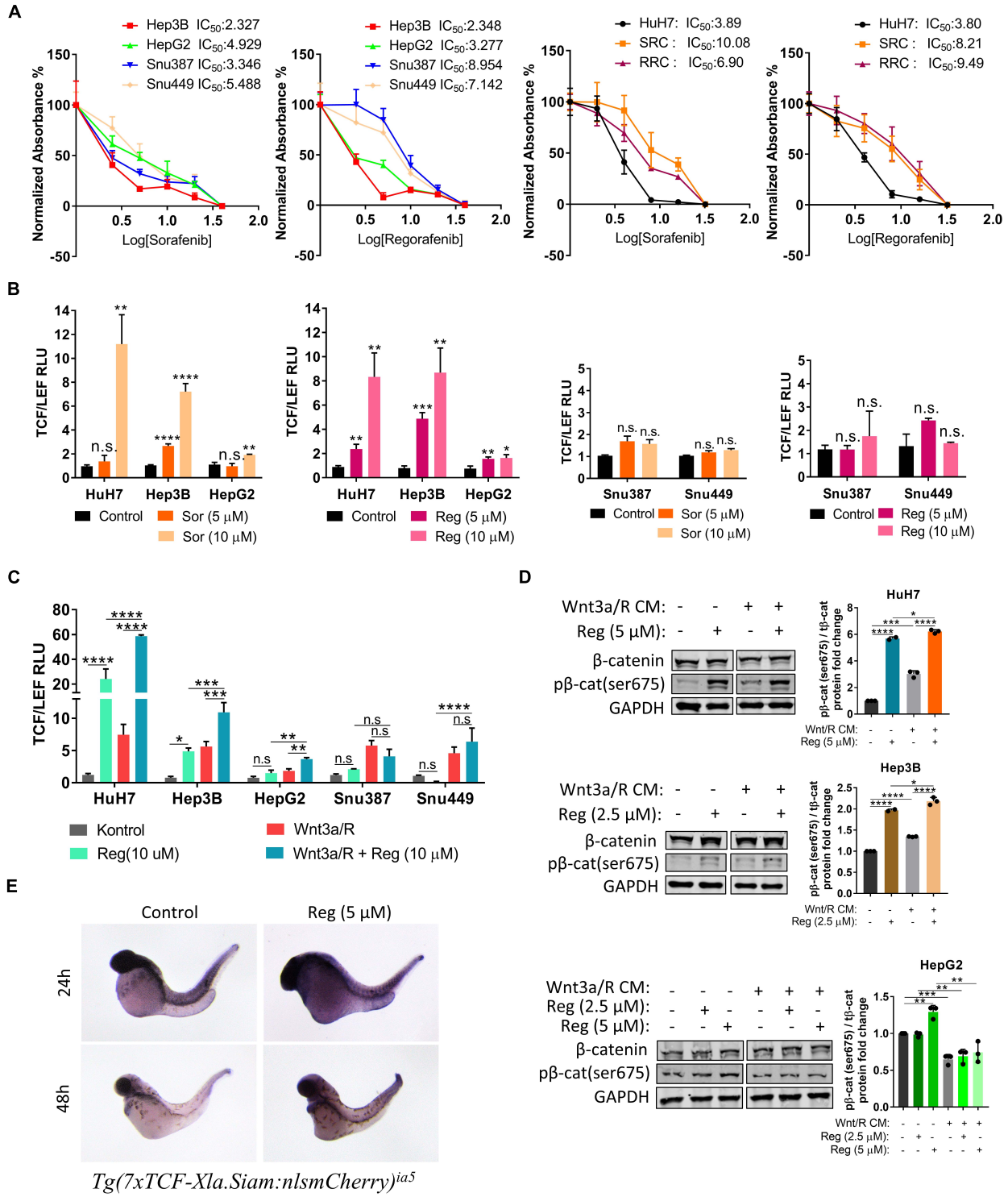


FIGURE 1 | Regorafenib increases TCF/LEF reporter activity both *in vitro* and *in vivo*. **(A)** MTT analysis demonstrates IC50 values for various HCC cell lines. RRC: Regorafenib resistant clone SRC: Sorafenib resistant clone **(B)** TCF/LEF reporter assay shows that sorafenib and regorafenib significantly increase the β-catenin transcriptional activity in hepatoblast-like HCC cell lines but not in mesenchymal-like HCC cell lines. **(C)** Regorafenib enhances Wnt3a/R-Spo1 induced TCF/LEF reporter transcriptional activity in hepatoblast-like HCC cell lines**. (D)** Regorafenib treatment increases phospho-β-catenin in hepatoblast-like HCC cells. **(E)** Whole-mount *in situ* hybridizations using mCherry probe on untreated and regorafenib treated *Tg(7xTCF-Xla.Siam)* Wnt/β-catenin reporter fishes demonstrate that regorafenib treatment increases the TCF/LEF activity *in vivo*. Data represent the average of at least three independent experiments. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

in zebrafish treated with regorafenib supporting the activation of Wnt/β-catenin signaling by this drug *in vivo* (**Figure 1E**).

Wnt/β-Catenin Activation Protects HuH7 Cells From Regorafenib Induced Apoptosis While Wnt/β-Catenin Inhibition Enhances Cell Death Upon Regorafenib Treatment

To further understand the regulation of the Wnt/β-catenin pathway by regorafenib, we treated HuH7 cells with 10 μM IWR-1 which stabilizes the destruction complex member Axin2 and thus silences the Wnt/β-catenin pathway. When combined with regorafenib, IWR-1 treatment increased Annexin V+/PI- cell population and augmented expression of cleaved PARP (**Figures 2A–C** and **Supplementary Figure 2**). Moreover, IWR-1 treatment significantly decreased basal and regorafenib-induced TCF/LEF reporter activity (**Figure 2D**) and β-catenin phosphorylation (**Figure 2E**) in the HuH7 cell line. On the other hand, when regorafenib was used in combination with Wnt3a/R-Spo1, regorafenib-induced cell death was greatly reduced (**Figure 2F**). We also detected a significant decrease in cleaved PARP levels in Wnt3a/R-Spo1treated cells (**Figure 2G**) and a decrease in sub-G1 cell population (**Supplementary Figure 2**). Taken together, these findings indicate that Wnt/β-catenin signaling activation prevents regorafenib-induced apoptosis while its inhibition can enhance cell death upon regorafenib treatment.

Regorafenib Treatment Induces Epithelial and Stemness-Related Gene Expression

To evaluate the effect of regorafenib treatment on EMT/MET transition, which is one of the essential hallmarks of cancer progression and metastasis, we detected the expression of epithelial and mesenchymal markers in regorafenib treated cells. Importantly, *E-CAD* expression increased when cells were treated with 5 μM regorafenib while the expression of mesenchymal markers decreased upon regorafenib treatment except *VIM* (**Figure 3A**). There was a decrease in the actin stress fibers in regorafenib treated cells (**Supplementary Figure 3**). Moreover, regorafenib treatment also induced the expression of hepatic stem/progenitor markers *LGR5*, *AXIN2*, *CCND1*, *EpCAM* and CK19 (**Figure 3B**). Also, the expression of *ANXA3* which promotes angiogenesis, drug resistance, and stemness in HCC (Tong et al., 2015, 2018), and the expression of *KLF4* which is one of the Yamanaka factors that also regulates liver cancer stem cell plasticity (Karagonlar et al., 2020), increased upon regorafenib treatment (**Figure 3B**). EpCAM is a known Wnt/β-catenin signaling target gene and an important liver cancer stem cell marker (Yamashita et al., 2007; Terris et al., 2010). Regorafenib treatment increased membranous expression of EpCAM (**Supplementary Figure 3**). Strikingly, flow cytometry analysis showed that when cells were treated with Wnt3a/R-Spo1 in combination with regorafenib, the induction of EpCAM+ cell population was greatly enhanced (from 42.4 to 68.5%). On the other hand, CD133+ and CD24+ cell populations were reduced under combined treatment (**Figure 3C**). Importantly, when we knocked down β-catenin expression using a shβ-catenin plasmid (**Figure 3D**), the regorafenib induced increase in EpCAM was abolished. Although the mRNA expression of E-CAD also dropped, the protein level did not seem to be significantly altered (**Figures 3E,F**). Knock-down of β-catenin also affected the 3-D growth of cells and their colony forming ability. Moreover, knock-down of β-catenin rendered 3-D spheroids more sensitive to regorafenib (**Figures 3G,H**).

TGF-β1 Treatment Decreases Regorafenib Induced E-CAD and p-β-Catenin Expression While Vimentin Expression Stays High

EMT is known to be a critical step in acquisition of drug resistance (Aiello and Kang, 2019; Derynck and Weinberg, 2019) and TGF-β signaling is a master regulator of EMT (Reichl et al., 2012; Papageorgis, 2015; Hao et al., 2019). Interestingly, we detected an increase in the expression of TGF-β1 upon regorafenib treatment, although the expression of TGFβ-R1 was decreased (**Figure 4A**). When we treated cells with both TGF-β1 and regorafenib, the increase in the expression of E-CAD and EpCAM upon regorafenib treatment was partly inhibited (**Figure 4A**). On the contrary, regorafenib induced expression of LGR5 and CK19 was further augmented by TGF-β1 treatment (**Figure 4A**). To analyze the effect of regorafenib treatment on TGF-β signaling, we utilized a reporter plasmid. While TGFβ-1 treatment significantly increased TGF-β signaling, regorafenib treatment suppressed TGFβ-1 induced activation (**Figure 4B**).

Confocal staining also confirmed that while regorafenib treatment induced the membranous expression of *E-CAD*, and p-β-*CAT*, upon TGF-β1 treatment regorafenib induced increase in their expression was abolished (**Figure 4C**). Consistent with the RNA expression data, Vimentin expression was also significantly induced by Regorafenib treatment in these cells. Moreover, upon TGF-β1 treatment, we saw a further increase in Vimentin expression and an increase in actin stress fibers consistent with the acquisition of a more-mesenchymal morphology (Deguchi and Sato, 2009; **Figure 4C**). Consistently, while regorafenib treatment decreased cell motility, TGF-β1 treatment alone increased the motility of cells. When two treatments combined, the inhibitory effect of regorafenib on cell motility was partly attenuated by TGF-β1 (**Figure 4D**).

Acquired Drug Resistance of HuH-7 Demonstrates Increased Mesenchymal Gene Expression and Augmented TGF-β Signaling

To analyze acquired drug resistance, we created Sorafenib and Regorafenib resistant cells by treating HuH7 cells with increasing doses of drugs starting with their IC50 values in long-term culture. Over 8–12 months, Sorafenib resistant (SRC) and Regorafenib resistant (RRC) cell lines were established and thereafter maintained constantly with media containing

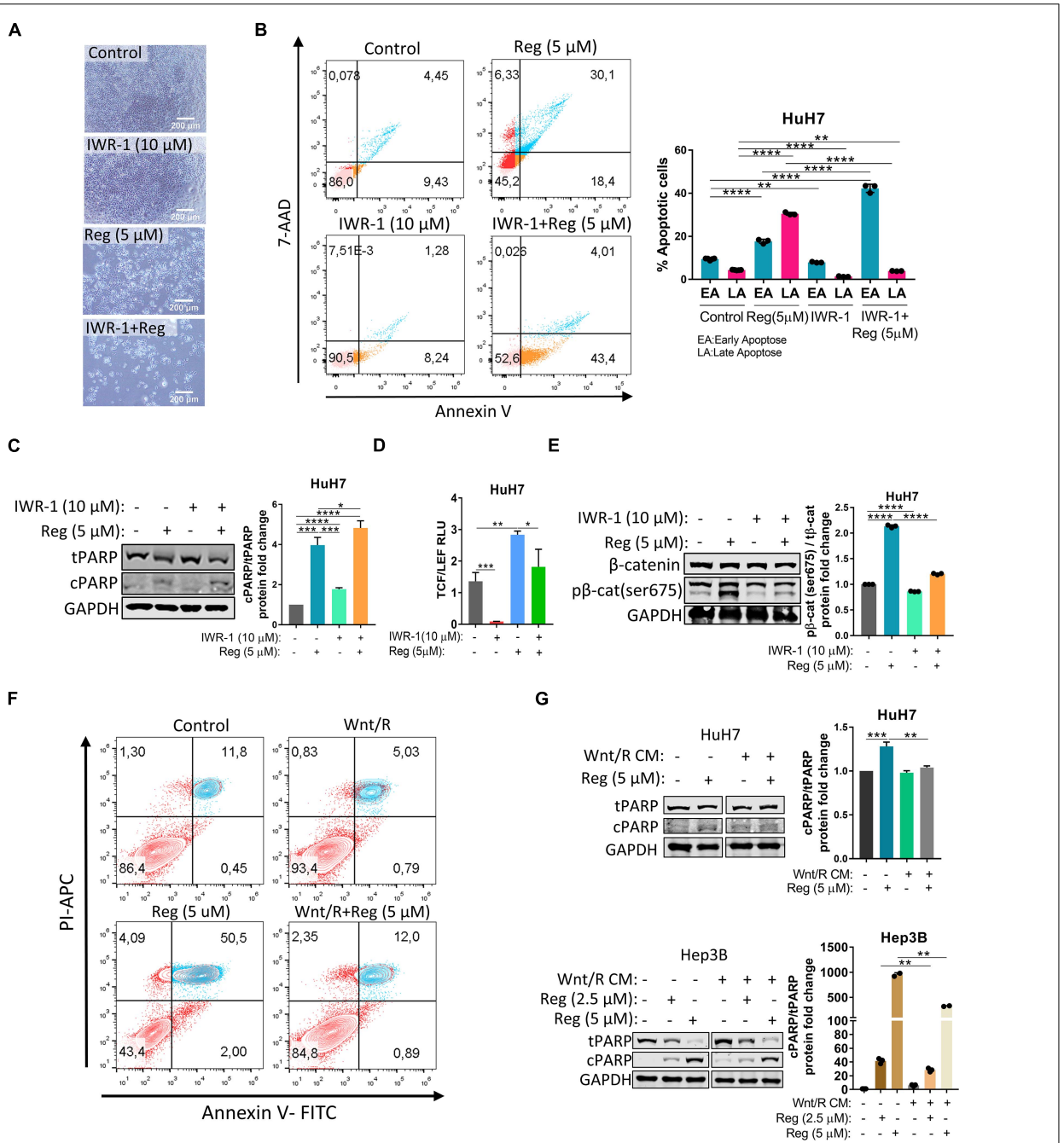


FIGURE 2 | Wnt/β-catenin activation *via* Wnt3a/R-Spo1 treatment protects cells from regorafenib induced apoptosis while Wnt/β-catenin inhibition by IWR-1 augments cell death upon regorafenib treatment. **(A)** Light microscope images indicate augmented cell death upon regorafenib and IWR-1 treatment. Scale bar: 200 μM **(B)** Apoptosis rates of HuH7 cells treated with IWR-1, regorafenib or their combinations were analyzed by Annexin V/PI staining using flow cytometry. **(C)** Western blot analysis of c-PARP and t-PARP in treated cells. **(D)** IWR-1 suppresses basal and regorafenib induced TCF/LEF reporter activity in the HuH7 cell line. **(E)** IWR-1 suppresses basal and regorafenib induced p-β-catenin expression in the HuH7 cell line **(F)** Apoptosis rates of HuH7 cells treated with Wnt3a/R-Spo1, regorafenib or their combinations were analyzed by Annexin V/PI staining using flow cytometry. **(G)** Western blot analysis of c-PARP and t-PARP in treated cells. Data represent the average of at least three independent experiments. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

7.2 μM Sorafenib (SRC line) or 8.4 μM Regorafenib (RRC line). MTT analysis confirmed that these resistant lines exhibited significantly higher IC50 values for both Sorafenib and Regorafenib, similar to mesenchymal-like HCC cell lines (**Figure 1A** and **Supplementary Figure 3**). Consistently, when compared to the parental HuH7 cell line, SRC and RRC cell lines

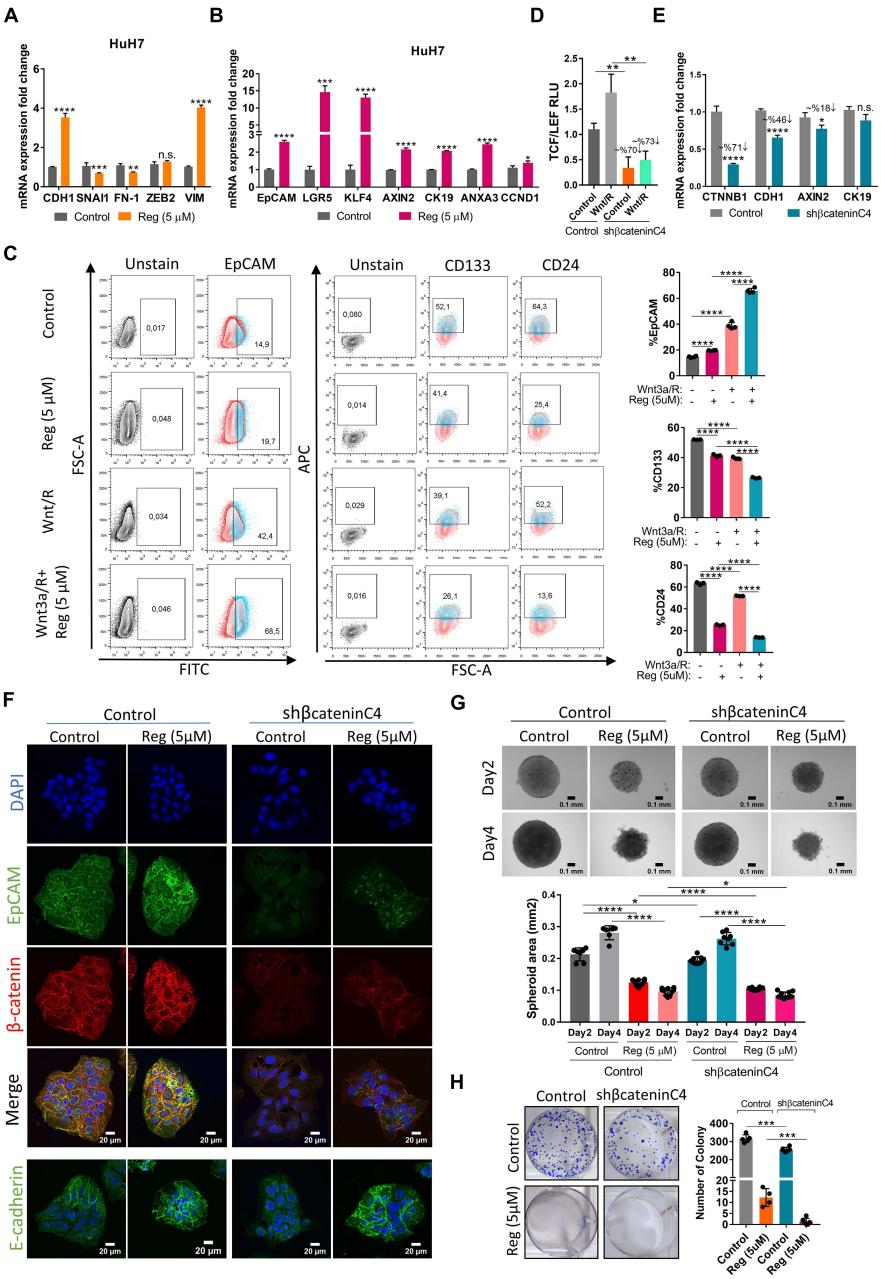


FIGURE 3 | Regorafenib treatment induces epithelial and stemness related gene expression. **(A)** Expression of mesenchymal and epithelial genes as well as **(B)** hepatic stem/progenitor genes were analyzed by q-PCR. Data represent the average of at least three independent experiments. **(C)** Cancer-stem cell marker (EpCAM, CD133 and CD24) positive cell populations were analyzed *via* BD LSR Fortessa flow cytometer with or without Wnt3a/R-Spo1 and regorafenib treatments. **(D)** TCF/LEF reporter activity in Wnt3a/R-Spo1 treated and untreated control and β-catenin knocked down cells **(E)** Expression of Wnt/β-catenin target genes were analyzed by q-PCR. **(F)** Expressions of EpCAM, E-CAD and β-catenin were analyzed in control and β-catenin knocked down cells by immunofluorescence. Images were acquired on a Carl Zeiss LSM 880 AxioObserver confocal microscope with a C-Apochromat 40x/1.2 W, Korr FCS M27 objective. **(G)** 3D spheroids were formed from control and β-catenin knocked down cells with or without regorafenib treatment. Spheroid area was calculated using Image J. **(H)** Colony formation assay was performed using control and β-catenin knocked down cells with or without regorafenib treatment. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

had upregulation of several mesenchymal markers such as *SNAI1, ZEB2* and *VIM* and α-SMA (**Figures 5A,C**), supporting the acquisition of a mesenchymal phenotype. Interestingly, *CDH1* expression was also increased in the RRC line. Although *CDH1* is a well-defined epithelial marker, distant metastases of invasive cancers were shown to re-express *CDH1* which contributes to the establishment of metastatic foci. Moreover, similar to acute regorafenib treated cells, *LGR5*, *CK19*, *KLF4*, *ANXA3,* and

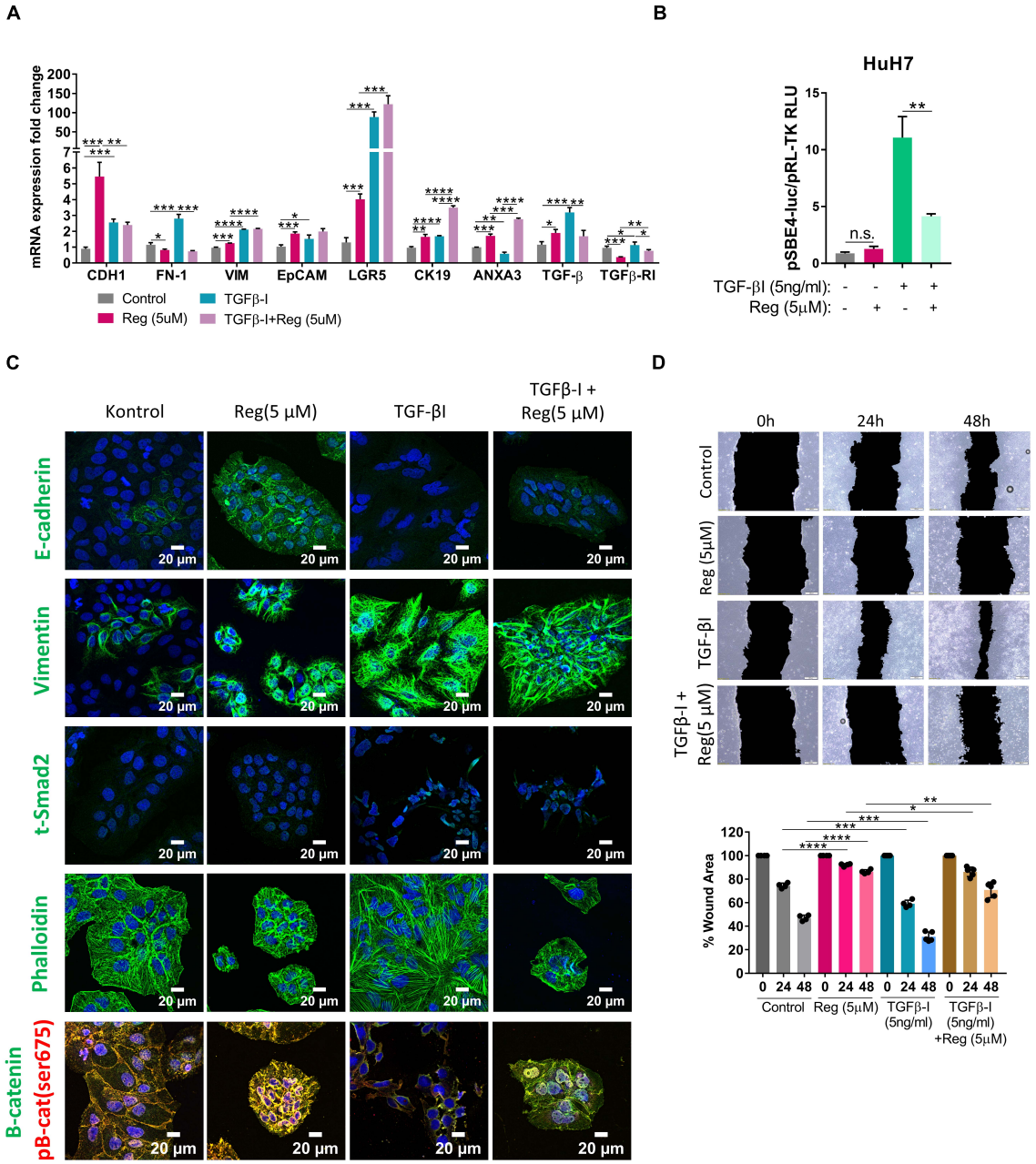


FIGURE 4 | TGF-β treatment partially reverses regorafenib induced effects. **(A)** Expression of mesenchymal and epithelial genes were analyzed by q-PCR. Data represent the average of at least three independent experiments. **(B)** TGF-β reporter activity in basal and regorafenib treated cells was measured using pSBE4-Luc/pRL-TK construct. **(C)** Expressions of E-CAD, VIM, SMAD2 and β-catenin were analyzed in control and treated (TGF-β1, regorafenib, or combined) cells by immunofluorescence. Images were acquired on a Carl Zeiss LSM 880 AxioObserver confocal microscope with a C-Apochromat 40x/1.2 W, Korr FCS M27 objective. **(D)** Scratch assay was performed in control and treated (TGF-β1, regorafenib, or combined) cells. Wound area was calculated using ImageJ MRI wound healing tool. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

CCND1 gene expressions were also still significantly higher in SRC and RRC lines compared to parental cells (**Figures 5B,C**). Also, there was a significant upregulation in TGF-β1 expression in the SRC and RRC lines. Consistently, the expressions of total and p-SMAD2 (S255) were increased in SRC and RRC cell lines, while the expression of p-β-catenin was reduced (**Figure 5C**). Reporter assays also confirmed reduced β-catenin signaling and increased TGF-β signaling in the resistant cell lines (**Figures 6A,B**).

