WW DOMAIN PROTEINS IN SIGNALING, CANCER GROWTH, NEURAL DISEASES, AND METABOLIC DISORDERS

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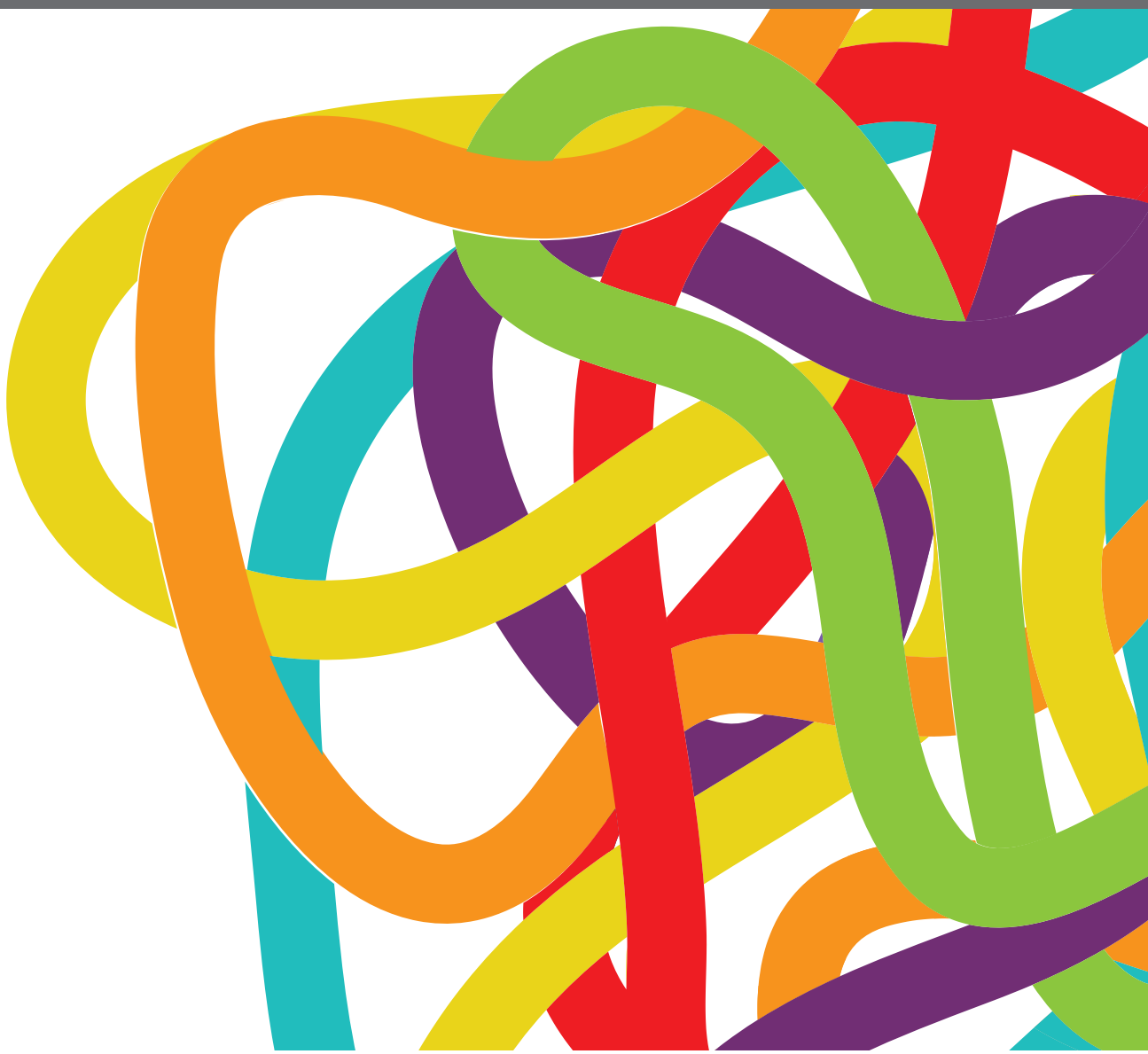

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WW DOMAIN PROTEINS IN SIGNALING, CANCER GROWTH, NEURAL DISEASES, AND METABOLIC DISORDERS

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EDITORIAL
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Editorial: WW Domain Proteins in Signaling, Cancer Growth, Neural Diseases, and Metabolic Disorders

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Keywords: WW domain, WWOX, signaling, Hippo, Smurf, neurodegeneration, cancer

Editorial on the Research Topic

WW Domain Proteins in Signaling, Cancer Growth, Neural Diseases, and Metabolic Disorders

First of all, the editorial team welcomes you to the specific Research Topic on "WW Domain Proteins in Signaling, Cancer Growth, Neural Diseases, and Metabolic Disorders." We appreciate the hard work and outstanding contributions from all authors. WW domain is well-known for its participation in mediating protein-protein interactions, especially its role in relaying many signaling cascades. WW domains mediate these interactions through recognition of proline-rich peptide motifs and phosphorylated serine/threonine-proline sites. They are found in many different structural and signaling proteins that are needed in a variety of cellular processes. In our recent analysis of the human proteome, there are at least 52 WW domain-containing proteins and more than 10,000 among all species that play various roles in vital cellular processes (1). Dysregulation of WW domain-mediated signaling cascades disrupts the normal physiology and results in disease states. Indeed, WW domain proteins and their binding-partner complexes have been implicated in major human diseases including cancer, neural diseases and metabolic disorders. For instance, WW-domain proteins YAP and TAZ of the Hippo pathway participate in the regulation of cell stemness maintenance, tissue homeostasis, and tumorigenesis, thus making them targets for new diagnostics and therapeutics (Chen et al.). Importantly, tumor suppressor *WWOX* gene has recently been recognized as one of the five new risk factors in Alzheimer's disease (2).

Yet, our understanding and the fundamental knowledge of the entire WW domain family proteins are very limited. This has prompted us to propose to the *Frontiers* journals a specific thematic issue discussing recent knowledge and advancement on WW domain proteins in physiology and diseases. Prior to this, we have launched a thematic issue on tumor suppressor WWOX (WW domain-containing oxidoreductase) that was published in *Experimental Biology and Medicine* in 2015. Over the recent few years, wakeup calls from parents of newborn patients with WWOX deficiency have pushed us to propose this specific issue. These ill-fated little angels suffer from severe neural diseases which unfortunately still have no cure. Our efforts, which we dedicate to WWOX patients and their parents, aim to enrich our discussion about this important topic and brainstorm new venues to help fight related diseases.

What's new? We will feature articles in the WW domain-regulated signal pathways, and then present articles dealing with WWOX in physiology and diseases. First of all, Koganti et al. reviewed the inhibitory Smurf family proteins for the bone morphogenetic protein (BMP) and the transforming growth factor beta (TGF-β) signaling pathways and addressed their crucial roles in

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cancer progression. As a C2-WW-HECT E3 ligase, Smurf1 is an oncogenic protein, whereas Smurf2 acts as a tumor suppressor and oncogenic protein. The oncogenic function of Smurf2 is due to its stabilization of KRAS, EGFP and upregulation of Wnt/β-catenin pathway. Smurf proteins in cancer cell migration, metastasis and autophagy are also described. Next, in a related pathway, Chen et al. reviewed the ubiquitous feature of the Hippo signal pathway for organ development, with special focus on the WW domain proteins YAP and TAD. Dysregulation of the Hippo signal pathway leads to organ outgrowth and cancer progress (Chen et al.). In physiological settings, YAP and TAZ orchestrate the embryonic development, organ growth, tissue regeneration, stem cell pluripotency, and tumorigenesis. Chen et al. addressed the crucial role of YAP/TAZ in balancing the stem cell niches, which is important for normal development, as well as cancer progression. Supporting research from WWOX also shows this protein may oversee the Hippo signaling pathway from the upstream via interacting with proteins in the TGF-β, hyaluronidase Hyal-2, and Wnt/β-catenin pathways (Chen et al.). In supporting this notion, a recent study reported that downregulation of WWOX results in tamoxifen resistance in breast cancer due to inactivation of Hippo signaling (3). Lee and Liou described the structure and the functional nature of Pin1. As a family of the peptidyl-prolyl *cis-trans* isomerase (PPIase), Pin1 catalyzes the *cis*/*trans* isomerization of the proline residue in the phosphorylated Serine/Threonine-Proline (S/T-P) motifs of substrates. The WW domain of Pin1 preferentially binds numerous protein substrates possessing the *trans* configuration of the phosphorylated S/T-P motif, which are needed in cell events such as cell cycle, transcription, DNA damage, and apoptosis. The PPIase catalyzes the *cis* to *trans* isomerization, whereas this may hinder WW domain in binding substrates.

Regarding the WWOX area, Jamous and Salah reviewed the role of WWOX and other WW domain proteins in breast cancer tumorigenesis. Similarly, Pospiech et al. described the history of WWOX research and association with breast cancer progression. Tanna and Aqeilan discussed the use of animal models to assess *in vivo* WWOX functions. The review covers the rodent, fish, and fly models. Defects in growth retardation, metabolism, reproduction, neural system, and early death are discussed. Saigo et al. reviewed the inhibitory proteins for WWOX, specifically with TMEM207. The WW domain of WWOX binds the PPxY motif in TMEM207. TMEM207 contribution to the pathogenesis of cancer was discussed. Hussain et al. utilized experimental approaches and identified WWOX-binding proteins. WWOX interactors are associated with metabolic pathways for proteins, carbohydrates, and lipids breakdown. In supporting the role of WWOX in maintaining DNA stability, McBride et al. reported *Wwox* deletion in mouse B cells leads to the development of genomic instability, neoplastic transformation, and monoclonal gammopathies. While loss of WWOX in newborns leads to severe neural diseases and early death, Liu et al. reviewed the cascade of WWOX downregulation-induced protein aggregation that causes neurodegeneration. Additionally, switch of the phosphorylation of WWOX at Tyr33 for anticancer response to Ser14 for disease progression (e.g., cancer and AD) is discussed. Suppression of Ser14 phosphorylation by a zinc finger peptide Zfra blocks cancer growth and restores memory loss in mice (4, 5).

Finally, what's urgent for the field? It would be of great importance to have a cure for the newborn patients who suffer severe neural diseases due to WWOX deficiency, and provide a complete termination for the severe progression of neurodegeneration in AD patients. For example, an effective drug to lessen seizure in the newborn patients would greatly benefit them. Preliminary findings from clinical treatment shows that despite mutations, forced transcription of *WWOX* gene appears to be a feasible approach to lessen the symptoms of seizure in patients with neurodegeneration (personal communications with Dr. D. S. Lin at the Taipei Medical University). Furthermore, blocking the downregulation of WWOX in the middle aged individuals would likely to prevent the development of AD.

In the concluding remarks, it is achievable to design WWOX-targeted therapy. Surface-enhanced Raman scattering (SERS) amplified Raman spectroscopy signal can be used for detecting and imaging biological specimens *in vitro* or *in vivo*. A recent success has utilized EGFR antibody to design Raman tags to target amplified EGFR in glioblastoma cells (6). By the same token, Raman tags can be designed to identify WWOX expression and its phosphorylation in normal neurons and glioblastoma cells in the brain, thus facilitating imaging, diagnosis, and treatment. However, there are expected difficulties in the brain imaging for patients, which requires further technical innovations. Small molecules such as synthetic chemicals or peptides can directly support treating patients in clinics, once they are functionally validated and approved for clinical use. Zfra peptides can be used as therapeutic options and strategies to target cancer and neural diseases associated with WWOX deficiency (4, 5).

AUTHOR CONTRIBUTIONS

N-SC initiated writing the original manuscript, revised, proof read, discussed with co-authors, and finalized the manuscript. RL and C-IS read, revised and proofed the manuscript. RA contributed in part to writing, revised and proofed the manuscript.

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ORIGINAL RESEARCH
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Wwox Deletion in Mouse B Cells Leads to Genomic Instability, Neoplastic Transformation, and Monoclonal Gammopathies

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WWOX (WW domain containing oxidoreductase) expression loss is common in various cancers and characteristic of poor prognosis. Deletions, translocations, and loss of expression affecting the *WWOX* gene are a common feature of various B cell neoplasms such as certain B cell lymphomas and multiple myeloma. However, the role of this common abnormality in B cell tumor initiation and/or progression has not been defined. In this study, we conditionally deleted *Wwox* early in B cell development by means of breeding *Cd19-Cre* transgenic mice crossed to *Wwox* floxed mice (*Cd19 Wwox KO*). We observed a significant reduced survival in *Cd19 Wwox KO* mice and the development of B cell neoplasms including B cell lymphomas, plasma cell neoplasias characterized by increased numbers of CD138+ populations as well as monoclonal gammopathies detected by serum protein electrophoresis. To investigate whether *Wwox* loss could play a role in genomic instability, we analyzed DNA repair functions during immunoglobulin class switch joining between DNA segments in antibody genes. While class switch recombination (CSR) was only slightly impaired, Wwox deficiency resulted in a dramatic shift of double strand break (DSB) repair from normal classical-NHEJ toward the microhomology-mediated alternative-NHEJ pathway, a pathway associated with chromosome translocations and genome instability. Consistent with this, Wwox deficiency resulted in a marked increase of spontaneous translocations during CSR. This work defines for the first time a role for Wwox for maintaining B cell genome stability during a process that can promote neoplastic transformation and monoclonal gammopathies.

Keywords: Wwox, B cells, monoclonal gammopathies, plasmacytomas, multiple myeloma, genomic instability

INTRODUCTION

WWOX (WW domain containing oxidoreductase) is a ubiquitously expressed tumor suppressor gene mapping to chr16q23, which spans one of the most common chromosomal fragile sites in the human genome, *FRA16D* (1–4). Tumor copy number alterations analyses revealed *WWOX* to be one of the most frequently deleted genes in cancer (5, 6) and loss of WWOX expression is characteristic of poor prognosis [Reviewed in (7)]. Although loss of WWOX is correlated with cancer development and progression, it does not behave as a highly penetrant classical

tumor suppressor in most mouse models. Complete *Wwox* deletion results in post-natal death by 3–4 weeks of age (8, 9) and tissue specific deletion using a variety of Cre expression mouse models did not result in spontaneous tumor formation in mice from mixed genetic background [Reviewed in (7)]. Recently however, increased mammary carcinogenesis has been reported in a cancer susceptible mouse genetic background, supporting the hypothesis of Wwox operating as a tumor suppressor (10). We have previously observed that hypomorphic *Wwox* mice developed B cell lymphomas at old age (11) and this appears in agreement with observations indicating that heterozygous mice with only a functional *Wwox* allele (i.e., *Wwox* $^{+/-}$) develop an increased B cell lymphoma incidence when exposed to the carcinogen ethyl-nitrosourea (8). Thus, both of these studies (8, 11) suggest a propensity of B cells for neoplastic transformation upon Wwox deficiency.

In humans, alteration and loss of WWOX has been associated with certain B cell tumors (12–15), and most notably Multiple Myeloma (MM). In MM, the t(14;16) (q32;q23) involving *IGH* (Immunoglobulin heavy chain) and *WWOX* is a primary genetic event that results in upregulation of *MAF* and characteristic of a subgroup of high-risk MM (1, 2, 16, 17). Importantly, besides of *WWOX* participation in t(14;16), lower *WWOX* expression appears to be associated with poor MM prognosis (18–21). Loss of heterozygosity at the *WWOX* locus was shown to correlate with loss of *WWOX* expression in MM cases (21). Homozygous *WWOX* deletions are indicative of poor prognosis (22) and alteration of 16q, which includes deletion of *WWOX*, were recognized by International Myeloma Working Groups as a recurrent secondary genetic event in high-risk MM (19, 20). Furthermore, in recent analyses of data from the Myeloma Genome Project, Walker et al. identified deletion 16q23.1 affecting *WWOX*, among the most common recurrent minimal copy number changes, detected in 252 out of 1,074 (23.5%) newly diagnosed MM cases characterized by whole-exome sequencing. Thus, *WWOX* deletion is one of the most common genomic abnormalities observed in MM overall (23, 24). In addition, *WWOX* gene promoter methylation was also reported to associate with disease progression (25). Despite the overwhelming evidence suggesting a connection between WWOX loss of function and B cell neoplasia, the pathophysiological role of WWOX in B cells remains unclear.

In B cell lymphomas and MM, translocations between the *IGH* locus and oncogenes are recurrent events that drive transformation (26, 27). They are thought to be primarily generated by aberrant double strand break (DSB) formation during the antibody diversification processes of class-switch recombination (CSR) and V(D)J recombination (28, 29). Site-specific break formation at the *IGH* locus and off-target sites are determinants that impact the location, while break frequency and persistence impact the rate of translocations (30). As such, efficient DNA damage response and repair of DSBs is important in suppressing translocations (28). The major DSB pathway that operates during CSR and VDJ is the Ku70/80-dependent classical non-homologous end-joining (C-NHEJ) pathway. In the absence of C-NHEJ factors, DSBs are repaired by alternative end-joining pathways (Alt-NHEJ). These ill-defined pathways are thought to be less efficient, requiring more extensive end-processing and are biased toward microhomology based repair. In this context, studies suggest that Alt-NHEJ is prone to generating translocations while efficient repair by C-NHEJ suppresses translocations and genomic instability (28, 31). In this study, we examine the consequence of conditional B cell *Wwox* deletion in mice and find reduced survival, tumor formation, and evidence of plasma cell neoplastic transformation. Analysis of CSR from *Wwox*-deficient B cells reveals inappropriate utilization of the Alt-NHEJ pathway together with an increase in the generation of oncogenic translocations. Thus, this study demonstrates a direct role for Wwox in B cell genome stability during processes that lead to neoplastic transformation.

MATERIALS AND METHODS

Animals

All animal research was conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International at the University of Texas MD Anderson Cancer Center, Science Park and all research was approved by the Institutional Animal Care and Use Committee. *BK5-Cre*^{TG} and *Cd19*^{Cre} mice (32, 33) and the protocol to generate *Wwox*$^{flox/flox}$ and *Wwox knock-out (KO)* mice have been previously described (9, 34). In brief, to generate *Wwox KO* mice we crossed *Wwox*$^{flox/wt}$ males with *BK5-Cre*$^{TG/TG}$, *Wwox*$^{flox/wt}$ females (both in mixed 129SV/C57Bl/6 background). Cre expression and deletion occurs in the oocyte and embryo resulting in general recombination and deletion (33). As controls (denoted *Wwox WT) BK5-Cre*$^{TG/0}$ *Wwox*$^{+/+}$ were used. Two-week-old *Wwox KO* mice and age matched control *Wwox WT* were compared in experimental analyses. For the generation of *Cd19 Wwox KO* we crossed *Wwox*$^{flox/wt}$ with *Wwox*$^{flox/wt}$ *Cd19*$^{Cre/+}$ mice. For control mice (littermates denoted *Cd19 Wwox WT*) *Wwox*$^{wt/wt}$, *Cd19*$^{Cre/+}$ mice were used. Mice of both genders were used in analysis. Genotypes were confirmed by PCR using primers previously described (9, 34) and see **Supplementary Figure 1**.

Antibodies

The following antibodies were used: Anti-CD3 (Biolegend 100237), anti-CD11b, (Biolegend 101237), anti-IgM (Biolegend 406525), anti-CD19 (BD Biosciences 561739), anti-CD95 (BD Biosciences 561856), anti-GL7 (BD Biosciences 553666), anti-CD21 APC (BD Biosciences 561770), anti-CD138 (BD Biosciences 553714), Horseradish peroxidase conjugated anti-mouse Light Chain Specific goat polyclonal antibody (Jackson Immunoresearch #115-035-174), anti-IgG1 (BD Biosciences 560089), and for histology anti-CD45R/B220 (Bio-Rad, MCA1258G) and anti-CD138 (Biolegend 142502).

Flow Cytometry and *Wwox* Expression Analysis in Primary Lymphocytes

The following cell populations were isolated from spleen or bone marrow by FACS sorting**:** Pro-B B220^{+}/CD43^{hi}/IgM^{-}, Pre-B B220^{+}/CD43^{lo}/IgM^{-}, Plasmablasts B220^{+}/CD138^{+}/CD19^{lo}, Plasma cells B220^{-}/CD138^{+}/CD19^{-},

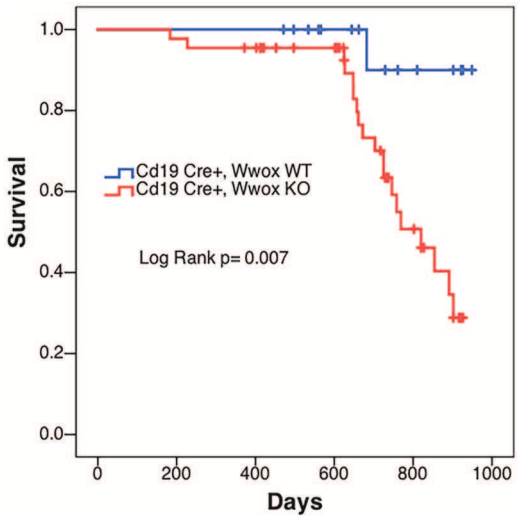

FIGURE 1 | Decrease survival of *Cd19 Wwox KO* mice. Comparative survival in days of a cohort of 17 $Cd19^{Cre/+}$ $Wwox^{+/+}$ control mice (*Cd19 Wwox WT*, blue line) vs. 44 $Cd19^{Cre/+}$ $Wwox^{flox/flox}$ (*Cd19 Wwox KO*, red line) mice, *p*-value of Log Rank (Mantel-Cox) analysis shown. *Cd19 Wwox KO* mice had a statistically significant lower survival rate than control ($p = 0.007$). *Cd19 Wwox KO* mice displayed a mean survival of 777 vs. 922 days for control mice.

Follicular $B220^{+}/CD19^{+}/CD23^{hi}/CD21^{lo}$, Marginal zone $B220^{+}/CD19^{+}/Cd23^{lo}/CD21^{hi}$, Germinal center $CD19^{+}/GL7^{+}/CD95^{+}$. Viable cells were identified by forward and side scatter as well as propidium iodide dye exclusion. $CD3^{+}$ and $CD11b^{+}$ cells were used to exclude non-B cell populations. Samples were sorted using a BD FACSARIA Fusion. RNA from each B lymphocyte subpopulation was isolated using Trizol Reagent (Ambion) and cleaned up with RNeasy kit (Qiagen). Quantitative gene expression by reverse transcription real time PCR was determined using primer/probe Taqman set spanning exons 6–7 of *Wwox* (Thermo Fisher Scientific Assay ID Mm01247384, 4351372) and normalized to 18S RNA.

Histology and Immunohistochemistry

Full necropsy was performed on all mice and samples from major organs and tumors (whenever available) were collected. Tissues were processed by means of formalin fixation, paraffin embedding and hematoxylin and eosin (H&E) staining. Tumor samples were also analyzed by immunohistochemistry (IHC) following standard procedures and staining with anti-CD45R/B220 and anti-CD138. Histological analyses were performed by two pathologists without prior knowledge of genotypes. Based on IHC staining pattern and cell morphology,

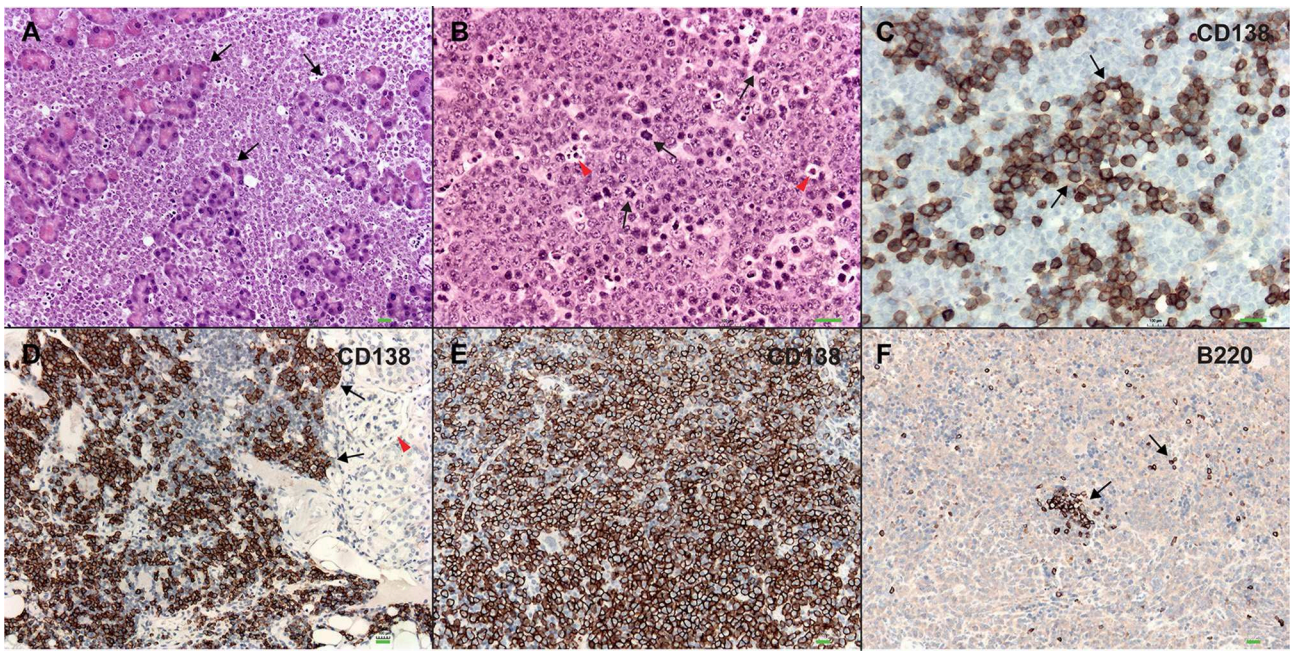

FIGURE 2 | Plasmablastic plasmacytomas in *Cd19 Wwox KO* mice. **(A)** Microphotograph of plasmablastic plasmacytoma from a *Cd19 Wwox KO* mouse infiltrating pancreas, magnification 10x with H&E staining. Note how pancreatic tissue architecture is totally destroyed and replaced by infiltrating plasmacytoma cells. Black arrows point to pancreatic acinar cells. **(B)** Plasmacytoma cells infiltrating para-pancreatic lymph nodes of the same case as in **(A)**. Black arrows point to some of multiple mitoses, red arrowheads point to apoptotic bodies. Magnification 20x, H&E staining. **(C)** Anti-CD138 immunostaining of tumor shown in **(B)**. Black arrows point to CD138 positive (brown) cells. As previously described not all plasmacytoma plasmablast cells stain with anti-CD138 antibody (36). Magnification 20x, counterstained with light hematoxylin. **(D)** Heavy CD138+ cells infiltrate in kidney from another *Cd19 Wwox KO* mouse, red arrowhead points to glomerulus. **(E)** Anti-CD138 immunostaining of spleen from a different *Cd19 Wwox KO* mouse to those shown in **(A–D)**. Note that most cells are positive for the CD138+ plasma cell surface marker (brown stained cells). **(F)** Same spleen sample as in **(E)** immunostained with anti-B220 (CD45R), as can be observed very few scattered cells are positive for this B lymphocyte marker (black arrows). Both of the mice (represented in **D–F**) displayed M spikes in SPEP analyses. Microphotographs **(D–F)** taken at 10x, light hematoxylin counterstaining. Horizontal green bars at lower right corner of each photograph represent 100 μm scale.

TABLE 1 | Histopathology of detected tumors and status of SPEP results.

Mouse ID	Genotype [Cd19 Wwox]	Age (ds)	Histopathology	M SPIKE in SPEP
15	KO	735	Mature B cell lymphoma	+
16	KO	735	Mature B cell lymphoma	n/d
68	KO	648	Plasmablastic plasmacytoma (in MLN)	–
70	KO	648	Plasmablastic plasmacytoma (in MLN)	+
93	KO	827	Anaplastic plasmacytoma	+
95	KO	820	Anaplastic plasmacytoma	+
97	KO	820	Plasmacytoma (in MLN)	+
103	KO	626	Anaplastic plasmacytoma	–
104	KO	625	Precursor B cell lymphoma	n/d
161	KO	602	Precursor B cell lymphoma	n/d
189	KO	769	Precursor B cell lymphoma	+
231	KO	737	Mature B cell lymphoma	–
254	KO	725	Precursor B cell lymphoma	–
261	KO	746	Plasmablastic plasmacytoma (in MLN)	n/d
269	KO	717	No macroscopic tumor	+
312	KO	921	Mature B cell lymphoma	+
332	KO	917	No macroscopic tumor	+
334	KO	891	Anaplastic plasmacytoma	+
180	WT	729	No macroscopic tumor	+
295	WT	926	Mature B cell lymphoma	–
325	WT	922	Mature B cell lymphoma	n/d

MLN, Mesenteric Lymph Node; + indicates positive for M spikes; – indicates negative for M spikes as per SPEP analysis; n/d, not determined.

tumors were classified following guidelines of the Bethesda classification of lymphoid neoplasms in mice and a more recent classification of mouse plasmacytomas (35, 36).

Serum Protein Electrophoresis (SPEP)

Mice blood samples were collected at time of euthanasia. Samples were allowed to coagulate at room temperature and spun at 3000×G for 10 min; 0.5 μl of sera were loaded in precast QuickGels (Helena Laboratories, 3505T) and run on a QuickGel Chamber (Helena Laboratories, 1284) according to the manufacturer's instructions. Samples were analyzed in triplicate with M-spike positive samples visible in all three analyses.

Statistical Analysis for Mouse Survival and Tumor Incidence

Analyses were performed using SPSS statistical software. Log-rank test was applied to compare survival curves. Fisher's exact test was used to compare tumor incidence rates. *P*-values of < 0.05 were considered significant.

B Cell Culture and Analysis

B cell isolation and culture have been previously described (37). Naïve B cells were purified from spleens of wild-type and *Wwox KO* 16–17 day-old mice (38, 39) by anti-CD43 bead depletion (Miltenyi Biotec). Cells were cultured in LPS (25 μg/ml, Sigma-Aldrich) and IL-4 (5 ng/ml, RD) for 72 h. To determine CSR to IgG1, cultured B cells were stained with anti-IgG1 antibodies and flow cytometric analysis of surface Ig expression was performed on a LSRFortessa (BD) with scatter gating and propidium iodide staining to exclude dead cells (40). Results were analyzed with FlowJo (Tree Star) and averages were obtained from triplicate cultures of six individual spleens in four independent experiments. Cell proliferation was analyzed with Cell Trace Violet (Invitrogen) according to manufacturer's protocol (41). Fluorescent intensity was measured at culture initiation and at 72 h. Two independent experiments were performed. The chromosome translocation assay has been previously described (42, 43). Genomic DNA was isolated from naïve B cells cultured to undergo CSR with LPS and IL-4 for 72 h. PCR with genomic DNA of 10^5 cells per reaction was performed. 1st round PCR primers:(5′-ACTATGCTATGGACTACTGGGGTCAAG-3′ and 5′-GTGAAAACCGACTGTGGCCCTGGAA-3′) and 2nd round PCR primers (5′-CCTCAGTCACCGTCTCCTCAGGTA-3′ and 5′-GTGGAGGTGTATGGGGTGTAGAC-3′) were used to amplify *Myc/Igh* translocations. Amplicons were confirmed as translocations with reactivity to both southern blot probes *Myc* (5′-GGACTGCGCAGGGAGACCTACAGGGG-3′) and Igh (5′-GAGGGAGCCGGCTGAGAGAAGTTGGG-3′). The data shown is the summary of three independent experiments and the *p*-value was calculated by two-tailed Fisher's exact test.

Class Switch Recombination Junction Analysis

CSR junctions were amplified from genomic DNA by PCR with primers and conditions previously described (44): 36 cycles of PCR (94°C 30 s, 62°C 30 s, 68°C 8 min) using first round primers (5′-CAGGCTAAGAAGGCAATCCTGG-3′) and (5′-TTGACCTGTAACCTACCCAGGAGAC-3′); and 36 cycles of PCR (94°C 30 s, 64°C 30 s, 68°C 8 min) using second round primers (5′-GATCCAAGGTGAGTGTGAGAGGACA-3′) (5′-CATCCTGTCACCTATACAGCTAAGCTG-3′). PCR products between 0.5 and 3 Kb were purified and cloned into pCR4-TOPO (Invitrogen), individual bacterial clones were picked up and sequenced. Sequences were analyzed from three independent experiments for microhomology and mutation. To identify junctions, donor and acceptor switch regions were aligned. MH was determined by identifying the longest overlap region at the junction. A single mismatch or gap was permitted if at least 5 bp from end of the overlapping sequence in the alignment (45, 46). For mutational analysis, 100 bp from junction were analyzed on both acceptor and donor regions.

RESULTS

Wwox Ablation in Cd19$^+$ B Cells Reduces Survival and Induces Neoplastic Transformation

In order to better understand the role of WWOX in B cell neoplasms we targeted deletion of this gene early in B-cell

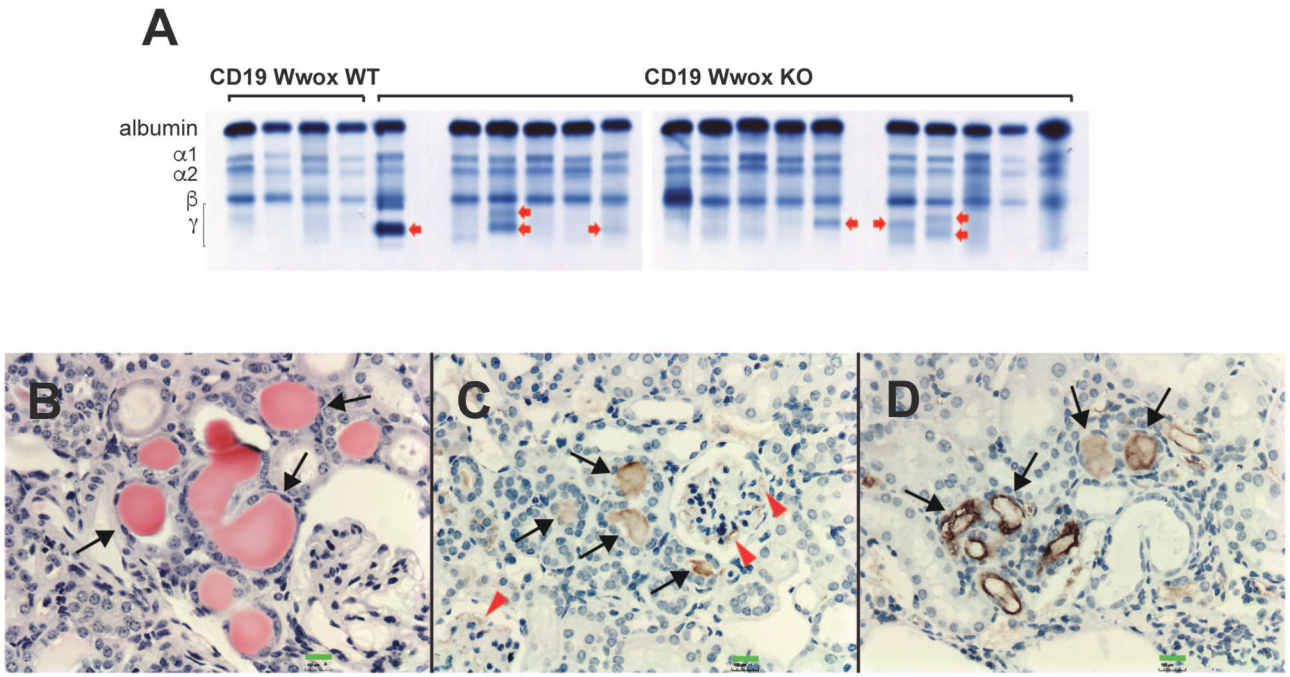

FIGURE 3 | SPEP gels and MM cast nephropathy. *Cd19 Wwox KO* mice displayed evidence of monoclonal gammopathies. **(A)** Representative SPEP of serum samples from *CD19 Wwox KO* and *WT* control mice. Red arrows point to some representative M spikes. All samples were analyzed in triplicate. Ten of 27 (37%) *Cd19 Wwox KO* mice displayed detectable M-spikes vs. only 1 of 9 (11%) tested *Cd19 Wwox WT* control mice. **(B–D)** Myeloma Cast Nephropathy. H&E staining and anti-mouse IgG of kidney tissue sections from a *Cd19 Wwox KO* displaying an M spike and poor health at 820 days of age. **(B)** Black arrows indicate eosinophilic (protein) deposits obstructing kidney tubules. **(C,D)** same kidney sample as shown in **(B)** immunostained using a goat polyclonal anti-mouse IgG, light chain-specific peroxidase conjugated antibody, counterstaining with light hematoxylin. Brown staining of the intratubular protein deposits are formed by Ig light chain aggregates (black arrows). Ig light chain deposits can also be observed in the mesangial area of glomeruli [red arrowheads in **(C)**]. All microphotographs taken at 20x magnification, 100 μM scale bars are shown.

development by crossing $Wwox^{flox/flox}$ mice (9) to $Cd19^{Cre}$ transgenic mice (32). We confirmed *Wwox* protein ablation in $Cd19^{+}$ B cells from $Cd19^{Cre/+}$ $Wwox^{flox/flox}$ *(Cd19 Wwox KO)* mice by means of immunoblot (**Supplementary Figure 1**). Wwox protein was not detected in B cell samples from all analyzed *Cd19 Wwox KO* mice indicating efficient deletion in B cells (data not shown). Survival of *Cd19 Wwox KO* mice was compared with $Cd19^{Cre/+}$ $Wwox^{+/+}$ *(Cd19 Wwox WT)* control littermates. Overall survival was plotted using the Kaplan-Meier method and analyzed by the Log-rank test. *Cd19 Wwox KO* mice had a statistically significant lower survival rate than control ($p = 0.007$, **Figure 1**). *Cd19 Wwox KO* mice displayed a mean survival of 777 vs. 922 days for control mice. Animals were necropsied and histology samples obtained at either the end of the experiment or when moribund. Importantly, we observed that several *Cd19 Wwox KO* mice developed intra-abdominal tumors with the characteristics of B cell neoplasms (**Figure 2**). Tumors were broadly classified into lymphomas and plasmacytomas based on the IHC staining pattern (**Table 1**). Neoplastic lesions that were exclusively B220+ indicative of B cell lymphomas were further classified into mature and immature lymphomas based on published guidelines (35). Several of the observed intra-abdominal tumors were positive for B220 and CD138, suggestive of plasmablastic plasmacytomas predominantly affecting mesenteric and retroperitoneal lymph nodes, and were histologically classified as previously described (36) (**Figure 2** and **Table 1**). Tumors were also tested for Wwox immunoreactivity and were negative (not shown). Of 34 *Cd19 Wwox KO* mice, 8 (23.4%) developed ($B220^{+}$) B cell lymphomas and 8 other mice developed plasmacytomas ($B220^{+}$,$CD138^{+}$) predominantly affecting mesenteric and retroperitoneal lymph nodes. Thus, a total of 16 of 34 (47%) *Cd19 Wwox KO* mice developed B cell neoplasms. In the *Cd19 Wwox WT* control group, only 2 of 14 (14.3%) mice developed B cell lymphomas and none developed plasmacytomas. In summary, the B-cell tumor incidence rates between the *Cd19 Wwox KO* group and the *Cd19 Wwox WT* group were significantly different ($p < 0.05$).