We also analyzed the expression of cancer stem cell surface markers *via* flow cytometry. We detected that EpCAM+ cell population decreased in both SRC and RRC lines. On the other

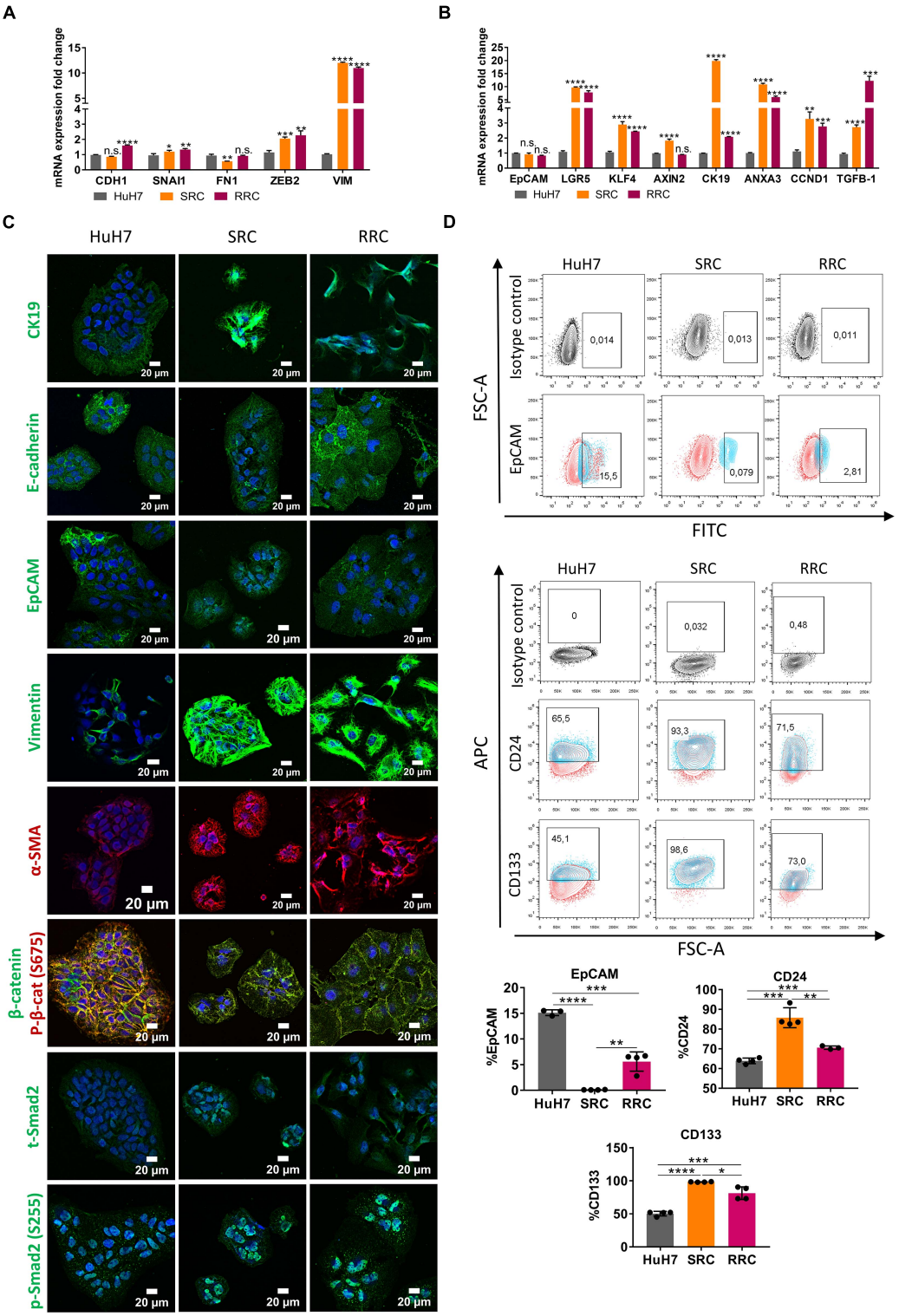


FIGURE 5 | Regorafenib resistant cells have increased mesenchymal gene expression and cancer stem-cell marker expression. **(A)** Expression of mesenchymal and epithelial genes as well as **(B)** hepatic stem/progenitor genes were analyzed by q-PCR. Data represent the average of at least three independent experiments. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD). **(C)** Expressions of CK19, E-CAD, EpCAM, VIM, a-SMA, SMAD2 and β-catenin were analyzed in parental and resistant cell lines by immunofluorescence. Images were acquired on a Carl Zeiss LSM 880 AxioObserver confocal microscope with a C-Apochromat 40x/1.2 W, Korr FCS M27 objective. **(D)** Cancer-stem cell marker (EpCAM, CD24 and CD133) positive cell populations were analyzed *via* BD LSR Fortessa flow cytometer in sorafenib resistant (SRC) and regorafenib resistant (RRC) cells.

hand, CD133 and CD24 expressing cell populations significantly increased in the resistant lines (**Figure 5D**). Moreover, although under 3-D growth conditions, resistant cell lines formed smaller spheroids, RRC spheroids were significantly more resistant to regorafenib treatment (**Figure 7A**). In addition, RRC cell line demonstrated significantly higher *in vitro* motility than parental and SRC cells in scratch assay (**Figure 7B**). Similarly, although basal colony forming capacity of SRC and RRC lines were lower than parental HuH7 cells, upon regorafenib treatment the colony forming ability of parental HuH7 cell line was greatly lost, while the colony forming abilities of SRC and RRC cells were not affected (**Figure 7C**).

Acquired Drug Resistance Enhances the *in vivo* Migration Capacity of HuH7 Cells as Opposed to Acute Regorafenib Treatment in a Zebrafish Xenograft Model

To evaluate our *in vitro* results, we also tested the migration ability of SRC and RRC lines in a zebrafish xenograft model. 5 μM regorafenib treated HuH7 cells as well as SRC and RRC lines were implanted into the yolk sac of 2 dpf zebrafish embryos. 5 days after injection, migration to the tail was quantified. In total, 106 fishes from the control group, 117 fishes from the 5 μM regorafenib treated group, 77 fishes from the SRC group, and 81 fishes from the RRC group were counted. We detected migrated cells in 27% of zebrafish injected with HuH7 cells, while only 15 % of zebrafish injected with HuH7 cells treated with 5 μM regorafenib had migrated cells suggesting that regorafenib treated cells have reduced migration ability. Interestingly SRC line did not exhibit significantly higher migration ability (around 32% of zebrafish injected with SRC line had migration). However, we detected migrated cells around 53% of zebrafish injected with RRC line suggesting RRCs have significantly enhanced *in vivo* migration ability (**Figure 7D**).

TGF-β RI Inhibition Significantly Prevents *in vitro* and *in vivo* Migration Ability of Regorafenib Resistant Cells

Wnt/β-catenin signaling increases upon acute regorafenib treatment. However, in SRC and RRC lines, we detected significantly reduced basal TCF/LEF activity (**Figure 6A**). Moreover Wnt3a/R-Spo-induced Wnt/β-catenin signaling was also diminished in SRC and RRC lines (**Supplementary Figure 3**). On the other hand, luciferase reporter assay using a reporter plasmid consisting of TGF-β responsive Smad-binding elements (pSBE4-Luc) demonstrated that basal TGF-β pathway activity is significantly higher in SRC (∼7.5-fold) and RRC (∼ 9-fold) lines compared to parental HuH7 cells (**Figure 6B**) consistent with significantly higher levels of TGF-β1 expression in these cells. We then utilized a TGFβ-R1 inhibitor that reduces TGF-β signaling in these cells (**Figure 6C**) and compared the various abilities of SRC and RRC cells with and without this inhibitor. Upon treatment of SRC and RRC spheroids with regorafenib and/or sorafenib and TGFβ-R1 inhibitor, cell death was augmented in the spheroids (**Figure 6D** and **Supplementary Figure 4**). Also compared to regorafenib alone, the combined treatment with TGFβ-R1 inhibitor and drugs significantly reduced colony formation (**Figure 6E** and **Supplementary Figure 4**) and *in vitro* migration of resistant cells (**Figure 6F** and **Supplementary Figure 4**). We also tested the effect of TGFβ-R1 inhibitor on the *in vivo* migration ability of RRC cells in the zebrafish model. In total, migration to the tail was counted in 143 fishes from the control group, in 139 fishes from the RRC group, and in 139 fishes from the RRC group treated with TGFβ-RI inhibitor (**Figure 6G**). We detected migrated cells in 21.6% of zebrafish injected with HuH7 cells, while 43% of zebrafish injected with the RRC group had migration again demonstrating the high migration ability of the RRC line. On the other hand, only 12.9% of zebrafish injected with the TGFβ-RI inhibitor treated RRCs had migrated suggesting that TGF-β pathway inhibition significantly prevents *in vivo* migration ability of regorafenib resistant cells.

Activation of the TGFβ pathway by TGF-β1 treatment is known to induce senescence in HCC cells (Senturk et al., 2010). In accordance with previous literature, we demonstrated that TGF-β1 treatment induces senescence in parental HuH7, SRC and RRC lines (**Supplementary Figure 5**). Interestingly, however, when we knocked down TGFβ-R1 in parental and resistant cells using a lentiviral plasmid, the majority of RRCs entered senescence while senescence was not detected in parental and SRC lines (**Figure 6H**). The induction of senescence by TGFβ-R1 knockdown in RRC line suggests that TGF-β pathway promotes growth and survival of regorafenib resistant cells. Phosphorylation of Smad2 at Ser255 *via* ERK was shown to serve as a STAT3 co-activator (Yoon et al., 2015). Western blot analysis demonstrated that in the RRC line, there are higher levels of phospho-STAT3, and phospho-SMAD2 (S255) (**Figure 8**). Importantly, regorafenib treatment reduced phosphorylations of SMAD2 and STAT3. However, this inhibition was even more significantly augmented when resistant cells were treated with regorafenib in combination with TGFβ-R1 inhibitor. Also, upon regorafenib treatment, phosphorylation of ERK1/2 was completely inhibited in HuH7 cells whereas in the resistant cell lines, the inhibition of pERK1/2 by regorafenib was not significant. However when regorafenib was combined with TGFβ-R1 inhibition, phosphorylation of ERK1/2 was completely inhibited even in SRC and RRC lines. On the other hand, upon regorafenib treatment phospho-β-catenin level increased in parental HuH7 and in the resistant lines. However when TGFβ-R1 inhibitor was applied in addition to regorafenib, the regorafenib induced increase in phospho-β-catenin was abolished while GSK3β phosphorylation increased (**Figure 8**).

DISCUSSION

Despite the recent advancements in HCC treatment, low response rates, drug toxicity, and treatment resistance usually followed by tumor relapse are still profound problems for HCC patients. The inter and intra-tumor heterogeneity of HCC plays a critical role in determining the patient's therapy

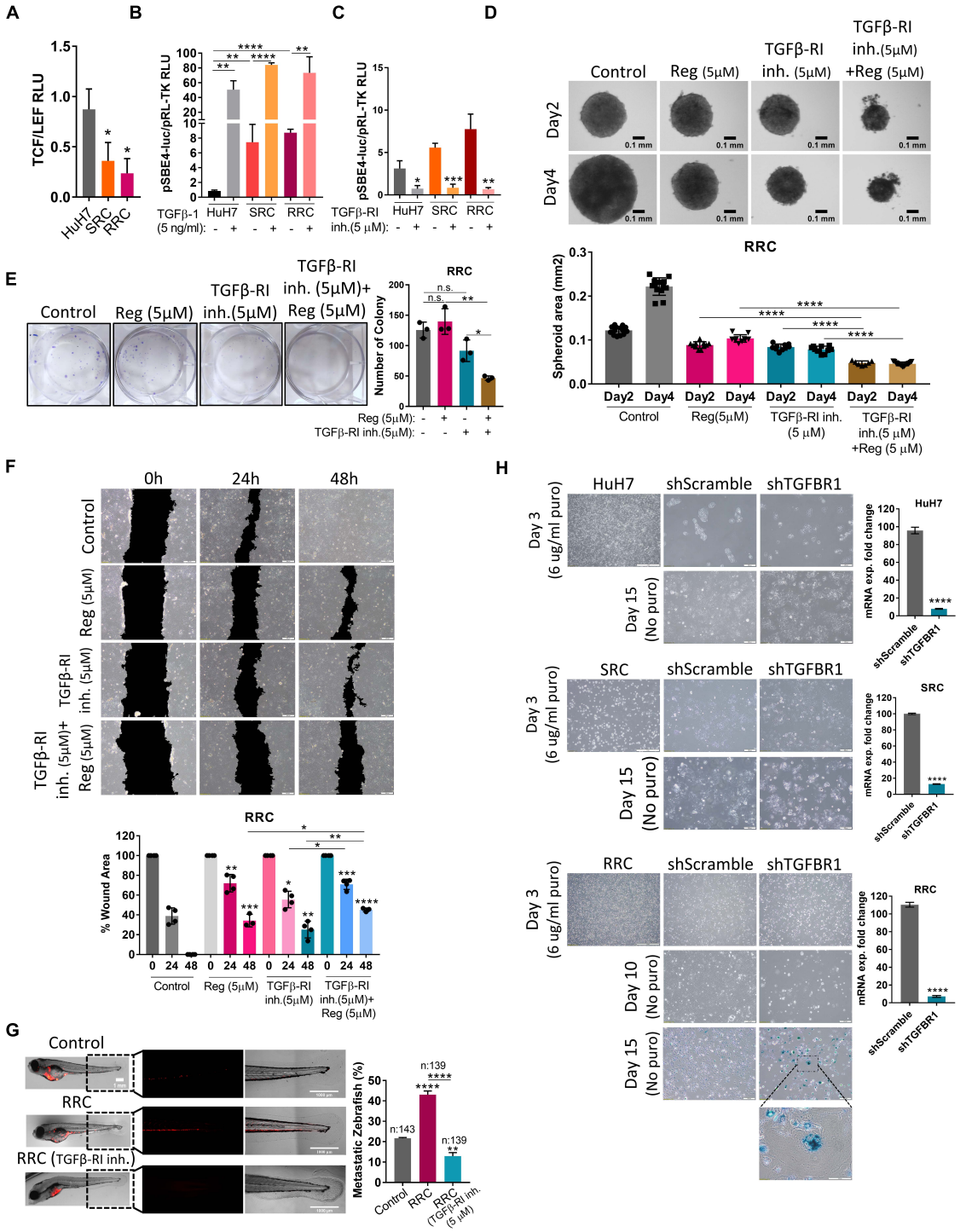


FIGURE 6 | The augmented migration capacity of regorafenib resistant cells is mediated by the TGF-β pathway. **(A)** TCF/LEF reporter assay of resistant cells exhibit decreased TCF/LEF reporter activity. **(B)** TGF-β pathway activity was detected in HuH7, SRC and RRC cell lines after treatment with TGF-β1 or **(C)** TGFβ-RI inhibitor using pSBE4-Luc/pRL-TK reporter plasmid. **(D)** 3-D spheroids were formed from RRC cells with or without regorafenib and TGFβ-R1 treatment. Spheroid area was calculated using ImageJ. **(E)** Colony formation and **(F)** Scratch assay was performed in RRC cells with or without regorafenib and TGFβ-R1 treatment. Wound area was calculated using ImageJ MRI wound healing tool. **(G)** HuH7 cells, untreated RRC cells and TGFβ-R1 inhibitor treated RRC cells were implanted into the yolk sac of 2 dpf zebrafish embryos. 5 days after injection, migration to the tail was quantified. Representative images of zebrafish embryos were acquired on a Carl Zeiss LSM 880 AxioObserver confocal microscope with an EC Plan-Neofluar 10x/0.30 M27 objective. **(H)** SA-β-gal staining of control and TGFβ-R1 knocked down HuH7, SRC and RRC cells. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

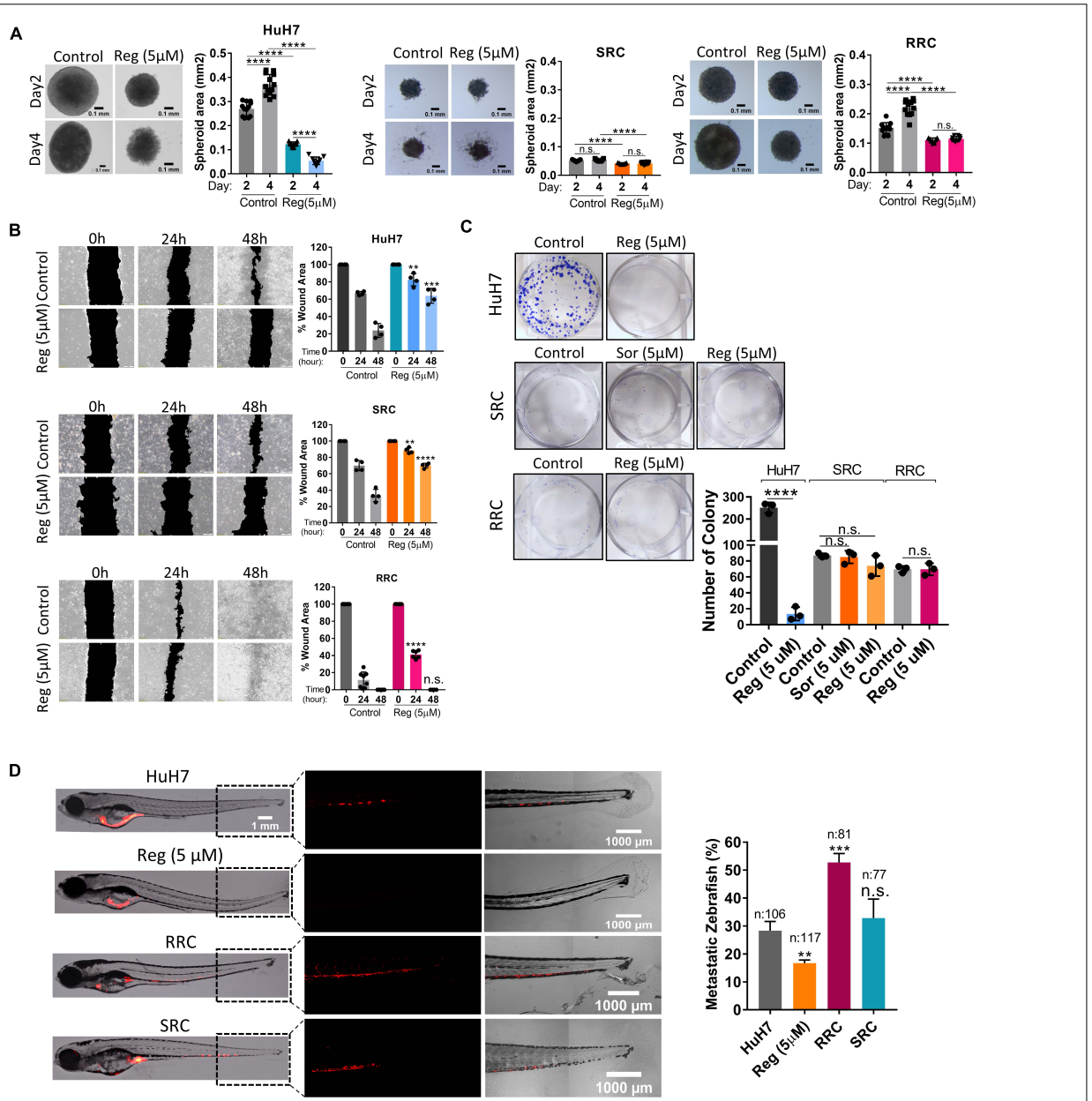


FIGURE 7 | Regorafenib resistant cells demonstrate increased *in vitro* and *in vivo* migration ability. **(A)** 3-D spheroids were formed from parental HuH7, SRC and RRC cells with or without regorafenib treatment. Spheroid area was calculated using Image J. **(B)** Scratch assay and **(C)** Colony formation assay was performed in parental HuH7, SRC and RRC cells with or without regorafenib treatment. Wound area was calculated using Image J MRI wound healing tool. **(D)** Parental HuH7 cells, 5 μM regorafenib treated HuH7 cells, SRC and RRC lines were implanted into the yolk sac of 2 dpf zebrafish embryos. 5 days after injection, migration to the tail was quantified. Representative images of zebrafish embryos were acquired on a Carl Zeiss LSM 880 AxioObserver confocal microscope with a EC Plan-Neofluar 10x/0.30 M27 objective. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

response. Thus, the future therapy direction of HCC should be focused on personalized treatment regimens and stratification of patients based on the efficacy of targeted therapies on HCC tumors with different molecular signatures (i.e., driver mutations, activated pathways) (Couri and Pillai, 2019; Pérez et al., 2020; Wang et al., 2020).

Human tumor-derived cell lines recapitulate HCC diversity and are widely used for studying mechanisms that drive drug resistance and sensitivity in HCC. Recent studies indicate that the various drug response rates among liver cancer cell lines were associated with the differences at transcriptomic level and the cell differentiation state. Among liver cancer cell lines, the most differentiated "hepatoblast-like" subgroup demonstrates the highest drug sensitivity (Caruso et al., 2019; Rebouissou and Nault, 2020). In our study, we also observed that hepatoblast-like HCC cell lines HuH7, HepG2, and

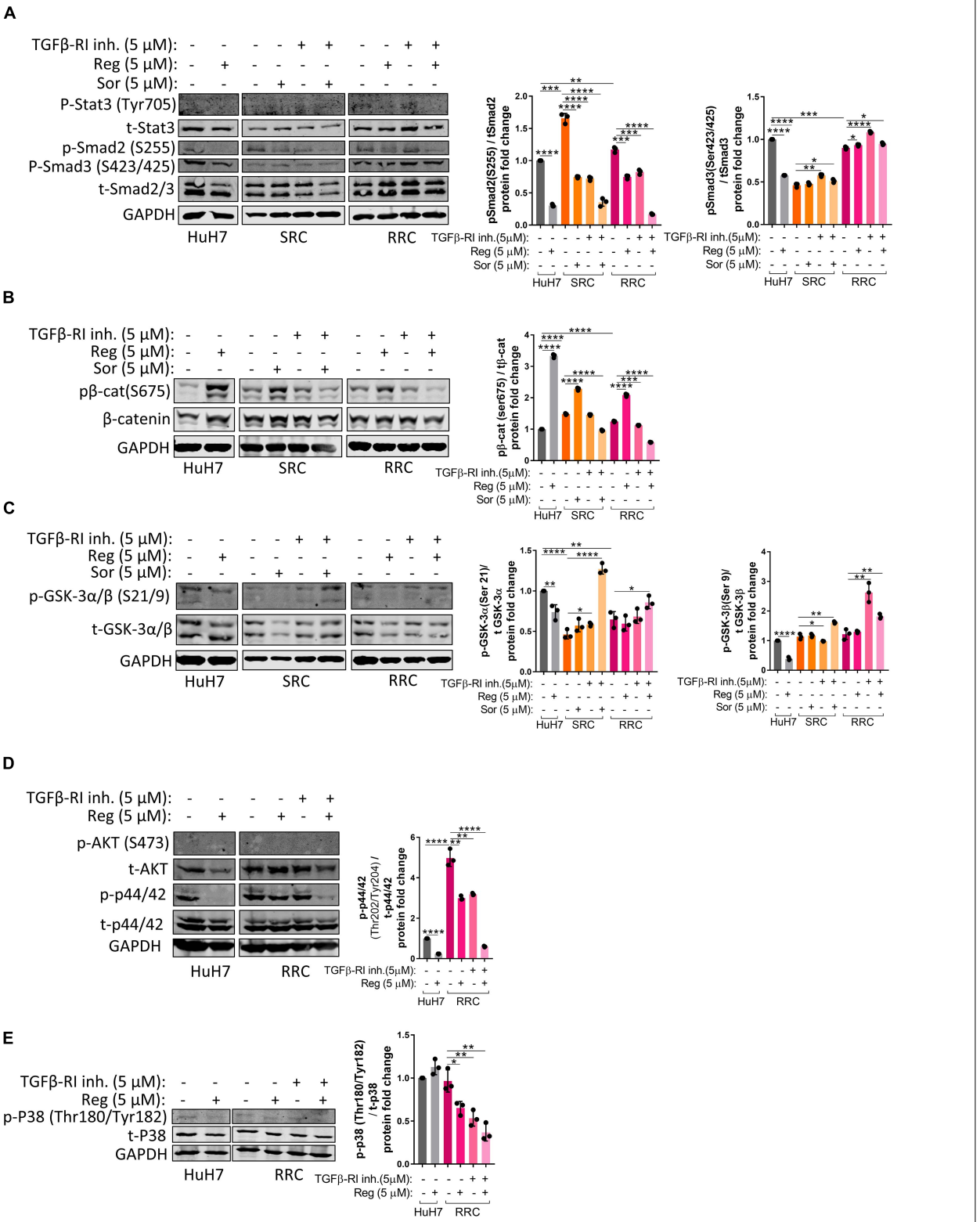


FIGURE 8 | Western blot analysis of resistant cells. **(A–E)** Western blot analysis of untreated and treated (sorafenib, regorafenib, TGFβ-R1 or combined) parental HuH7, SRC and RRC cells. Bar graph represents the densitometric plot of protein expression in various groups. $p > 0.05$ (n.s.), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Error bars indicate standard deviation (SD).

Hep3B are more sensitive to regorafenib treatment compared to mesenchymal cell lines Snu387 and Snu449 (**Figure 1A**). However, we showed that although regorafenib induces cell death at lower doses in the "hepatoblast-like" cells, regorafenib treatment also activates Wnt/β-catenin signaling in these cells and induces enrichment of markers associated with hepatic stem/progenitor cells. Importantly, the activation of Wnt/β-catenin signaling can protect these cells from regorafenib induced apoptosis while the inhibition of Wnt/β-catenin signaling ameliorates cell death upon regorafenib treatment. Taken together, our results suggest that tumors with mutations that result in Wnt/β-catenin activation might have higher intrinsic regorafenib resistance. Importantly, a recent study by Harding et al. (2019) indicates that in HCCs patients treated with immune checkpoint inhibitors, the activating mutations of WNT/β-catenin signaling is associated with a decrease in drug response, shorter OS and innate resistance. Regorafenib is currently under clinical investigation as first-line therapy in combination with immunotherapy for advanced HCC (ClinicalTrials.gov. U.S. National Library of Medicine) and our data also supports the idea that stratification of patients based on the Wnt/β-catenin activation for high drug resistance could increase patient outcome in combinational therapies involving regorafenib.

On the other hand, upon long-term regorafenib treatment, Wnt signaling activity was diminished in HuH7 cells. Over 8–12 months, regorafenib treated HuH7 cells developed acquired regorafenib resistance and exhibited increased mesenchymal gene expression along with an induction of CD24 and CD133 cancer stem cell markers. Moreover, regorafenib resistant cells had enhanced TGF-β signaling activity and significantly higher migration capacity *in vivo* which can be reversed upon TGFβ-R1 inhibition. Thus, HuH7 cells, although they still express hepato-specific genes and fetal/progenitor markers, after long-time regorafenib treatment, also started to show properties of "mixed epithelial-mesenchymal" subgroup and the "mesenchymal-like" subgroup which are less differentiated and exhibit activation of the TGF-β pathway.

TGF-β exhibits multi-faceted roles and even opposite functions in distinct stages of cancer. During early stages of tumorigenesis TGF-β acts as a tumor suppressor, while in late stages it assumes an oncogenic role, promoting metastasis of tumor cells (Elliott and Blobe, 2005). Consistently, serum and tissue levels of TGF-β1 were found higher in metastatic HCC (Giannelli et al., 2002). In addition, high TGF-β1 levels correlate with tumor vascularity, metastasis and poor survival in HCC making TGF-β pathway a promising target in HCC therapy (Ito et al., 1995; Okumoto et al., 2004). However, although TGF-β pathway blockade has given promising results in preclinical models (Connolly et al., 2012; Katz et al., 2013), various anti-TGF-β agents that selectively and/or un-selectively inhibit TGF-β signaling tested in clinical trials, exhibited limited efficacies (Colak et al., 2017; Ciardiello et al., 2020; Nemunaitis et al., 2020). Due to its known roles in mediating inflammation, fibrogenesis, and immunomodulation in the tumor microenvironment, TGF-β inhibition is still a valuable target in combinational therapies. In a recent Phase 2 trial, Galunisertib, a small-molecule selective inhibitor of TGFβ-R1, was tested in combination with sorafenib in first-line patients with advanced HCC. The combination of two treatments showed a prolonged OS outcome compared to sorafenib alone (Kelley et al., 2019). The promising results of this study support further exploration of TGFβ-R1 inhibition in combination with other therapeutic agents. Importantly, a recent study indicated that HCC with active TGF-β signaling can be used as a potential biomarker for identifying the immune resistant tumors with poor prognosis (Chen et al., 2019). Immune-checkpoint inhibitors (ICIs) have been approved as second-line or first-line therapies for a list of malignancies including liver cancer (Gong et al., 2018; Havel et al., 2019). However, tumor response rates for these immune-checkpoint inhibitors are low, being less than 20% in HCC (Sangro et al., 2013; El-Khoueiry et al., 2017; Zhu et al., 2018). The highly immunosuppressive tumor environment in advanced HCC is believed to contribute to low treatment response of HCC to immune-checkpoint inhibitors (Prieto et al., 2015). Since TGF-β signaling has a role in immunomodulation of the tumor microenvironment, TGF-β blockade recently became the target of attention to enhance the ICI therapy especially for TGF-β-activated tumors. Several preclinical studies demonstrated that inhibition of TGF-β pathway could increase ICI drug response (Bai et al., 2019) and current ongoing clinical trials are exploring immunotherapeutic targeting of TGF-β signaling (Giannelli et al., 2011; Bai et al., 2019; Chen et al., 2019; Ungefroren, 2019; Groeneveldt et al., 2020; Li et al., 2020).

Our data by showing the role of TGF-β signaling in drug resistance of regorafenib resistant cells also merits the strategies involving TGF-β blockade in combination with regorafenib and immunotherapy in advanced HCC. TGF-β, as a potent regulator of tumor microenvironment, is a valuable candidate for therapies targeting the cross talk of cancer cells with the immune system and the stroma. Thus, despite the encouraging results, the clinical relevance of our study remains to be further established in preclinical animal models and in human patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC).

AUTHOR CONTRIBUTIONS

MK, ZF, and EE: conceptualization, original draft preparation, and review and editing. MK and YA: methodology and experimentation. ZF, EE, GO, and SS: supervision. EE and GO: project administration and funding acquisition.

All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.639779/full#supplementary-material

Supplementary Figure 1 | Regorefenib treatment significantly inhibits **(A)** *in vitro* migration, **(B)** spheroid growth and **(C)** colony formation of hepatoblast-like cell lines.

Supplementary Figure 2 | (A) Sub-G1 analysis of Wnt3/RSpo treated HuH7 cells **(B)** cleaved PARP analysis of Wnt3/RSpo treated HepG2 cells. **(C)** cleaved PARP analysis of IWR-1 treated cells Hep3B and HepG2 cells.