A characteristic of multiple myeloma, plasmacytomas and other plasma cell neoplasms is the secretion of immunoglobulin (Ig), detected in serum protein electrophoresis (SPEP) as Ig monoclonal bands (M-spikes) which is a routine method used in the clinic for the diagnosis of plasma cell dyscrasias. Serum samples were obtained from morbid mice prior to euthanasia whenever possible and those that survived to the end of the experimental period. SPEP analyses revealed M-spikes in multiple *Cd19 Wwox KO* mice (**Figure 3A**). In total 10 of 27 (37%) *Cd19 Wwox KO* mice but only 1 of 9 (11%) tested *Cd19 Wwox WT* control mice had detectable M-spikes. Full histopathologic analyses also indicated that some

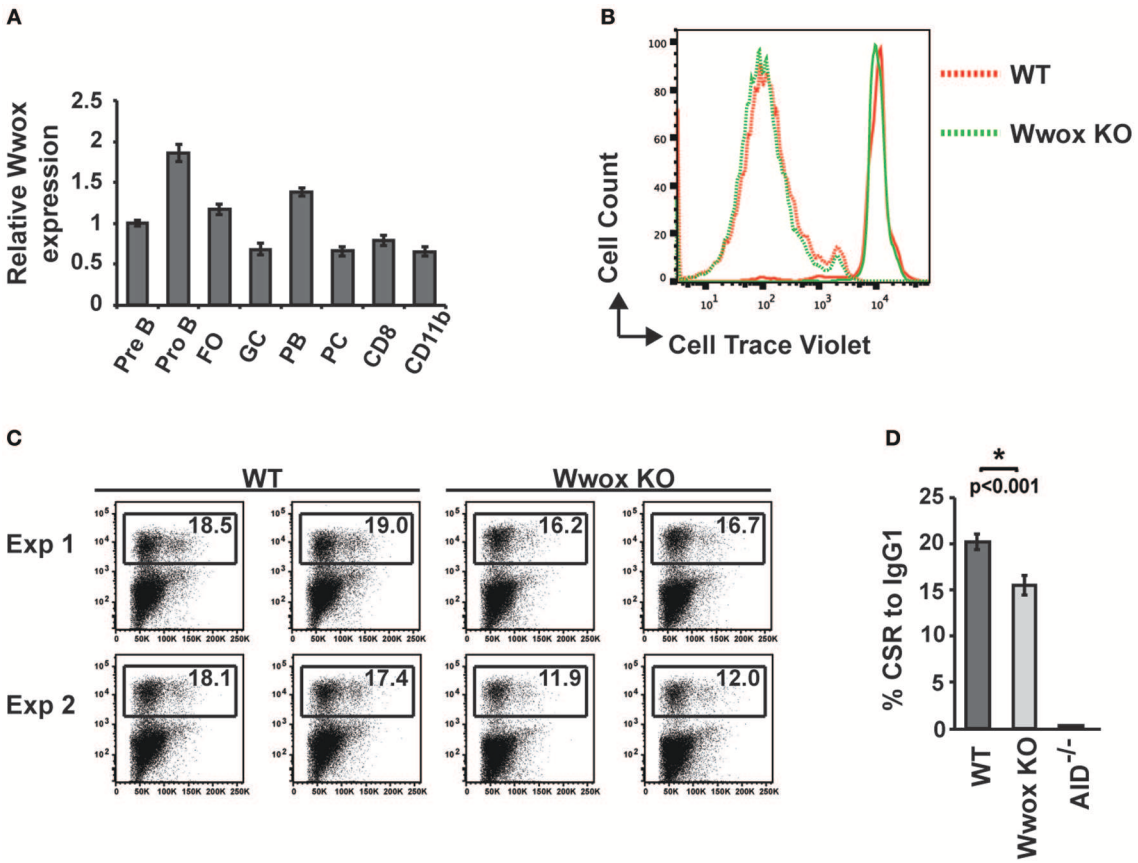

FIGURE 4 | Wwox B cell expression and function during Ig class-switching. **(A)** Wwox transcript level analysis at various B cell differentiation stages. Pre B ($B220^+/CD43^{lo}/IgM^-$), Pro B ($B220^+/CD43^{hi}/IgM^-$), follicular ([FO], $B220^+/CD19^+/CD23^{hi}/CD21^{lo}$), germinal center ([GC], $CD19^+/GL7^+/CD95^+$), Plasmablast ([PB], $B220^+/CD138^+/CD19^{lo}$), and Plasma cell ([PC], $B220^-/CD138^+/CD19^-$) B cells; $CD8^+$ T cells; and $CD11b^+$ populations were FACS sorted from the spleen or bone marrow of unimmunized wild-type mice. Relative levels of Wwox were assessed by RT-qPCR (Taqman assay) and normalized to 18S RNA levels. Results shown are mean values of triplicate analyses (error bars SEM). **(B)** Representative histogram of B cells proliferation analysis. Fluorescent intensity of baseline labeling (solid lines) of naïve B cells isolated from *Wwox WT* or *Wwox KO* spleens and after 72 h culture with IL4 and LPS (dotted lines). **(C)** Representative flow cytometry plots of CSR to IgG1 after 72 h culture. Relative percentage of cells expressing IgG1 is indicated on each plot. Duplicate plots from two experiments displayed. **(D)** Mean values of CSR to IgG1 in activated B cells from $n = 4$ independent experiments, (error bars SEM). *P* value was determined by a two-tailed *t*-test assuming unequal variance, * indicates significant *p* value.

of the mice with M-spikes displayed evidence of myeloma cast nephropathy (myeloma kidney). This is characterized by the presence of obstructing casts in the lumen of kidney tubules. These eosinophilic (pink) deposits are formed by aggregates of monoclonal Ig light chains (**Figures 3B–D**).

Wwox Is Expressed in B Cells and Functions During Ig Class Switching

To address when during B cell development Wwox may be suppressing tumorigenesis, we first assessed normal *Wwox* expression in various B cell compartments. B cells from Pro-B, Pre-B, marginal zone, follicular B, germinal center (GC), plasmablast, and plasma cell compartments were purified from the spleen or bone marrow of wild-type mice. *Wwox* expression was analyzed by qPCR and compared to that of splenic $CD8^+$ lymphocytes and $CD11b^+$ leukocytes (**Figure 4A**). We find *Wwox* expression throughout B cell development including germinal center cells where *Ig* genes undergo mutagenic rearrangement. During *Igh* CSR, the enzyme AID (activation-induced cytidine deaminase) induces DSB in the switch regions, a process that is a significant source of genomic instability. To determine if Wwox functions during CSR we analyzed B cells for the ability to undergo proper CSR. Naïve (IgM^+) B cells were isolated from the spleens of wild-type, *Wwox KO* and $Wwox^{+/-}$ 15 days old mice. Cells were cultured with LPS and IL-4 for 72 h to stimulate proliferation and CSR to *IgG1*. Cell proliferation was normal and flow cytometric analysis of cell surface antibody isotype expression revealed that *Wwox KO* cells supported *IgG1* CSR at a rate of ∼75% of wild-type controls (**Figures 4B–D**).

Wwox Deficiency Impairs C-NHEJ and Promotes Alt-NHEJ During Ig Class Switching

CSR requires end-joining between DSBs to exchange one isotype constant region for another. DSBs are induced and resolved

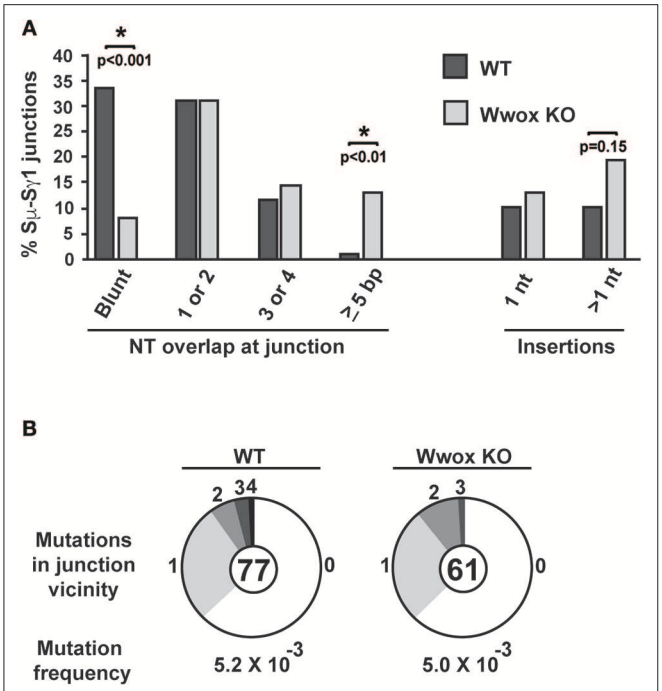

FIGURE 5 | Analysis of class switch recombination junctions. **(A)** Naïve B cells isolated from the spleens of *Wwox WT* or *Wwox KO* mice were induced to undergo CSR to IgG1 with IL4 and LPS for 72 h. Genomic DNA was isolated, Sμ-Sγ junctions were PCR amplified, cloned and sequenced. The percentage of junctions with the denoted number of nucleotide (NT) overlap or insertion is indicated (a total of 76 WT and 61 *Wwox KO* unique clones, from $n = 4$ experiments were analyzed, see also **Supplementary Figure 2**). A significant decrease in blunt end joins can be observed in *WWOX KO* B cells (*Wwox WT*, 26 blunt of 77 total junctions [i.e., 33.8%] vs. *Wwox KO,* 5 blunt [8.2%] of 61 total junctions analyzed). Long MHs (>5 bp MH) were rare in *WT* B cells but significantly increased in *Wwox KO* B cells (*Wwox WT*, 1 of 77 [1.3%] vs. *Wwox KO,* 8 of 61 [13.1%]). *P* values were determined using two-tailed Fisher's exact test, * indicates significant *p* value. **(B)** Mutations within the vicinity of CSR junctions. 100 bp flanking the 5′ and the 3′ of the Sμ-Sγ breakpoint were analyzed for mutations. Segment sizes in the pie charts are the proportion of clones that contained the number of mutations indicated in the periphery of the charts. The total number of independent junctions analyzed is indicated in the center of each chart. Analysis of mutation frequency within 100 bp of the CSR junction revealed no difference between *Wwox WT* and *Wwox KO* cells (5.2×10^{-3} vs. 5.0×10^{-3} mutations/bp, respectively, $p = 0.8$ two-tailed *t*-test, assuming unequal variance). Frequency denoted as mutations per base pair.

during phase G1 of the cell cycle predominantly by the C-NHEJ pathway. In the absence of C-NHEJ, CSR is supported at a slightly reduced rate by alternative end-joining activity (Alt-NHEJ), with recombination junctions biased toward microhomology-mediated (MH) end-joining (MMEJ) (38, 45–49). To determine if Wwox deficiency altered C-NHEJ engagement we analyzed CSR junctions for MH usage following standard methods (44–49). To this end, the Sμ-Sγ1 junctions were amplified, PCR products were purified, cloned and sequenced from pools of B cells induced to undergo CSR to *IgG*. Sequence analysis of individual clones representative of both conditions (i.e., *Wwox WT* vs. *Wwox KO* B cells) revealed significant differences in the overall use of donor/acceptor homology at the CSR junctions (two-tailed Mann-Whitney test, $p < 0.001$). *Wwox*-deficient B cells displayed a significant shift toward microhomology (average MH *Wwox WT* 1.2 bp vs. *Wwox KO* 2.6 bp). Importantly, there was a significant decrease in blunt end joins (*Wwox WT*, 26 blunt of 77 total junctions [i.e., 33.8%] vs. *Wwox KO,* 5 blunt [8.2%] of 61 total junctions analyzed, $p < 0.001$ Fisher's exact test). Long MHs (>5 bp MH) were rare in *WT* B cells but significantly increased in *Wwox KO* B cells (*Wwox WT*, 1 of 77 [1.3%] vs. *Wwox KO,* 8 of 61 [13.1%], $p < 0.01$ Fisher's exact test) (**Figure 5A** and **Supplementary Figure 2**). The overall frequency of nucleotide insertions at CSR junctions was not significantly different, although *Wwox KO* B cells displayed a slight increase of insertions >1 bp (**Figure 5A**). Mutations occur in the vicinity of the junctions and are thought to be due to the AID induced mutational processes engaged during CSR (50). Analysis of mutation frequency within 100 bp of the CSR junction revealed no difference between *Wwox WT* and *Wwox KO* (5.2×10^{-3} vs. 5.0×10^{-3} mutations/bp respectively, $p = 0.8$ *t*-test) (**Figure 5B**). We conclude that Wwox deficiency results in a significant shift toward MMEJ between switch regions, consistent with a shift toward the Alt-NHEJ pathway in the repair of AID induced DSBs.

Increased Translocations in *Wwox KO* B Cells Undergoing Class Switch Recombination

Although AID preferentially targets *Ig* genes, off-target AID activity can induce DSBs at other sites providing recombination substrates that result in characteristic chromosome translocations frequently found in B cell tumors (51, 52). One example is the oncogenic *MYC/IGH* translocation found in Burkitt's lymphoma and other B cell tumors. This translocation is mediated by AID induced DSBs at both *MYC* and *IGH*, with breaks in the *MYC* gene being rate-limiting (43, 53). Chromosome translocations display signatures of Alt-NHEJ repair in B cells while C-NHEJ suppresses translocation formation (47). To determine if *Wwox* had a role in the incidence of spontaneous chromosome translocations during CSR, we used a previously described PCR/Southern blot assay to measure the frequency of AID induced *Myc/Igh* translocations (43, 53) (**Figure 6A**). It has been shown that naïve B cells do not harbor *Myc/Igh* translocations while cells induced to undergo CSR display translocations in an AID dependent fashion (43, 54). We observed that compared to *Wwox WT*, *Wwox*-deficient B cells had a significant 2.5-fold increase in spontaneous translocations (**Figures 6B,C**). Thus, we find that lack of Wwox expression is affecting the generation of spontaneous chromosome translocations, a process known to be promoted by the Alt-NHEJ pathway (47).

DISCUSSION

The incidence of plasma cell dyscrasias observed in our mouse model demonstrates that ablation of *Wwox* contributes to disease. Several mechanisms can mediate the loss of WWOX expression in human plasma cell dyscrasias including promoter methylation (25), genomic deletions (18, 21–24) and translocations (16, 17). One translocation example, t(14;16), is known as a MM high-risk indicator (20). The chromosome 16

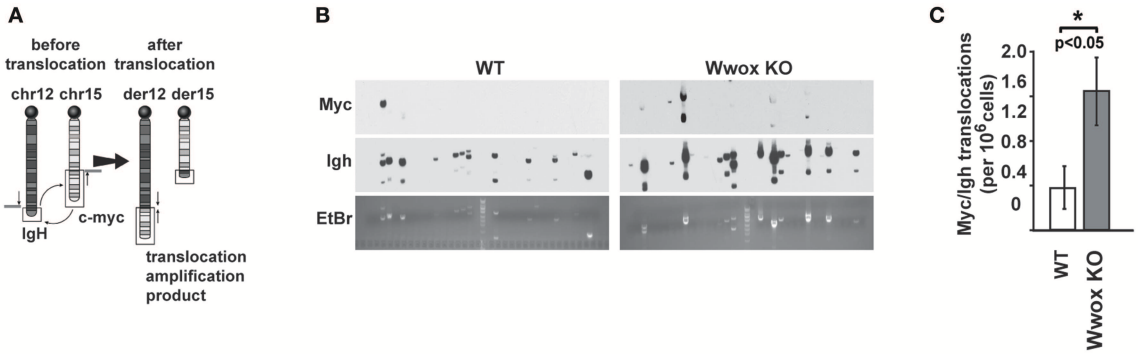

FIGURE 6 | Wwox-deficient B cells display increased frequency of spontaneous *Myc/Igh* translocations. **(A)** Schematic for the *Myc/Igh* translocation assay. PCR amplification primers for mouse chromosomes 12 (*Igh* locus) and 15 (*Myc* locus) are represented by black arrows and Southern probes by gray bars. Naïve B cells from 15 day old *Wwox WT* or *Wwox KO* mice were cultured with LPS and IL-4 for 72 h and assayed for translocations. **(B)** Representative translocation detection assay performed by means of Southern blots using *Myc* and *Igh* probes and corresponding ethidium bromide stained gels. Each lane represents the amplification result from 1×10^5 cells. **(C)** Summary of translocation events detected. Total frequency from $n = 4$ independent experiments. *P* value determined using two-tailed Fisher's exact test, *indicates significant *p* value.

breakpoints of t(14;16) are found within the *WWOX* gene and as a consequence structurally disruptive of the affected allele. The *MAF* locus, located 3' of *WWOX*, is brought within the influence of chromosome 14 *IGH* control elements and the resulting MAF overexpression has a causative role in MM. It is unclear however whether *WWOX* haploinsufficiency plays any role, or if silencing of the remaining allele via additional structural disruption (e.g., deletion) or epigenetic mechanisms is required in order to contribute to disease progression.