Supplementary Figure 3 | (A) Phase-contrast image, **(B)** phalloidin staining and **(C)** EpCAM staining of acute regorafenib treated cells. **(D)** Phase contrast images and **(E)** TCF/LEF activity of resistant cell lines.

Supplementary Figure 4 | (A) *In vitro* migration, **(B)** spheroid formation and **(C)** colony formation of TGFβ-R1 treated SRC cells.

Supplementary Figure 5 | (A) SA-β-gal staining of TGF-β1 treated, TGF β-R1 treated and DOX treated cells. **(B)** Spheroid formation of sh-TGF β-R1 cells.

Supplementary Table 1 | qPCR Taqman specific primers.

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BRIEF RESEARCH REPORT
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Receptor/Raft Ratio Is a Determinant for LRP6 Phosphorylation and WNT/β-Catenin Signaling

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Microdomains or lipid rafts greatly affect the distribution of proteins and peptides in the membrane and play a vital role in the formation and activation of receptor/protein complexes. A prominent example for the decisive impact of lipid rafts on signaling is LRP6, whose localization to the same lipid rafts domain as the kinase CK1γ is crucial for its successful phosphorylation and the subsequent activation of the signalosome, hence WNT/β-catenin signaling. However, according to various experimental measurements, approximately 25 to 35 % of the cell plasma membrane is covered by nanoscopic raft domains with diameters ranging between 10 to 200 nm. Extrapolating/Translating these values to the membrane of a "normal sized" cell yields a raft abundance, that, by far, outnumbers the membrane-associated pathway components of most individual signaling pathway, such as receptor and kinases. To analyze whether and how the quantitative ratio between receptor and rafts affects LRP6 phosphorylation and WNT/β-catenin pathway activation, we present a computational modeling study, that for the first time employs realistic raft numbers in a compartment-based pathway model. Our simulation experiments indicate, that for receptor/raft ratios smaller than 1, i.e., when the number of raft compartments clearly exceeds the number of pathway specific membrane proteins, we observe significant decrease in LRP6 phosphorylation and downstream pathway activity. Our results suggest that pathway specific targeting and sorting mechanism are required to significantly narrow down the receptor/raft ratio and to enable the formation of the LRP6 signalosome, hence signaling.

Keywords: rule-based modeling and simulation, Wnt/β-catenin signaling, lipid rafts, LRP6 receptor, LRP6 phosphorylation, CK1γ, receptor/raft ratio, compartmental modeling

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1. INTRODUCTION

WNT signaling regulates central developmental processes of the cell, including cell fate, cell proliferation, cell migration and adult homeostasis. At the same time, aberrant or deregulated forms of WNT signaling are involved in a number of human cancers and developmental disorders (Logan and Nusse, 2004; Moon et al., 2004; Clevers and Nusse, 2012). Several studies suggested an involvement of lipid rafts in the WNT /β-catenin pathway. Accordingly LRP6 , the main receptor of the canonical WNT signaling pathway, is only phosphorylated by the kinase CK1γ when both proteins are located in (the same) lipid raft domain (Bilic et al., 2007; Sakane et al., 2010; Özhan et al., 2013). Even though LRP6 is homogenously distributed in the membrane and only a minor

fraction is raft-associated, its localization to lipid rafts is vital for the activation of the LRP6 signalosome, hence wnt/β-catenin signaling.

Lipid rafts are local assemblies of highly concentrated sphingolipids and cholesterol in the cell membrane. They emerge as differences in the interaction affinities between various lipids and proteins, that are sufficient to produce heterogeneous lipid distribution leading to macroscopic phase separation, i.e., the formation of lipid raft (liquid-ordered)—and non-raft (liquid-disordered) domains in the membrane (Sezgin et al., 2017a). This process depends on lipid composition (Veatch and Keller, 2003; Levental et al., 2009, 2016), and environmental conditions such as the temperature (Magee et al., 2005; Veatch et al., 2008). According to various experimental measurements, approximately 25 to 35 % of the cell plasma membrane is covered by nanoscopic domains with diameters ranging between 50 to 100 nm (Varma and Mayor, 1998; Pralle et al., 2000; Prior et al., 2003). For a typical cell this translates to a number of 10,000–100,000 lipid rafts, which is more than five to ten times the number of a typical membrane-associated protein (e.g., for LRP6 and CK1γ a number of 4,000 and 5,000 molecules per cell were experimentally determined, respectively, Bafico et al., 2001). For most individual signaling pathway, this quantitative point of view would imply, that rafts clearly outnumber the membrane-associated pathway components, such as receptor and kinases. Here, we apply computational modeling to analyze whether and how the quantitative ratio between receptor and rafts affects LRP6 phosphorylation and WNT/β-catenin pathway activation. Therefore, a simulation model is needed, that takes a realistic number of lipid rafts as compartments into account.

The vast majority of existing models and simulation studies of lipid rafts focus on the molecular nature of these domains at the nanoscale. These approaches apply molecular dynamics or coarse grained approaches to analyze the spontaneous phase separation in lipid bilayers of varying lipid and cholesterol composition/mixtures (Risselada and Marrink, 2008; Bennett and Tieleman, 2013) as well as the interaction with transmembrane proteins on the molecular level (Parton et al., 2013). To our knowledge only a few computational models exist, that aim to analyze the impact of raft domains on signaling. These studies, however, either comprise significantly less rafts than proteins/receptors under study (Nicolau et al., 2006; Fallahi-Sichani and Linderman, 2009; Hsieh et al., 2010; Haack et al., 2013), or consider microdomains as a single compartment in the membrane (e.g., in the case of pathway studies) (Barua and Goldstein, 2012; Haack et al., 2015). This way neither the actual quantitative ratio between microdomains and proteins nor the interactions and dynamics, such as co-localization, diffusional association, or bimolecular reactions are adequately represented. To fill in these gaps, we adapt our previously published model of WNT/β-catenin signaling, in which microdomains have been described as a single compartment inside the membrane (Haack et al., 2015), and successively increase the number of lipid rafts compartments. Thereby we are, for the first time, able to analyze whether and how the quantitative ratio between microdomains and membrane proteins affects the raft-dependent phosphorylation of raft-dependent LRP6 and eventually the pathway's activity in terms of beta-catenin accumulation.

Indeed, our simulation experiments indicate, that for receptor/raft ratios smaller than 1, i.e., when the number of raft compartments exceeds the number of pathway specific membrane proteins the model dynamics in terms of LRP6 phosphorylation and downstream pathway activity are significantly changed. For realistic amounts of lipid rafts observe a an almost complete decline in LRP6 phosphorylation and β-catenin accumulation. This result suggests that the general existence of microdomains does not optimize, but rather inhibit WNTβ-catenin signaling, despite their ascribed beneficial properties. Instead, pathway specific targeting and sorting mechanism are necessary to significantly narrow down the receptor/raft ratio and ensure the signaling.

2. MATERIALS AND METHODS

2.1. Combined Model of Intracellular WNT Signaling and Membrane Dynamics

We base our simulation study on the computational model of canonical WNT signaling published in Haack et al. (2015). For the detailed description of the model and the corresponding calibration and validation experiments we refer to the aforementioned publication. Note that several (fitted) reaction rates in the original model have been replaced by values from literature. As depicted in the provenance graph (**Figure 1**), we replaced the shuttling rate k1 based on Lauffenburger and Linderman (1996) and the WNT association and dissociation constants k4/k5 based on Bourhis et al. (2010). Being the scope of this simulation study, we vary and increase the number of lipid rafts compartments (nLR). Here it is important to note, that in our approach nLR represents the number of raft compartments and not the actual number of lipid rafts. Please see the considerations in the next subsection "Compartmental modeling approach."

The model can be roughly divided into two main model components: (i) the intracellular signaling cascade, including the interaction between β-catenin and Axin as part of the β-catenin destruction complex; and (ii) the membrane-associated signaling events, such as ligand-receptor binding between WNT and LRP6 and the subsequent, raft-dependent activation, and assembly of the LRP6 signalosome.

Central parts of the intracellular model are derived from the original work of Lee et al. (2003). As suggested by Mirams et al. (2010) we use a highly simplified version of the Lee model, that still captures the essential dynamics of the signaling cascade. Accordingly in the model, β-catenin is constantly produced (k14) and may shuttle between the nucleus and the cytosol (k17/18). Aggregated β-catenin in the nucleus induces the production of Axin (k13). In its phosphorylated form Axin induces the degradation of β-catenin in the cytosol (k16). Due to its low abundance in most cell types (Tan et al., 2012), Axin is the rate limiting element in the β-catenin destruction complex Therefore, Axin may serve as sole representative of the entire β-catenin

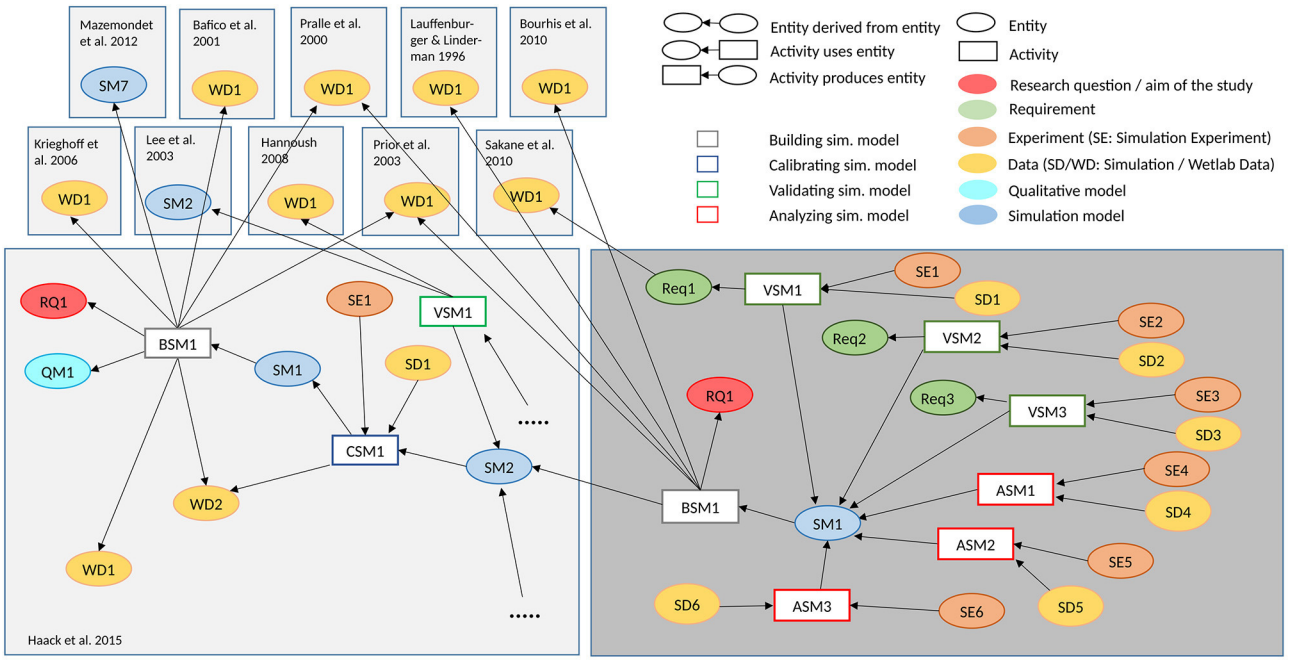

FIGURE 1 | Provenance graph of the conducted simulation study. The provenance graph shows central entities of the conducted simulation study, and what activities and other entities contributed to their generation, based on the provenance standard W3C-Prov (Missier et al., 2013). The WNT simulation model (Haack et al., 2015) is adapted, to study how different numbers of lipid rafts influence LRP6 phoshorylation and β-catenin accumulation (Research question RQ1). The model building activity takes wet-lab measurements from various sources into account. Several validation experiments are executed. It is checked whether the ratio of LRP6 within and outside of lipid rafts (Requirement Req1) as described by Sakane et al. (2010) is still valid in the new model (Validating Simulation Model VSM1), whether the WNT concentration is not affected by increasing the number of lipid rafts (Req2 and VSM2), and whether the β-catenin remains constant (Req3 and VSM3), if the membrane model is decoupled from the intra-cellular model. Afterwards parameter scans, in which the number of lipid rafts varies between 1 and 30,000 with low (Analyzing Simulation Model ASM1), medium (ASM2) and high concentration of WNT (ASM3), respectively, are executed to analyse the impact on LRP6 phosphorylation and β-catenin (see **Figure 3**). The entities can be found in our git repository[1] https://git.informatik.uni-rostock.de/mosi/receptor-raft-ratio-model. The provenance graph also illuminates the relation to the earlier simulation study (Haack et al., 2015) (for more detailed provenance of this and other WNT signaling models see Budde et al., 2021).

destruction complex. This applies in particular for simulation studies, in which the activity of the canonical WNT signaling pathway in terms of beta-catenin aggregation shall be monitored. In contrast to the original Lee model (Lee et al., 2003), where WNT stimuli were represented as exponential decay functions and directly inhibited the phosphorlyation of Axin, our model also considers the signaling events at the membrane. Here extracellular WNT ligands bind to membrane-associated LRP6 receptors and form a receptor-ligand (LRP6 /WNT) complex (k4/k5). Note, that we do not explicitly consider Frizzled (FZ) as part of the LRP6 /WNT complex, because crucial events in canonical WNT signaling primarily depend on LRP6 and its activation through phosphorylation (Niehrs and Shen, 2010). Regarding the phosphorylation of LRP6 we consider solely the interaction between CK1γ and LRP6 (k6/k7), whereas a detailed representation of DVL mediated unspecific phosphorylation of LRP6 by GSK3β is omitted. This assumption is justified by several studies indicating that the LRP6 phosphorylation site targeted by GSK3β, S1490, is constitutively phosphorylated and not or only weakly responsive to WNT stimulation, while the phosphorylation of the CK1γ specific phosphorylation site, T1479, is clearly induced by WNT stimulation (Davidson et al., 2005; Zeng et al., 2005; Niehrs and Shen, 2010). In addition several studies revealed that CK1γ-mediated phosphorylation of LRP6 is confined to lipid rafts (Sakane et al., 2010; Özhan et al., 2013). We include this finding in our model by restricting the phosphorylation to rafts-associated proteins, i.e., only LRP6 that are located within a lipid raft may be phosphorylated by CK1γ.

2.2. Compartmental Modeling Approach

To capture the structural organization of the membrane as well as raft-specific or raft-dependent reactions in a pathway model we use a compartmental modeling approach. This means we consider the cell and all entities, such as the membrane and the nucleus as hierarchically nested compartments. We assume that molecules within a compartment are well mixed. In our model, we consider one cell that contains the nucleus and the membrane as individual, intracellular compartments; the membrane further contains a varying number of lipid rafts or microdomain compartments. Molecules may shuttle between compartments by diffusion and thereby change their localization.

[1] A permanent DOI and repository will be provided after paper acceptance.

TABLE 1 | Parameter and reference values of the model as depicted in **Figure 2**.

Parameter	Description	Value	References
nWnt	total Wnt	1	
nLRP6	total LRP6	4,000	Bafico et al., 2001
CK1γ	total CK1γ	5,000	Haack et al., 2015
nbetacyt	β-catenin in cytosol	12,992	Lee et al., 2003; Mazemondet et al., 2012
nbetanuc	β-catenin in nucleus	5,283	Lee et al., 2003; Mazemondet et al., 2012
nAxin	unphosphorylated Axin	220	Lee et al., 2003; Mazemondet et al., 2012
nAxinP	phosphorylated Axin	253	Lee et al., 2003; Mazemondet et al., 2012
nLR	total number of Lipid rafts	1–30,000	Pralle et al., 2000; Prior et al., 2003
k1	Raft Shuttling	$3.61 \cdot 10^5 \frac{1}{\mathrm{M\,s}}$	Lauffenburger and Linderman, 1996
k2	Wnt synthesis	$4.5 \cdot 10^{-9}$ to $4.5 \cdot 10^{-10} \frac{\mathrm{M}}{\mathrm{min}}$	
k3	Wnt degradation	$0.27 \frac{1}{\mathrm{min}}$	Lee et al., 2003; Haack et al., 2015
k4	Wnt LRP6 association	$2.16 \cdot 10^6 \frac{1}{\mathrm{M\,min}}$	Bourhis et al., 2010
k5	Wnt LRP6 dissociation	$0.02 \frac{1}{\mathrm{min}}$	Bourhis et al., 2010
k6	LRP6 phosphorylation	$2.412 \cdot 10^9 \frac{1}{\mathrm{M\,min}}$	Haack et al., 2015
k7	LRP6 dephosphorylation	$4.7 \cdot 10^{-2} \frac{1}{\mathrm{min}}$	Haack et al., 2015
k8	Axin/LRP6 binding	$2.629 \cdot 10^{12} \frac{1}{\mathrm{M\,min}}$	Haack et al., 2015
k9	Axin/LRP6 dissociation	$3 \cdot 10^{-4} \frac{1}{\mathrm{min}}$	Haack et al., 2015
k10	Axin phosphorylation	$0.03 \frac{1}{\mathrm{min}}$	Lee et al., 2003; Mazemondet et al., 2012
k11	Axin dephosphorylation	$0.03 \frac{1}{\mathrm{min}}$	Lee et al., 2003; Mazemondet et al., 2012
k12	Axin degradation	$4.48 \cdot 10^{-3} \frac{1}{\mathrm{min}}$	Lee et al., 2003; Mazemondet et al., 2012
k13	β-catenin induced Axin synthesis	$7.6086 \cdot 10^{-10} \frac{1}{\mathrm{M\,min}}$	Lee et al., 2003; Mazemondet et al., 2012
k14	β-catenin synthesis	$1.14 \cdot 10^{-9} \frac{\mathrm{M}}{\mathrm{min}}$	Lee et al., 2003; Mazemondet et al., 2012
k15	β-catenin basal degradation	$1.13 \cdot 10^{-4} \frac{1}{\mathrm{min}}$	Lee et al., 2003; Mazemondet et al., 2012
k16	Axin induced β-catenin degradation	$1.104 \cdot 10^8 \frac{1}{\mathrm{M\,min}}$	Lee et al., 2003; Mazemondet et al., 2012
k17/18	β-catenin nucleus shuttling	$2.886 \cdot 10^{11} \frac{1}{\mathrm{M\,min}}$	Lee et al., 2003; Mazemondet et al., 2012

M *corresponds to mol/l.*

This applies for example to molecules entering or leaving the nucleus, that associate or dissociate from the membrane as well as to membrane bound proteins and receptors that shuttle into or out of individual lipid rafts. We approximate the rate constant for the lipid rafts shuttling process (k1) by adapting the rate of receptors shuttling between clathrin pits, as described in Lauffenburger and Linderman (1996).

Note, that for all simulations performed in this study, we assume a raft coverage of 25% and always consider the sum/unity of all lipid rafts for rate calculations of reactions inside lipid rafts compartments. To ensure, that the raft shuttling is also independent of the raft number, we have to divide by the number of rafts compartments, when increasing the number of raft compartments (since the rule for shuttling corresponds to a second order reaction).

```
Membrane[LRP6] + LR -> Membrane[] +
LR[LRP6] @ k1 / (a*0.75*vm*nLR);
```

This way we can, starting from the "one raft compartment model" successively increase the number of raft compartments without introducing a bias due to unrealistic properties, such as very small raft coverages or very high raft radii. Note, that this is an approximation used to study the impact of the receptor/raft ratio and to make different model approaches comparable to each other, such as the simplified model, where we have only a single compartment representing the sum of all rafts.

2.3. Modeling Approach and Simulation Setup

The model is specified using a *rule-based* modeling language. In particular, we have chosen our *domain-specific-language* called ML-Rules, because it supports writing nested structures, simplifying the abstractions needed for this study (Maus et al., 2011; Helms et al., 2017). An example rule is `LRP6 + LR -> LR[LRP6]`, where an LRP6 moves into a Lipid Raft (LR). The simulator takes all the rules and enumerates all possible species variants that might be constructed as well as their possible transitions. As LRP6 has two attributes, i.e., whether or not being phosporalized and being bound to WNT respectively, for the example rule, one such transition would be `LRP6_phosporalized_unbound -> LRP6_phosporalized_unbound_in_Lipid_Raft_7`. This set of enumerated species and their transitions is called the reaction network, and such enumerations are well-established (for example, via the Biological Network Generator BioNetGen, Harris et al., 2016). With this approach, more complex interactions and structural properties could be included and described in future extensions of our model, such as post-translational modifications and specific protein-lipid interactions that are required to specifically direct the membrane pathway components, such as WNT, LRP6,

or CK1y, to the same domain of the plasma membrane for co-localization (Davidson et al., 2005; Bourhis et al., 2010; Perrody et al., 2016; Azbazdar et al., 2019; Sada et al., 2019).

For our particular implementation, we use two optimizations to improve the runtime of this expansion. Firstly, the network generation uses multiple threads. Secondly, we store cache files of the previously expanded networks and check if an identical system has been expanded before to safe runtime on replications. The simulator itself runs sequentially, although multiple replications are run in parallel.

To facilitate efficient execution, a dependency graph between reactions is needed. For the larger models, this dependency graph limits possible performance as it takes large amounts of memory (e.g., 42 GB for 30,000 Lipid Rafts). Throughput is also highly dependent on the number of Lipid Rafts. For ten lipid rafts, we have a total of 3e7 steps in about 4 s, whereas 10,000 lipid rafts take about 6 min for their respective 7e7 steps. The total number of transitions does not increase drastically, but the effort per transition and the memory use. Therefore we have used hundreds of replications for the fast, small runs and 10s of replications for the very large systems The plots are created using python and the pandas and matplotlib libraries. The plotting scripts and the simulator (written in rust) are provided alongside the paper.

Further we analyzed the robustness of the model. For this we determined single and total-order sobol indices to determine the impact resulting from changes in individual or combination of parameters on the model output (Jansen, 1999; Saltelli et al., 2010). The results of the analysis are depicted in the **Supplementary Figure 1**. We analyzed four model parameters that are crucially involved in the signal transduction at the membrane: The WNT stimulation in terms of WNT synthesis rate (kWsyn), the receptor/raft shuttling rate (ksh, k1 in **Figure 2**), phosphorylation and dephosphorylation of LRP6 receptor (kLp/kLdp, k6/k7 in **Figure 2**). To test, whether the robustness of the model changes with an increasing number of raft compartments, we applied the analysis to the original model (with one lipid rafts compartment) and to a model configuration with high raft numbers (i.e., low receptor-raft ratio). In both cases, single- and total-order indices clearly show that, in contrast to the other parameters, the WNT stimulation has - by far - the strongest impact on the model output. This result indicates, that the model is very robust against changes in the model parameters, but sensitive to changes in the WNT stimulus, i.e., the input parameter.

3. RESULTS

Lipid Rafts or microdomains are typically considered as small, circular shaped entities within the membrane. Depending on the cell type, the membrane composition and the surrounding environmental conditions (such as temperature) microdomains approximately cover between 20% and 40% of the membrane, with a radius ranging between 25 to 50 nm (Pralle et al., 2000; Prior et al., 2003). Based on these properties, the membrane of a "normal sized" cell, such as HeLa cells or fibroblasts, with a cell volume of 2.000 to 3.000 μm^3 and a radius of 8 to 9 µm comprises more than 100.000 microdomains or lipid rafts. This outnumbers the typical amount of receptor and membrane-associated proteins by far and drastically changes the common picture of how receptors and lipid rafts interact.

In the following we analyze how increasing the number of raft compartments affect the raft-dependent phosphorylation of LRP6 and the pathway's activity in terms of β-catenin accumulation. Further we apply three different WNT stimuli: high, medium, and low. For this we observe the localization, binding and phosphorylation states of LRP6 as well as the accumulation of β-catenin in the nucleus.

To verify our approach and to monitor changes in the membrane dynamics, we first perform a simulation experiment, in which we uncoupled the membrane model from the intracellular model by inhibiting the AXIN/LRP6 binding (i.e., we set k8 to zero). Shown in the upper row of **Supplementary Figure 2** are the simulation results of the uncoupled membrane model, which illustrate the direct impact on the raft distribution, WNT binding and phosphorylation of LRP6 receptors for each WNT stimulation scheme (columns), depending on the number of lipid rafts. We observed the fraction of total LRP6 vs. raft-associated (gray), WNT-bound (orange stripes), and phosphorylated LRP6 (blue circles). Regardless of WNT stimulation or raft number, the fraction of LRP6 localized in lipid rafts remains mostly unaffected. As expected, the fraction of LRP6 receptors bound to WNT increases with the WNT stimulus. However, the phosphorylation of LRP6, in contrast to its localization and binding state, seems to be strongly affected by an increasing raft number. Since the distribution of LRP6 between raft and non-raft domain as well as the LRP6/WNT binding dynamics are not significantly affected, we infer that the significant decline in LRP6 phosphorylation is primarily caused by the change in model structure.

In the following we aim to explore how this change in model structure and resulting phosphorylation dynamics affects the entire WNT pathway. The result of our simulation are depicted in **Figure 3**. In the upper row the composition of LRP6 states after 12 h of stimulation is depicted for each WNT stimulation scheme. In addition to the raft distribution (gray area), WNT binding (orange stripes) and phosphorylation state of LRP6 receptors (blue circles), the amount of LRP6 species localized in the signalosome (red stripes, bound AXIN) is displayed. Here, we note two important features. First, the amount of phosphorylated LRP6 being part of the AXIN/LRP6 complex is much higher than in the uncoupled membrane model; second beyond a certain threshold of lipid raft numbers ($\sim$ 1,200, see dotted line), the number of AXIN/LRP6 complexes and effective LRP6 phosphorylation decrease rapidly with increasing raft numbers.

The lower row in **Figure 3** shows the predicted β-catenin accumulation in the nucleus within the first 12 h of WNT stimulation, with varying parameter values for lipid raft numbers and WNT stimulation. The accumulation of β-catenin is denoted as fold change, i.e., for each time point of the trajectory the actual number of β-catenin in the nucleus is related

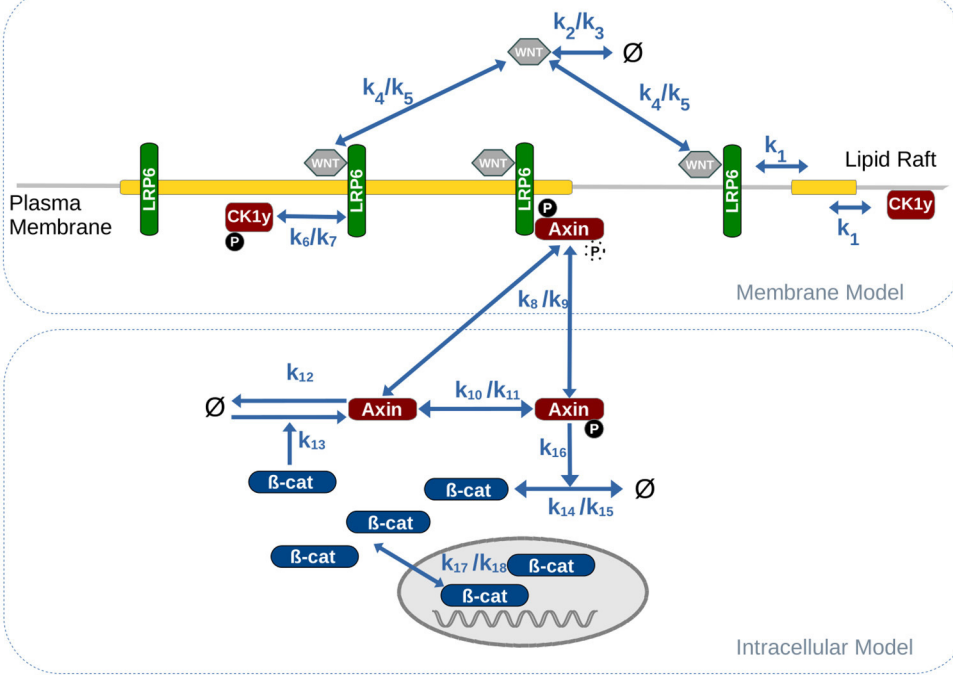

FIGURE 2 | Combined model of intracellular WNT signaling and membrane dynamics. Parameter values for the reaction rates are listed in **Table 1**.

to its initial concentration at the start of the simulation (time point 0).

Overall we were surprised to see a strong decrease in fold change of β-catenin accumulation in the nucleus with increasing numbers of raft compartments. This applies for all WNT stimulation scenarios. In fact, for low and medium WNT concentrations, almost no β-catenin accumulation is detectable within 12 h of stimulation for raft numbers above 10,000. Even with a high WNT stimulus, the maximum fold change drops from more than six, to less than 1.5.

Further, we observe a slight temporal shift of the trajectory's peak, i.e., the time point when β-catenin accumulation reaches its maximum fold change. For high WNT stimulation, we observe a transient signal for raft numbers up to 1000, and a temporal shift of the trajectory's peak from $\sim$4 to $\sim$6 h. For higher raft numbers, the β-catenin accumulation is no longer transient, but is rather characterized by a gradual increase, followed by a constant plateau.

4. DISCUSSION

As shown in **Supplementary Figure 1** the fraction of receptors localized in a raft compartment stays the same in all model configurations, regardless of the number of raft compartments. The effective WNT/LRP6 binding rate changes only slightly with increasing raft abundance. This confirms, that neither the shuttling and distribution of LRP6 between raft and non-raft domains nor the binding of WNT to LRP6 is affected or disturbed by the increment of raft compartments in our model. Instead, the strong decline in LRP6 phosphorylation solely results from the increased number of raft compartments i.e., the change in the model structure. In order to interact, i.e., to collide and react with each other, LRP6 and CK1γ need to be located in the same raft compartment. However, the chances of LRP6 and CK1γ molecules being located in the same compartment at the same time diminishes with increasing amount of compartments. This becomes particularly evident when the number of compartments exceeds the molecular count of LRP6 and CK1γ , i.e., when the ratio of molecule number vs. raft compartments is $<$ 1. In the following we refer to this ratio as receptor/raft ratio. **Figure 3** illustrates this effect, as the LRP6 phosphorylation starts to decline considerably when raft abundance exceeds a value that roughly corresponds to the amount of membrane-associated LRP6 receptor and its kinase CK1γ . This implies that the quantitative ratio between rafts, receptor and kinases plays a pivotal role for the receptor activation, and more importantly the realistic raft abundance clearly exceeds the molecular count of the membrane-associated components of the WNT signaling pathway, i.e., the receptor/raft ratio is well below 1. In our system, which is calibrated to a cell volume of $1.37 \cdot 10^{-15}\,\mathrm{m}^3$, a realistic raft number lies above 70k, yielding a receptor/raft ratio that would prevent any beta-catenin accumulation. This means, the pure existence of lipid rafts and their ascribed features do not promote WNT/β-catenin signaling, but would rather prevent the receptor

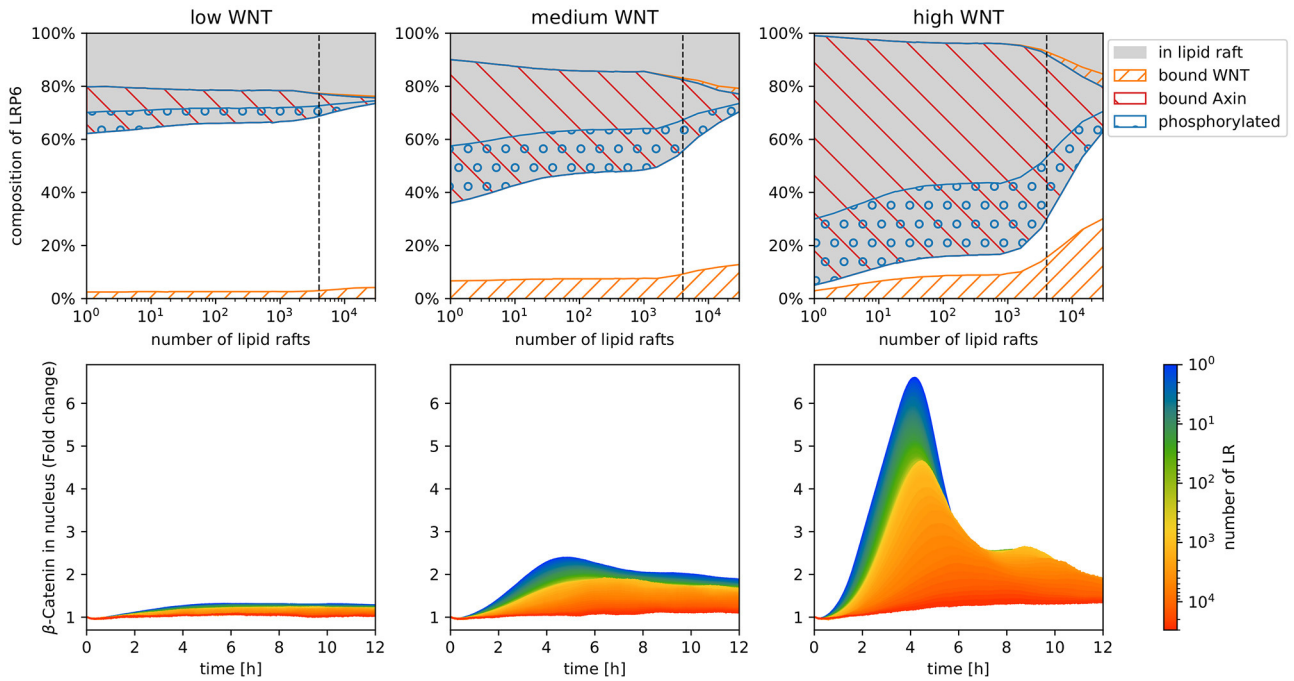

FIGURE 3 | Simulation results of the model displayed in **Figure 2**, with increasing raft numbers and with three different WNT stimuli: high, medium, and low. In the upper row the composition of LRP6 states after 12 h of stimulation is depicted for each WNT stimulation scheme. Depicted are raft-associated (gray), phosphorylated (blue circles), WNT-bound (orange stripes), and Axin-bound (red stripes) LRP6 molecules. Note that in the stacked area chart of the upper row, the amount of bound and phosphorylated LRP6 are displayed separately for non-raft (lower area, white background) and raft (upper area, gray background) domains and have to be added/summed up to obtain the total number of each receptor state. The lower row illustrates the fold change of β-catenin concentration in the nucleus [compared to the initial number of β-catenin in the nucleus (nbetanuc)] during 12 h of constant WNT stimulation for increasing numbers of lipid rafts. Each colored line represents the mean trajectory of several simulation runs (replications) of our model parameterized with the corresponding lipid rafts amount (encoded in the color).

activation and signal transduction. For an effective interaction between the membrane-bound components of the pathway (LRP6 and CK1γ) and the successful activation of the pathway (β-catenin accumulation) a reduction of the amount of raft domains available for membrane-bound pathway components is essential.

In the case of canonical WNT signaling, a number of mechanisms have already been proposed, that target the localization of WNT ligands and LRP6 to specific membrane domains to promote (or inhibit) the interaction between CK1y and subsequent receptor phosphorylation. One of the first mechanisms described in this context is receptor clustering and signalosome formation. Early studies on LRP6 activation demonstrated, that overexpression (Bilic et al., 2007) or truncation of LRP6 molecules (Brennan et al., 2004) promotes the self-aggregation of LRP6 receptors into large multiprotein complexes, that contain WNT pathway components like Frizzled, Disheveled and CK1γ to provide a stable binding platform for Axin. Indeed, our simulations confirm, that the recruitment and binding of cytosolic pathway components, such as AXIN, promotes the LRP6 phosphorylation and subsequent signaling. Considering a configuration of our model, in which AXIN cannot bind to the phosphorylated LRP6 complex, yields significantly less phosphorylated LRP6 (cf. **Supplementary Figure 1**). This is due to the fact, that LRP6 being part of the signalosome is less prone to dephosphorylation than individual, phosphorylated LRP6 molecules (cf. **Figure 3**). Additionally, WNT induces the recruitment and aggregation of DVL at the plasma membrane, which in turn leads to a co-clustering of LRP6 on DVL platforms and the recruitment of other pathway components (Bilic et al., 2007). This increases the local LRP6 density, which promotes LRP6 phosphorylation and AXIN binding and compensates for the diluting effect of a low receptor/raft ratio. This effect is not included in our model, but it further promotes LRP6 phosphorylation and subsequent signal transduction.

For a successful LRP6 phosphorylation and subsequent induction of signalosome formation at endogenous LRP6 concentrations, however, further guiding/targeting mechanisms are required. Various recent studies emphasize the importance of localizing WNT and LRP6 to specific membrane domains. On the one hand several membrane-associated proteins and ligands have been discussed to affect the localization of LRP6 and thereby regulate the pathway activity. For example, CD44 (Schmitt et al., 2015) and LYPD6 (Özhan et al., 2013), both being glycoproteins, physically interact with LRP6, modulate its membrane localization and promote its phosphorylation; whereas DKK (Yamamoto et al., 2008) and Waif1/5T4 (Kagermeier-Schenk et al., 2011) modulate the localization and internalization route of LRP6. On the other hand it has been shown, that palmitoylation of WNT (Azbazdar et al., 2019) and LRP6 (Abrami et al., 2008; Sada et al., 2019) target ligand and receptor to specific membrane domains. In

this context (Sezgin et al., 2017a) demonstrated, that WNT preferentially localizes to raft-domains, where it binds to LRP6. and induces signaling.

In regard to our simulation results a scenario, in which immobilized palmitoylated proteins recruit saturated lipids and thus nucleate ordered domains at specific cellular sites, instead of raft-like domains recruiting palmitoylated proteins (Tulodziecka et al., 2016), might take on a greater significance. While this mechanisms is still rather hypothetical and needs to be confirmed in other cellular context, related work in different cellular context (Biernatowska et al., 2017; Zhou et al., 2017) provide evidence for a more general mechanism, in which pathway-specific, lipidated proteins or lipids act as domain sorter and considerably regulate the localization and stability of organized membrane domains (Sezgin et al., 2017b).

In general, distinct perturbations in membrane composition or temperature are prone to change the balance between protein and lipid interaction and raft distribution, hence the receptor/raft ratio, and thereby change the membrane dynamics of membrane associated pathway components.

DATA AVAILABILITY STATEMENT

The datasets, models and simulation experiment specifications to reproduce **Supplementary Figures 1, 3** for this study can be found in the following repository: doi.org/10.17605/OSF.IO/NCGV3.

AUTHOR CONTRIBUTIONS

FH performed the modeling. FH and TK performed the simulation experiments. FH and AU conceived the study. FH, TK, and AU wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.706731/full#supplementary-material

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REVIEW
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Metabolic Contributions of Wnt Signaling: More Than Controlling Flight

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The canonical Wnt signaling pathway is ubiquitous throughout the body and influences a diverse array of physiological processes. Following the initial discovery of the Wnt signaling pathway during wing development in *Drosophila melanogaster,* it is now widely appreciated that active Wnt signaling in mammals is necessary for the development and growth of various tissues involved in whole-body metabolism, such as brain, liver, pancreas, muscle, and adipose. Moreover, elegant gain- and loss-of-function studies have dissected the tissue-specific roles of various downstream effector molecules in the regulation of energy homeostasis. This review attempts to highlight and summarize the contributions of the Wnt signaling pathway and its downstream effectors on whole-body metabolism and their influence on the development of metabolic diseases, such as diabetes and obesity. A better understanding of the Wnt signaling pathway in these tissues may aid in guiding the development of future therapeutics to treat metabolic diseases.

Keywords: Wnt signaling, β-catenin, TCF7L2, GSK-3β, metabolism, embryogenesis, organogenesis

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INTRODUCTION

During embryogenesis, the Wnt signaling pathway has active and critical roles in tissue development, including, but not limited to, axon guidance, body segmentation, limb development, and stem cell differentiation (Yang, 2003; Dunty et al., 2008; Geetha-Loganathan et al., 2008; Nusse, 2008; Stanganello et al., 2019). While its importance during embryogenesis cannot be understated, the influence of active Wnt signaling on the postnatal growth of tissues and their physiological

Abbreviations: APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; TCF/LEF, transcription like factor lymphocyte enhancer factor; LRP, lipoprotein receptor-related protein; CK, casein kinase; Mtgr1, myeloid translocation gene related-1; PP, periportal hepatocytes; PV, perivenous hepatocytes; MyoD, myoblast determination protein; Myf, myogenic factor; NMJ, neuromuscular junction; AChR, acetylcholine receptor; BCL9, B-cell CLL/lymphoma protein 9; CBP, CREB-binding protein; NF-kB, nuclear factor kB; PTCH1, protein patched homolog 1; MHC, myosin heavy chain; LTCF7L2KO, liver specific TCF7L2 knockout; IBMX, isobutylmethylxanthine; PITX2, paired like homeodomain 2; MIN6, mouse INsulinoma 6 cells; CDK4, cyclin dependent kinase 4; PPAR, peroxisome proliferater activated receptor; CEBP, CCAT enhancer binding proteins; Brdu, bromodeoxyuridine; PDGFRa, platelet derived growth factor; DDR2, discoidin domain receptor; FSP-1, fibroblast specific protein 1; DKK1, Dickopf; TNF, tumor necrosis factor; IRS, Insulin receptor substrate; LiCl, lithium chloride; BIO, 6-bromoindirubin; GLP, glucagon like peptide; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; WAT, white adipose tissue; NEFA, non-esterified fatty acid; FABP, Fatty acid binding protein; HMG, high mobility group; DIO, diet induced obesity.

functions are now gaining wide appreciation. Some of the lesser appreciated functions of the Wnt signaling pathway are its influence on systemic, organ, and tissue metabolism and energy homeostasis.

Whole-body metabolism is regulated by various tissues, including brain, liver, pancreas, muscle and adipose, and each contributes significantly to carbohydrate and/or lipid metabolism. The brain requires a constant uptake of oxygen and glucose, where astrocytes convert glucose to lactate and neurons use glucose and lactate in an oxidative manner (Deitmer et al., 2019). The liver is a critical metabolic tissue, as it is capable of glucose and glycogen metabolism, gluconeogenesis, fatty acid metabolism, and drug metabolism (Rui, 2014). The pancreas is responsible for secreting digestive enzymes and hormones, most notably insulin and glucagon, to control glucose homeostasis (Roder et al., 2016). Skeletal muscles require glucose and fatty acids to function, and can themselves be metabolized and converted to amino acids for use by other tissues. Insulin promotes glucose uptake in muscle where it can also be stored as glycogen (McPherron et al., 2013). Adipose tissue not only functions as energy reservoirs, but also secretes key hormones and metabolites to help control systemic energy balance (Choe et al., 2016). For example, it releases leptin to promote satiety (McDuffie et al., 2004). Thus, the goal of this review is aimed at highlighting the important contributions of the Wnt signaling pathway and its effectors within these metabolic tissues.

OVERVIEW OF THE WNT SIGNALING PATHWAY

The naming of Wnt is derived from the gene *wingless (wg)* in *Drosophila melanogaster*, where its mutation resulted in a wingless phenotype (Sharma and Chopra, 1976). Its vertebrate homolog *Int1* encodes for a retrovirus docking site and was first discovered in mice (Nusse and Varmus, 1982). Wnts are thought to primarily mediate their effects via autocrine and paracrine mechanisms, as the hydrophobic palmitate group near the N-terminus serves to tether the protein to the plasma membrane of its secreting cell (Kaemmerer and Gassler, 2016). However, recent findings have detected Wnt within exosomes, which suggests that Wnts could exert effects in distal sites in the body (Gross et al., 2012; Beckett et al., 2013).

The initiation of the canonical Wnt signaling pathway, sometimes referred to as the Wnt/β-catenin pathway, occurs by the binding of Wnt proteins to complexes consisting of the cell-surface Frizzled (Fz) receptor and lipoprotein-related proteins (LRP5/6). This leads to a cascade of downstream events, that culminate in the dissociation of the β-catenin "destruction complex" comprised of Glycogen Synthase Kinase 3 (GSK-3), Axin, Casein-kinase 1 α (CK1-α), and Adenomatosis Polypsis Coli (APC) (**Figure 1**; Thompson and Williams, 2018). In the absence of Wnt signaling, GSK-3 binds β-catenin, the transcriptional co-activator integral to Wnt signaling, to promote its phosphorylation. The interaction of β-catenin with GSK-3 is initiated by CK1-α-mediated phosphorylation of Ser47, which allows GSK-3 to bind and subsequently phosphorylate additional serine and threonine residues (Thompson and Williams, 2018), leading to the eventual ubiquitination and degradation of β-catenin. Disheveled (DSH) is also recruited to the Fz-LRP5/6 complex and recruits the components of the destruction complex to the cell membrane (MacDonald and He, 2012). This aids in the stabilization and accumulation of β-catenin in the cytoplasm. Once β-catenin accumulates in the cytosol, it translocates to the nucleus where it binds the TCF family of transcription factors that influence downstream Wnt target genes and those that exert negative- or positive-feedback effects on the Wnt signaling pathway itself (Jho et al., 2002). The mechanism by which β-catenin enters and exits the nucleus is currently unknown, although some studies suggest the involvement of the scaffold protein 14-3-3ζ (Li et al., 2008).

Wnts can also bind to Fz and activate the non-canonical pathway, also known as the β-catenin-independent pathway. Non-canonical Wnts include Wnt1, Wnt2, Wnt5a, Wnt5b, and Wnt11 (Nie et al., 2020). This sub-grouping includes two distinct pathways called the Planar Cell Polarity (PCP) and Wnt/Ca^{2+} pathways (Komiya and Habas, 2008). PCP was discovered through loss of function studies of Fz and Dvl in *Drosophila*. The ensuing phenotype displayed randomized orientations of epithelial structures (Seifert and Mlodzik, 2007). This pathway has also been shown to be involved in cytoskeletal reorganization and coordination of cellular polarization (Komiya and Habas, 2008). It is activated by Wnt binding Dsh and Fz, which activates Rho through Daam1, activating the Rho kinase. Daam1 also activates Rac and JNK to initiate transcription via the actions of c-jun (Le Floch et al., 2005). The Wnt/Ca^{2+} pathway is activated by the same cytoplasmic receptors; however, it is coupled to a G-protein, leading to intracellular Ca^{2+} release by the actions of PLC and IP3 (Kohn and Moon, 2005). Collectively, the actions of CAMKII and Calcineurin lead to the accumulation of NFAT in the nucleus to initiate the transcription of target genes (Saneyoshi et al., 2002). This pathway is believed to affect early gastrulation cell movements and in some cases may inhibit the canonical Wnt pathway (Gilland et al., 1999; Wallingford et al., 2001).

Additional negative and positive modulators of the canonical pathway have been identified. For example, Frizzled-related protein family (SFRP) proteins and Wnt inhibitory factors, such as Dickkopf (DKK1), bind LRP5/6 directly to inhibit its association with the Fz receptor (Niehrs, 2006). On the other hand, antagonists like Cerberus bind Wnts extracellularly, inhibiting their ability to complex with the Fz/LRP5/6 receptor complex (Piccolo et al., 1999). Corepressors like Groucho/TLE, MTGR1 or COOP exert inhibitory effects by dissociating β-Catenin from TCFs (Cavallo et al., 1998; Moore et al., 2008; Song et al., 2010; Valenta et al., 2012). Nuclear APC has also been reported to sequester β-Catenin and prevent its interactions with TCFs (Hamada and Bienz, 2004). Positive modulators like serine-threonine phosphatase 2A (PP2A) increase β-Catenin's activity by antagonizing the effects of GSK3 through de-phosphorylation of β-Catenin (Kimelman and Xu, 2006). This allows β-Catenin to translocate into the nucleus without being degraded. PP2A is also able to dephosphorylate Axin and prevent its association with β-Catenin (Willert et al., 1999). When taken together, multiple

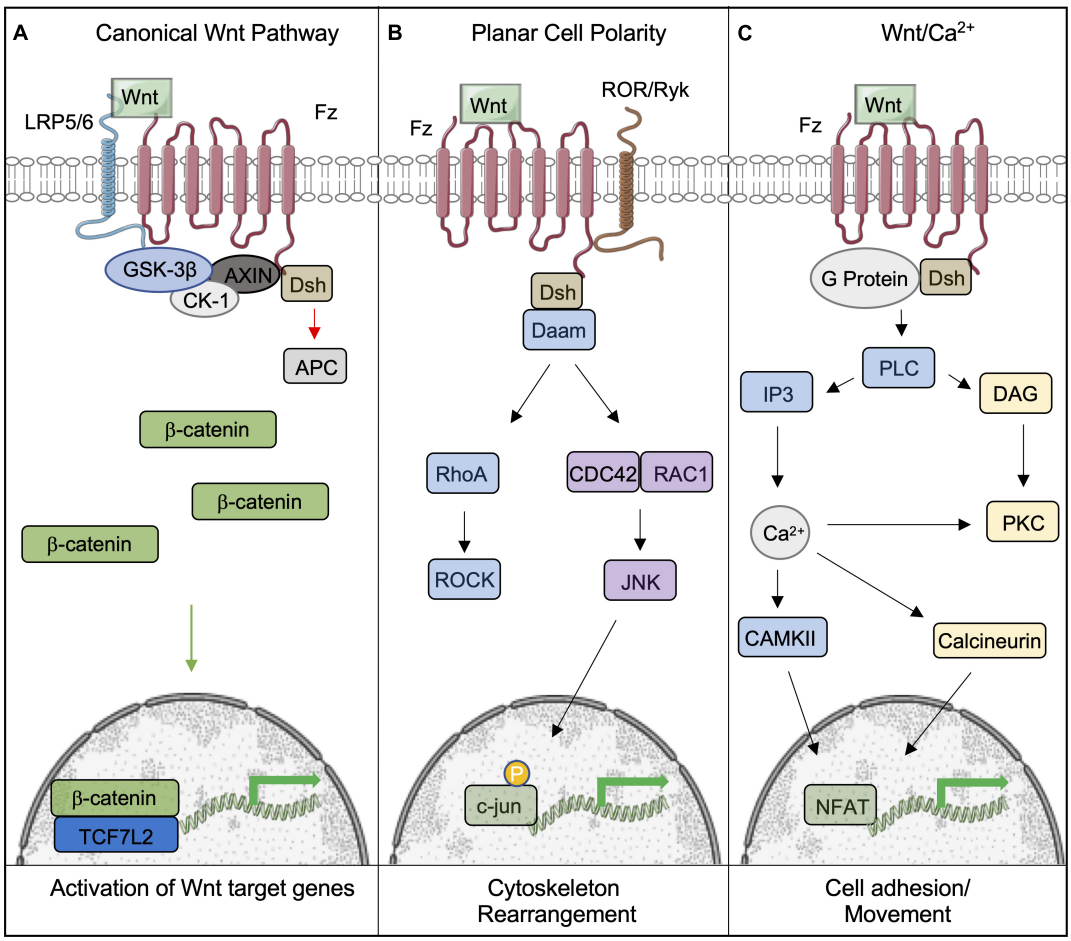

FIGURE 1 | Overview of the canonical and non-canonical Wnt signaling pathways. **(A)** Following binding of Wnt to the Frizzled-LRP5/6 complex and the activation of the signaling pathway, the destruction complex (comprising of GSK-3β, CK-1, AXIN, and APC) is tethered to the plasma membrane, which permits β-catenin to accumulate and translocate to the nucleus, where it bind to TCF7L2 to activate downstream genes. **(B,C)** Wnts can also signal through non-canonical pathways to induce cell responses. **(B)** The Planar Cell Polarity pathway is activated upon Wnt binding to the Fz receptor. Dsh is subsequently activated, initiating the activites of Rho and Rac through Daam1. This leads to the eventual activation of ROCK and Jnk, which phosphorylates c-jun within the nucleus and allows it to initiate the transcription of target genes. **(C)** The Wnt/Ca^{2+} pathways is also activated by Fz; however, it is coupled to a G-protein, leading to intracellular Ca^{2+} release through PLC/IP3 activity. Ca^{2+} promotes the activity of CAMKII and Calcineurin to provoke the accumulation of the transcription factor NFAT in the nucleus. This figure was made with modified images from Servier Medical Art (Creative Commons Attribution 3.0 Unported License).

endogenous mechanisms exist that exert inhibitory effects on Wnt-β-catenin signaling.

ROLES OF WNT SIGNALING IN THE DEVELOPMENT OF METABOLIC TISSUES

Through the use of genetic approaches, the contributions of different cell types and tissues, such as adipose tissue, skeletal muscle, brain, pancreas, and liver, to overall metabolic homeostasis have been identified (Kitamura et al., 2003; von Maltzahn et al., 2012; Kubota et al., 2017), and Wnt signaling, which influences spatial patterning and organogenesis, is required for the development of these metabolic tissues (**Tables 1–3** and **Figure 2**).

Wnts

Numerous mammalian Wnt proteins have been identified, with each isoform having distinct physiological functions in different tissues [for a detailed list, please refer to van Amerongen and Berns (2006)]. Deletion of one allele of a given Wnt does not necessarily lead to an overt phenotype, as demonstrated by the outcome of homo- and hetero-zygous *Wnt1* deletion on brain development and viability (Thomas and Capecchi, 1990; van Amerongen and Berns, 2006). The Wnt ortholog in *Drosophila*, *wg*, has been shown to be important for brain development (**Table 1**; Richter et al., 1998), as deletion of *wg* in fly embryos resulted in partial development of the protocerebrum. Loss of function of *wg* also led to enhanced apoptosis in the ventral ganglia, and gain-of-function experiments using a hsp70 promoter exhibited an increase in CNS development in flies, with a two-fold increase in cerebral mass (Richter et al., 1998).

TABLE 1 | Developmental phenotype resulting from over-expression or deletion of Wnt signaling effectors in *Drosophila*.

Effector	Model	Effect	Reference
Wingless	*wgIL114*	– Impaired protocerebrum development – Apoptosis of ventral ganglia cells	Richter et al., 1998
	HSwg/TM3, hb-LacZ	– Doubling of cerebral mass	Richter et al., 1998; Lee et al., 2014
	wgIIID23	– Absence of fat body in ventral section – Enhanced fat body in dorsolateral segment	Riechmann et al., 1998

TABLE 2 | Developmental phenotype resulting from over-expression or deletion of Wnt signaling effectors in *Xenopus.*

Effector	Model	Effect	Reference
Dickkopf	mRNA injection	– Anteriorized embryos – Large heads – Short trunks	Glinka et al., 1998
	Antibody targeting Dkk-1	– Microcephaly – Lack of cement glands	Glinka et al., 1998
Wnt8	Plasmid injection in D1 cells	– Inhibited development of pancreas, liver, and intesting	Schohl and Fagotto, 2002
	dnWnt8 in dorsal marginal zone of gastrula	– Induced MyoD expression	Hoppler et al., 1996
β-Catenin	mRNA injection	– Supressed foregut markers	Schohl and Fagotto, 2002
GSK3B	mRNA injection in lateral region of embryo	– Diminished expression of MyoD and Myf5	Shi et al., 2002

TABLE 3 | Developmental phenotype resulting from the over-expression or deletion of Wnt signaling effectors in mice.

Effector	Model	Effect	Reference
Wnt1	OE (*Pdx1*-driven transgenic)	Agenesis of pancreas and spleen	Heller et al., 2002
Wnt5a	OE (*Pdx1*-driven transgenic)	Reduced size of pancreas, stomach, and duodenum	Heller et al., 2002
Frizzled receptor-8 (Fz8)	OE (*Pdx1*-driven expression of a dominant negative)	– Reduced pancreatic mass – Increased insulin secretion and content in β-cells – Decrease in pancreatic cell proliferation	Papadopoulou and Edlund, 2005
β-Catenin	*Pdx1*-Cre: *Ctnnb1*$^{flox/flox}$	– Embryonic lethality – Acinar hypoplasia	Wells et al., 2007
	OE (*aP2*-Cre driven constitutively active)	Fibrosis in subdermal tissue	Zeve et al., 2012
	OE (*Pparg*-tTA; TRE-Cre driven constitutively active)	– Reduction of VAT – Elevated serum triglyceride – Fibrosis in SCAT – Depletion of adipocytes	Zeve et al., 2012
APC	*Ngn3*-Cre: *APC*$^{flox/flox}$	Pancreas lacking β/α-cells	Sharon et al., 2019
	Pdx1-Cre: *APC*$^{flox/flox}$	– Enlargment of pancreas – Acinar hyperplasia	Strom et al., 2007
TCF7L2	*Tcf7l2*$^{GFPCre+neo/fl}$	Mediates myogenic maturation	Mathew et al., 2011
	Pax7-Cre: *Tcf7l2*$^{flox/flox}$	Reduction in hind limb and diaghpragm myogenesis	Mathew et al., 2011

KO, knockout; OE, over-expression.

Aside from roles in brain development, Wnt ligands also have essential roles in primitive streak formation and at various stages of embryogenesis (van Amerongen and Berns, 2006). For example, Wnt7a mediates the formation of ventral cell types during limb development in the central nervous system of mice, whereas Wnt4 is involved in the formation of the anterior-posterior axis during spinal cord development and is responsible for directing the migration of neurons and their axons (Parr et al., 1993; Hollis and Zou, 2012).