It has been shown that *Wwox* does not behave like a highly penetrant tumor suppressor gene [Reviewed in (7)]. The limited tumor incidence, long latency and heterogeneity of neoplastic transformation in our mouse model suggests that additional secondary genetic events have to occur for full-blown malignancy to develop. Nevertheless, the lack of tumorigenesis when *Wwox* is conditionally deleted in various other tissues in mice with the same mixed genetic background as those here used (7) together with previous observations (8, 11), suggests that B cells are indeed a target tissue in which *Wwox* ablation contributes to neoplastic transformation.

Lymphocytes are unique in that they undergo programmed DNA damage during their receptor diversification processes. While T and B cells undergo V(D)J recombination only B cells undergo CSR and somatic hypermutation. The genomic instability induced by these B cell specific processes likely drives the imbalance of 95% of lymphomas being B cell derived with the majority being of post-germinal center origin (26). During CSR, activation-induced cytidine deaminase (AID) induces DSB in the *IGH* switch regions to trigger recombination between isotype constant regions. The generation and resolution of these breaks occurs during the G1 phase of the cell cycle when NHEJ repair, but not homologous recombination, is readily available. Breaks randomly occur throughout each isotype switch region which are sequence diverse from each other. The result generates incompatible DSB end structures that require end-processing for ligation. The Ku70/80-mediated C-NHEJ pathway is the major pathway engaged during CSR. Recombination junctions normally lack microhomology consistent with the ability of C-NHEJ factors including XRCC4-DNA Ligase IV (Lig4) to process non-compatible ends (47). If C-NHEJ is unavailable, CSR proceeds with reduced efficiency via Alt-NHEJ, which tends to utilize MMEJ (46). We find that *Wwox*-deficient B cells support CSR at a slightly reduced rate and display MMEJ at recombination junctions consistent with Alt-NHEJ repair (46). MMEJ is always mutagenic since extensive end resection occurs to find microhomologous regions with repair deleting some intervening sequence (55). Alt-NHEJ has been implicated in chromosome translocations, as junction MH is a frequent feature of breakpoints and loss of C-NHEJ activity increases translocations (47, 56). For example, *Ku70* or *Lig4*-deficient B cells generate frequent *Myc/Igh* translocations via an Alt-NHEJ joining mechanism (49). Therefore, inappropriate usage of the Alt-NHEJ pathway has the potential to destabilize the genome. In this study, we find that *Wwox* deficiency increased generation of spontaneous B cell *Myc/Igh* translocations. This translocation is generated by AID induced DSBs at both Myc and *Igh* in primary B cells undergoing CSR (43, 53). Such translocation is a primary event in lymphomas such as Burkitt's and a common MM secondary event. Translocation capture assays show *Myc/Igh* to be indicative of genome wide *Igh* translocation and so determination of the spontaneous occurrence of such translocation event serves as a surrogate marker for overall genome instability (51, 52). In *Wwox* deficient B cells AID levels are not higher than WT (**Supplementary Figure 3**) and mutation frequency in the vicinity of recombination junctions is not increased. This further supports that *Wwox* deficiency influences DSB repair and not the mutation-generating machinery. Although AID is not expressed in MM cell lines, interaction of MM with dendritic cells in the *in vivo* microenvironment provides conditions that could induce AID expression and genomic instability (57). Furthermore, mutational signatures from APOBEC deaminase family members are found in MM and are associated with poor prognosis (58). Therefore, a primary

event involving loss of WWOX could influence genome stability during periodic AID or APOBEC expression in MM.

The mechanism by which Wwox influences C-NHEJ and Alt-NHEJ during B cell CSR is not clear. Previous studies have indicated that *WWOX* deficiency in human cell lines results in genome instability and abnormalities in DNA damage repair pathways (59, 60). Abu-Odeh et al. have reported that WWOX depletion can lead to reduced ATM checkpoint kinase activation and impaired DNA repair (59). In support of the observations here described, we have previously reported that WWOX depletion decreased C-NHEJ efficiency in human cell lines and is associated to the generation of phenotypes displaying increased resistance to the effects of DNA damaging agents (60, 61). However, *WWOX* function in B cells had not been previously examined. Since the DNA damage response is often dysregulated in cell lines, we here analyzed Wwox deficiency in primary B cells both in culture and *in vivo*. These results not only provide a clear and novel role for Wwox in B cell transformation and plasma cell dyscrasias but also further strengthen the notion of WWOX as an important player in maintaining genome integrity.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All animal research was conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International at the University of Texas MD Anderson Cancer Center, Science Park and all research was approved by the Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

CA and KM designed research. HK, YM, JP, JL, and MZ performed research. MS and CA performed pathology analyses. CA, MA, and KM analyzed the data. CA and KM wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00517/full#supplementary-material

Supplementary Figure 1 | Mouse genotyping and Wwox expression ablation in B cells from *Cd19 Cre+*, $Wwox^{flox/flox}$ mice. **(A)** PCR genotype analysis of DNA from two $Cd19^{Cre/+};Wwox^{wt/flox}$, two $Cd19^{Cre/+};Wwox^{wt/wt}$, and two $Cd19^{Cre/+};Wwox^{flox/flox}$ mice as indicated. Genotyping was performed using Cre primers F; 5′ GCC TGC ATT ACC GGT CGA TGC AAC G 3′ and R; 5′ GTG GCA GAT GGC GCG GCA ACA CCA T 3′ generating a PCR product of 700 bp in size. For amplifying the *Wwox wt* and *Wwox floxed* loci we used primers *Wwox*-N1; 5′ ATG GGA CGA AAC TGG AGC TCA GAA 3′, *Wwox*-N2; 5′ TCA GCA ACT CAC TCT GGC TTC AAC 3′ and *Wwox*-L; 5′ GCA TAC ATT ATA CGA AGT TAT TCG AG 3′, as previously described (9). The *Wwox wt* amplicon generates a 463 bp PCR product while the *Wwox* floxed allele generates a 344 bp product as indicated. **(B)** Representative immunoblot using Wwox antibody on protein extracts from FACS isolated Cd19+ B cells (first and fifth lanes) and from various other tissues (cerebellum, lung and kidney) from a $Cd19^{Cre/+};Wwox^{flox/flox}$ *(Cd19 Wwox KO)* mouse and a $Cd19^{Cre/+};Wwox^{wt/wt}$ *(Cd19 Wwox WT)* counterpart. As can be observed Wwox protein ablation is exclusive of B cells in the *Cd19 Wwox KO* mouse with other tissues expressing normal Wwox protein levels. Loading control using Actin antibody, lower panel.

Supplementary Figure 2 | Summary and alignments of CSR junctions. Summary table of number of clones with each type of junction. Representative alignments of a blunt, a 1 bp, a 2 bp, and all CSR junctions with more than 4 bp microhomology (MH). Sequence of a CSR junction (Blue, **Middle**) is aligned with germline switch donor **(Top)** and acceptor **(Bottom)** regions. Vertical lines denote identity between germline switch region and the sequenced CSR junction, bold lines mark continuous identity used to identify the breakpoint. Red denotes overlap between switch donor and acceptor region.

Supplementary Figure 3 | AID levels are not altered in *Wwox KO* B cells. Mouse splenocytes from wild-type (WT), *AID KO* (*Aicda*), or *Wwox KO* mice were cultured in LPS and IL-4 for 3 days. Anti-AID (Cell Signaling Technology L7E7) and anti-tubulin (Sigma) Western blot was performed on cell lysates and the relative signal ratio of AID to tubulin is displayed. Experiment representative of 3 independent experiments.

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REVIEW
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WW Domain-Containing Proteins YAP and TAZ in the Hippo Pathway as Key Regulators in Stemness Maintenance, Tissue Homeostasis, and Tumorigenesis

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The Hippo pathway is a conserved signaling pathway originally defined in *Drosophila melanogaster* two decades ago. Deregulation of the Hippo pathway leads to significant overgrowth in phenotypes and ultimately initiation of tumorigenesis in various tissues. The major WW domain proteins in the Hippo pathway are YAP and TAZ, which regulate embryonic development, organ growth, tissue regeneration, stem cell pluripotency, and tumorigenesis. Recent reports reveal the novel roles of YAP/TAZ in establishing the precise balance of stem cell niches, promoting the production of induced pluripotent stem cells (iPSCs), and provoking signals for regeneration and cancer initiation. Activation of YAP/TAZ, for example, results in the expansion of progenitor cells, which promotes regeneration after tissue damage. YAP is highly expressed in self-renewing pluripotent stem cells. Overexpression of YAP halts stem cell differentiation and yet maintains the inherent stem cell properties. A success in reprograming iPSCs by the transfection of cells with Oct3/4, Sox2, and Yap expression constructs has recently been shown. In this review, we update the current knowledge and the latest progress in the WW domain proteins of the Hippo pathway in relevance to stem cell biology, and provide a thorough understanding in the tissue homeostasis and identification of potential targets to block tumor development. We also provide the regulatory role of tumor suppressor WWOX in the upstream of TGF-β, Hyal-2, and Wnt signaling that cross talks with the Hippo pathway.

Keywords: Hippo pathway, WW domain proteins, tissue homeostasis, regeneration, stem cell, induced pluripotent stem cells, tumorigenesis

INTRODUCTION

The Hippo pathway was originally identified by genetic screens of tumor suppressors for tissue growth control in *Drosophila melanogaster*. Recent advances in the identification of the mammalian Hippo pathway components and functional implications highlight the role of the pathway in organ development, tissue regeneration, stem cell maintenance, and tumorigenesis (1–4). The pathway is evolutionarily conserved.

The stem cell has indeed generated a great interest for scientists in the past decades and attracted more and more attention from the public recently. The unique properties of stem cells give substantial clues for clinical doctors, biologists, and scientists to solve problems in fundamental biology during the developmental process and the aberrant progression of degenerative diseases and cancer. Despite extensive investigations into the underlying mechanisms of stemness properties, what has not been known is the key signaling pathway, which orchestrates the network for conferring stemness maintenance and tissue homeostasis. Exploration of the Hippo pathway on stem cell biology has shed light on the developmental path (5–8). Here, we focus on discussing how the key WW domain-containing YAP and TAZ of the Hippo pathway contribute their regulatory role via the WW domain and PY (proline-tyrosine) motif interactions.

THE HIPPO PATHWAY

The Hippo pathway took the name from aberrant tissue overgrowth and neoplasia that generates a unique "hippopotamus"-like phenotype. Beginning in 1995, two studies first discovered the Warts (Wts) gene deletion, which caused robust multiple tissue overgrowth in *D. melanogaster* (9, 10). Later, researchers uncovered more components within this pathway, including scaffolding protein Salvador (Sav) (11), Ste20-like kinase Hippo (Hpo) (12–14), and Mob as tumor suppressor (Mats) (15). These mutant proteins may cause tissue overgrowth in *Drosophila*. Soon after the discovery of the Salvador/Warts/Hippo pathway, Yorkie (Yki) was shown to be one of the key functional effectors of this pathway from the screening of Wts-interacting proteins (16). The Hippo pathway-regulated overgrowth in organs has quickly attracted broad attention and led to the development of transgenic mouse models (16, 17).

Abbreviations: YAP, Yes-associated protein; TAZ, Transcriptional co-activator with PDZ binding motif; iPSCs, Induced pluripotent stem cells; Oct3/4, Octamer-binding transcription factor 4; Sox2, SRY (sex determining region Y)-box 2; PY motif, Proline-tyrosine motif; MST1/2, Mammalian sterile 20-like 1/2; SAV1, Salvador; LATS1/2, Large tumor suppressor homolog 1/2; MOB1A/B, MOB kinase activator 1A/B; TEAD, Transcriptional enhancer factor domain; VGLL4, Vestigial-like family member 4; NF2, Neurofibromin 2/ Merlin; MAP4Ks, Mitogen-activated protein kinase kinase kinase kinase; PRM, Proline-rich peptide motifs; TB, TEAD-binding region; PPXY, Proline/proline/any amino acid/tyrosine; PP2A, Protein Phosphatase 2A; Tgi, Tondu-domain-containing growth inhibitor; VGLL4, Vestigial-like family member 4; CTGF, Connective tissue growth factor; Cyr61, Cysteine-rich 61; TE, Trophectoderm; ICM, Inner cell mass; AMOT, Angiomotin; ESCs, Embryonic stem cells; BMP, Bone Morphogenetic Protein; TICs, Tumor initiating cells.

Core Components of Hippo Pathway

The Hippo pathway is essentially a cascade of kinases, comprising transcription coactivators and DNA-binding proteins. Although the pathway was first discovered in *Drosophila*, it is evolutionarily conserved in mammals. The mammalian orthologs of Hpo, Sav, Wts, Mats, and Yki are Mammalian sterile 20-like 1/2 (MST1/2, also named as STK4/3), Salvador (SAV1), Large tumor suppressor homolog 1/2 (LATS1/2), MOB kinase activator 1A/B (MOB1A/B), and Yes-associated protein (YAP)/Transcriptional co-activator with PDZ binding motif (TAZ), respectively (**Figure 1** and **Table 1**) (15, 18–21, 29). The best-known role of the Hippo pathway is to orchestrate organ development and control tissue homeostasis through modulation of cell proliferation, apoptosis, migration, and differentiation (7, 8). Hippo pathway also regulates stem cell self-renewal and expansion and tissue regeneration (30–32).

Numerous Initiating Signals for the Responsive Kinase Cascade: An Integrated Action or a Conundrum in Chaos?

The Hippo pathway receives a broad range of signals, including extracellular mechanical force, hormonal cues, and intrinsic cell physical mechanism, so as to control cell polarity and cytoskeletal organization, and interactions with proteins in the extracellular matrix (33–35). Numerous proteins participate in the initiation of the Hippo signaling. For example, protein kinases, including Src kinase, protein kinase A (PKA), PAR-1/MARK kinase, and TAO kinase are involved (1, 36). Extracellular soluble factor Amphiregulin (37), matrix Agrin proteoglycan and Integrins (38), and cell junctional proteins Echinoid and Cadherin-Catenin complex (39, 40) participate in the signaling network. Furthermore, cell polarity proteins Crumbs, and Scribble complexes (41, 42), G-protein-coupled receptor (GPCR) and Wnt/beta-catenin signal pathways (43, 44), and cytoskeletal Spectrin, Myosin II, and F-actin (45–49) are integrated in the signaling network (**Figure 2**). Among these signals, many of them collaborate with each other, whereas others may be in conflict. How each cell selectively sorts out which signal(s) to respond and/or converge all signals, or ignore and terminate them, remains to be a critical issue to resolve.

When cells receive the extracellular upstream activation signals, the signal receiver MST1/2 (or *Drosophila* Hpo) phosphorylates LATS1/2 (or *Drosophila* Wts) and MOB1 (or *Drosophila* Mats) in a canonical manner, with the assistance of cofactor SAV1 (or *Drosophila* Sav). SAV1 is a WW domain-containing protein needed for integrating the upstream signal(s). Then, the activated LATS1/2, in turn, triggers the phosphorylation of the major coactivators YAP/TAZ (two homologs of *Drosophila* Yki) at multiple residues (**Figure 1**). Phosphorylation of YAP at S127 (corresponding to S89 on TAZ) promotes its binding with 14-3-3, thus resulting in the cytoplasmic retention (20). Phosphorylation of YAP/TAZ at S381 and S311, respectively, creates a binding site for casein kinase 1 (CK1) and subsequent phosphorylation by CK1δ/ε at the DSGxS motif. Then SCFβTrCP, a multi-subunit SKP-CULLIN-F-box (SCF) ligase complex specifically recognizes the phosphodegron

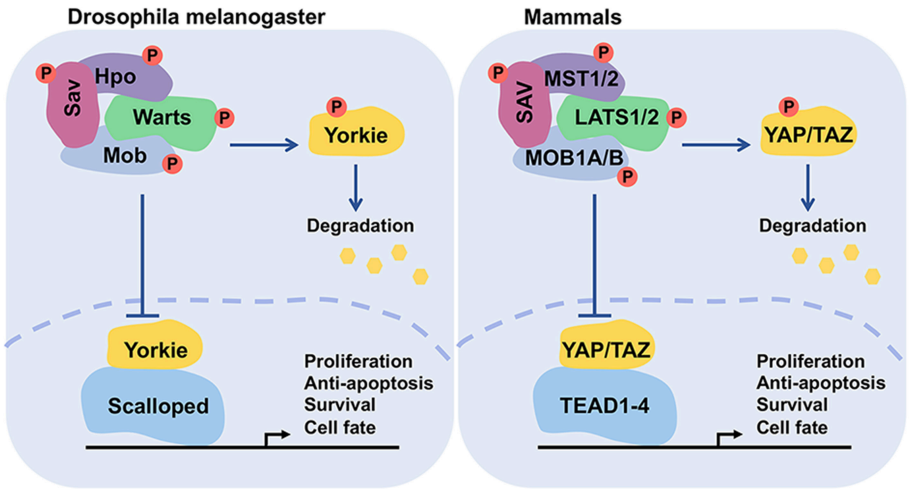

FIGURE 1 | Functional conservation of the core components of Hippo pathway. The functionally conserved factors of *Drosophila melanogaster* and mammals are matched by color. This network controls the transcriptional events for regulating cell proliferation, survival, and death.

TABLE 1 | Hippo pathway components and major functions.

Drosophila	Mammals	Major function in Hippo pathway	References
Hippo (Hpo)	MST1 MST2	Phosphorylate LAST1/2, MOB1, and SAV, leading to LAST1/2 activation	(18)
Salvador (Sav)	SAV1	Interact with MST1/2, promotes phosphorylation of LAST1/2 by MST1/2	(19)
Warts (Wts)	LATS1 LATS2	Phosphorylate and inactivate YAP/TAZ	(20)
Mats	MOB1A/B	Scaffold protein of LAST1/2	(15)
Yorkie (Yki)	YAP TAZ	Transcription co-activator, major effectors of the Hippo pathway	(21)
Scalloped (Sd)	TEAD1-4	Transcription factors mediate the effect of YAP/TAZ	(21)
Tgi	VGLL4	Competes with YAP/TAZ for TEADs, inhibits YAP/TAZ functions	(22)
Misshapen (Msn)	MAP4K4	May form a complex and mediates upstream signals. (from plasma membrane) to MST1/2. Activates LAST1/2	(23)
Merlin (Mer)	NF2	Signal transduction between cytoskeletal proteins and cell membrane. May bring LAST1/2 to plasma membrane and facilitate its activation by MST1/2.	(24)
Kibra	KIBRA	Phosphoprotein involved in cell polarity, mitosis and cell migration	(25)
Expanded (Ex)	FRMD6 AMOT RASSF1	Protein linking cytoskeleton to plasma membrane Sequesters YAP/TAZ to cell junctions, binding and indirectly activates LAST1/2, a substrate of LAST1/2 Signal transducer, microtubule stabilization, cell cycle arrest	(26) (27) (28)

DpSGxpS of YAP and TAZ for leading to eventual YAP/TAZ ubiquitination and degradation (20, 50, 51). YAP protein is also degraded via autophagy (52). Unphosphorylated YAP/TAZ complex translocates to the nucleus to drive transcriptional activation (**Figure 2**). The phosphorylation/degradation strategy has been seen in many biological molecules for their turnover. For example, tumor suppressor p53 is subjected to Mdm2-mediated degradation in the cytoplasm, whereas phosphorylated p53 is stabilized in the nucleus.

MST1/2 in Hippo pathway can be activated without upstream kinases. The phosphorylation cascade is enhanced by MST1/2 dimerization (53). Active MST1/2 phosphorylates SAV1 and MOB1A/B (19, 29), which assists MST1/2 to recruit and phosphorylate LATS1/2 at their hydrophobic motifs (T1079 for LATS1 and T1041 for LATS2) (24, 54). Another key component in this action is NF2 (or Merlin), which directly interacts with LAST1/2 and promotes their phosphorylation (24). LATS1/2 subsequently undergoes autophosphorylation (18), and

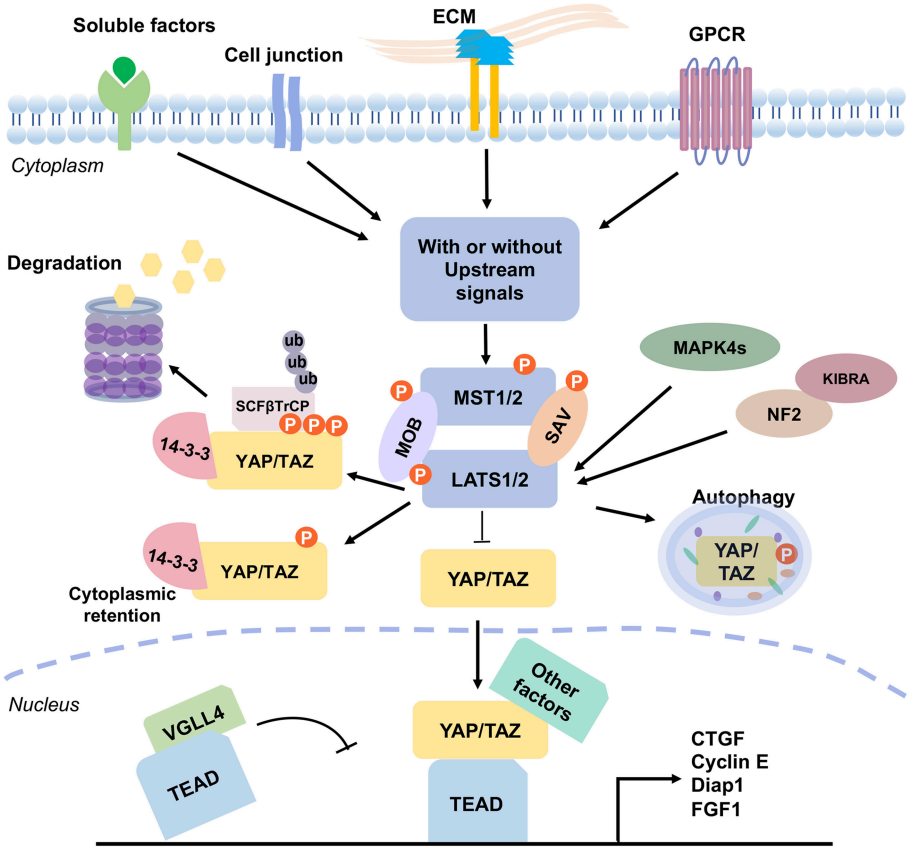

FIGURE 2 | The mammalian Hippo pathway signaling. Various signal stimulators activate the Hippo pathway, which includes GPCR, cell junction and ECM proteins, and soluble factors. These signals regulate the phosphorylation of MST1/2 and LATS1/2 kinases to further induce YAP/TAZ activation. Without the upstream signals, KIBRA interacts with NF2 to promote the pathway activity. Also, NF2 and MAPK4s directly interact with LAST1/2 and promote LATS1/2 phosphorylation (pLATS1/2). Then, pLATS1/2 phosphorylates YAP/TAZ. And, the phosphorylated YAP/TAZ presents a 14-3-3 biding site and SCFβTrCP specific recognition site, which causes cytoplasmic retention of YAP/TAZ and results in YAP/TAZ degradation. YAP protein is also degraded via autophagy. VGLL4 acts as a transcriptional repressor by competing with YAP/TAZ for binding TEADs. Unphosphorylated YAP/TAZ complex translocates into the nucleus, and acts as transcription coactivators by forming complexes with various transcriptional factors, such as TEADs. Binding of YAP/TAZ with TEAD prevents VGLL4 from associating with the complex, and consequently drives the expression of numerous genes which are related to cell proliferation, survival, and migration. The signaling outcome includes induction of cell cycle regulator cyclin E, cell-death inhibitor Diap1, connective tissue growth factor (CTGF), and fibroblast growth factor (FGF1), and etc.

triggers the phosphorylation of YAP and TAZ for functional inactivation (55). Moreover, in parallel to MST1/2, two groups of MAP4Ks (mitogen-activated protein kinase kinase kinase kinase), MAP4K1/2/3/5 [homologs of *Drosophila happyhour* (Hppy)] and MAP4K4/6/7 [homologs of *Drosophila misshapen* (Msn)] directly phosphorylate LATS1/2 at their hydrophobic motifs and result in LATS1/2 activation, which consequently inactivates YAP/TAZ (23, 56, 57).

Overall, like many signaling pathways, the Hippo phosphorylation cascade is well-orchestrated and evolutionarily conserved. However, the ultimate outcome can be altered, either enhanced, or altered, by various signal stimulators. Conceivably, a single stimulator Wnt or growth factor, for example, may activate not only the Hippo pathway but also other molecular paths, thereby either toning down or escalating the outcomes. Nonetheless, there are multiple signal initiators for the Hippo pathway. The signal flow could be in either a concerted manner or ends up in chaos.

Among all the factors, how can those signals possibly work in a concert or contradictory manner? In short, GPCR either activates or inhibits the Hippo-YAP pathway depending on the signaling effected by the soluble Serum-borne lysophosphatidic acid and sphingosine 1-phosphophate (44). Soluble factor Amphiregulin binds EGFR and acts as an autocrine growth factor for establishing a positive autocrine regulatory feedback loop between EGFR and YAP1, which is important in cancer progression (37). Cell junction proteins Echinoid and E-cadherin inhibit YAP/TAZ activation. Echinoid physically binds and stabilizes the Hpo-binding partner Sav at adherens junctions. Loss of Echinoid compromises Yki phosphorylation, resulting in elevated Yki activity that increases Hpo-targeted gene expression and drives tissue overgrowth (39). Also, E-cadherin inhibits YAP/TAZ activation

without involving the upstream signals of the Hippo pathway. This is achieved via the regulation of alpha/beta-catenin pathway (40).

YAP AND TAZ ARE WW DOMAIN-CONTAINING PROTEINS

The WW domain is a structural module that mediates protein-protein interactions through recognition of proline-rich peptide motifs (PRM) and phosphorylated serine/threonine-proline sites (58). WW domains are found in at least 52 different structural and signaling proteins in the human proteome. They participate in a variety of cellular processes and have been implicated in major human diseases such as cancer and neurodegeneration. In the Hippo pathway, SAV, KIBRA, YAP, and TAZ are the critical WW domain-containing proteins (59–61). While WW domains are responsible for protein/protein binding, the actual functional areas in the WW domain-containing proteins may not necessarily be on the WW domain areas. Phosphorylation in particular amino acid residues outside of the WW domain(s) may confer crucial molecular functions both *in vivo* and *in vitro*.

The Configurations and Binding Motifs of YAP/TAZ

The human *YAP* gene is located on chromosome11q22. YAP protein was first identified as a proline-rich phosphoprotein, and is capable of binding the SH3 domain of Yes and Src protein tyrosine kinases (62). *YAP* mRNA is not only ubiquitously expressed in a broad range of tissues, but also is expressed during the entire developmental process (63). The human *TAZ* gene is mapped at chromosome 3q23-q24, and encodes TAZ protein (also known as WW-domain containing transcriptional regulator 1, WWTR1). Similarly, the *TAZ* gene is expressed in various tissues and amplified in many cancer cells (64).

Structurally, YAP and TAZ proteins share nearly half of the protein sequence identity and have a very similar topology. YAP protein has 488 amino acids, possessing a TEAD-binding region (TB), one or two WW domains in the isoforms, an SH3-binding motif, a coiled-coil domain, a transcription activation domain, an *N*-terminal proline-rich domain, and a *C*-terminal PDZ-binding motif. The WW domain has two-conserved tryptophan (W) residues separated by 20–23 amino acids. TAZ protein consists of 400 amino acids and has a similar domain organization with YAP, although it lacks the second WW domain, the SH3-binding motif, and the proline-rich domain (**Figure 3**).

The WW domains of YAP and TAZ physically interact with the "PPXY motifs (proline/proline/any amino acid/tyrosine)" in many proteins, including transcriptional factors (65). The TEAD-binding domain (TB) of YAP and TAZ recognizes the transcriptional enhancer factor domain family (TEAD) family of transcription factors and activates target gene expression, whereas the 14–3–3 binding motif is involved in the degradation of YAP and TAZ. As mentioned previously, in a canonical pathway, LATS phosphorylates YAP/TAZ and promotes the binding of 14-3-3 with YAP/TAZ. 14-3-3 binds YAP/TAZ via the phosphorylation sites S127 of YAP and S89 of TAZ, which results in cytoplasmic retention of YAP/TAZ. Certain upstream regulators, such as α-catenin, require the binding of 14-3-3 with the YAP pS127 site for subsequent inhibition of cell proliferation (66). Protein Phosphatase 2A (PP2A) dephosphorylates the 14-3-3 docking site on YAP, resulting in the nuclear import of YAP. The PDZ-binding motif is required for binding with another PDZ domain, and this can be found in many transmembrane or cytoskeleton-associated proteins. The PDZ-binding domains display to direct the cellular distribution of YAP and TAZ (67, 68). Functionally, YAP and TAZ are transcriptional co-activator and work synergistically with transcription factor companions such as TEAD and RUNX1/2, SMAD family members (69–71).

YAP/TAZ Action in Downstream Gene Targets

YAP and TAZ cannot physically bind chromosomal DNA and thus act as co-activators with DNA-binding transcription factors to regulate a large number of target genes (72–75). Both proteins regulate the gene expression mainly by binding TEAD1-4 [homologs of *Drosophila* Scalloped (Sd)] (21). The interaction between YAP/TAZ and TEAD drives the expression of numerous genes for cell proliferation, survival, and migration. The gene products are the cell cycle regulator cyclin E, cell-death inhibitor Diap1, connective tissue growth factor (CTGF), cysteine-rich 61 (Cyr61), fibroblast growth factor (FGF1), and bantam microRNA (37, 69). Mouse models harboring the deletion of Mst1/2, Sav1, Mob1A/B, NF2, or Last1/2, or Yap overexpression, exhibit up-regulated *Tead* target gene expression, and increased expansion of progenitor cells and tissue overgrowth (76–79). thereby manifesting the importance of these genes in the Hippo pathway (**Figure 2**).

Importantly, Tondu-domain-containing growth inhibitor (Tgi) and Vestigial-like family member 4 (VGLL4, an ortholog of Tgi) are well known regulators of Yki and YAP/TAZ, respectively. Tgi directly competes with Yki for Sd binding in nucleus, restrains the Yki-regulated transcription (22, 69). When Hippo signaling cascade is on, Tgi and Sd form a complex to exert transcriptional repression. Conversely, when Hippo signaling is off, Yki translocates into the nucleus and takes the place of Tgi from Sd, permitting the expression of Yki target genes (69). By the same token, in mammals, VGLL4 competes with YAP/TAZ for TEAD binding (80, 81).

The YAP/TAZ complex also regulates the expression of certain genes via binding specific factors. For instance, the YAP/TAZ complex interacts with the nucleosome remodeling and histone deacetylase (NuRD) complex, and decreases the transcription of target genes (82). Moreover, YAP is identified as a regulator for miRNA biogenesis by modulating miRNA 'processing enzymes microprocessor or Dicer complex (83, 84). Further, YAP directly induces the production of miR-130a, which represses VGLL4 expression, and forms a positive feedback loop for promoting the amplification of YAP activity (85). Hence, the observations suggest that YAP/TAZ regulate gene transcription through coordination of multiple mechanisms (86).

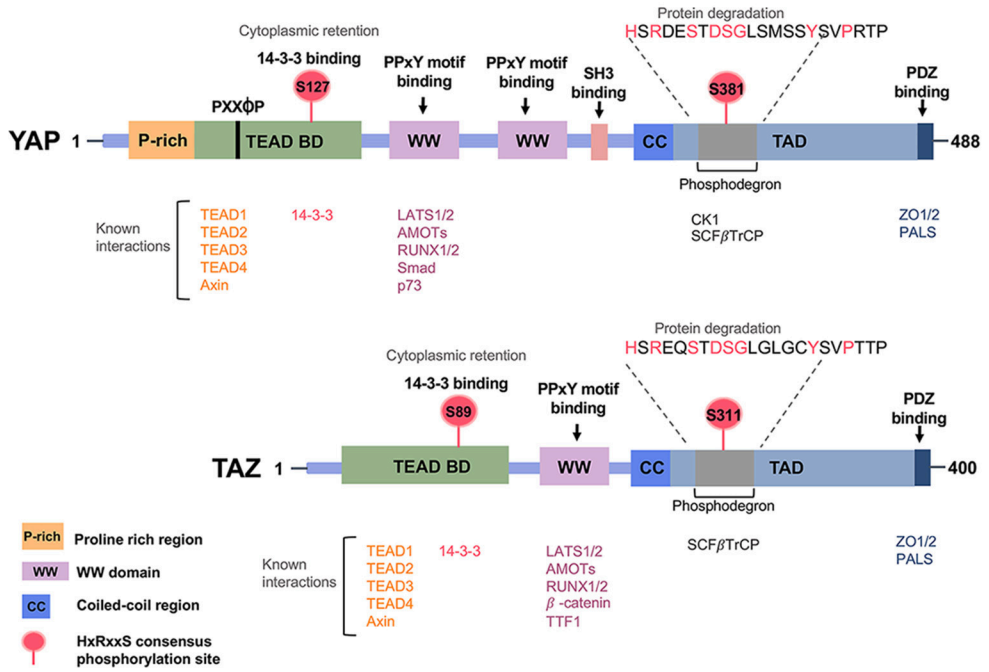

FIGURE 3 | Regulatory domains of the Hippo pathway effector proteins YAP/TAZ. Prominent regions include the WW domain(s), the coiled-coil (CC) domain, the SH3-binding domain, the TEAD transcription factor-binding domain, the transcriptional activation domain (TAD) and the PDZ-binding motif.

HIPPO SIGNALING IN ORGAN SIZE CONTROL AND TISSUE HOMEOSTASIS

The remarkable effect on organ size and tissue homeostasis regulation is the best-known feature of the Hippo pathway. How the Hippo pathway senses the physiological cues to modulate organ size is largely unknown. It is generally believed that the mechanical force or tension may determine the tissue growth and restrain YAP/TAZ activation when the organ reaches its proper size. Organ sizes are restricted/induced by some soluble factors via the autocrine/paracrine mechanism in a concentration-dependent manner (87). Still, a crucial point is that how the cells on the outer surface of an organ sense the overgrowth from the inner mass of the organ, and vice versa. Does the Hippo pathway coordinate the even cell growth, cell shape, and spatial relationship of all cells in an expanding organ?

Effects of Hippo Signaling on Organ Size Regulation

In *Drosophila*, dysregulation of the Hippo pathway kinases (*Hpo* and *Wts*) or upstream regulators (*Ex*, *Mer*, *Kibra*, *Ft*, etc.) promotes Yki-induced cell proliferation and finally leads to tissue/organs overgrowth (16, 25, 26, 28). Under physiologic conditions, the liver returns to its normal size while Yap expression is restored at the background level (88). The long-term persistent Yap activation causes the onset of hepatocellular carcinoma (30). Liver conditional knockout of *Mst1/2*, *Sav1*, or *Nf2* causes liver enlargement (76, 89). Moreover, deletion of *Sav1*, *Mst1/2*, or *Lats1/2* gene brings upon embryonic heart enlargement.

The genetic manipulation of *Yap* robustly affects the proliferation and survival of cardiomyocytes (90–92). Substantial evidence revealed that tissue-specific deletion of *Yap* gives rises to abnormalities in several organs, including heart, skin, and kidney (93–95). Abnormal activation of YAP/TAZ makes aberrant influences in multiple organs, such as liver, heart, stomach, and spleen. However, tissue-specific deletion of *Yap* does not cause defects in the breast and intestine (78, 96). Mammary glands and intestine remain relatively normal upon *Yap* deletion (96–98). These findings imply that differential regulation of the Hippo pathway is utilized for size regulation among organs. Despite the outstanding findings, the mechanical force from within or outside the organ to balance the cellular size, shape, and tension, along with regulation by the Hippo pathway and/or others, is not well-defined.

Hippo Signaling Regulation in Tissue Regeneration

For adult organisms, damage, and impair can barely be avoided during the lifetime. Thus, tissue regeneration is especially important. The Hippo signaling pathway, especially Yap, participates in organ regeneration. For example, liver regenerates efficiently post damage (e.g., partial hepatectomy), due to the proliferation of hepatocytes to restore liver mass. The transcriptional activity of Yap/Tead is increased during liver regeneration, meanwhile Mst1/2 and Lats1/2 are down-regulated toward the completion of tissue repair. When the regeneration is finished, the Yap activity is repressed and Mst1/2 and Lats1/2 inhibition is released automatically (89, 99–101).

Similarly, the study in the mouse model showed that intestines can effectively repair and regenerate from colitis induced by dextran sulfate sodium (DSS). However, the regenerative capability is severely obstructed upon tissue-specific knockout of *Yap* (96). Marvelously, overexpression of *Yap* restores some myocardial regenerative capability, although the regeneration of the adult heart is very limited. In contrast, specific deletion of *Yap* obstructs the regeneration of the neonatal heart (91, 92, 102). Again, the studies imply that organ formation and morphogenesis is not only just the proper function of YAP but also other cellular factors are involved.