Orthologs of Wnt also exist in different species, where they exhibit evolutionarily conserved functions. For example, in *C. elegans*, the orthologous *Wnt* gene, *mom-2* is responsible for maintaining polarity of the embryo during development (Bischoff and Schnabel, 2006). In mammals, several Wnt proteins have also been found to have similar roles in maintaining cell polarity and the development of various metabolic organs, as seen by deletion of Wnt3 that results in the failure of Anterior-Posterior axis formation in the developing vertebrate embryo (Liu et al., 1999). Deletion of Wnt proteins are also associated with defects

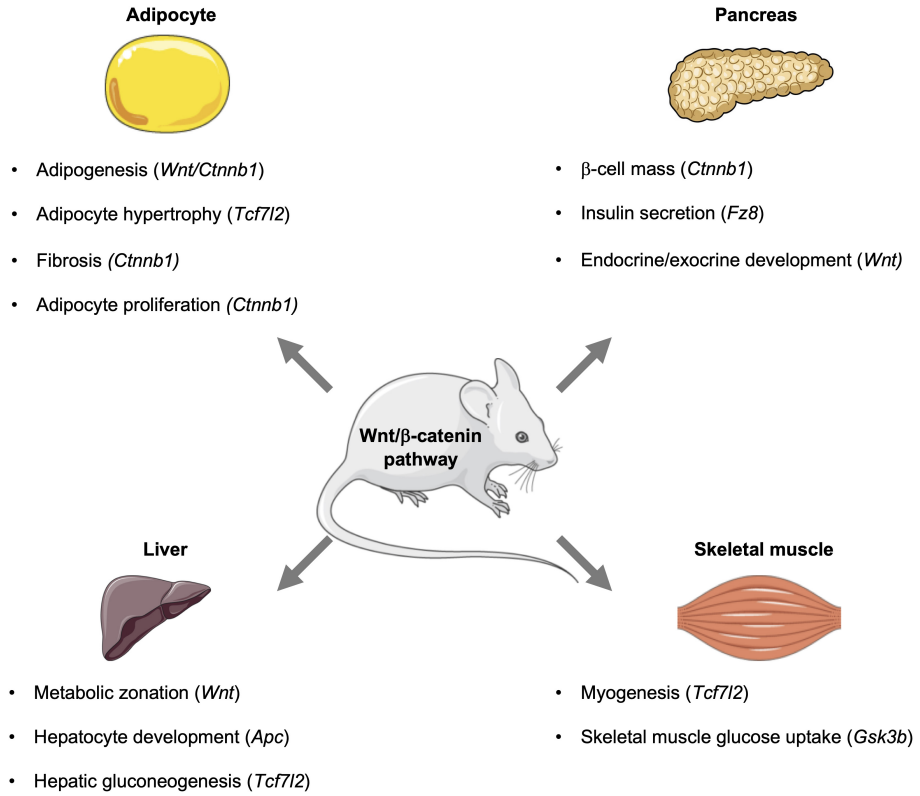

FIGURE 2 | Influence of the Wnt signaling pathway and its downstream effectors in metabolic tissues. In mice, the Wnt signaling pathway influences metabolic events in key tissues, including the pancreas, adipose, liver, and skeletal muscle. Activation of this pathway can influence the development of tissues and organs, as well as signaling events that control glucose homeostasis and metabolism. This figure was made with modified images from Servier Medical Art (Creative Commons Attribution 3.0 Unported License).

in organogenesis, as demonstrated by the failure of mesenchymal stem cells to differentiate into epithelial cells in kidneys of mice deficient in Wnt4. This results in the inability to form nephrons (Stark et al., 1994).

In the early stages of *Xenopus* embryo development (**Table 2**), inhibition of the Wnt signaling pathway appears to be essential, as injection of mRNA for *dkk-1*, the Wnt inhibitor, into blastomeres of *Xenopus* embryos led to anteriorized embryos displaying large heads, enlarged cement glands, and short trunks (Glinka et al., 1998). On the other hand, embryos injected with antibodies targeting *dkk-1* exhibited the opposite phenotype of microcephaly accompanied with a lack of cement glands. The embryonic axis remained unaffected, highlighting the importance of Wnt inhibition for *Xenopus* brain development. Toward the later stages of development, Wnt signaling is necessary for the downregulation of BMP-4 expression to induce the formation of the dorsal ectoderm (Baker et al., 1999). Antagonizing BMP-4 further allows for neural induction, a function found to be mediated by TCF7L2. For a more detailed anaylsis of the Wnt signaling pathway in the developing *Xenopus* brain, please see the review by Patapoutian and Reichardt (Patapoutian and Reichardt, 2000).

Wnt proteins are highly expressed in neurons in the posterior segment of the *Planarian* brain (Adell et al., 2009). They plays an inhibitory role on brain expansion, as evidenced by the increase in brain cells following RNAi mediated suppression of *wnt11-6* (Kobayashi et al., 2007; Hill and Petersen, 2015). Effectors of the Wnt signaling pathway can also affect organogenesis independent of β-catenin, as RNAi-mediated silencing of Dvl in *Planarians* compromised the development of the posterior ectopic brain (Almuedo-Castillo et al., 2011). Knockdown of β-catenin led to trunk loss, lack of tail identity, and loss of gut anterior-posterior polarity. However, brain development appeared to be phenotypically normal, indicating Dvl exerts its effects through a non- canonical Wnt pathway (Gurley et al., 2008).

During embryogenesis, various Wnt genes exhibit a biphasic pattern of expression. Some family members are upregulated before embryonic day-15 (e15) in mouse embryos, followed by their rapid downregulation by e16 (Heller et al., 2002). Wnt1 over-expression under the control of the *Pdx1* promoter disrupted spleen development and caused pancreas agenesis (Heller et al., 2002; **Table 3**). Similarly, over-expression of Wnt5a, the non-canonical Wnt, also altered pancreatic development, resulting in reduced pancreas size and the production of atypical endocrine structures (Heller et al., 2002), showing that both Wnt pathways can share common functions. Another example of the requirement of Wnt signaling in pancreatic development has been observed in mice expressing a dominant-negative Fz

receptor (*Pdx1-Fz8CRD*) (Papadopoulou and Edlund, 2005). Transgenic mice expressing the dominant-negative FZ receptor in PDX1-positive cells displayed reduced pancreatic mass, but did not develop hyperglycemia or diabetes. β-cells of transgenic mice had a 50% increase in insulin content, suggesting a compensatory mechanism for the loss of pancreatic mass. The reduction in pancreatic mass of transgenic mice is partially attributed to decreased pancreatic cell proliferation (Papadopoulou and Edlund, 2005).

In the liver, Wnt ligands have been found to influence liver growth and regeneration, as well as regulating cell proliferation and apoptosis (Monga, 2011). Various Wnt ligands, are expressed in the liver during development (Lade and Monga, 2011), and while it is not known if all Wnts have roles in liver development, Wnt2b has been implicated in liver organogenesis during the embryogenesis (Ober et al., 2006). The conservation of Wnt2b in liver development has also been observed in zebrafish embryos, as morpholinos against *wnt2bb* led to agenesis of hepatic tissue (Ober et al., 2006). The Wnt signaling pathway is active in *Xenopus* blastula as well; however, its activity decreases during gastrulation, suggesting it is not essential for early stage organogenesis (Schohl and Fagotto, 2002). Development of the pancreas, liver and intestine can be impaired by injection of Wnt8 plasmids into D1 cells of late stage *Xenopus* embryos. Moreover, micro-injection of stabilized forms of β-catenin mRNA into blastomeres were sufficient to supress foregut markers and recapitulated the phenotypes observed under Wnt8 administration. Administering DKK1 enhanced expression of *for1* and *pdx1*, markers of the liver and pancreas, respectively. Enlarged liver and pancreas buds were observed at stage 42 of embryogenesis. Similarly, overexpression GSK3-β enhanced expression of *for1* and *pdx1* (Schohl and Fagotto, 2002).

One critical function of the hepatic Wnt signaling pathway is its role in liver zonation, which is the distribution of proteins along a protocentral axis defined by periportal (PP) and perivenous (PV) hepatocytes (Sekine et al., 2006; Yang et al., 2014). PP hepatocytes are situated near the portal vein and are exposed to high concentrations of oxygen and nutrients when compared to PV hepatocytes. Thus, enzymes requiring a high demand of oxygen and nutrients, such as gluconeogenic enzymes, are predominantly expressed in in PP hepatocytes, as they are more aerobic than PV hepatocytes (Jungermann and Katz, 1989; Braeuning et al., 2006). Meanwhile, enzymes involved in glutamine synthesis are enriched in PV hepatocytes, which provide glutamine to PP hepatocytes for urea biosynthesis (Jungermann and Katz, 1989).

Claudin-2 is a tight junction protein important for the regulation of bile composition and flow (Ma, 2020), and deletion of Claudin-2 in mice results in decreased bilary flow and impaired generation of the osmotic water gradient in bile ducts (Matsumoto et al., 2014). While it is predominantly located in PV hepatocytes, its expression pattern can be altered by inhibiting Wnt exocytosis by deleting *Wls*, the gene encoding the Wnt receptor GPR177, in murine endothelial cells (Ma, 2020). This results in decreased Claudin-2 expression in adult PV hepatocytes (Ma, 2020). However, more recent studies suggest Wnt signaling may also play a role in liver zonation of newborn mice (Boj et al., 2012). Microarray analysis of hepatocytes isolated from whole-body TCF7L2 knockout mice revealed differential expression of liver zonation genes (Boj et al., 2012). In particular, *Glu1* (Glutamine synthase) and *Rnase4* (Ribonuclease 4) were found to be upregulated at the neonatal stage, with no differences during the embryonic phase (Boj et al., 2012). These studies highlight the importance of the Wnt signaling pathway in postnatal liver development and zonation, as well as demonstrating cellular crosstalk between hepatocytes and endothelial cells to regulate Wnt activity in adult hepatocytes.

Myogenesis is the development of muscle tissue, originating in precursor satellite cells expressing PAX3/7. Satellite cells undergo commitment and differentiation into myoblasts, followed by differentiation to myotubes. The fusion of myotubes leads to the formation of specific muscle fibers (Buckingham et al., 2003; Ganassi et al., 2018; Ultimo et al., 2018). Each step of myogenesis requires the input of multiple signaling pathways that control the expression of myogenic regulatory factors, such as Myogenin, MyoD and Myf5 (Zammit, 2017), and the Wnt signaling pathway influenced the expression of these myogenic factors (Ridgeway et al., 2000; Vertino et al., 2005). Whole-body deletion of Wnt10b in mice is associated with increased myogenic potential, as defined by higher levels of MyoD and Myogenin, and this is partially mediated by an upregulation of Wnt7b (Vertino et al., 2005).

Xenopus myogenesis requires enhanced Wnt signaling activity, as dominant-negative Wnt-8 supresses induction of MyoD in embryos. Gain of function experiments in the dorsal marginal zone of the gastrula showed ectopic expression of Wnt8 is sufficient to induce MyoD expression (Hoppler et al., 1996). GSK3-β mRNA injections in the lateral region of embryos diminished expression of MyoD and Myf5, independent of their expression in the dorsal mesoderm (Shi et al., 2002). These studies suggest Wnt has local, specific effects on myogenesis during embryogenesis.

Formation of neuromuscular junctions, which are responsible for the contraction of skeletal muscle, are also dependent on Wnt ligands. Acetylcholine receptors (AChRs) are highly enriched within neuromuscular junctions (Cisternas et al., 2019), and over-expression of Wnt3 in chick wing muscles increased AChR aggregates by up to 70% (Henriquez et al., 2008). In contrast, inhibition of the Wnt signaling pathway due to over-expression of Sfrp1 decreased the number of AChR aggregates and reduced the formation of neuromuscular junctions (Henriquez et al., 2008). The actions of Wnt3 were mediated by a non-canonical Wnt signaling mechanism that required the actions of the Rho GTPase, Rac (Henriquez et al., 2008). Collectively, these findings indicate Wnt ligands are required for skeletal muscle formation and function.

β-Catenin

β-Catenin is a member of the Armadillo protein family and has been shown to have pleiotropic functions, including cell adhesion to gene transcription. One of the earliest, identified functions of β-catenin is its involvement in cell-cell adhesion via its association with E-cadherin to form adherens junctions (Valenta et al., 2012). β-catenin has also been reported to serve

as a transcriptional co-activator of the Wnt signaling pathway (Sineva and Pospelov, 2014). In early embryonic development, β-catenin is involved in establishing the body axis and is critical for tissue and organ development, including but not limited to enamel, lung, kidney, and cartilage (Eberhart and Argani, 2001; Grigoryan et al., 2008; Prakash and Swaminathan, 2015). In the postnatal period, β-catenin contributes to cell renewal, as it has been shown to mediate the regeneration of hair follicles and retinal cells (Grigoryan et al., 2008; Steinhart and Angers, 2018).

β-catenin is characterized by the presence of 12 Armadillo repeats, which help to form a rigid center with flexibility at the C- and N- termini (Huber et al., 1997). Armadillo repeats generally share 30% homology, and this provides β-catenin with the ability to have a wide range of binding partners, including proteins associated with its destruction complex, inhibitors of the Wnt pathway, and transcription factors, such as BCL9, CBP, and Pontin-52 (Peifer et al., 1994; Xu and Kimelman, 2007). Depending on its role in the cell, β-Catenin can undergo conformational changes or dimerize. For instance, dimerization with α-catenin occurs in the context of cell-cell adhesion, but with gene transcription, a monomeric, backfolded conformation is required (Pokutta and Weis, 2000; Gottardi and Gumbiner, 2004). The C-terminal domain has been found to be integral for signaling activities, whereas its Helix-C motif is required for adhesion (Xing et al., 2008). In fact, most of β-catenin's binding partners, such as 14-3-3ζ, require a Helix-C motif recognition sequence (Fang et al., 2007; Mosimann et al., 2009). Lastly, the motif recognized by GSK3-β, which marks β-catenin for degradation, is located on the N-terminal domain (Wu et al., 2003). Mutations in this region of the N-terminal domain leads to a constitutively active form β-catenin, which is found in multiple cancers (Buendia, 2002; Nejak-Bowen and Monga, 2011; Lade et al., 2012).

Proteins related to β-catenin include plakoglobin (γ-catenin), δ-catenin, and α-catenin, and like β-catenin, they associate with E-cadherin (Zhao et al., 2011). Among the related proteins, only plakoglobin exhibits a high degree of homology to β-Catenin, and it also contains 12 Armadillo repeats. Evolutionary analyses have revealed that plakoglobin is derived from a gene duplication of β-Catenin in vertebrates (Zhao et al., 2011), and despite the high degree of homology, plakoglobin, unlike β-catenin, is primarily localized to desmosomes (Lewis et al., 1997). Instead of armadillo repeats, α-catenin contains 3 vinculin domains, which has led to its classification as its own catenin subfamily (Zhao et al., 2011). Although δ-catenin has ten Armadillo repeats, it belongs to its own subfamily of catenins, named the p120 family. δ-catenin's primary binding partner is the NF-kB transcription factor (Perez-Moreno et al., 2006).

Deletion of β-catenin in PDX1-positive cells in the pancreas revealed that β-catenin is necessary for acinar development, but not for the development of endocrine cells. Knockout mice exhibited early lethality and were smaller in size than wildtype mice, and they developed pancreatic hypoplasia (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007). Histological analysis of pancreata from β-catenin-deficient, PDX1-positive cells resembled hepatocytes, suggesting that trans-differentiation had occurred (Wells et al., 2007). Conversely, transgenic mice over-expressing a degradation-resistant form of β-Catenin displayed a hypoplastic pancreas that lacked endocrine and exocrine tissue when compared to wild-type controls (Heiser et al., 2006). Immunohistological analysis of pancreata revealed that these mice displayed significant reductions in PDX1-positive pancreatic progenitor cells and increased expression of the hedgehog signaling pathway receptor, PTCH1, which is known to regulate postnatal pancreatic β-cell mass (Heiser et al., 2006; Nakayama et al., 2008). Thus, modulating intracellular levels of β-catenin in the pancreas during embryogenesis can having lasting effects on the formation of a functioning pancreas.

β-catenin's role in myogenic differentiation is not well-defined, as different groups have reported different effects of over-expression or depletion on the initiation of myogenesis (Cossu et al., 1996; Tajbakhsh et al., 1998; Goichberg et al., 2001; Mermelstein et al., 2007; Brack et al., 2008). Retroviral over-expression of β-catenin in C2 and L8 cells were sufficient to inhibit myogenesis by reducing the expression of myogenin (Goichberg et al., 2001), and this has been proposed to be mediated by adherens junctions, as myogenin levels were rescued through the co-transfection of N-cadherin (Goichberg et al., 2001). This observation was recently supported by the finding that dimerization of β-catenin with α-catenin at adherens junctions is necessary for myogenesis (Cui et al., 2019). Moreover, RNA sequencing of control and β-catenin-null myocytes treated with Wnt3a revealed an upregulation of myogenic genes in control cells but not in knockout cells (Cui et al., 2019). It should be noted that expression of a β-catenin mutant unable to bind TCF7L2 did not impair myogenesis, suggesting that β-catenin exerts its myogenic effects via a TCF7L2-independent manner (Cui et al., 2019). In contrast, depletion of β-catenin in C2C12 cells inhibited myotube differentiation (Kim et al., 2008), and β-catenin depletion in 10T1/2 fibroblasts was also found to reduce the expression of MHC, a marker of muscle cell differentiation, indicating an impairment in myogenesis (Kim et al., 2008). Critical to β-catenin's role in myogenesis was the finding that interactions of β-Catenin to MyoD via its C-terminal domain was necessary for the transcriptional activity of MyoD (Kim et al., 2008).

Human $CD56^+$ muscle cell progenitors express low levels of nuclear β-catenin prior to the induction of myogenesis, followed by a marked increase post-differentiation (Agley et al., 2017). Pharmacological inhibition of GSK-3β with BIO, CHIR, or LiCl was sufficient to inhibit differentiation, demonstrating inhibitory effects of active β-catenin myogenesis (Agley et al., 2017). Over-expression of a dominant-negative TCF7L2 in $CD56^+$ cells also reduced intracellular levels of β-catenin and impaired the onset of myogenesis, which suggests that TCF7L2 is required for β-catenin-mediated myogenesis (Agley et al., 2017).

Adenomatosis Polypsis Coli (APC)

In addition to its roles as a tumor suppressor and a component of the β-catenin destruction complex, APC is also responsible for regulating chromosome formation, DNA replication, cell cycle progression, and apoptosis (Groden et al., 1995; Morin et al., 1996; Green et al., 2005). Nonsense mutations in the *APC* gene, which are generally associated with the generation of truncated forms of APC, contribute to the development of colon cancer

(Powell et al., 1992; Laken et al., 1999; Fearnhead et al., 2001; Hankey et al., 2018).

Liver-specific deletion of APC produces an inverted expression of β-catenin along the proto-central axis and is associated with hepatomegaly and increased mortality in mice (Benhamouche et al., 2006). Knockout mice were also found to have increased circulating ammonia and elevated glutamine in the brain, which is suggestive of hepatic encephalopathy (Benhamouche et al., 2006).

In contrast to β-catenin, which is responsible for the development of the exocrine pancreas, APC appears to be important for both endocrine and exocrine cells (Sharon et al., 2019). Deletion of APC in cells expressing NEUROG3, an endocrine marker in the pancreas, impaired pancreatic endocrine cell development, as insulin- and glucagon-containing β-cells and α-cells, respectively, failed to develop (Sharon et al., 2019). In contrast, ablation of APC in exocrine cells led to an enlargement of the pancreas, characterized by acinar cell hyperplasia, and no changes in glucose homeostasis, or insulin and glucagon content were detected (Strom et al., 2007). Overall, APC is able to regulate the development of both exocrine and endocrine cells of the pancreas.

TCF7L2

TCF7L2 (also known as TCF4) is an important component of the Wnt signaling pathway, as it binds to β-catenin to mediate the expression of critical Wnt-target genes. Genome-wide association studies have revealed a strong association with SNPs close to the TCF7L2 locus and the risk of developing of type 2 diabetes (Grant et al., 2006; Boj et al., 2012). These SNPs have been associated with impaired insulin secretion and increased hepatic glucose production under periods of fasting (Florez et al., 2006; Saxena et al., 2006; Lyssenko et al., 2007; Boj et al., 2012).

Systemic deletion of TCF7L2 (TCF7L2KO) in mice results in early lethality, as newborns were considerably hypoglycemic 3 h postpartum (Boj et al., 2012). Notably, embryonic development of the endocrine pancreas was not affected, but glycogen storage, triglyceride synthesis, fatty acid oxidation, and ketone body synthesis were diminished in livers of TCF7L2KO mice (Boj et al., 2012). Indeed, *Gys2* (Glycogen Synthase 2) mRNA levels were decreased, while mRNA levels of *Pck1, G6pc*, and *Fbp1* were increased, which may account for changes in glycogen synthesis and gluconeogenesis at birth (Boj et al., 2012).

Postnatal deletion of TCF7L2 in β-cells was not associated with any effects on body weight or glucose homeostasis in mice fed normal chow or high-fat diets (Boj et al., 2012). Histological examination of pancreata did not reveal any differences in β-cell mass or β-cell proliferation, suggesting that TCF7L2-dependent Wnt signaling was not required for pancreatic β-cell development (Boj et al., 2012).

Deletion of TCF7L2 in hepatocytes (LTCF7L2KO) resulted in mild fasting hypoglycemia, similar to what was observed with systemic TCF7L2KO mice (Boj et al., 2012). Microarray analysis revealed diminished expression of genes involved in glycolysis, fatty acid metabolism, and in the Wnt signaling pathway in livers of LTCF7L2KO mice (Boj et al., 2012). Under high-fat diet conditions, LTCF7L2KO mice displayed a more severe hypoglycemic phenotype, as hepatocytes from LTCF7L2KO mice demonstrated impaired gluconeogenesis following glucagon or IBMX treatment and reduced mRNA levels of gluconeogenic genes *G6pc* and *Aldh3a2* (Boj et al., 2012). Opposite to the LTCF7L2KO mice, adenoviral-mediated over-expression of TCF7L2 increased fasting glucose concentrations, in addition to enhanced hepatic glucose production following pyruvate injections (Boj et al., 2012). Genes associated with gluconeogenesis, such as *Pck1, G6pc,* and *Fbp1,* require functional TCF7L2, as hepatic over-expression of a dominant-negative TCF7L2 mutant under the control of the *Alb* promoter, resulted in their upregulation (Ip et al., 2015). Moreover, over-expression of the TCF7L2 mutant was associated with a progressive worsening in gluconeogenesis and glucose tolerance, despite no changes in insulin sensitivity (Ip et al., 2015).

In skeletal muscle, TCF7L2 expression is low when compred to other tissues (Osmark et al., 2009); however, TCF7L2 appears to be highly expressed in muscle connective tissue fibroblasts (Mathew et al., 2011). Fibroblast-specific deletion of TCF7L2 in mice was associated with reduced expression of *Myh7*, a marker of myogenic maturation, in slow-muscle fibers. However, its expression was upregulated in the skeletal muscle of adult mice, with the exception of the soleus muscle, suggesting that TCF7L2 exerts developmental effects on myogenic maturation. Deletion of TCF7L2 in fetal myogenic cells using a *Pax7*-Cre driver was associated with reduced *Myh7* and *Myh2* gene expression in hind limb muscles and the diaphragm, supporting the hypothesis that TCF7L2 is important for myogenesis (Mathew et al., 2011).

CONTRIBUTIONS OF WNT-β-CATENIN SIGNALING EFFECTORS TO METABOLISM

Since establishing the roles of Wnt signaling and its downstream effectors in the development of metabolic tissues and organs, the individual contributions of effectors to cellular and whole-body metabolism is now gaining wide appreciation. This includes their metabolic contributions in various metabolic organs and tissues, including liver, brain, pancreas, and adipose tissue (**Tables 4**, **5** and **Figure 2**).

Wnt

Activation of Wnt signaling has been found to increase pancreatic β-cell proliferation and the expression of cell cycle genes via the actions of the transcription factor PITX2 (**Table 4**; Rulifson et al., 2007). β-cell-specific over-expression of a constituitively active form of β-catenin was found to stimulate β-cell expansion. Conversely, inhibition of Wnt signaling by over-expression of the Wnt signaling inhibitor, AXIN, led to β-cell hypoplasia and dysregulated islet architecture (Rulifson et al., 2007). Increasing or decreasing Wnt signaling was associated with enhanced or defective insulin secretion, respectively, along with changes in glucose tolerance (Rulifson et al., 2007).

Adipogenesis, or adipocyte differentiation, in white adipose tissue is a two-step process by which mesenchymal stem cells commit to preadipocytes, followed by differentiation into mature

TABLE 4 | Metabolic phenotype resulting from the over-expression or deletion of Wnt signaling effectors in mice.

Effector	Model	Effect	Reference
β-Catenin	*Pdx1*-Cre: *Ctnnb1*$^{flox/flox}$	– Fasting hyperglycemia – Reduced glucose clearance – Increased insulin resistance	Elghazi et al., 2012
	OE (*Pdx1*-driven expression of a constitutively active)	– Hypoplasic pancreas – Lacking endocrine and exocrine tissue	Heiser et al., 2006
	OE (Ad-Cre: *Ctnnb1*$^{flox/flox}$)	– Reduced hepatic glucose production – Improved insulin tolerance – Improved glucose tolerance	Liu et al., 2011
	OE (adenovirus in hepatocytes)	– Stimulate hepatic gluconeogenesis	Liu et al., 2011
	OE (*RIP*-Cre driven)	– Expansion of β-cell mass – Decreased fasting glucose	Rulifson et al., 2007
	Adipoq-Cre: *Ctnnb1*$^{flox/flox}$	– Reduced body weight – Reduced body size – Improved insulin sensitivity	Chen et al., 2019
	OE (*aP2*-Cre driven constitutively active)	Fibrosis in subdermal tissue	Zeve et al., 2012
	OE (*Pparg*-tTA; TRE-Cre driven constitutively active)	– Reduction of VAT – Elevated serum triglyceride – Fibrosis in SCAT – Depletion of adipocytes	Zeve et al., 2012
	HAS-MCM-Cre: *Ctnnb1*$^{flox/flox}$	– Reduced insulin-stimulated glucose transport – Mild glucose intolerance – Mild insulin resistance	Masson et al., 2020
APC	*TTR*-Cre^{Tam}: *APC*$^{flox/flox}$	– Hepatomegaly – Increased mortality – Increase in circulating ammonia/glutamine levels in the brain	Benhamouche et al., 2006
TCF7L2	*Tcf7l2*$^{-/-}$	– Lethality – Hypoglycemia – Impaired hepatic function	Boj et al., 2012
	RIP-Cre-ER^{T2}: *Tcf7l2*$^{flox/flox}$	No effect	Boj et al., 2012
	OE (Ad-Cre: *Tcf7l2*$^{flox/flox}$)	– Increase fasting glucose – Increase hepatic glucose production	Boj et al., 2012
	SA-Cre-*ER*^{T2}: *Tcf7l2*$^{flox/flox}$	Mild fasting hypoglycemia	Boj et al., 2012
	Adipoq-Cre: *Tcf7l2*$^{flox/flox}$	– Glucose intolerant – Hepatic insulin resistance	Chen et al., 2018
	Adipoq-Cre: *Tcf7l2*$^{flox/flox}$	Weight gain	Geoghegan et al., 2019
	Adipoq-Cre: *Tcf7l2*$^{flox/flox}$	Reduced insulin secretion	Nguyen-Tu et al., 2021
GSK3B	*Gsk3B*$^{-/-}$	Embryonic lethality	MacAulay and Woodgett, 2008
	Gsk3B$^{-/-}$	Embryonic lethality	Hoeflich et al., 2000
	OE (*RIP*-Cre driver, crossed with *Ir*$^{\pm}$)	– Impaired glucose tolerance – Smaller β-cells	Liu et al., 2008
	SA-Cre: *Gsk3B*$^{flox/flox}$	No effect	Patel et al., 2008
	Mlc1f-Cre: *Gsk3B*$^{flox/flox}$	– Enhanced glucose sensitivity – Improved insulin sensitivity	Patel et al., 2008

KO, knockout; OE, over-expression.