YAP/TAZ in Progenitor Cell

Progenitor cells play an important role in tissue homeostasis and regeneration. A high YAP/TAZ activity has been observed in the stem cells or progenitor cells of multiple tissues. Activation of YAP, which usually leads to the expansion of progenitor cells, impairs cell differentiation of target tissues such as intestine, liver, skin, and nervous system (96, 97, 103, 104). In intestinal crypts, YAP is highly localized in the nucleus promoting target gene expression in basal progenitor cells and in intestinal stem cells (30, 96, 105). In chick embryo, YAP and its transcriptional targets CTGF and CYR61, which are known to stimulate cell proliferation, are abundant during early development of the stomach (106). Moreover, YAP is highly expressed in the nuclei of single-layered basal epidermal progenitors. YAP is crucial in promoting the expansion of basal epidermal progenitors, proliferation, and inhibition of terminal differentiation through transcriptional target Cyr61. After initiation of hair follicle morphogenesis, YAP translocates to the cytoplasm of differentiating cells (107). Taken together, the capability of YAP/TAZ on cell proliferation and progenitor cell expansion suggests its decisive role in organ development, tissue homeostasis, and injury-induced regeneration. As a negative control mechanism, YAP/TAZ has to be under proper phosphorylation in order to be subject to degradation by the 14-3-3/SCFβTrCP/ubiquitin/proteasome system.

HIPPO PATHWAY IN EARLY EMBRYONIC DEVELOPMENT, STEMNESS MAINTENANCE AND PROMOTING THE PRODUCTION OF INDUCED PLURIPOTENT STEM CELLS (iPSCs)

Stem cells possess the unique properties of self-renewal and differentiation potency, which undergo asymmetric replication to divide one daughter cell identically to the parental cell and another daughter cell differentiating into destined cell type (108). In mammals, there are two major types of stem cells: pluripotent stem cells and somatic stem cells (109). Pluripotent stem cells, such as embryonic stem cells (ESCs), which are isolated from the inner cell mass (ICM) of blastocysts during development. These cells are capable of differentiating into near all cell types of three germ layers—ectoderm, endoderm, and mesoderm. Somatic stem cells are isolated in various adult tissues. The somatic stem cells and progenitor cells function in the repair system in different adult tissues and maintain tissue growth, homeostasis and regeneration through expanding cell numbers and replacing old or injured cells. Stem cells play critical roles in both embryonic developmental and adult stages. Therefore, it is important to understand the molecular mechanisms underlying the stemness maintenance. Appropriate tools are needed to pry into the perfect prospectus regarding how one single cell grows into a multicellular organism under a concerted and yet a regulatory signaling network.

YAP/TAZ in TE and ICM Specification

Stem cells possess the drive to proliferate, differentiate, and migrate during development and tissue regeneration, in which the Hippo pathway is involved. The first cell lineage specification during embryonic development is a special feature of mammalian development with the emergence of the inner cell mass (ICM) and trophectoderm (TE).

In mice, after the gametes are fertilized in the oviduct, the embryo divides into an eight-cell morula with a relatively slow dividing rate. Each cell of the morula is called a blastomere. During the preimplantation stage, the blastomeres form adherens and tight junctions, and acquire apical-basal polarity, as well as an increase in the surface contact with its neighbors in a process called compaction (110). This creates the polarization of the cells within the morula. As these cells divide into the 16-cell stage, the innermost and more compacted cells lose their polarity. The differences between the inner and the outer cells bring about a disparate distribution of TAZ/YAP proteins, followed by further division to yield a blastocyst composed of about 32 cells. By entering the blastocyst stage, the subcellular localization of TAZ/YAP determines the first cell fate choice in the embryo—the settlement of embryonic cells to become either TE or ICM. For TE cells, TAZ/YAP is accumulated in the nuclei but distributed all over the cytoplasm of the ICM cells (111). The TE cells at the outer layer of the blastocyst form extraembryonic tissues, whereas ICM gives rise to the definitive structures of the fetus (112) (**Figure 4**).

YAP/TAZ in Embryonic Development

Yap knockout mouse exhibits arrest in the division in the blastomere before entering the morula stage (16–32 cells) and embryonic lethal at E8.5 with a vascular defect of yolk sac (63, 111, 113–115). Similarly, deletion of both *Yap* and *Taz* results in cell fate specification defects, leading to embryo death at the morula stage prior to the specification of TE or ICM (87, 111), again suggesting the Hippo pathway and YAP/TAZ play vital roles at early embryonic development.

When the Hippo signaling pathway is inactive, YAP promotes trophectoderm fates in the embryo. However, when the Hippo signaling is activated, YAP relocates from the cell nucleus to the cytoplasm to promote pluripotent cell fates (116, 117). The differential distributions of YAP/TAZ in the TE and ICM causes distinct gene expression signatures, where the nuclear TAZ/YAP complex directs a TE-specific transcriptional program by interacting with TEAD transcription factors. For example, at the blastocyst stage, YAP/TAZ induces *Cdx2* and *Gata3* transcription by modulating the activity of TEAD in the outer TE cells (111, 118). High YAP activity is necessary for TE

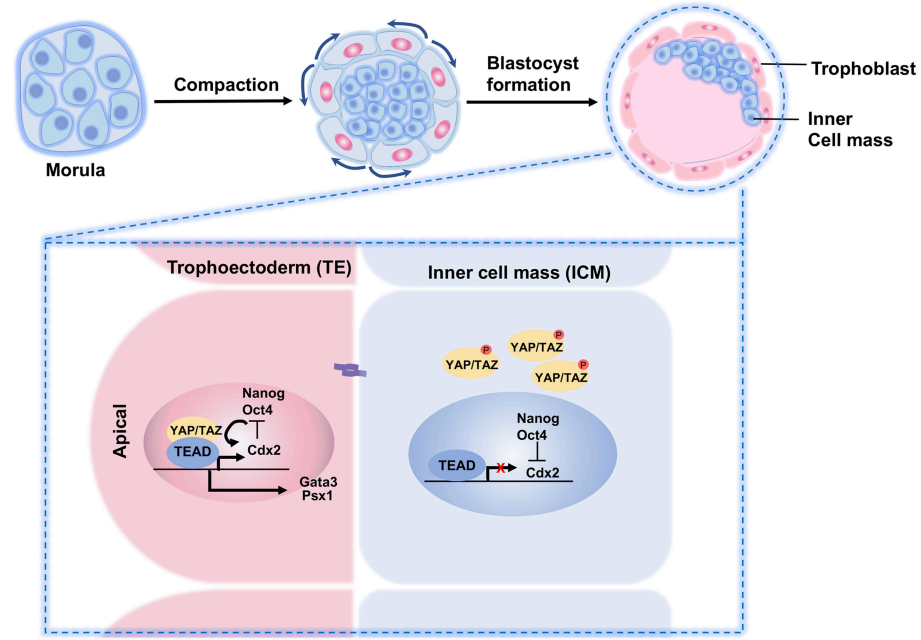

FIGURE 4 | A schematic illustration of YAP/TAZ regulation of TE and ICM specification in preimplantation embryo. During preimplantation, the outer blastomeres of the embryo form an outer epithelial trophoectoderm (TE) that envelopes the remaining blastomere and the inner cell mass (ICM). The Hippo pathway plays a crucial role in the cell fate determination. In the outer cells, nuclear YAP and TEAD4 regulate specification of the TE lineage through activation of the TE-specific genes such as Cdx2 and Gata3. In the inner cells, cell-cell adhesions influence the Hippo signaling. Activated Hippo pathway impairs YAP nuclear localization in the ICM lineage, thereby limiting TEAD4 transcription and abrogating the expression of outer TE-specific genes.

specification. For example, in *Tead4*$^{-/-}$ embryos, all blastomeres are specified to the ICM lineage (119–121).

TEADs

TEADs are the earliest genes highly expressed during embryo development, and are the transcription factors with YAP/TAZ as cofactors. Among them, TEAD4 is required for specification of the TE lineage during preimplantation in mammals. TEAD4 activity depends on the nuclear localization of YAP, which is regulated by cell-cell contacts and LATS1/2-mediated phosphorylation for negative regulation. Phosphorylated YAP undergoes degradation in the cytoplasm. Increased nuclear localization of TAZ/YAP, resulting from the deletion of *Lats1/Lats2*, leads to amplified *Cdx2* expression, which prevents proper specification of the ICM (111). In addition, the junction-associated angiomotin (AMOT) family proteins angiomotin (Amot) and angiomotin-like 2 (Amotl2) are essential for Hippo pathway activation and appropriate cell fate specification (27). Depleting LATS1/2, NF2, or AMOT/AMOTL2 turns all cells into TE, and these embryos fail to establish ICM-derived tissues (119–121). In contrast, knockout of TEAD leads to the loss of *Cdx2* expression and prohibits the embryos to form the TE with a consequence of all cells transforming into ICM (24, 122).

Depletion of both *Mob1a* and *Mob1b*, which are regulators of LATS1/2 activity, also results in developmental defects, with embryo arrest at E6.5 prior to gastrulation (110). Analysis of *MOB1A/B*-deficient blastocysts revealed aberrant nuclear YAP localization and modest growth failure of ICM, with few defects associated with the TE. Similar to the *Lats1/2*-deficient embryos, depletion of both *Amot* and *Amotl2* also increases nuclear YAP localization and *Cdx2* expression throughout both inner and outer cell populations, thereby resulting in severe pre-implantation defects. However, depletion of either *Amot* or *Amotl2* alone did not cause pre-implantation defects, indicating redundancy between AMOT family members (117).

Additionally, Neurofibromin 2 (NF2) (or Merlin) and AMOT, two upstream components of the Hippo pathway, facilitate YAP phosphorylation via LATS1/2 during cell fate specification of mouse preimplantation development. Mechanistic studies have revealed that LATS1/2 induces the phosphorylation of AMOT in the inner cells of the pre-implantation embryo, promoting its association with NF2 at cell membranes, and consequently amplifying the TAZ/YAP phosphorylation (117). Altogether, these results demonstrate a critical role for YAP and Hippo signaling cascade in the process of cell fate specification and development in early embryos.

TAZ/YAP in Stem Cell Pluripotency

The Hippo pathway regulates pluripotent stem cells *in vitro*. TAZ/YAP participates in the differentiation of embryonic stem cells (ESCs) derived from the ICM of the blastocyst. ESCs are pluripotent stem cells capable of self-renew and differentiating into near all functional cell types in an adult organism. YAP is highly expressed in self-renewing ESCs. Downregulation of YAP protein and mRNA levels correlates

with significantly decreased pluripotent markers along the line of ESC differentiation. In addition, YAP is sequestered and thereby inactivated in the cytoplasm during ESC differentiation, and consequently a large number of major genes for stem cell maintenance and function, including *Nanog, Oct3/4,* and *Sox2*, are repressed. Overexpression of *Yap* inhibits ESC differentiation and maintains stem cell properties even under differentiation culture conditions, while knockdown of *Yap*/*Taz* is sufficient to result in the impairment of ESC phenotype (123–126). Furthermore, restricting YAP and TEAD2 expression or inhibiting TEAD function induces differentiation toward the endoderm lineage. In contrast, deletion of *Mst1/2* in ESCs induces cell proliferation and breaks the process of differentiation (127). Further, bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) signals maintain mouse ESCs in an undifferentiated and pluripotent state, whereas human ESCs require fibroblast growth factors (FGF), BMP, and transforming growth factor-β (TGF-β) to maintain pluripotency.

A fine-tune of growth factor-induced and cytoskeleton-associated cues are crucial in the maintenance and balance between differentiation and self-renewal in ESCs. These signals ultimately control the levels and actions of a core transcriptional circuitry consisting of OCT4 (also known as POU5F1), NANOG and SOX2 (128). YAP and TEAD2 activate the expression of ESC master transcriptional regulators OCT4 and NANOG in mammalian ESCs. Also, nuclear TAZ/YAP activity is required to integrate growth factor signals with these core transcriptional regulators to maintain the ESC pluripotent state. Human ESCs require signals induced by FGFs and members of the TGFβ family (129). TGFβ stimulates the action of serine/threonine kinase receptors that phosphorylate and activate the SMAD2/3 class of transcription factors (130). TAZ/YAP proteins form complexes with phosphorylated SMAD2/3 (123, 126). In the nucleus, the TAZ/YAP-SMAD2/3 complexes bind TEAD transcription factors, and the core stem cell regulator OCT4, and together mediate the pluripotent state (124). Mechanistically, this complex assembles with factors that make up the nucleosome remodeling and deacetylation (NuRD) complex to buffer the expression of pluripotency genes and repress genes that define mesoendoderm. Upon mesoendoderm specification, the TAZ/YAP-TEAD-OCT4 complex dissociates from the SMADs, allowing the SMADs to activate the forkhead transcription factor FOXH1 and drive differentiation (124).

Similarly, mouse ESCs also require precise *Yap* levels to maintain their pluripotent state. Knockdown of *Yap* in mouse ESCs leads to loss of OCT4 and SOX2 expression, and consequent differentiation (125).

YAP may induce the expression of pluripotency-associated genes, which promote ESC self-renewal. It is suggested that YAP is dispensable for self-renewal but is required for the differentiation of ESCs. Knockdown or knockout of *Yap* does not alter ESC self-renewal but impairs their differentiation (131, 132). TAZ and the TEADs are also dispensable for ESC self-renewal (132). Deletion of *Lats2* in ESCs impairs their pluripotency and ability to differentiate (133). The discrepancy among these studies is likely due to the presence of unknown factors, which affect the Hippo pathway in different cell lines, as well as variations in cell culture and experiments.

YAP in Induced Pluripotent Stem Cells (iPSCs)

The role of YAP in controlling pluripotency is seen in induced pluripotent stem cells (iPSCs), which was established by using the ectopic expression of *Oct3/4, Sox2, Klf4,* and *c-Myc* genes closely resemble that of ESCs in phenotype and differentiation potential (124, 134). YAP is activated during the reprogramming process of human embryonic fibroblasts into iPSCs, and the addition of YAP to original four factors increases the reprogramming efficiency in mouse embryonic fibroblasts, further confirming a positive role of YAP in stemness (135). As in human ESCs, the interaction between YAP and TEADs directs transcriptional circumstances important for maintaining pluripotency. Ectopic expression of a nuclear-localized mutant YAP promotes mouse ESC self-renewal and increases the efficiency of iPSC reprogramming (125). In human iPSC, knockdown of *LATS2* increases the reprogramming efficiency of iPSCs (136). Moreover, a recent study reported the success in reprograming of iPSCs derived from primary human amniotic epithelial cells (HuAECs) through the introduction of OSY (*OCT3/4, SOX2,* and *YAP*) to activate the Hippo-Yap signaling pathway (135).

While the nuclear TAZ/YAP complex exhibits crucial roles in ESCs cultured *in vitro*, studies using mouse blastocysts indicate that TAZ/YAP is in the cytoplasm of the ICM, the region from which ESCs are derived. The observations suggest that pluripotent ESCs exist very transiently *in vivo*, and that changes in TAZ/YAP localization might provide a mechanism to integrate microenvironmental cues during cell differentiation. Indeed, a mechanical microenvironment dramatically affects stem cell fate (137).

HIPPO SIGNALING IN TUMORIGENESIS AND TUMOR INITIATING CELLS

The Hippo pathway is critical in homeostasis, and is utilized by cancer cells for growth and progression. Mechanically, cytosolic phosphorylated YAP/TAZ inhibits tumor growth. Upon de-phosphorylation, YAP/TAZ undergoes nuclear accumulation and promotes cell and tumor growth. In mouse, long-term YAP activation results in liver cell transformation and tumor development (138). The oncogenic activity of YAP is highly dependent on TEAD-mediated gene transcription. Dominant negative TEAD inhibits YAP-induced liver cancer by sequestering YAP and TAZ in the cytoplasm (138). Clearly, these findings suggest that the action of YAP/TAZ fails to distinguish what is a normal or an abnormal cellular event, and there is no apparent regulatory mechanism in the nucleus to override the action of YAP/TAZ.

YAP/TAZ Gene Alterations and Protein Fusions

Upregulated expression or nuclear accumulation of YAP/TAZ has been observed in various types of human cancers, including liver, breast, lung, colon, ovary (139–141). Gene alteration and fusion genes involving *YAP* and *TAZ* have also been examined in human cancers. For example, *NF2* mutation causes neurofibromatosis 2, schwannomas, meningiomas, and

mesothelioma (142). Heterozygous deletion of Yap completely blocks liver tumorigenesis in *Nrf2* knockout mice (47). Statistics with clinical epithelioid hemangioendotheliomas specimens revealed that almost all cases carry *TAZ-CAMTA1*, *TAZ-FOSB*, or *YAP-TFE3* fusion genes (101, 143–147). Besides, in a portion of ependymal tumors, *YAP* gene is found to fuse with MAMLD1 or *C11orf95* (145, 148).

A similar finding is also observed in neuroblastoma, neural stem cells carrying the *YAP-C11orf95* fusion gene tent to effectively form brain tumors when transplanted into mice (148). In addition, a familial *YAP* point mutation (R331W) has also been reported to correlate with a high incidence of lung adenocarcinomas (149). Although in these fusion genes, YAP/TAZ proteins are not unabridged, all YAP/TAZ fusions proteins preserve their *N*-terminal TEAD binding domain. This indicates that the fusion proteins still have the binding ability to TEAD and are able to activate the TEAD-dependent transcriptional program for promoting tumorigenesis. Aberrant mutations of components within the Hippo pathway affect the function of YAP/TAZ leading to oncogenesis. *LATS1/2* mutations or gene fusion, which may cause YAP/TAZ activation, have also been sporadically identified in different cancers (150–154). Evidence of the Hippo pathway in tumorigenesis based on mouse models is summarized in **Table 2**. In addition, crosstalk with other cancer-related signaling pathways, such as *KRAS*, *APC,* and *LKB1* mutations, have all been reported to contribute to upregulate the YAP/TAZ activity in cancers (156–158).

YAP/TAZ in Cancer Metastasis

Cancer metastasis is responsible for a huge portion of cancer-associated deaths. It has been reported that high YAP or TAZ activity enables the cells to escape contact inhibition and anoikis, and support anchorage-independent growth (55, 156, 159).

TABLE 2 | Genetic alteration of Hippo pathway in human cancer.

Gene	Alteration	Cancer type	References
NF2	Mutation or deletion	Mesothelioma Neurofibromatosis type 2 Schwannoma Meningioma	(142)
LATS1/2	Gene Fusion (*LATS1-PSEN1*)	Mesothelioma	(154)
	LATS2 deletion	Mesothelioma	(152)
	LATS2 mutation	Sporadic in different cancers	(151)
YAP	Amplification	Hepatocellular carcinoma Medulloblastoma Esophageal squamous cell carcinoma	(153)
	Mutation (R331W)	Lung adenocarcinoma	(149)
	Gene Fusion (*YAP-TFE3*, *YAP-ESR1*, *YAP-C11orf95*, and *YAP-MAMLD1*)	Epithelioid Hemangioendothelioma Luminal breast cancer Ependymal tumors	(146) (144) (147) (145) (148)
	Deletion	Hematological cancer	(155)
TAZ	Gene Fusion (*TAZ-CAMTA1*, and *TAZ-FOSB*)	Epithelioid hemangioendothelioma	(143) (144) (101)

YAP or TAZ activation promotes metastasis by influencing many of processes related to metastasis, such as epithelial-to-mesenchymal transition (EMT), invasion, extravasation, and escaping from the immune system. Substantial evidence indicates that in addition to promoting tumor growth, YAP activation is sufficient to drive cancer metastasis (160). For example, YAP promotes tumor metastasis through inducing the expression of Zinc finger E-box-binding homeobox 1 and 2 (ZEB1/2) for stimulating EMT (161). Activation of YAP through the loss of leukemia inhibitory factor receptor (LIFR) promotes metastatic colonization of breast cancer cells (162, 163). In fact, YAP/TAZ and TEADs in metastasis of numerous cancer types have also been implied, including melanoma (162), lung cancer (164–166) breast cancer (163, 167–169), cholangiocarcinoma (170), gastric cancer (171, 172), ovarian cancer (173), colorectal cancer (174, 175). Clinical data showed that YAP or TAZ expression or nuclear localization is increased in metastatic tumors compared to primary tumors in pancreatic cancer (176), breast cancer (177, 178), and prostate cancer (179).

YAP/TAZ in Drug Resistance and Tumor Relapse

In addition to metastasis, drug resistance, and tumor relapse are the hard cracking nuts for cancer therapy. YAP/TAZ activity also correlates with drug resistance and cancer recurrence. One study has analyzed the gene expression profile of several cancer cell lines, and uncovered the immune checkpoint molecule PD-L1 as a target of Hippo signaling (180). In this process, PD-L1 expression is suppressed by the upstream kinases MST1/2 and LATS1/2 of the Hippo pathway, while TAZ and YAP enhance PD-L1 levels in breast and lung cancer cell lines. Critically, TAZ-induced PD-L1 upregulation in human cancer cells is sufficient to inhibit T cell function (181). Further, *in vitro* studies indicate that cultured breast cancer cells exhibit high YAP/TAZ activity and confer drug-resistance to many routinely used chemotherapeutic drugs, such as taxol, 5-fluorouracil, and doxorubicin (182–184). Lung and colon cancer cells with high YAP activity exhibit resistance to RAF- and MEK-targeted therapies (185). Furthermore, tamoxifen is one of the commonly used chemo-drug for the treatment of estrogen receptor (ER)-positive breast cancer. Recent study has shown that tamoxifen may activate YAP/TAZ by stimulating the membrane estrogen receptor GPER, which may explain why certain ER-positive breast cancers are insensitive to tamoxifen (186). Elevated gene expression of *YAP* is associated with cancer relapse in *KRAS*-driven colon and pancreatic cancers (155, 187). Taken together, inhibition of YAP/TAZ not only restrains tumorigenesis and tumor progression, but also has the potential to sensitize tumor cells to drugs in chemotherapies or target therapies and prevent cancer recurrence.

Tumor Initiating Cells and the Hippo Pathway

A special subpopulation of highly tumorigenic cells, known as tumor initiating cells (TICs), has been proposed to harbor unique properties such as self-renewal and tumor-initiating potential. TICs are believed to be responsible for drug resistance, metastasis, recurrence, and major causes of cancer death

(188). Chemotherapy and target therapy are currently the major treatments for patients with cancer. Unfortunately, these conventional therapies often fail to eradicate TICs, thereby permitting TIC-mediated cancer relapse. The YAP/TAZ complex has been shown to be a key regulator of TICs in various cancer types.

Tumor cells with a high YAP/TAZ activity display resistance to chemotherapeutics. YAP activation promotes the transformation of prostate epithelial cells to become an androgen-insensitive state and castration resistance *in vivo* (189). YAP facilitates the dedifferentiation and expands undifferentiated stem/progenitor cells, e.g., transformation of the mature hepatocytes into progenitor cells (89). YAP/TAZ activation leading to induction of TIC properties has been observed in multiple human cancers. In breast cancer, YAP occupies mammary stem cell signature gene promoters to induce breast TICs (190). TAZ expression is enriched in breast TICs with $CD44^{high}/CD24^{low}$ phenotype, which is required to sustain self-renewal and tumor-initiating properties (191). In addition, glucocorticoid hormone-induced YAP activation expands chemo-resistant breast TICs.

YAP is one of the major inducers of TIC properties, which directly upregulates the SOX9 transcription factor for controlling the differentiation of many cell types (190). Similarly, in osteosarcoma and glioblastoma, SOX2 promotes YAP activation to maintain TICs capacity (192). Glucocorticoid hormone-induced YAP activation induces expansion of chemo-resistance in breast TICs (191). In clinical specimens of urothelial carcinoma of bladder (UCB), the expression of YAP positively correlates with the expression of SOX2 and COX2. The tumor cells draw support from the proinflammatory role of the COX2/PGE2 pathway, and the growth-regulatory YAP recruits the stem cell factor SOX2 in expanding and sustaining the accumulation of TICs. In mouse xenograft study, activation of the COX2/PGE2 and YAP pathways promote acquired resistance to EGFR inhibitors (193). Taken together, YAP/TAZ plays an important role in tumorigenesis, cancer progression, and TICs maintenance. TICs are highly related to tumor spread, drug-resistance, and cancer relapse, and are responsible for treatment failure and poor prognosis. TIC-specific regulatory mechanisms of YAP/TAZ and Hippo pathway remain further elucidation.

TARGETING THE HIPPO SIGNALING PATHWAY FOR THERAPY

Nowadays, cancer becomes one of the notorious leading causes of mortality globally. A plenty of studies indicate that the Hippo pathway plays a key role in tumorigenesis. Aberrant Hippo pathway activity promotes cancer cells growth and leads cancer cells to acquire drug resistance (183). YAP and TAZ activity is often upregulated in cancer cells, but their activity is typically silent in normal resting tissue, suggesting that systemic YAP/TAZ inhibition allows cancer treatment without causing significant side effects (30, 117) (**Figure 5**).

Small Molecular Drugs

Small molecular drugs are intended to disrupt the YAP/TAZ-TEAD complex and block the complex-mediated gene transcription. Verteporfin, an FDA-approved photosensitizer of porphyrin family molecules for treating macular degeneration, is currently the most widely used YAP inhibitor in research laboratories (138). Verteporfin binds YAP and disrupts the binding of YAP/TAZ with TEADs, and, alternatively, upregulates 14-3-3 expression to facilitate YAP/TAZ degradation (194–196). Verteporfin suppresses liver overgrowth induced by YAP overexpression or NF2 inactivation in mice (138). However, YAP-independent cytotoxic effect of Verteporfin has been reported (197–199). Moreover, pharmacological modulation of signal transduction pathways that crosstalk with the Hippo pathway or inhibition of YAP/TAZ target genes by combination therapy may provide promising approaches to target YAP/TAZ activity. Together, the potential of YAP/TAZ-TEAD as a therapeutic target may improve the current treatment strategies (200–202).

Boosting the Hippo Pathway

In stark contrast, given the fact that the Hippo pathway is essential for organ development and regeneration, manipulating the components of the Hippo pathway would enhance drug design for regenerative medicine. Regenerative medicine refers to medical approaches to promote functional repair and regeneration of damaged tissues or organs, the practices can be separated into two major parts to reach the goal. One is stimulation of intrinsic repair process by molecular therapy, and the other is transplantation of tissues or stem/progenitor cells cultured in laboratories. Clinical doctors always face the hard situation with the shortage of donors compared to the increasing needs of tissue/organ transplantation. Thus, development of novel regenerative therapy is imperative.

YAP/TAZ activity is generally high during embryonic development, but soon declines to a basal level after birth. Upon tissue injury, YAP/TAZ activity is immediately reactivated in a transient manner. Transient activation of YAP/TAZ promotes the expansion of progenitors or dedifferentiation of mature cells to facilitate tissue regeneration. Thus, activation of YAP/TAZ is a potential strategy to promote tissue regeneration (**Figure 5**).

Inhibition of upstream kinases, such as MAP4K4, MST1/2, or LATS1/2, in the Hippo pathway, represents an ideal approach to induce YAP/TAZ activity and facilitate the process of tissue repair or regeneration, and possibly for treating degenerative diseases. Systematic or local delivery of Hippo pathway kinase inhibitors can induce this regenerative program. Recently, an MST1/2 inhibitor has been discovered and exhibits a good response in promoting liver and intestinal regeneration (203). Gene therapy is also an effective strategy (204), either by regulating YAP by the viral or viral-free system or introducing siRNA or miRNA for tissue regeneration (205).

Transplantation of *in vitro* expanded progenitors, organoids, or tissues are feasible methods. In recent years, a variety of organoids have been generated successfully *in vitro*, including stomach, liver, kidney, lung, intestine, brain, and retina (206). Nonetheless, it is a puzzle to command the complicated biological parameters, including cell types, organization, and microenvironment within an organoid system (207). Supporting evidence revealed the outstanding effects of YAP/TAZ transient activation in dedifferentiating mammary, neuronal, and pancreatic cells into a progenitor

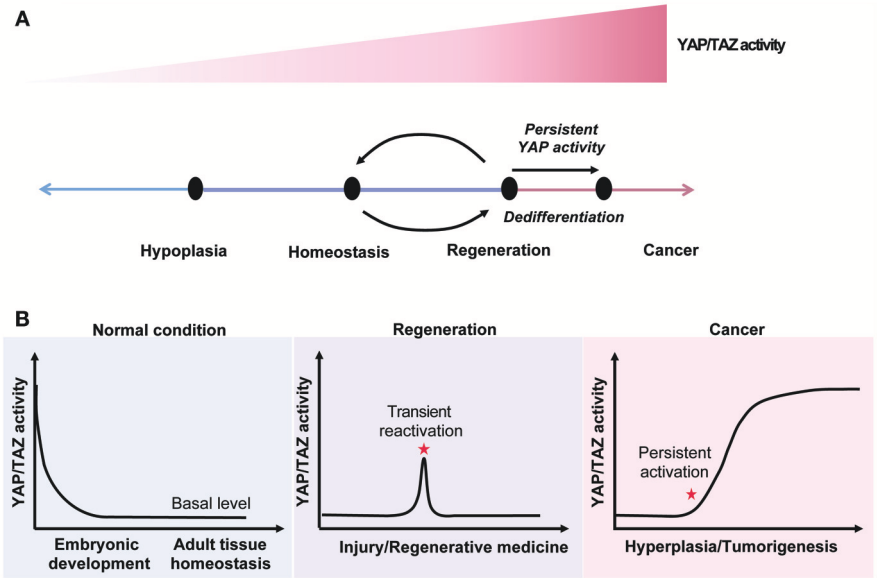

FIGURE 5 | The Hippo signaling in tissue homeostasis, regeneration and cancer. **(A)** YAP/TAZ activity regulates tissue homeostasis and modulates the cell state. **(B)** YAP/TAZ activity is declined to a relative low level after birth. The YAP/TAZ activity is reactivated after tissue injuries and constitutively upregulated in cancer.

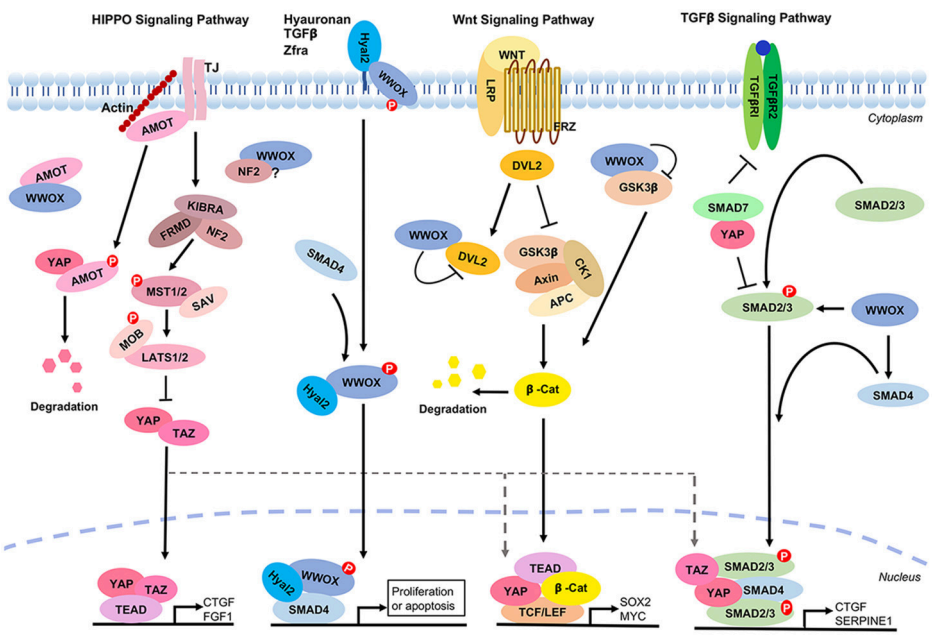

FIGURE 6 | WWOX controls TGF-β, Hyal-2, and Wnt signaling pathways that cross talks with the Hippo signaling. A panel is shown for the Hippo, Hyal-2/WWOX/Smad4, Wnt and TGF-β signaling pathways. WWOX supports the Hyal-2 and TGF-β signal pathways. However, WWOX blocks the Wnt pathway by binding GSK3β and DvL2. WWOX binds AMOT and probably NF2 in the Hippo pathway, whereas the functional consequence of this binding is unknown.

state, and these cells can be used to generate organoids for transplantation (208). Thus, modulating the Hippo pathway may represent a useful strategy for enrichment of progenitor cells or differentiated organoids for regenerative medicine.

In conclusion, the marvelous regulation of the Hippo pathway and YAP/TAZ in progenitor expansion and cell differentiation is forceful and feasible for promoting tissue regeneration. The Hippo pathway is generally considered as an effector in tumor suppression, and many anticancer drugs been developed (209).

In facilitating regenerative medicine, small molecular drugs for Hippo activation can be implemented.

IS WW DOMAIN AN ALL-TIME PARTY COORDINATOR?

All-Time Party Coordinator?

The WW domain is a well-known structural module that is responsible for protein/protein binding interactions (210, 211). The WW domain has three anti-parallel beta-sheets, which appears to facilitate protein/protein binding. In the human proteome, there are at least 52 WW domain-containing proteins, and more than 10,000 among all species. Each protein may have one or up to 4 repeats of WW domains in the amino acid sequence. The WW domain binds a proline-rich motif such as PPxY or PPPY in a target protein. Phosphorylation in specific residues within the WW domain enhances its binding capability as seen in WWOX (210, 211). In the Hippo pathway (212), there are 4 WW domain-containing proteins, namely SAV, KIBRA, YAP, and TAZ. Amazingly, YAP, TAZ, and/or the YAP/TAZ complex binds approximately 20 proteins, including p73, RUNXs, SMADs, PRGP2, Pax3, ErbB4, ASPP2, AMOTs, Wbp2, β-catenin, Parafibromin, RORα, and SET1A (212). Without a doubt, WW domain can be considered as an all-time party coordinator. Each WW domain-containing protein interacts with many proteins. The binding signals biological events for cell survival, death, and others. However, how can a single cell handle so many downstream signaling targets from an upstream stimulator during a single signaling run? And, this may end up with desired outcomes. Presumably, a specific downstream effector(s) stands out to solely carry out the signaling mission and exhibits the results, and meanwhile eliminates the outcome from other signaling co-effectors.

The signaling branches derived from the upstream signaling could exhibit conflicts among the downstream effectors. For example, Hippo pathway has a close connection with SMAD signaling (213). YAP enhances pSmad1-dependent gene transcription. In contrast, as a self-control mechanism, YAP promotes Smad7-mediated inhibition of TGF-β signaling (213). Nonetheless, there is a converge among Wnt, TGF-β, and YAP/TAZ pathway (214, 215). YAP takes the center of the signaling stage by participating in the gene transcription in the nucleus among these pathways (216).

Tumor Suppressor WWOX in the TGF-β, Hyal-2, and Wnt Signaling That Cross Talks With the Hippo Pathway

Tumor suppressor WWOX is a potent inhibitor of the Wnt/β-catenin signaling via binding with Disheveled Dvl-2 to enforce the cytoplasmic retention of Dvl-2 and increased degradation of β-catenin (210, 217). WWOX binds Smad2, 3, and 4 in the TGF-β/Smad and Hyal-2 signal pathways to modulate cell proliferation or death (218–220) (**Figure 6**). Hyaluronan, TGF-β and Zfra (zinc finger-like protein that regulates apoptosis) utilize the Hyal-2/WWOX/Smad4 pathway (218, 219). Activation of the Wnt/β-catenin signaling allows nuclear translocation of TAZ to carry out gene transcription with TEAD. WWOX intervenes the event by directly binding and blocking the function of GSK-3β in the upstream complex of APC/AXIN/β-catenin/GSK-3β/YAP (221) (**Figure 6**). Indeed, YAP is involved in the transcription stage of the TGF-β, Wnt/β-catenin and Hippo signaling (222). WWOX controls the aforementioned signaling from the upstream steps and thereby affects the function of YAP in the downstream. Indeed, WWOX binds AMOT and probably NF2 in the upstream of the Hippo pathway.

TABLE 3 | Outstanding questions.

Gene and proteins	
Gene and protein isoforms	• What are the tissue-specific gene products from alternative splicing for the Hippo pathway? • The Hippo pathway is evolutionarily conserved. Why do these proteins in the pathway readily respond to diversified stimulators? What are the key purposes for the biological outcomes? • Do proteins isoforms compete with each other during the Hippo signaling? • How can each cell handle the diversified signals? Signal integration or elimination?
WW domain proteins	• What are the functional differences between two-WW-domain YAP1 and one-WW-domain YAP2? • Further, YAP1 has 4 isoforms due to alternative splicing in the TAD domain. What are the functional differences among these proteins in terms of regulation of gene expression? Can which isoform act as a decoy protein to block the signal flow? • KIBRA participates in both the Wnt/Hedgehog/Notch and Hippo signaling pathways. Which pathway does KIBRA have the most effect on the biological outcome? • KIBRA binds LATS1 and LATS2. Which complex KIBRA/LATS1 or KIBRA/LATS2 is more effective in blocking YAP/TAZ? • SAV1 binds MST1 and promotes apoptosis. How does apoptosis get activated?
Upstream of the Hippo signal pathway	• Which upstream signaling molecules act in concert to suppress the YAP/TAZ activity, and which are in antagonistic manners? Which stimulator can override the signaling from others? • Since the pathway is sensitive to diversified stimulators, are there unidentified agonists?
The Hippo signal flow	• How many signal proteins in the Hippo pathway readily crosstalk with proteins from Wnt, TGF-β, and ERK pathways? Tumor suppressor WWOX interacts with these pathways. Can WWOX act as a modulator for optimizing the signaling outcomes? • Lacking a real-time mode for cellular signaling pathways is hard to unravel the hidden events (e.g., promoter activation, protein/protein interactions, and etc.). For a signaling with so many branches in the downstream, or a signaling with many upstream stimulators, is there a dominant signal flow?