TABLE 5 | Metabolic phenotype resulting from over-expression or deletion of Wnt signaling effectors in *Drosophila.*

Effector	Model	Effect	Reference
Axin	FRT82B Axn127/TM6b	– Decreased adipogenesis – Reduction of diacylglycerol, free fatty acid and trigylcerides	Zhang et al., 2017
Wingless	Mef2-Gal4Mhc-Gal4Tin-Gal4	– Increased whole body triglyceride	Lee et al., 2014
Ck1-α	RNAi-mediated knockdown	Upregulation of haemolymph glucose	Ugrankar et al., 2015

adipocytes. The differentiation of preadipocytes is governed by the transcription factors PPARγ and CEBP-α, which drive committed preadipocytes toward terminal differentiation (Cristancho and Lazar, 2011). In general, activation of the Wnt-β-catenin pathway in pre-adipocytes inhibits adipogenesis with the exception of the non-canonical Wnt5b-related

signaling, which potentiates adipocyte differentiation of 3T3-L1 preadipocytes (Kanazawa et al., 2005; Cristancho and Lazar, 2011). Moreover, some Wnt proteins, such as Wnt6 and Wnt10, can also inhibit the maturation of thermogenic brown adipocytes (Tseng et al., 2005). Wnt proteins have also been reported to increase leptin production, as acute administration of Wnt3a to differentiated 3T3-L1 cells is sufficient to enhance leptin mRNA levels (Chen et al., 2015).

Wingless (*wg*) has also been implicated in obesity in fruit flies. Abdominal fat body mass was increased following knockdown of *wg* using a Mef2-Gal4 driver (**Table 5**; Lee et al., 2014). Mhc-Gal4 and Tin-Gal4 drivers were also used to supress *wg* in the muscle and heart, respectively, and in both models, whole body triglyceride content was increased (Lee et al., 2014). Overexpression of *wg* in muscle exhibited the opposite phenotype, with a decrease in total fat accumulation, indicating Wingless can function as a repressor of adipogenesis (Riechmann et al., 1998). Indeed, deletion of *wg* in embryos showed a complete absence of fat in the ventral section. Conversely, the dorsolateral segment displayed high expression of adipocyte progenitors. Thus, *wg* appears to be essential for ventral adipogenesis and is required to supress dorsolateral mesodermal fat development. Gonadal agenesis was also reported in the mutant embryos, suggesting all dorsolateral cells were converted to adipocyte progenitors (Riechmann et al., 1998).

Non-canonical Wnt pathways have also been implicated in metabolic diseases. Wnt5A was found to be upregulated in visceral adipose tissue (VAT) of obese individuals compared to subcutaneous adipose tissue (SCAT) (Fuster et al., 2015), and Wnt5a inhibition in mice fed a high-fat diet prevented the development of metabolic dysfunction, with a reduction in insulin and glucose levels (Fuster et al., 2015). However, no changes in body weight or body fat percentage were detected, suggesting no effects on adipogenesis. Interestingly, proinflammatory cytokines such as TNF-α and CCL2/MCP-1 were downregulated, indicating a potential reduction in inflammation. sFRP5, which sequesters and antagonizes Wnt5a was also found to produce anti-inflammatory effects in mice (Ouchi et al., 2010). Deletion of sFRP5 in leptin-deficient *Ob*/*Ob* mice on a high-fat diet elevated glucose and triglyceride levels. Additionally, increases in F4/80 cells were detected in epididymal white adipose tissue, indicating increased inflammation (Ouchi et al., 2010).

LRP6 is essential for Wnt binding to Fz in the canonical pathway, but it can also inhibit the non-canonical Wnt signaling pathway (Bryja et al., 2009). LRP6 mutant mice, with nucleotide substitutions at 10443 and 10445, were found to have an enhanced levels of cytokines, MPO, IL-6, and CD68, in the liver (Wang et al., 2015). Histological analysis revealed mutant mouse livers to have a greater accumulation of lipid droplets, indicative of hepatic steatosis. Modest reductions in β-catenin were detected in the liver, with increased expression of RhoA and ROCK2 mRNA, suggesting enhanced activity of the non-canonical pathway. Fibrosis was also noted in the liver of mutant mice following high-fat diet feeding. Notably, recombinant Wnt3a administration was able to rescue the mutant phenotype and reduced inflammation (Wang et al., 2015). These findings demonstrate contrasting and opposing functions of the canonical and non-canonical Wnt pathways within the same tissue.

β-Catenin

Gain- and loss-of-function approaches have revealed that β-catenin can influence hepatic metabolism (Liu et al., 2011). Adenovrial-mediated depletion of β-catenin in hepatocytes attenuated hepatic gluconeogenesis, resulting in hypoglycemia. Conversely, over-expression of β-catenin had opposite effects on hepatic glucose production. Levels of mRNA corresponding to the rate-limiting gluconeogenic enzymes Phosphoenolpyruvate carboxykinase (*Pck)* and Glucose-6-phosphatase (*G6p*) *in vivo*, as well as in Hepa1-6 hepatoma cells, were decreased or increased following depletion or over-expression of β-catenin, respectively (Liu et al., 2011). Under high-fat diet feeding conditions, depletion of β-catenin in hepatocytes was associated with improved glucose tolerance and reduced hepatic gluconeogenesis (Liu et al., 2011).

In pancreatic endocrine cells, β-catenin is essential for β-cell function and survival. Targeted deletion of β-catenin in murine β-cells during embryogenesis resulted in severe hypoglycemia and premature death. Moreover, β-cells from knockout mice displayed impaired insulin release (Dabernat et al., 2009). In various *in vitro* β-cell models, acute depletion of β-catenin has also been shown to impair glucose-stimulated insulin secretion (GSIS) due to negative effects on re-modeling of the actin cytoskeleton (Sorrenson et al., 2016). Deletion of β-catenin in the exocrine pancreas resulted in mice that were smaller in size and weight when compared to controls, in addition to impaired development of exocrine tissues (Elghazi et al., 2012). Deletion of β-catenin in exocrine tissue was also associated with fasting hyperglycemia, in addition to lower rates of glucose clearance and insulin sensitivity (Elghazi et al., 2012). Depletion of β-catenin also led to impaired pancreatic function and growth, as knockout mice displayed reduced β-cell proliferation and β-cell mass (Wells et al., 2007; Elghazi et al., 2012).

Recently, β-catenin levels have been found to be elevated in subcutaneous white adipose tissue from individuals with obesity (Chen et al., 2019). Similarly in mice, over-feeding was sufficient to increase β-catenin expression in murine adipose tissue, suggesting pro-adipogenic or -obesogenic roles in adipose tissues (Chen et al., 2019). Indeed deletion of β-catenin in adipocytes was found to be protective against high-fat diet-induced weight gain and improved insulin sensitivity. The attenuation of fat mass expansion was associated with significant reductions in proliferating PDGFRA-positive pre-adipocytes (Chen et al., 2019).

Over-expression of β-catenin in mature adipocytes under the control of the aP2 promoter was associated with fibrosis in subdermal tissues, with no other significant changes under normal chow diet conditions (Zeve et al., 2012). In contrast, β-catenin over-expression in adipocyte progenitor cells led to significant reductions in visceral white adipose tissue and circulating levels of leptin and adiponectin. Interestingly, over-expression of β-catenin in adipocyte progenitor cells was not

associated with glucose intolerance or insulin resistance, as would be expected from a lipodystrophic phenotype (Zeve et al., 2012).

Inhibitors of the Wnt/β-catenin pathway have been shown to promote adipogenesis. For example, the β-catenin antagonist Chibby, which removes unphosphorylated β-catenin from the nucleus (Killoran et al., 2015), is necessary for adipogenesis. Depletion or ectopic over-expression of Chibby can inhibit or promote spontaneous adipogenesis, respectively (Li et al., 2007). Similarly, the Wnt antagonist, Dickkopf1 (DKK1), has similar effects in promoting adipogenesis. DKK1 inhibits the Wnt pathway by binding the receptors LRP5/6, and *in vitro* assays using human pre-adipocytes have shown that DKK1 expression increases during the early stages of adipogenesis (Christodoulides et al., 2006). In conditions associated with sub-optimial differentiation of 3T3-L1 adipocytes, ectopic expression of human DKK1 could restore differentiation to the same extent as complete adipogenic conditions due to inhibition of Wnt signaling (Christodoulides et al., 2006).

Skeletal muscle is responsible for 70–90% of glucose uptake from the circulation, and this process is impaired in individuals with type 2 diabetes (Evans et al., 2019). Although the Wnt signaling pathway is known to be involved in myogenesis, it has also been found to participate in skeletal muscle glucose metabolism. Deletion of β-catenin in skeletal muscle was found to diminish insulin-stimulated glucose transport in mice, and this was also associated with mild glucose intolerance and insulin resistance (Masson et al., 2020). *In vitro* studies with isolated muscle and myocytes demonstrate that knockdown of β-catenin can also reduce GLUT4 protein abundance upon insulin stimulation, demonstrating impaired insulin action (Masson et al., 2020).

Axin

Removal of exon 11 of Axin (*Axn)* in *Drosophila* and its replacement with a repetitive heterochromatin sequence resulted in a hyperactive Wnt signaling response, as Armadillo (B-catenin's *Drosophila* ortholog) expression increased (Zhang et al., 2017). Homozygous *Axn* mutants displayed decreased accumulation of abdominal adipocytes at mid to late larval stages, along with lower levels of diacylglycerol, free fatty acid, and triglycerides. Furthermore, RNAi-mediated knock down of Armadillo successfully rescued the phenotype (Zhang et al., 2017).

The axin ortholog in *C.elegans*, PRY-1, was recently shown to be involved in lipid metabolism (Ranawade et al., 2018). Worms with a nonsense *pry-1* mutation had enhanced Wnt activity, demonstrating that β-catenin activity on lipid metabolism is conserved across species. Mutants exhibited a reduction in lipid content, egg laying and survival following starvation. Genetic studies revealed a downregulation in *vits* and *fats*, the genes responsible for yolk lipoproteins and fatty acid desaturases (Ranawade et al., 2018).

CK1-α

Ck1-α emerged as a candidate gene with high association to hyperglycemia in *Drosophila* (Ugrankar et al., 2015). RNAi-mediated knockdowns in adipose tissue or muscle of third instar larvae led to an increase in haemolymph glucose levels. Most notably, *CSNK1a1* knockouts in murine pre-adipocytes recapitulated the hyperglycemic phenotype, indicating a species-conserved function of CK1-α (Ugrankar et al., 2015).

GSK3

GSK-3 is an important regulator of glycogen synthesis, as it is responsible for inhibiting glycogen synthase to attenuate glycogenolysis and glucose production (MacAulay and Woodgett, 2008). GSK-3 has two paralogs, GSK-3α and GSK-3β, and while systemic knockouts of GSK-3β are embryonically lethal due to enhanced TNF-α-associated hepatocyte apoptosis, systemic GSK-3α knockouts were found to be viable (Hoeflich et al., 2000; MacAulay and Woodgett, 2008). In addition, improved glucose tolerance, as well as higher insulin sensitivity, were observed, which may be attributed to increased mRNA levels of Glycogen Synthase and Insulin Receptor Substrate-1 (MacAulay and Woodgett, 2008).

Deletion of the insulin receptor (IR) in mice is known to cause perinatal lethality due to diabetic ketoacidosis, and mice heterozygous for *Ir* display mild insulin resistance and compensatory increases in β-cell mass. Partial deletion of the insulin receptor is also associated with significant hyperglycemia and hyperinsulinemia, despite exhibiting no changes in insulin signaling (Bruning et al., 1997). GSK3β is involved in the insulin signaling pathway, as it inactivates glycogen synthase by phosphorylating serine residues on its C-terminal domain. GSK3β can also inhibit the insulin pathway by phosphorylating the insulin receptor substrate-1 (IRS-1) directly (Liberman and Eldar-Finkelman, 2005). Mice heterozygous for *Gsk3b* (*Gsk-3*$\beta^{\pm}$) exhibited decreased fed and fasting insulin levels, and when bred with mice heterozygous for *Ir*, haploinsufficiency of GSK3-β was able to inprove the metabolic phenotype of heterozygous *Ir* mice (Tanabe et al., 2008). Previous studies have shown that whole body-IRS2 knockout mice resemble insulin receptor knockout mice, as they displayed insulin resistance, hyperglycemia, reduced β-cell mass, and decreased body weight (Uchida et al., 2005). When compared to systemic IRS2 knockout mice, GSK-3β haploinsufficient mice on an *Irs2*-null background did not exhibit loss of β-cell mass due to increased β-cell proliferation and reduced rates of β-cell apoptosis (Uchida et al., 2005). Expansion of β-cell mass was regulated by reductions in the Cyclin-dependent kinase inhibitor, $p27^{Kip1}$, a rate limiting factor in cell proliferation (Uchida et al., 2005; Rachdi et al., 2006).

Deletion of GSK-3β in β-cells is sufficient to partially rescue the diabetic phenotype of IRS2 knockout mice, as mice were normoglycemic despite being hyperinsulinemic (Tanabe et al., 2008). This was attributed to the deletion of GSK-3β resulting in reducing the incidence of apoptosis and promoting β-cell proliferation (Tanabe et al., 2008). Over-expression of a constitutively active GSK3-β (RIP-GSK3βCA) mutant in β-cells led to impaired glucose tolerance, which was only observed in male mice. Histological analysis of pancreata revealed that transgenic mice had decreased β-cell area, as well as impaired β-cell proliferation. Moreover, over-expression of a constituitively active GSK3-β mutant led to defects in insulin secretion and resulted in impaired glucose tolerance (Liu et al.,

2008). In conjunction, these studies demonstrate the importance of GSK-3β in regulating β-cell mass and proliferation.

In liver-specific GSK-3β knockout mice, no significant metabolic changes were observed, thereby suggesting that GSK-3β in the liver is dispensable for whole-body metabolism (Patel et al., 2008). Skeletal muscle-specific knockout of GSK-3β mediated by *Mlc1f*-promoter driven Cre-mediated recombination results in mice with enhanced glucose tolerance. Morover, these mice had increased glycogen synthase, resulting in improved insulin signaling and actions, and this highlights GSK-3β's importance in regulating insulin action in a tissue specific manner (Patel et al., 2008).

In the context of obesity, GSK-3β inhibition has been found to have beneficial metabolic effects. Administration of the GSK3 inhibitors SB216763 or CHIR99021 to high-fat diet-induced obese mice improved insulin sensitivity without any changes in fat mass or body weight (Wang et al., 2018). Furthermore, GSK-3 inhibition was found to influence macrophage polarization such that increased numbers of anti-inflammatory M2 macrophages could be detected (Wang et al., 2018). Additionally, *in vitro* studies performed on human adipose- derived stem cells showed that GSK3 inhibitors, such as LiCl and BIO, have the ability to inhibit cell proliferation, and BIO-treated human adipose-derived stem cells were unable to differentiate to adipocytes in the presence of adipogenic stimuli (Zaragosi et al., 2008).

TCF7L2

Glucagon-like peptide-1 (GLP-1), which is derived from intestinal endocrine L cells, potentiates glucose-stimulated insulin secretion through actions on the GLP-1R on the surface of β-cells (Lim and Brubaker, 2006). In L cells, TCF7L2 participates in GLP-1 synthesis by regulating the transcription of *Gcg*, thereby representing an alternative mechanism whereby the Wnt signaling pathway can influence insulin secretion from β-cells (Shao et al., 2013). Indeed, targeted transgenic expression of a dominant-negative TCF7L2 mutant in neurons and enteroendocrine cells led to significant reductions in *Gcg*-positive neurons and GLP-1-positive cells, respectively

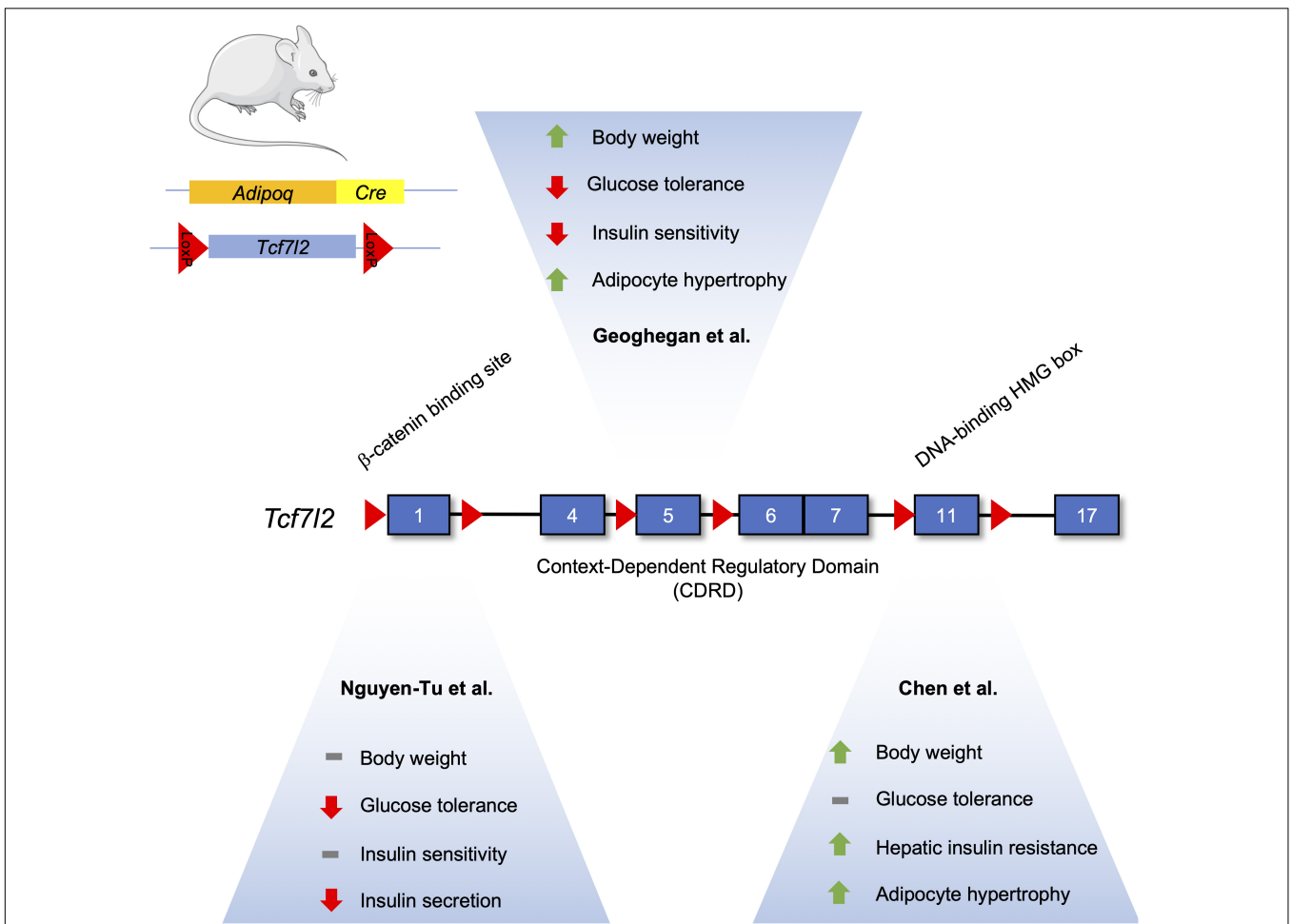

FIGURE 3 | Differences in the metabolic phenotype of adipocyte-specific TCF7L2 knockout mouse models. The *Tcf7l2* gene contains 17 exons, as denoted by numerated blue rectangles. Recombination of floxed alleles for Exon 1 (Nguyen-Tu et al., 2021), Exon 5 (Geoghegan et al., 2019), and Exon 11 (Chen et al) by Cre recombinase under the control of the *Adipoq* promoter results in different metabolic phenotypes. This figure was made with modified images from Servier Medical Art (Creative Commons Attribution 3.0 Unported License).

(Shao et al., 2013, 2015). Although these mutant mice were found to be glucose intolerant and insulin resistant, compensatory increases in β-cell mass were detected (Shao et al., 2013). In adult mice, GLP-1 was found to induce the phosphorylation of β-catenin by cAMP/PKA or insulin/PAK-1 signaling pathways to facilitate β-cell proliferation (Xiong et al., 2012; Shao et al., 2013).

To examine TCF7L2's effects on type 2 diabetes in zebrafish, heterozygous knockouts were generated by introducing a mutation in intron 1, which produced a truncated protein (Facchinello et al., 2017). Adult fish displayed postprandial hyperglycemia. Reduction of the overall size of the fish was observed, as well as reductions in the sizes of the endocrine and exocrine pancreas. Interestingly, the mutant exocrine pancreas was also found to have an accumulation of adipose tissue, along with a decrease in number of β-cells, suggesting a mechanism by which deficiency in TCF7L2 may lead to a diabetic phenotype protein (Facchinello et al., 2017).

TCF7L2 has been demonstrated to have important roles in adipogenesis, but it is not without controversy (**Figure 3**). One study suggests its expression increases during adipogenesis, which may seem contradictory as activated Wnt signaling is known to inhibit adipocyte differentiation (Cristancho and Lazar, 2011; Chen et al., 2018). Indeed, knockdown of TCF7L2 via short-hairpin RNA in 3T3-L1 cells inhibited adipogenesis and downregulated *Slc2a4* (GLUT4) mRNA. Adipocyte-specific TCF7L2 knockout mice were glucose intolerant and showed signs of hepatic insulin resistance that was associated with increased gluconeogenesis. Under high-fat diet conditions, knockout mice gained more weight and showed increased hypertrophy of inguinal white adipose tissue, and gene expression analysis of inguinal white adipose tissue displayed an increase in the Wnt target gene, *Axin2*, suggesting that the reduction of TCF7L2 activated the Wnt signaling pathway to initiate adipocyte hypertrophy.

In contrast, Geoghegan et al. (2019) have reported that TCF7L2 expression is reduced during adipogenesis in both *in vitro* and *in vivo* models. Mice fed a high fat diet exhibited an increased abundance of TCF7L2 and adiponectin expression in epididymal white adipose tissue, and this increase in TCF7L2 abundance could be recapitulated in adipose tissue from *ob/ob* and *db/db* mice (Geoghegan et al., 2019). Adipocyte-specific deletion of TCF7L2 led to weight gain when compared to control mice fed a normal chow diet. On a high-fat diet, knockout mice displayed significantly higher weight gain, in addition to impaired glucose tolerance and insulin resistance. Histological analysis revealed hypertrophy in inguinal WAT depots and lipolytic genes, such as *Tgh* and *Tgh2*, were downregulated. Adipocyte-specific knockout of TCF7L2 was associated with increased lipid storage, due in part to changes in *Tgh1* (triacylglycerol hydrolase) and *Tgh2* gene expression, and adipocyte hypertrophy (Geoghegan et al., 2019).

Further studies done by Nguyen-Tu et al. (2021) on TCF7L2 knockout mice using an *Adipoq*-Cre driver recapitulated the hyperglycemic phenotype seen by Chen et al. (2018). However, this phenotype was only seen in 16-week old males, and they were not insulin resistant. Insulin secretion was abrogated in isolated islet *ex vivo*, possibly due to the decreased islet expression of *Glut2* mRNA. No differences in insulin secretion were seen *in vivo* under normal chow-fed conditions, possibly due to compensatory increases in circulating levels of NEFAs that can potentiate glucose-stimulated insulin secretion. Furthermore, under high-fat diet, no weight changes were seen but TCFL2 knockout diminished insulin secretion (Nguyen-Tu et al., 2021).

All three studies examining TCF7L2's effects on adipocyte function and adipogenesis were performed on male mice using different Adipocyte-specific Cre driver mice. However, the exons targeted for recombination were also different for all three studies. Chen et al., floxed exon 11, known to encode the DNA binding HMG box (Hansson et al., 2010). On the other hand, Geoghegan et al. (2019) used mice with lox-p sites flanking exon 5, while Nguyen-Tu et al. (2021) used exon 1 floxed mice. Both found TCF7L2 to have an inhibitory role on adipogenesis. Taken together, the common and differing phenotypes associated with TCF7L2 deletion demonstrate the complexity of the biological actions of the Wnt signalng pathway in adipocytes.

CONCLUSION AND PERSPECTIVES

Although the Wnt signaling pathway is conserved across species and is necessary for embryogenesis and development, it is now clear that it also has essential metabolic roles in mammals. Moreover, aberrant expression or activity of various downstream effectors in the Wnt signaling pathway is associated with the development of chronic diseases, including diabetes and obesity. Given the pathway's wide ranging effects and multiple downstream targets, more research is required to further our understanding of its involvement across different metabolic diseases.

AUTHOR CONTRIBUTIONS

FAA wrote the manuscript. GEL edited the manuscript and the guarantor of this work. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Regulation of the Low-Density Lipoprotein Receptor-Related Protein LRP6 and Its Association With Disease: Wnt/β-Catenin Signaling and Beyond

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Wnt signaling plays crucial roles in development and tissue homeostasis, and its dysregulation leads to various diseases, notably cancer. Wnt/β-catenin signaling is initiated when the glycoprotein Wnt binds to and forms a ternary complex with the Frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Despite being identified as a Wnt co-receptor over 20 years ago, the molecular mechanisms governing how LRP6 senses Wnt and transduces downstream signaling cascades are still being deciphered. Due to its role as one of the main Wnt signaling components, the dysregulation or mutation of LRP6 is implicated in several diseases such as cancer, neurodegeneration, metabolic syndrome and skeletal disease. Herein, we will review how LRP6 is activated by Wnt stimulation and explore the various regulatory mechanisms involved. The participation of LRP6 in other signaling pathways will also be discussed. Finally, the relationship between LRP6 dysregulation and disease will be examined in detail.

Keywords: LRP6, Wnt, cancer, metabolism, signaling

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INTRODUCTION

Wnt signaling has crucial roles in development and tissue homeostasis (Nusse and Clevers, 2017). The interaction between Wnt, Frizzled, and lipoprotein receptor-related protein 5/6 (LRP5/6) activates Wnt signaling. If the main output of Wnt signaling activation is stabilization of the transcriptional activator β-catenin, the pathway is known as canonical Wnt or Wnt/β-catenin signaling (hereafter referred to as "Wnt/β-catenin signaling"). In the absence of Wnt, the scaffold protein Axin together with adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase 1 alpha (CK1α) form the so called destruction complex that binds cytoplasmic β-catenin, leading to its phosphorylation by CK1α and GSK3β. Phosphorylated β-catenin is ubiquitinated by the $SCF^{\beta\text{-}Trcp}$ E3 ubiquitin ligase complex, a process that targets it for proteasomal degradation (Aberle et al., 1997; Kitagawa et al., 1999; Liu et al., 2002). In the presence of Wnt, the β-catenin destruction complex is recruited to the plasma membrane

and inactivated (**Figure 1A**). As a result, β-catenin is stabilized and then translocates to the nucleus to activate the expression of target genes involved in cell proliferation, differentiation, stem cell self-renewal and many other biological processes (MacDonald et al., 2009). In non-canonical Wnt signaling, Wnt (e.g., Wnt5a) transduces signaling without β-catenin stabilization by activating alternative downstream cascades such as JUN kinase, planar cell polarity (PCP), or calcium signaling (Kikuchi et al., 2009). It is well known that dysregulation of Wnt signaling causes developmental disorders and several diseases such as cancer (Nusse and Clevers, 2017). Notably, hyper-activation of β-catenin, due to mutations in *APC*, *AXIN*, or *CTNNB1* (gene for β-catenin), is a well-known risk factor for carcinogenesis, especially colon cancer (Bugter et al., 2021).

The type I single transmembrane protein LRP6 is a member of the LDLR gene family of receptors that is highly conserved among species (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). The extracellular region of LRP6 contains four YWTD (Tyr-Trp-Thr-Asp)-type β-propellers, followed by EGF-like domains (E1–E4), and three LDLR type A domains (Cheng et al., 2011), and its intracellular region contains five PPPS/TP (Pro-Pro-Pro-Ser/Thr-Pro) motifs (Tamai et al., 2004). Formation of the Wnt-FZD-LRP6 ternary complex at the plasma membrane (i.e., Wnt-on state) induces phosphorylation of the intracellular region of LRP6 (MacDonald and He, 2012). Phosphorylation of LRP6 is therefore considered a hallmark of Wnt/β-catenin signaling activation. Contrary to Wnt, the secreted Wnt modulator Dickkopf (Dkk) binds to LRP6 and promotes its membrane clearance, thereby functioning as an LRP6 antagonist (Mao B. et al., 2001; Mao et al., 2002). Owing to its importance in Wnt/β-catenin signaling transduction, mutation or dysregulation of LRP6 is implicated in several diseases (Joiner et al., 2013). LRP5, which is a paralog of LRP6 and shares 71% sequence conservation (Tamai et al., 2000), plays a similar role as LRP6 in transducing Wnt/β-catenin signaling (Mao J. et al., 2001); however, the biological functions of LRP6 and LRP5 differ significantly (Joiner et al., 2013). In this review we will mainly focus on LRP6. We will describe the molecular mechanisms governing Wnt-dependent LRP6 activation, and discuss how LRP6 function is regulated by various factors. We will also discuss LRP6's role in the regulation of downstream Wnt/β-catenin signaling, provide examples of its involvement in Wnt/β-catenin-independent pathways, and illustrate how dysregulation or mutation of LRP6 can lead to several diseases.

CORE MECHANISMS OF LRP6 ACTIVATION VIA Wnt STIMULATION

In 2000, LRP6 was identified as a co-receptor for Wnt and FZD to transduce Wnt/β-catenin signaling (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). The extracellular domain of LRP6 interacts with Wnt and activates Wnt/β-catenin signaling at the plasma membrane. LRP6 with a truncated extracellular domain is constitutively active and can potentiate Wnt/β-catenin signaling independently of Wnt (Liu et al., 2003). Conversely, LRP6 with a truncated intracellular domain acts as a dominant-negative form, inhibiting Wnt/β-catenin signaling (Tamai et al., 2000). There are five PPPS/TP motifs in the LRP6 intracellular domain, and the serine/threonine residues in these motifs are phosphorylated upon Wnt stimulation (Tamai et al., 2004). GSK3β and CK1γ are the main kinases that phosphorylate the PPPS/TP motifs and their flanking regions, respectively (Davidson et al., 2005; Zeng et al., 2005). Dishevelled (DVL) proteins are essential for Wnt-induced LRP6 aggregation with FZD, and the complex formed between LRP6, FZD, and DVL relies on the DIX and PDZ domains of DVL (Zeng et al., 2008; **Figure 1B**). In the Wnt-on state, additional Wnt/β-catenin signaling components such as Axin, CK1α, and GSK3β form a complex with LRP6 known as the signalosome (Bilic et al., 2007). Signalosome formation leads to further LRP6 phosphorylation by GSK3β that in turn promotes more aggregation of Wnt/β-catenin signaling components (Zeng et al., 2008). The end result is increased dissociation of β-catenin away from the destruction complex, allowing it to accumulate in the cytoplasm and then translocate to the nucleus (Cselenyi et al., 2008; Wu et al., 2009). Wnt3a-induced activation of LRP6 is rapid, and aggregation of components involved in Wnt/β-catenin signaling can be observed as early as 30 min by live cell imaging (Bilic et al., 2007). Another important player in the signalosome is PIP5K1, a phosphatidylinositol phosphate kinase whose activation is mediated by FZD and DVL (Pan et al., 2008). Activation of PIP5K1 leads to production of phosphatidylinositol 4,5-bisphosphate (ptdIns(4,5)P_2), which in turn induces aggregation and phosphorylation of LRP6 (Pan et al., 2008; **Figure 1C**). Hence, non-protein components such as phospholipids can also play crucial roles in LRP6-mediated Wnt/β-catenin signaling.

REGULATION OF LRP6 FUNCTION AND DOWNSTREAM SIGNALING

Phosphorylation

As described above, in the presence of Wnt, the five PPPS/TP motifs in the intracellular domain of LRP6 are mainly phosphorylated by GSK3β and CK1γ. However, additional ligands, kinases or interacting proteins have also been shown to regulate LRP6 phosphorylation and thus influence Wnt/β-catenin signaling. First, we review how these components affect LRP6 phosphorylation and positively regulate Wnt/β-catenin signaling. Similar to GSK3β, G protein-coupled receptor kinases 5 and 6 (GRK5/6), mitogen-activated protein kinases (MAPKs), such as p38, extracellular signal regulated kinase 1 and 2 (ERK1/2), and c-Jun N-terminal kinases 1 (JNK1) also interact with LRP6 and induce phosphorylation of its PPPS/TP motifs (Chen et al., 2009; Červenka et al., 2011; **Figure 2**). For example, fibroblast growth factor 2 (FGF2)-induced ERK activation leads to phosphorylation of the S1490 or T1572 residues of LRP6, resulting in Wnt/β-catenin signaling activation (Krejci et al., 2012). Parathyroid hormone (PTH), a crucial factor for bone formation, acts as another LRP6 regulator by forming a ternary complex with PTH1 receptor (PTH1R) and LRP6 to facilitate PPPS/TP phosphorylation (Wan et al., 2008). In addition, several proteins interact with LRP6 and thereby

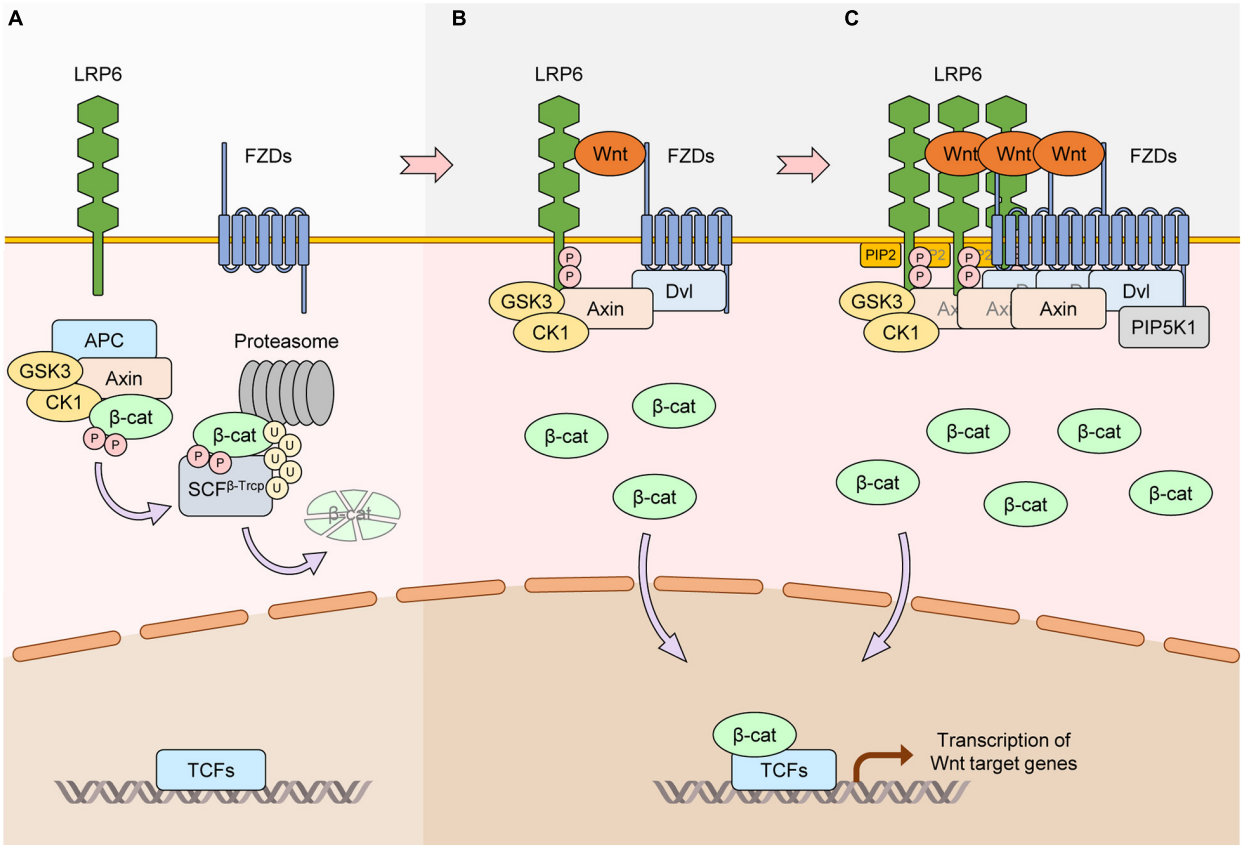

FIGURE 1 | Core activation mechanisms of LRP6 via Wnt stimulation. **(A)** In the absence of Wnt, the scaffold protein Axin together with APC, GSK3, and CK1 form the β-catenin destruction complex. β-catenin interacts with the complex and is phosphorylated by GSK3 and CK1α (CK1). Phosphorylated β-catenin is ubiquitinated by $SCF^{β\text{-}Trcp}$ and degraded by the proteasome. Protein levels of β-catenin thus remain low, and β-catenin-dependent transcription of Wnt/β-catenin target genes is suppressed. **(B)** Wnt interacts with the FZD and LRP6 receptors. Axin-bound GSK3 and CK1γ phosphorylate PPPS/TP motifs in the intracellular domain of LRP6. Phosphorylated LRP6 serves as a docking site for Axin, facilitating the interaction between Axin and LRP6 and inhibiting the kinase activity of GSK3. This causes the dissociation and inactivation of the β-catenin destruction complex, leading to β-catenin stabilization and activation of Wnt/β-catenin target gene transcription. **(C)** Treatment of Wnt induces LRP6 aggregates in a DVL-dependent manner. In this condition, FZDs, Axin, and GSK3 can also aggregate with LRP6, generating LRP6 signalosomes. PIP_2 is generated via DVL-bound PIP5K1. PIP_2 accelerates the formation of LRP6 signalosomes and phosphorylation of LRP6, resulting in further activation of Wnt/β-catenin signaling.

enhance its phosphorylation and Wnt/β-catenin signaling by modulating LRP6 localization or acting as a scaffold for LRP6 and other Wnt components. For instance, the G protein $Gβ_1γ_2$ promotes GSK3 localization to the plasma membrane, which in turn promotes LRP6 phosphorylation (Jernigan et al., 2010). DVL is well-known for playing a crucial role in signalosome formation. Ectopic expression of the DVL DIX domain fused to the LRP6 intracellular domain promotes Wnt/β-catenin signaling via formation of cytoplasmic signalosomes and induction of LRP6 phosphorylation, suggesting that stable LRP6-DVL interactions are essential for the maintenance of LRP6 phosphorylation (Metcalfe et al., 2010). Interestingly, transmembrane protein 198 (TMEM198), a previously uncharacterized seven-transmembrane protein, acts as a scaffold protein for CK1γ and LRP6 (Liang et al., 2011) and thus enhances phosphorylation of LRP6 (**Figure 2**).

Changes in plasma membrane lipid composition can also affect the phosphorylation of LRP6 and subsequent Wnt/β-catenin signaling activation. APC membrane recruitment protein 1 (Amer1) translocates to the plasma membrane in a $PtdIns(4,5)P_2$-dependent manner, where it recruits Axin, CK1γ, and GSK3β to promote LRP6 phosphorylation (Tanneberger et al., 2011). ADP-ribosylation factors 1 and 6 (Arf1/6) switch to the GTP-bound active form upon Wnt3a treatment, which facilitates the production of $PtdIns(4,5)P_2$ (PIP_2) and subsequent LRP6 phosphorylation (Kim W. et al., 2013). LY6/PLAUR domain-containing 6 protein (Lypd6) interacts with LRP6 and induces its localization to lipid rafts (Özhan et al., 2013). A lipid raft is a specific region in the plasma membrane where lipid components such as sphingolipid and cholesterol are enriched and cellular signaling is activated (Sezgin et al., 2017). Therefore Lypd6 potentiates LRP6 phosphorylation and activates Wnt/β-catenin signaling (**Figure 2**).

Next, we review other proteins that influence LRP6 phosphorylation and negatively regulate Wnt/β-catenin signaling. In contrast to CK1γ, whose phosphorylation of LRP6

enhances Wnt/β-catenin signaling, CK1ε inhibits Wnt/β-catenin signaling by interacting with and phosphorylating LRP6 at the S1420 and S1430 residues that are not present in PPPS/TP motifs (Swiatek et al., 2006; **Figure 2**). Moreover, Src and Fer tyrosine kinases phosphorylate LRP6 tyrosine residues near the PPPS/TP motifs, which leads to reduction of LRP6 cell surface levels and blockage of signalosome formation (Chen et al., 2014). It has been shown that several ligands for LRP6 inhibit its phosphorylation and suppress Wnt/β-catenin signaling. Wnt5a is mainly involved in non-canonical Wnt signaling. However, by recruiting Wnt receptors away from canonical Wnts (e.g., Wnt3a), Wnt5a can inhibit the phosphorylation of LRP6 and therefore act as a negative regulator of Wnt/β-catenin signaling (Grumolato et al., 2010; Sato et al., 2010). Pigment epithelium-derived factor (PEDF) interacts with the extracellular domain of LRP6, inhibiting LRP6-FZD interaction and phosphorylation of LRP6 (Park et al., 2011). Protein interactions in the intracellular region of LRP6 also mediate the inhibition of LRP6 phosphorylation. Merlin, a main player in the Hippo signaling pathway, interacts with LRP6 and inhibits its phosphorylation (Kim et al., 2016). Merlin-induced inhibition of LRP6 phosphorylation is counteracted by Wnt3a treatment, which, by inducing phosphorylation of merlin through P21 activated kinase 1 (PAK1), promotes merlin dissociation from LRP6 (Kim et al., 2016; **Figure 2**). Overall, these studies suggest that several proteins, by acting as kinases, ligands, or binding partners for LRP6, are crucial for regulating LRP6 phosphorylation and Wnt/β-catenin signaling, either in a positive or negative manner.

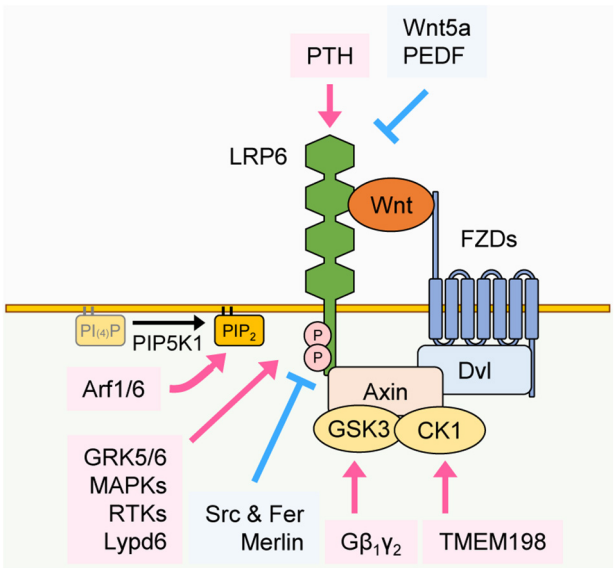

FIGURE 2 | Regulation of LRP6 phosphorylation. Phosphorylation of LRP6 can be regulated by various proteins through distinct mechanisms. PTH interacts with the extracellular domain of LRP6 and facilitates its phosphorylation. In contrast, Wnt5a and PEDF interact with the extracellular domain of LRP6 and inhibit LRP6 phosphorylation. GRK5/6, MAPKs, and RTKs facilitate phosphorylation of LRP6 in the cytoplasm, and Lypd6 facilitates phosphorylation of LRP6 in the plasma membrane. Src and Fer and Merlin inhibit phosphorylation of LRP6 in the cytoplasm. $G\beta_1\gamma_2$ and TMEM198 promote phosphorylation of LRP6 in GSK3 and CK1-dependent manners, respectively. Arf1/6 facilitates the phosphorylation of LRP6 in a PIP_2-dependent manner.

Internalization

Receptor-mediated internalization plays a crucial role in signal transduction. LRP6 is internalized after binding to ligands, and internalized LRP6 can either positively or negatively regulate Wnt/β-catenin signaling. For instance, the secreted Wnt modulator Dkk1, by forming a ternary complex with the single transmembrane protein Kremen1/2 and LRP6, internalizes LRP6 and decreases its plasma membrane levels, leading to Wnt/β-catenin signaling inhibition (Bafico et al., 2001; Mao B. et al., 2001; Semënov et al., 2001; Mao et al., 2002; **Figure 3A**). Internalization of LRP6 via Dkk1 also leads to decreased LRP6 phosphorylation by CK1γ (Sakane et al., 2010). Angiopoietin-like 4 protein (ANGPTL4) is another secretory protein that, by forming a complex with syndecan and LRP6, induces LRP6 internalization and decreases its surface levels (Kirsch et al., 2017). Similar to ANGPTL4, the secretory protein Bighead interacts with LRP6 and promotes its endocytosis and lysosomal degradation, resulting in suppression of Wnt/β-catenin signaling (Ding et al., 2018). Glycosylation of LRP6 can also influence its internalization. LRP6 can be fucosylated, a process that promotes the internalization of lipid raft-localized LRP6. This process prevents formation of the Wnt-FZD-LRP6 complex and thus inhibits Wnt/β-catenin signaling (Hong et al., 2020; **Figure 3A**). Wnt-activated inhibitory factor 1 protein (Waif1), a transmembrane protein, interacts with LRP6 and inhibits Wnt3a-induced LRP6 internalization into endocytic vesicles, thereby reducing Wnt/β-catenin signaling (Kagermeier-Schenk et al., 2011; **Figure 3B**).

Clathrin, a protein with a prominent role in cellular vesicle formation, promotes Dkk-mediated LRP6 internalization and thus acts as a negative regulator Wnt/β-catenin signaling (Yamamoto et al., 2008). Interestingly, clathrin can also promote LRP6 internalization in the presence of Wnt. This is because Wnt3a treatment, by inducing S1579 phosphorylation of LRP6, enhances LRP6 binding to disabled-2 (Dab2), an interaction that promotes clathrin-mediated LRP6 internalization (Jiang et al., 2012). Similar to Dab2, long-term treatment of Wnt3a (6–8 h) induces phosphorylation of adaptor related protein complex 2 subunit mu 1 (AP2M1) through AP2-associated kinase 1 (AAK1), and phosphorylated AP2M1 activates clathrin-mediated LRP6 internalization, once again leading to negative regulation of Wnt/β-catenin signaling (Agajanian et al., 2019). Therefore Dab2 and AAK1 seem to alleviate hyper-activation of Wnt/β-catenin signaling induced by Wnt stimulation (**Figure 3A**). Whereas clathrin is known for having a role in internalization of LRP6 and inhibition of Wnt/β-catenin signaling, it is reported that clathrin and AP2 act as components of the LRP6 signalosome, being recruited to the signalosome in a PtdIns(4,5)P_2-dependent manner (Kim I. et al., 2013). In this context, clathrin and AP2 seem to play a role in cell surface signalosome formation for activation of Wnt/β-catenin signaling, as well as in LRP6

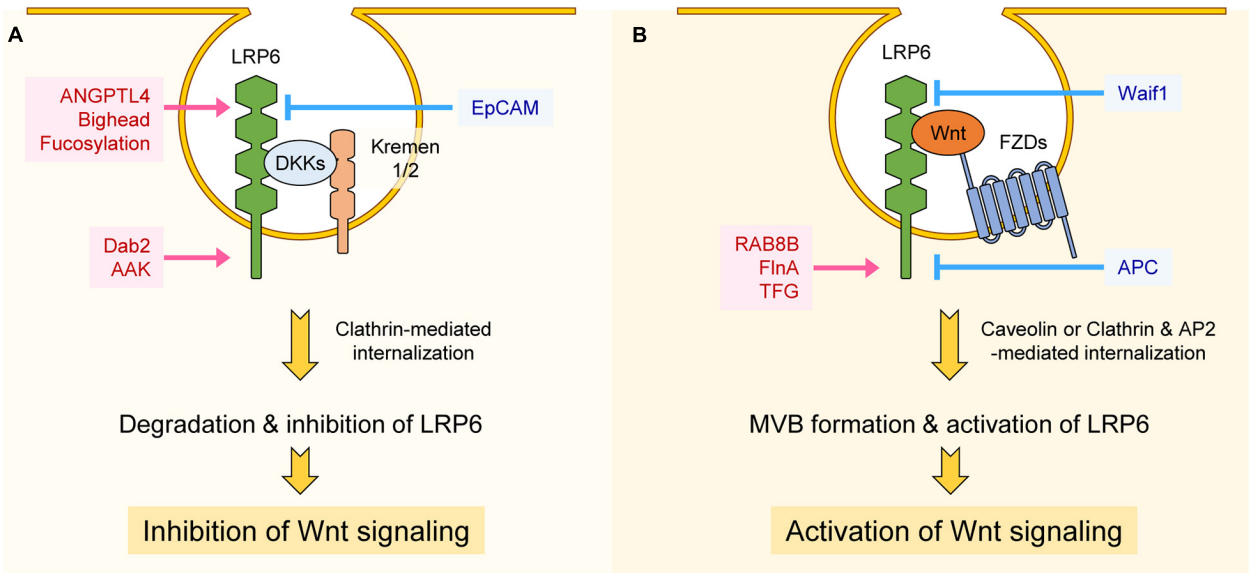

FIGURE 3 | Regulation of LRP6 internalization. **(A)** Interaction between LRP6, DKKs, and Kremen1/2 promotes clathrin-mediated internalization, resulting in degradation or inhibition of LRP6. ANGPTL4, Bighead, and fucosylation of LRP6 promotes internalization of LRP6 at the extracellular level, thereby inhibiting LRP6 function and Wnt/β-catenin signaling. Dab2 and AAK function at the intracellular level and promote clathrin-mediated internalization of LRP6, resulting in Wnt/β-catenin signaling suppression. On the other hand, EpCAM inhibits LRP6-DKK-Kremen1/2 complex formation, resulting in Wnt signaling activation. **(B)** Interaction among LRP6, FZDs, and Wnt promotes caveolin-mediated internalization, resulting in LRP6 activation and MVB formation. RAB8B, FinA, and TFG promote Wnt3a-mediated internalization of LRP6 and Wnt/β-catenin signaling at the intracellular level. However, Waif1 compromises Wnt-LRP6 interaction and inhibits Wnt/β-catenin signaling. APC inhibits Clathrin and AP2-mediated internalization of LRP6 at the intracellular level, resulting in suppression of Wnt/β-catenin signaling.

internalization. Interestingly, it is reported that APC, a main component of the β-catenin destruction complex, is also involved in LRP6 internalization. APC directly interacts with clathrin and AP2 to inhibit clathrin-induced LRP6 internalization, a process that generally leads to constitutive ligand-independent Wnt/β-catenin activation (Saito-Diaz et al., 2018). APC thus blocks Wnt/β-catenin signaling in two different contexts: in the cytoplasm, by forming destruction complex, and in the plasma membrane, by preventing LRP6 internalization (Saito-Diaz et al., 2018; **Figure 3B**).

It is well known that several components positively regulate Wnt/β-catenin signaling by modulating internalization of LRP6. Epithelial-cell-adhesion molecule (EpCAM) interacts with Kremen1 and inhibits Kremen1-Dkk2-LRP6 complex formation and internalization, resulting in activation of Wnt/β-catenin signaling (Lu et al., 2013; **Figure 3A**). In the presence of Wnt, LRP6 together with FZD, Axin, and GSK3β are internalized in caveolin-enriched vesicles (Yamamoto et al., 2006). GSK3β is sequestered in complex with LRP6 in multivesicular bodies (MVBs) and vastly reduces its phosphorylation of β-catenin, leading to activation of Wnt/β-catenin signaling (Taelman et al., 2010). There are many components involved in the activation of Wnt/β-catenin signaling via internalization of LRP6. These include Rab GTPase RAB8B protein and actin-binding protein filamin A (FlnA), both of which promote caveolin-mediated LRP6 internalization (Demir et al., 2013; Lian et al., 2016). Upon Wnt3a treatment, RAB8B interacts with LRP6 and is recruited to the signalosome complex, where it enhances caveolin-mediated internalization of LRP6 and subsequent Wnt/β-catenin signaling activation (Demir et al., 2013). FlnA interacts with LRP6, and loss of FlnA impairs internalization of LRP6 and activation of Wnt/β-catenin signaling, resulting in decreased proliferation of neural progenitor cells (Lian et al., 2016; **Figure 3B**). Recently, LRP6 proximity proteins induced upon short-term Wnt3a treatment were identified using an LRP6-Apex2 fusion protein (Colozza et al., 2020). Among them, Trk fused gene protein (TFG) appears to have an important role in Wnt3a-mediated LRP6 internalization and activation of Wnt/β-catenin signaling (Colozza et al., 2020; **Figure 3B**).

Regulation of LRP6 Maturation and Stability

Proper folding and maturation are essential for LRP6 to carry out its functions at the plasma membrane, and there are several components involved in these processes. Although mature LRP6 is known to be a stable protein (Perrody et al., 2016), its stability can be altered by extracellular stimuli or regulatory factors. Mesoderm development LRP chaperone protein (Mesd) localizes to the endoplasmic reticulum (ER), where it enhances the maturation and plasma membrane localization of LRP6 (Hsieh et al., 2003; **Figure 4**). Several proteins are involved in Mesd-mediated maturation of LRP6. The ER heat shock protein Grp94 promotes interaction between LRP6 and Mesd, and Grp94-deficiency suppresses LRP6 maturation (Liu et al., 2013). The transmembrane glycoprotein CD44 interacts with

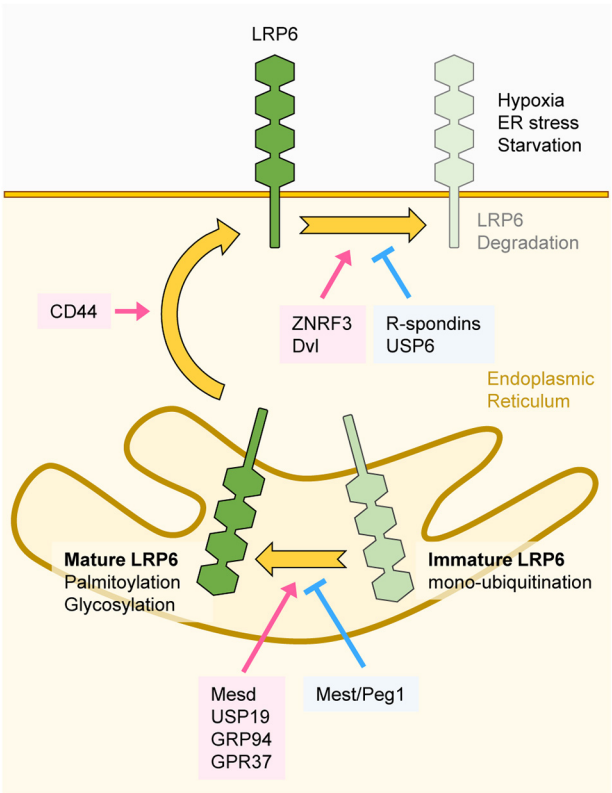

FIGURE 4 | Regulation of LRP6 maturation and stability. The transmembrane protein LRP6 undergoes several folding and maturation processes to translocate to the plasma membrane and properly function as a Wnt co-receptor. Mesd, USP19, GRP94, and GPR37 promote proper folding of LRP6. Glycosylation and palmitoylation of LRP6 are essential for its maturation. Mest/Peg1 inhibits glycosylation of LRP6 and immature LRP6 is mono-ubiquitinated, resulting in ER retention. Folded and mature LRP6 localizes to the plasma membrane, and CD44 promotes membrane localization of LRP6. ZNRF3 and DVL downregulate LRP6 protein levels, and USP6 and R-spondins antagonize ZNRF3 function. Extracellular stimuli such as hypoxia, ER stress, and starvation also degrade LRP6.

LRP6 and promotes Mesd-mediated membrane localization of LRP6 (Schmitt et al., 2015). The Parkinson's disease-associated receptor (GPR37) acts as an additional chaperone for LRP6 and promotes the maturation and membrane localization of LRP6. Additionally, GPR37 also inhibits ER-associated degradation of LRP6 and thereby enhances the protein levels of LRP6 (Berger et al., 2017).

Post-translational modifications (PTMs) have also been found to be important for regulating LRP6 folding and maturation. By using an endogenous antibody against LRP6, it has been found that LRP6 is N-glycosylated, and that N-glycosylation is necessary for the membrane localization of LRP6 (Khan et al., 2007). On the other hand, mesoderm-specific transcript/paternally expressed gene 1 (Mest/Peg1) represses glycosylation and plasma membrane localization of LRP6 (Jung et al., 2011), resulting in repression of Wnt/β-catenin signaling. Moreover, palmitoylation on a juxtamembrane cysteine of LRP6 enables its translocation from the ER to the plasma membrane (Abrami et al., 2008; **Figure 4**). If this process is hindered, mono-ubiquitination on the K1403 residue of LRP6 is promoted, leading to ER retention (Abrami et al., 2008). Further studies revealed that LRP6 mono-ubiquitination can be negatively regulated by the deubiquitinase USP19. Deubiquitination of LRP6 by USP19 facilitates LRP6 translocation to the plasma membrane through proper folding and palmitoylation (Perrody et al., 2016).

The R-spondin family members are secreted proteins that influence LRP6 stability (Wei et al., 2007). R-spondins are high affinity ligands for the Leucine-rich repeat-containing G-protein coupled receptors 4/5 (LGR4/5) and the transmembrane E3 ubiquitin ligases ZNRF3/RNF43 (Carmon et al., 2011; Hao et al., 2012; Koo et al., 2012). In the absence of R-spondins, ZNRF3/RNF43 ubiquitinate Wnt receptors and promote their clearance from the plasma membrane. Binding of R-spondins to LGR4/5 and ZNRF3/RNF43 induces ZNRF3/RNF43 internalization, leading to Wnt receptor stabilization. R-spondins thus regulate the activity and phosphorylation of LRP6 by stabilizing it at the plasma membrane (Carmon et al., 2011; Hao et al., 2012; Koo et al., 2012). Further studies revealed that DVL recruits ZNRF3 to the plasma membrane and mediates ZNRF3-dependent downregulation of LRP6 (Jiang et al., 2015). Consistently, upregulation LRP6 protein levels was observed in DVL1/2/3 knockout cells, owing to lack of LRP6 plasma membrane clearance by ZNRF3 (Jiang et al., 2015). Therefore, DVL seems to have dual role in the regulation of Wnt/β-catenin signaling since it promotes both aggregation and destabilization of LRP6 at the plasma membrane. Contrary to ZNRF3, the deubiquitinase USP6 increases LRP6 membrane levels and potentiates Wnt/β-catenin signaling by antagonizing the function of ZNRF3 (Madan et al., 2016).

Cellular stress can influence LRP6 stability. Chemically induced ER stress or hypoxia reduces the stability of LRP6, resulting in inhibition of Wnt/β-catenin signaling (Xia et al., 2019). Moreover, *O*-GlcNAcylation, a PTM that induces the attachment of *N*-acetylglucosamine (GlcNAc) to Ser/Thr residues, also plays a crucial role in LRP6 stability. During serum starvation, *O*-GlcNAcylation of LRP6 is reduced, which is followed by lysosomal degradation of LRP6 (Jeong et al., 2020; **Figure 4**).

β-CATENIN-INDEPENDENT SIGNALING VIA ACTIVATION OF LRP6

It is generally assumed that the primary output of LRP6 activity is directly associated with alterations in Wnt/β-catenin signaling. However, several studies have revealed that LRP6 affects not only Wnt/β-catenin signaling, but other signaling pathways as well. These include non-canonical Wnt signaling, Wnt-dependent stabilization of proteins (Wnt/STOP) signaling, G protein-coupled receptor (GPCR) and Hippo signaling.

The interaction of GPCR ligands with their associated receptors initiates GPCR signaling via activation of the G protein Gα, which mediates the activity of downstream effector proteins. LRP6 interacts with and promotes membrane localization of the G protein $G\alpha_s$ (Wan et al., 2011). Moreover, in the presence

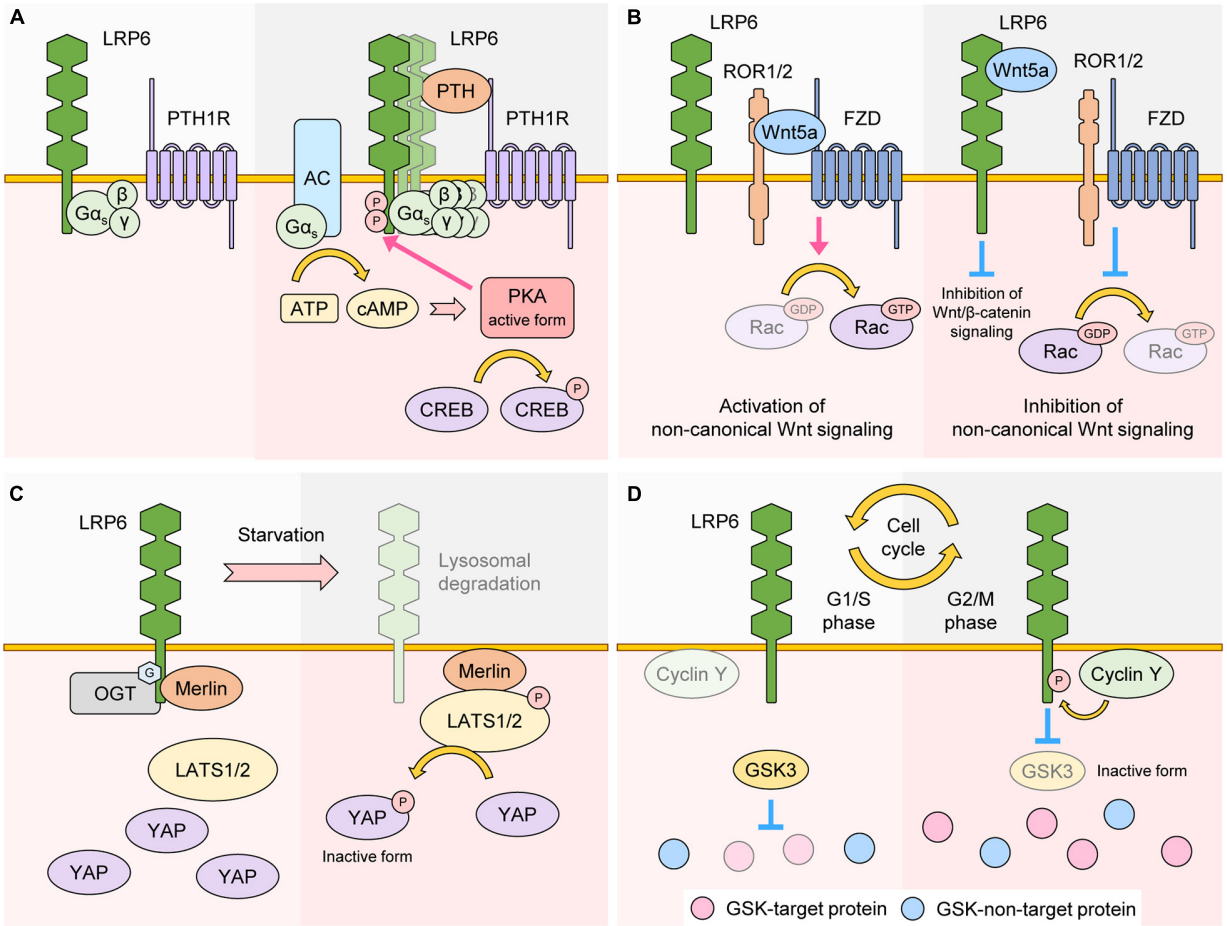

FIGURE 5 | Role of LRP6 as a regulator of other signaling. **(A)** LRP6 as a regulator of GPCR signaling. In the basal state, LRP6 interacts with G protein $G\alpha_s$. In the presence of the GPCR ligand PTH, a LRP6-PTH-PTH1R ternary complex is formed, which promotes aggregation of LRP6 and membrane localization of $G\alpha_s$. Production of cAMP is also upregulated in a $G\alpha_s$-AC-dependent manner. cAMP activates PKA, which promotes phosphorylation of LRP6 and CREB, two well-known downstream targets of PKA. **(B)** LRP6 as a regulator of non-canonical Wnt signaling. Wnt5a interacts with ROR1/2 and FZD, resulting in activation of Rac, a non-canonical Wnt signaling target. Wnt5a can also interact with LRP6. In these conditions, the binding affinity of ROR1/2 and FZD to Wnt5a is reduced. As a result, Rac becomes inactive and non-canonical Wnt signaling is inhibited. Because LRP6-Wnt5a binding weakens LRP6-Wnt3a interaction, Wnt/β-catenin signaling is also inhibited. **(C)** LRP6 as a regulator of Hippo signaling. In a nutrient rich state, LRP6 is *O*-GlcNAcylated and interacts with Merlin. In this condition, activity of LATS1/2 is maintained at low levels, resulting in stabilization and activation of YAP. In nutrient starvation conditions, *O*-GlcNAcylation and protein levels of LRP6 are both downregulated, and Merlin changes its binding partner from LRP6 to LATS1/2, resulting in activation of LATS1/2. YAP is phosphorylated by LATS1/2 and becomes inactive. **(D)** LRP6 as a regulator of Wnt/STOP signaling. In G1/S phase, cyclin Y protein levels are less abundant and the phosphorylation state of LRP6 is low, resulting in higher GSK3 activity. GSK3-target proteins are thus phosphorylated and targeted for proteasomal degradation. In G2/M phase, cyclin Y protein levels peak and promote LRP6 phosphorylation, resulting in inactivation of GSK3 and stabilization of GSK3-target proteins.

of GPCR ligands such as PTH, LRP6 stimulates the production of cyclic AMP (cAMP) via $G\alpha_s$, and newly generated cAMP activates protein kinase a (PKA). Previous reports have shown that PTH facilitates LRP6 phosphorylation and activation of Wnt/β-catenin signaling in osteoblasts (Wan et al., 2008), suggesting that LRP6 is involved in both, Wnt/β-catenin and GPCR signaling, in the context of bone formation (Wan et al., 2011; **Figure 5A**).

The extracellular region of LRP6 interacts with Wnt5a, and this interaction inhibits activation of Rac1, a target protein of non-canonical Wnt signaling (Bryja et al., 2009). In addition, Wnt5a treatment interferes with the interaction between Wnt3a and LRP6, resulting in not only inhibition of Wnt/β-catenin signaling, but activation of non-canonical Wnt signaling as well (Bryja et al., 2009; Grumolato et al., 2010; **Figure 5B**).

Hippo signaling is a crucial regulator of organ size and cellular homeostasis (Pan, 2010). Activation of Hippo signaling leads to serial phosphorylation and activation of STE20-like serine/threonine kinases 1/2 (MST1/2) and Large Tumor Suppressor 1/2 (LATS1/2). Activated LATS1/2 phosphorylates Yes-associated protein (YAP) and WW domain containing transcription regulator protein 1 (TAZ). As a result, phosphorylated YAP and TAZ undergo 14-3-3-mediated cytoplasmic retention or proteasomal degradation, a process which blocks their transcriptional activity and thereby inhibits cell proliferation and survival (Meng et al., 2016).

It has been revealed that LRP6 is involved in the regulation of Hippo signaling. YAP/TAZ are incorporated into the β-catenin destruction complex, and treatment with Wnt3a or overexpression of LRP6 stabilizes the protein levels of YAP/TAZ in a similar fashion to β-catenin, leading to increased YAP/TAZ transcriptional activity (Azzolin et al., 2014). Another study revealed that loss of LRP6 via serum starvation promotes the dissociation of Merlin from LRP6, which activates Hippo signaling by facilitating Merlin-LATS interaction. As a result, loss of LRP6 enables phosphorylation of YAP, inhibiting its transcriptional activity (Jeong et al., 2020; **Figure 5C**).

Recent studies have shown that LRP6 phosphorylation peaks during the G2/M phase of the cell cycle and that this peak is dependent on cyclin Y and its cyclin dependent kinase 14 (CDK14). Originally, identified via kinome-wide RNAi screening in *Drosophila* cells, the cyclin Y-CDK14 complex phosphorylates the PPPS/TP S1490 residue of LRP6 (Davidson et al., 2009). Cyclin Y protein levels peak during G2/M, which explains the cell cycle dependence of LRP6 phosphorylation (Davidson et al., 2009). Mechanistically, G2/M phosphorylation of LRP6 by cyclin Y-CDK14 primes LRP6 for incoming Wnts, which in turn suppresses the activity of GSK3 and prevents GSK3 target proteins from proteasomal degradation (Taelman et al., 2010; **Figure 2**). Suppression of GSK3 during G2/M thus leads to an overall increase in protein stabilization, ensuring proper cell division and growth (Acebron et al., 2014). Importantly, this new Wnt sub-branch, also known as Wnt/STOP pathway, is completely dependent on LRP6 (Acebron et al., 2014; Acebron and Niehrs, 2016). Another player in the Wnt/STOP pathway is Caprin-2, which acts as a scaffold for LRP6 and cyclin Y and thereby promotes LRP6 phosphorylation during G2/M (Wang et al., 2016). Moreover, B-cell CLL/lymphoma 9 protein (BCL9) is phosphorylated at the T172 residue by cyclin dependent kinase 1 (CDK1), and phosphorylated BCL9 inhibits LRP6 degradation thereby acting as a positive regulator of Wnt/STOP signaling (Chen et al., 2018). These data suggest that LRP6 phosphorylation-mediated Wnt signaling can be transduced in a β-catenin-independent manner (**Figure 5D**).

LRP6 DYSREGULATION AND DISEASE

Cancer

Dysregulation of Wnt/β-catenin signaling is highly associated with cancer, and mutations in AXIN, APC, and β-catenin often lead to increased cancer formation and metastasis (Bugter et al., 2021). Similarly, dysregulation of LRP6 is also involved in cancer. *LRP6* is highly expressed in several cancer cell lines and overexpression of *LRP6* promotes cancer cell proliferation (Li et al., 2004). More specifically, LRP6 is a well-known regulator of breast cancer: *LRP6* expression is frequently upregulated in breast cancer tissue, and respective overexpression or knockdown of *LRP6* induces or inhibits breast tumorigenesis (Li et al., 2004; Lindvall et al., 2009; Liu et al., 2010; Zhang et al., 2010). The role of LRP6 in breast cancer tumorigenesis is highly dependent on Wnt/β-catenin signaling. If antibodies that block LRP6-Wnt1 or LRP6-Wnt3a interactions are administered in mice, Wnt/β-catenin signaling is blocked and breast tumor growth is suppressed (Ettenberg et al., 2010). In breast cancer tissue, high expression of the Sry-related HMG box 9 protein (SOX9) activates Wnt/β-catenin signaling by inducing *LRP6* expression (Wang et al., 2013). LRP6 also plays a role in breast cancer metastasis. N-myc downstream regulated gene-1 protein (NDRG1) interacts with LRP6 and suppresses LRP6-mediated Wnt signaling activation, resulting in inhibition of breast cancer metastasis (Liu et al., 2012). Contrastingly, in the absence of Wnt3a, LRP6 inhibits FZD8-mediated non-canonical Wnt signaling by interacting with the extracellular domain of FZD8 (Ren et al., 2015). As a result, breast tumor metastasis, which is usually promoted by non-canonical Wnt signaling, is inhibited through the extracellular domain of LRP6 (Ren et al., 2015). Therefore, LRP6 seems to play a dual role in breast tumor metastasis that depends on the presence or absence of Wnt.

Another cancer with which LRP6 is highly correlated is liver cancer. *LRP6* is highly expressed in tumors of liver cancer patients, and overexpression of *LRP6* promotes liver cancer cell proliferation and tumor growth (Tung et al., 2012). Several components are involved in liver cancer progression via regulation of LRP6. For example, expression of stearoyl-CoA desaturase (SCD) is increased in liver tumors, where it promotes the production of monounsaturated fatty acids (MUFA) (Lai et al., 2017). MUFA induces expression of *LRP6* and activation of Wnt/β-catenin signaling, which then activates expression of *SCD*, functioning as a positive feedback loop (Lai et al., 2017). Connective tissue growth factor (CTGF) is highly expressed in liver cancer patients, and CTGF promotes phosphorylation of LRP6 (Jia et al., 2017). Finally, expression of long non-coding RNA DLGAP1-AS1 is increased in liver cancer tissue, where it inhibits miR-26a/b-5p, a negative regulator of *LRP6* expression (Lin et al., 2020).

In colorectal cancer, elevated activity of LRP6 has been reported. LRP6 phosphorylation was also found to be enhanced in colorectal cancer tissue from patients (Lemieux et al., 2015). In colorectal cancer cells, gain of function mutations in KRAS increase LRP6 phosphorylation, resulting in activation of Wnt/β-catenin signaling (Lemieux et al., 2015). CD110 receptor-expressing colorectal cancer tumor-initiating cells (TICs) are activated via thrombopoietin in blood vessels. In TICs, production of acetyl-CoA is promoted via degradation of lysine, and the LRP6 K802 residue is acetylated (Wu et al., 2015). Acetylation of LRP6 leads to its phosphorylation in a CK1γ-dependent manner, leading to activation of Wnt/β-catenin signaling. As a result, self-renewal and metastasis of colorectal cancer TICs are enhanced (Wu et al., 2015). V-set and transmembrane domain containing 2A (VSTM2A) is a secretory protein that is lowly expressed in colorectal cancer tissue (Dong et al., 2019). VSTM2A interacts with the extracellular domain of LRP6 and inhibits LRP6 phosphorylation, thereby inducing its lysosomal degradation, and suppressing colorectal cancer progression (Dong et al., 2019).

In addition to breast, liver, and colorectal cancer, the role of LRP6 in other cancers has been studied. In prostate cancer, high

expression levels of caveolin-1 and *LRP6* are detected, and these two proteins activate Wnt/β-catenin signaling and glycolysis through Akt signaling. The end result is increased prostate cancer cell proliferation (Tahir et al., 2013). Through mass spectrometry-based proteomic analyses of mass spectrometry data, it was identified that *LRP6* expression is high in oral squamous cell carcinoma (OSCC) (Yuan et al., 2017). In addition, LRP6 increases the protein levels of fibroblast growth factor 8 (FGF8), which can act as an oncogene and promote OSCC progression (Yuan et al., 2017). LRP6 is also involved in regulating the activity of cancer-associated fibroblasts (CAFs). In the stroma of breast, colon, and ovarian cancers, *Dkk3* expression and internalization with Kremen1/2 are enhanced, resulting in upregulation of LRP6 protein levels (Ferrari et al., 2019). Finally, LRP6 stabilizes not only β-catenin, but also YAP/TAZ. Stabilized YAP/TAZ enters the nucleus where it enhances tumorigenic activity in various cancer types (Ferrari et al., 2019).

Neurodegeneration

Cognitive and behavioral disorders caused by functional neuron failure and neuronal death are referred to as neurodegeneration. Representative examples include Alzheimer's, Parkinson's, and Huntington's diseases. The causes of neurodegeneration include genetic mutations, protein aggregation, mitochondrial dysfunction, etc. However, the molecular mechanisms underlying neurodegeneration still require further elucidation (Gan et al., 2018). The relationship between Wnt signaling dysregulation and neurodegeneration has been reported, and several studies have shown that mutations in LRP6 are associated with neurodegeneration.

Through genome-wide screening, it was identified that a single nucleotide polymorphism (SNP) in the 1062 residue of LRP6, which converts isoleucine to valine (hereafter referred to as Ile1062Val), leads to reduced Wnt/β-catenin signaling and is implicated in Alzheimer's disease (De Ferrari et al., 2007). In addition, it was also shown that an isoform that skips the third exon of LRP6 and displays reduced Wnt/β-catenin signaling activation is significantly augmented in the brains of patients with Alzheimer's disease (Alarcón et al., 2013). When *LRP6* is specifically deleted in the forebrain, synapse formation is suppressed while amyloid-β accumulation and neuronal apoptosis are promoted, altogether resulting in aggravation of Alzheimer's disease symptoms (Liu et al., 2014). Consistently, in Alzheimer's disease patients, *DKK1* is highly expressed and causes suppression of LRP6-amyloid precursor protein (APP)-mediated Wnt/β-catenin signaling, which results in accumulation of amyloid-β and synapse loss (Elliott et al., 2018). These data suggest that dysregulation of LRP6 function in the brain leads to suppression of Wnt/β-catenin signaling and exacerbation of Alzheimer's disease symptoms. A positive role of LRP6 for neuronal activity has also been reported. Through genetic screening, LRP6 was found to localize to excitatory synapses of mature neurons and promote synaptogenesis (Sharma et al., 2013), and Wnt3a and Wnt8 have been shown to cooperate with LRP6 in this process (Avila et al., 2010; Sharma et al., 2013). Moreover, APP, also known as precursor of amyloid-β, interacts with LRP6 and activates Wnt/β-catenin signaling, leading to enhanced synaptic stability (Elliott et al., 2018).

It is well-known that mutations in *PARK8* are implicated in Parkinson's disease (Kumari and Tan, 2009). LRRK2, a product of *PARK8* gene, interacts with LRP6 and acts as a scaffold between LRP6 and the β-catenin destruction complex (Berwick and Harvey, 2012). Pathogenic mutations in LRRK2 lead to reduced interaction with LRP6, suppressing Wnt/β-catenin signaling (Berwick and Harvey, 2012).

LRP6 also plays a protective role in brain ischemic injury (Abe et al., 2013). Compared to wild-type mice, more areas of the brain are damaged through ischemic injury in $LRP6^{+/-}$ mice. GSK3β activity and expression of inflammatory marker genes are also increased in the brains of $LRP6^{+/-}$ mice (Abe et al., 2013).

Metabolic Syndrome

Metabolic syndrome is characterized by abnormal levels of metabolites (e.g., sugars and lipids) in the body and is highly associated with cardiovascular disease and diabetes. Risk factors for metabolic syndrome are diet, low physical activity, aging, and genetics (Rochlani et al., 2017). The relationship between LRP6 dysfunction and metabolic syndrome has been widely studied.

It is well known that dysregulation of LRP6 is highly associated with coronary artery disease (CAD) and atherosclerosis. Through genome-wide analysis of CAD patients, R473Q, R360H, N433S, and R611C residue mutations in LRP6 were found to be correlated with CAD pathogenesis, as determined by high glucose, lipid, and low-density lipoprotein (LDL) levels in blood vessels (Mani et al., 2007; Singh et al., 2013b). In addition, the LRP6 R611C mutation, which does not effectively activate Wnt/β-catenin signaling compared to wild-type LRP6, leads to low LDL uptake and clearance. Taken together, these data suggest that LRP6 is a critical modulator of receptor-mediated LDL endocytosis (Mani et al., 2007; Liu et al., 2008; Ye et al., 2012).

Abnormal proliferation of vascular smooth muscle cells (VSMC) via activation of PDGF signaling is a well-known cause of atherosclerosis (Raines, 2004). Wild-type LRP6 interacts with PDGF receptor-β and causes its lysosomal degradation, a function that is impaired in the LRP6 R611C form (Keramati et al., 2011). As a result, VSMC proliferation through PDGF signaling is increased in $Lrp6^{R611C/R611C}$ mutants (Keramati et al., 2011). Moreover, VSMCs from $Lrp6^{R611C/R611C}$ mice exhibit suppressed Wnt/β-catenin signaling but increased non-canonical Wnt signaling, a shift that results in the activation of PDGF signaling via SP1 (Srivastava et al., 2015). Consequently, VSMCs are maintained in an undifferentiated form in the arterial wall, further increasing their proliferation and causing them to migrate at accelerated rates (Srivastava et al., 2015). In summary, impairment of LRP6 activity is highly correlated with CAD through PDGF signaling. Finally, the miRNA-17∼92 cluster targets LRP6 and downregulates Wnt/β-catenin signaling, and deficiency of miRNA-17∼92 in endothelial cells improves blood flow and arteriogenesis (Landskroner-Eiger et al., 2015).

The LRP6 R611C mutant form is also associated with altered insulin signaling. R611C mutation of LRP6 in skeletal muscle suppresses *TCF7L2*-dependent transcription of the insulin receptor (IR) and reduces its protein levels. This results in low

insulin sensitivity and high glucose level in blood vessels, both of which contribute to type II diabetes (Singh et al., 2013a).

Additionally, $Lrp6^{R611C/R611C}$ mice maintain a high level of LDL and lipids in the plasma, which induces fatty liver (Go et al., 2014). In $Lrp6^{R611C/R611C}$ mutant hepatocytes, IGF/Akt/mTORC1/2 signaling and lipid synthesis are activated, and treatment with the mTOR inhibitor rapamycin or recombinant Wnt3a rescue these pathogenic effects (Go et al., 2014).

Cardiac-specific knockout of LRP6 activates dynamin-related protein 1 (Drp1) in heart tissue and reduces carnitine palmitoyltransferase 1b (CPT1b) (Wang et al., 2020). Since CPT1b is an enzyme involved in lipid oxidation, downregulation of CPT1b levels due to LRP6 deficiency causes lipid accumulation in heart tissue and reduces left ventricular ejection fraction (LVEF), altogether leading to cardiac dysfunction (Wang et al., 2020).

Inflammation

Organ homeostasis is maintained through the coordinated action of inflammatory cytokines with host defense function, and dysregulation of inflammatory cytokines is implicated in immune disease or cancer (Greten and Grivennikov, 2019). Moreover, inflammatory cytokines can regulate Wnt/LRP6 signaling. For instance, long exposure to pro-inflammatory cytokine interferon-γ or TNF-α induces Dkk1 expression and inhibits Wnt/β-catenin signaling, leading to increased intestinal inflammation (Nava et al., 2010). Ileal Crohn's disease (CD) is a disease that causes pain, diarrhea, and malnutrition due to chronic inflammation in the intestine (Koslowski et al., 2012). Genome-wide analysis from CD patients revealed an association between the Ile1062Val LRP6 SNP with early disease onset. Lower levels of LRP6 mRNA were also detected in these patient samples (Koslowski et al., 2012). Dendritic cell (DC)-specific knockout of LRP5/6 promotes differentiation of effector T cells and represses regulatory T cell differentiation, resulting in enhanced anti-tumor immunity and inhibition of tumor growth, both of which show that fine regulation of LRP6 is essential for proper immune responses (Hong et al., 2016).

Skeletal Disease

Bone mass formation and maintenance is regulated by the activity of osteoblasts, which form bone, and osteoclasts, which degrade bone. Dysregulation of bone mass leads to osteoporosis or sclerosteosis, diseases that are heavily influenced by genetic factors (Regard et al., 2012). For instance, LRP5 mutations generally lead to decreased bone mass and osteoporosis due to reduced Wnt/β-catenin signaling (Gong et al., 2001). One exception is the G171V mutation in LRP5, which increases rather than decreases bone mass (Babij et al., 2003). LRP6 is a paralog of LRP5, and studies on the association between LRP6 and bone homeostasis have also been performed. For example, heterozygous deficiency of LRP6 in mice leads to a reduction in bone mineral density (BMD) (Holmen et al., 2004). Moreover, combination of LRP6 heterozygous deficiency with LRP5 homozygous deficiency, leads to a greater reduction in BMD compared to LRP5 homozygous deficiency alone (Holmen et al., 2004). Tissue-specific ablation of LRP5 and LRP6 in the mesenchyme, which is the precursor of skeletal tissue, leads to embryonic skeletal defects, similar to the phenotype seen upon mesenchyme-specific deletion of β-catenin (Joeng et al., 2011).

Several proteins that bind to LRP6 regulate bone formation via modulation of Wnt/β-catenin signaling. It is well-known that loss-of-functions mutation in sclerostin (expressed by the *SOST* gene) cause sclerosteosis (Balemans et al., 2001). Sclerostin inhibits Wnt/β-catenin signaling by binding to LRP6 and disrupting FZD-LRP6 interaction (Li et al., 2005; Semënov et al., 2005). Biglycan, a type of proteoglycan, activates Wnt/β-catenin signaling by maintaining the interaction between Wnt3a and LRP6, and deficiency of biglycan compromises bone formation (Berendsen et al., 2011).

Parathyroid hormone interacts with PTH1R to promote LRP6 phosphorylation and activate Wnt/β-catenin signaling in osteoblasts, leading to increased bone formation (Wan et al., 2008). Osteoblast-specific knockout of *LRP6* reduces the expression of osteoblast differentiation-related genes and suppresses bone formation, even in the presence of PTH (Li et al., 2013, 2016).

Oxidized phospholipids bind to LRP6 and reduce LRP6 plasma membrane levels via clathrin-dependent endocytosis (Wang et al., 2018). As a result, phosphorylation of LRP6 and Wnt/β-catenin signaling are reduced, inhibiting osteoblast differentiation (Wang et al., 2018). It has also been shown that oxidized phospholipid levels are high in patients suffering from hyperlipidemia, suggesting that LRP6 may have an important role in the occurrence of osteoporosis via hyperlipidemia (Wang et al., 2018).

PERSPECTIVES (CLOSING REMARKS)

It has been more than 20 years since LRP6 was discovered as a co-receptor for Wnt/β-catenin signaling. Most studies focusing on the mechanisms underlying LRP6-mediated Wnt/β-catenin signaling have concentrated on phosphorylation events in the intracellular domain of LRP6. However, recent reports have determined that additional PTMs such as ubiquitination, acetylation, and *O*-GlcNAcylation are also essential for regulating LRP6 activity. Moreover, LRP6 is involved in multiple signaling cascades apart from Wnt/β-catenin. These include, but are not limited to, non-canonical Wnt signaling, GPCR signaling, cell cycle-related signaling, and Hippo signaling (**Figure 5**). Until now, the occurrence of disease via dysregulation of LRP6 has been primarily linked to aberrant Wnt/β-catenin signaling. However, we suggest that future studies focusing on LRP6 and disease should also investigate the involvement of other signaling pathways. Moreover, since single point mutations in LRP6 are linked to multiple diseases, it would be worthwhile to analyze the cross-talk between these diseases, and to more thoroughly decipher their connections to LRP6. Considering the complex mechanisms surrounding the regulation and activation of LRP6, as well as its important role in disease occurrence, LRP6 is thus expected to be an attractive therapeutic target in future studies.

AUTHOR CONTRIBUTIONS

WJ planned and wrote the manuscript. EJ directed, edited, and finalized the manuscript. Both authors read and approved the final version of the manuscript.

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