Protein Isoforms

WW-domain isoforms could be another issue. Some proteins have one WW domain, and others have more than one. YAP1 has two WW domain and YAP2 has one. YAP1 has up to 9 isoforms according to the NCBI protein database. For a single protein possessing more than one WW domains in the amino acid sequence, these WW domains may act in a concerted manner to boost the protein function (223). However, during the Hippo signaling, it is not clear which YAP isoform is the key executor to generate the intended outcome in a specific cell. The complexity of the signaling events can be observed not only in the Hippo signaling pathway but also in many signaling pathways (e.g., TGF-β, MAPK/ERK, and others). Among these pathways, there are crosstalk between each other. Knockdown of a single protein could generate a desired phenotype. However, the observation could be a sum of many signaling pathways.

CONCLUDING REMARKS AND OUTSTANDING QUESTIONS

In summary, we have addressed the role of Hippo pathway in the regulation of embryonic development, organ growth, tissue regeneration, stem cell pluripotency, and tumorigenesis. As detailed in the above section, our concerns mainly point to the pathway-regulated biological effects and ultimate outcomes. Several outstanding questions are listed in the **Table 3**. Our hope is that more sophisticated tools are needed to predict and solve problems associated with multiple signal stimulators, crosstalk among the signal proteins in the pathway with others, functional efficacy of protein isoforms in the signaling, and problem-solving associated with conflict signaling.

AUTHOR CONTRIBUTIONS

Y-AC and C-YL carried out the literature review, prepared schematic graphs, and wrote the manuscript. T-YC participated in drafting and prepared high-resolution figures and organized the references section. N-SC, S-HP, and H-FC provided advisory points to the first and other authors. N-SC thoroughly revised added challenging concepts and outstanding questions, developed new ideas, and completed the final version of the manuscript. All authors read and approved the final version.

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REVIEW
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WW-Domain Containing Protein Roles in Breast Tumorigenesis

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Protein-protein interactions are key factors in executing protein function. These interactions are mediated through different protein domains or modules. An important domain found in many different types of proteins is WW domain. WW domain-containing proteins were shown to be involved in many human diseases including cancer. Some of these proteins function as either tumor suppressor genes or oncogenes, while others show dual identity. Some of these proteins act on their own and alter the function(s) of specific or multiple proteins implicated in cancer, others interact with their partners to compose WW domain modular pathway. In this review, we discuss the role of WW domain-containing proteins in breast tumorigenesis. We give examples of specific WW domain containing proteins that play roles in breast tumorigenesis and explain the mechanisms through which these proteins lead to breast cancer initiation and progression. We discuss also the possibility of using these proteins as biomarkers or therapeutic targets.

Keywords: WW domain, WWOX, hippo pathway, E3 ligases, breast cancer

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INTRODUCTION

Protein function is determined by the domains and motifs that it harbors. These domains can be functional domains such as the catalytic domains found in many enzymes, or structural domains that are important for protein-protein interactions or the assembly of multi-protein complexes. Thus, protein's function is determined by its catalytic activity and its partners. One of these important domains found in many proteins is the WW domain. WW domain, is the smallest naturally occurring module. It consists of ∼35–40 amino acids, including two highly conserved tryptophans (W), after which the module is named (WW). The two Ws are separated by 20–23 amino acids (1). Based on their ligand preference, WW domains are classified into five different classes. WW domains are very important domains that are involved in very critical cellular processes including; transcription, splicing, ubiquitination, apoptosis, cell growth, proliferation, and differentiation (2). Because of its link to many critical cellular processes and its widespread distribution among many proteins, WW module is linked to many diseases including; Liddle's syndrome of hypertension, muscular dystrophy, Alzheimer's and Huntington's diseases, and cancer (1, 2).

WWOX AND BREAST TUMORIGENESIS

WW domain oxidoreductase (WWOX) is a tumor suppressor gene, which is altered in different types of cancer, including breast cancer (3). In breast cancer, WWOX gene is lost even in pre-invasive stages (4, 5). Moreover, recent studies have shown a correlation between WWOX expression and the clinical outcome of breast cancer (6–10).

In addition to clinical data that supports WWOX role in breast tumorigenesis, different animal models have proved its function in proper mammary gland development and tumorigenesis. It was shown that WWOX is highly expressed in mammary gland tissue and that its targeted deletion results in mammary gland fibroadenoma (11), as well as impaired mammary gland development (12, 13). Moreover, it was shown that aging WWOX-heterozygous C3H knockout mice develop higher incidence of mammary tumors. These tumors bear altered gene expression that resembles altered gene expression in human breast cancer (14).

WWOX mediates its functions by interacting with and modulating the activities of different proteins either through its WW domains or in a WW domain independent manner. Nonetheless, WWOX has also an oxidoreductase domain that seems to play a role in tumorigenesis too. WWOX alters different cellular processes involved in tumorigenesis. As discussed below, these cell activities include apoptosis, genomic instability, metabolism and metastasis.

WWOX Function in Breast Cancer Cell Apoptosis

Different published findings have linked WWOX to apoptosis. It was revealed that WWOX overexpression or endogenous WWOX reactivation in breast cancer cells leads to apoptotic cell death *in vitro* and suppression of breast tumor growth *in vivo*. These effects are mediated by reducing the expression levels of the anti-apoptotic protein Bcl-2 and inducing the pro-apoptotic protein BCL2-associated X protein (Bax) (15) (**Figure 1**). Furthermore, WWOX was shown to interact with p73β in MCF7 breast cancer cell line (**Figure 1**). This interaction, although was not tested in breast cancer cells, leads to a cytoplasmic p73β dependent cell death (16). WWOX was also shown to interact and stabilize p53 and confer cellular sensitivity to apoptotic stress (17). Another study demonstrated that WWOX induces breast cancer cell apoptosis by triggering Smad4 transcriptional activity (**Figure 1**) (18). In the clinical context of cell death and apoptosis, WWOX was shown to interact with two very important factors involved in hormone treatment resistance. These factors include activating protein 2γ (AP2γ) and WW domain-binding protein 2(WBP2) (**Figure 1**). WWOX interacts with AP2γ and sequesters it in the cytoplasm and inhibits its transactivational function (19). For WBP2, WWOX physically interacts with WBP2 and suppresses ER transactivation pathways (**Figure 1**) (20).

WWOX and Genomic Instability in Breast Cancer

Genomic instability is one of the important cancer hallmarks involved in tumor initiation as well as tumor progression (21, 22). WWOX was recently assigned an important function in DNA damage response (DDR) (23). Abu-Odeh et al. demonstrated that WWOX interacts with Ataxia telangiectasia mutated (ATM) and activates both ATM and Ataxia telangiectasia mutated RAD3-related (ATR) following DNA damage. They also showed that WWOX knockdown is associated with less activation of DDR signaling molecules, and consequently less efficient DNA repair and thus more genomic instability (**Figure 2**) (23, 24). In the context of response and resistance of cells to DNA damaging therapies, a recent study found that WWOX deficiency increases survival of cells after ionizing radiation-induced double strand breaks (DSBs), and that WWOX restoration in MDA MB231 breast cancer cells, that lack endogenous WWOX, leads to reduced survival upon gamma irradiation. On the contrary, WWOX-silenced cells survived bleomycin treatments as compared to WWOX-expressing MCF10A cells (25). Moreover, in this study, WWOX interaction with Breast Cancer gene1 (BRCA1) was shown to affect DNA DSB repair pathway choice and thus affects cell response to DSB inducing agents (25, 26). These findings prove that WWOX plays an important role in DDR and genomic stability, which might make it a marker for the success of DDR-targeting biological therapies. Since WWOX seems to play a very important role in DDR, and that its loss impairs the repair process and leads to genomic instability, it will be interesting to see whether WWOX loss would enhance the activity of PARP inhibitors. Here, based on PARP inhibitors mechanism of action, it is expected that cells that lack WWOX will be more sensitive to this type of therapy.

WWOX and Cancer Metabolism

Another cancer hallmark connected to the tumor suppressor gene WWOX is cell metabolism. The connection between WWOX and cancer cell metabolism was established after linking WWOX protein to Hypoxia-inducible factor 1-alpha (HIF1α) function (27). In this study, it was found that WWOX interacts with HIF1α and modulates its transcriptional activity (**Figure 3**). In breast cancer cells, the authors showed that WWOX knock down increases HIF1α in MCF7 cells (27). Finally, they demonstrated that WWOX expression inversely correlates with HIF1α target gene Glucose transporter 1 gene (Glut1) in breast cancer tissue samples (27) (**Figure 3**). These findings and others all point to the fact that WWOX is very important in modulating cancer cell metabolism in general and breast cancer cells specifically. Since WWOX function is usually lost in breast cancer, it will be interesting to test whether a small molecule that resembles WWOX WW domain would inhibit the function of HIF1α and shifts cancer cell metabolism from a glycolysis dependent to a normal metabolism.

Metastasis

Most of cancer patients die from metastasis and not from primary tumors. WWOX was also found to play a role in breast cancer metastasis. Loss of WWOX expression in breast cancer is significantly associated with the number of metastatic axillary lymph nodes and poor survival (28). In addition, WWOX was shown to play an important role in hepatocyte growth factor (HGF) mediated mesenchymal to epithelial transition (MET) in breast cancer bone metastasis (29). Another study demonstrated that the chemotherapeutic drug 5-aza-2′-deoxycytidine (decitabine), increases WWOX expression and localization to the nucleus in bone metastasis xenograft model. It was proposed that the elevation in WWOX expression is responsible for altering the HGF/Met/E.cadherin signaling

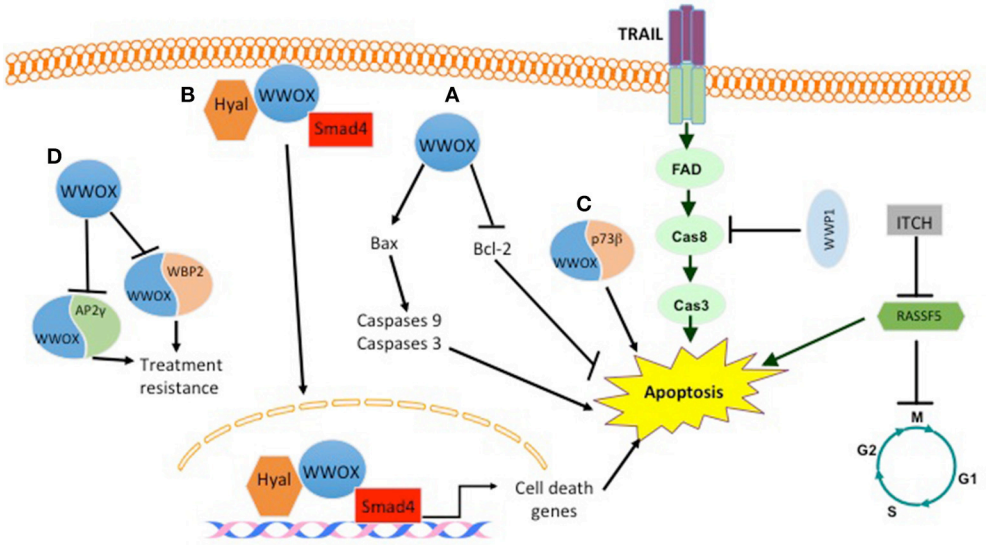

FIGURE 1 | Effect of some WW domain proteins on cell death. WWOX induces cell death by **(A)** inducing BAX levels and decreasing BCL2 levels. **(B)** Upon hylauronan treatment WWOX complexes with hyaluronidase and Smad4. This complex translocates to the nucleus and induces the expression of Smad4 pro-apoptotic target genes. **(C)** WWOX binds to P73b and induces its cytoplasmic dependent cell death. **(D)** WWOX binds and sequesters WBP2 and AP2γ in the cytoplasm. This inhibits their treatment resistance phenotype. E3 ligases also manipulate breast cancer cell death. WWP1 inhibits TRAIL mediated cell death. ITCH by inhibiting RASSF5 prevents apoptosis and activates cell cycle.

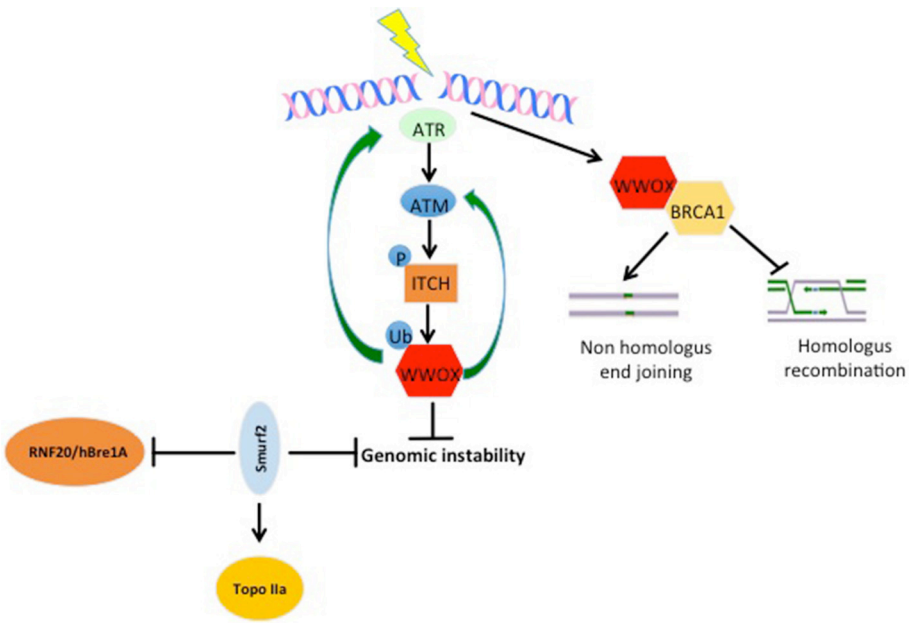

FIGURE 2 | WW domain protein roles in genomic stability. Upon DNA damage, ATR and ATM get activated. When activated, they can phosphorylate and activate the E3 ligase ITCH. Active ITCH can further ubiquitinate and activate WWOX leading to its translocation to the nucleus. In the nucleus, WWOX, in a positive feedback loop activates ATM and ATR and enhances DNA damage response and repair. Moreover, WWOX can interact with BRCA1 and enhance Non-homologous end joining over homologus recombination repair pathway**.** E3s also play roles in genomic stability too**.** SMURF2 increase genomic stability by inhibiting the activity of RNF20/hBre1A, the major ubH2B-specific E3 and by stabilizing topoisomerase IIa (Topo IIa). ITCH enhances genomic integrity by increasing the efficiency of DNA damage repair (DDR). Upon DNA damage ITCH is activated by ATM. Active ITCH can further ubiquitinate and activate WWOX leading to its translocation to the nucleus. In the nucleus, WWOX, in a positive feedback loop, activates ATM and ATR and enhances DNA damage response and repair.

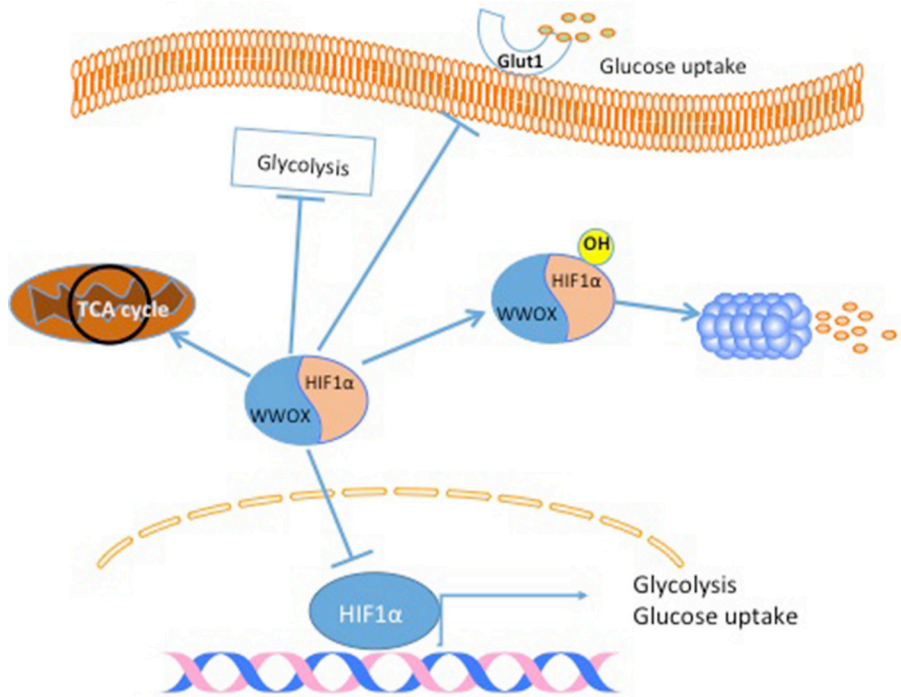

FIGURE 3 | WWOX modulates cancer cell metabolism. When WWOX is present it binds to HIF1α and inhibits its transcriptional activity. Moreover, WWOX increases HIF1α hydroxylation and increase its degradation. As a consequence WWOX reduces glucose uptake, decreases glycolysis and increase mitochondrial respiration.

axis and thus reducing breast cancer metastasis to the bone (30). In a different study that examined the role of WWOX and Transcriptional coactivator with PDZ-binding motif (TAZ) in breast metastasis to bone, WWOX and TAZ increased invasiveness of the bone metastasis-derived clone 1,833 (these cells were derived from MDA MB-231 metastasis to the bone) (31). This effect on 1,833 clone was explained by the fact that overexpression of WWOX and TAZ increases E.cadherin levels, which is an event believed to be important in MET and successful bone metastasis. Interestingly, in the same study, WWOX overexpression didn't alter E.cadherin levels and inhibited the invasion capability of the 1,833 clone parental cells, MDA MB-231 (31). These findings and others indicate that at least some of WWOX suppressive functions are cell context specific, and that experiments questioning WWOX functions should be better designed and defined.

Questioning WWOX Suppressive Functions

Although the compelling evidence that indicates with no doubt that WWOX is a tumor suppressor gene, the presence of WWOX in a fragile locus in the genome raises suspicion about its identity of being a classical tumor suppressor gene. As well, its localization in a fragile sequence raises another question on why such an important gene lies in a damage prone locus? The answer to this question might be that loss of WWOX can act as an early alarm for the cell to respond to different types of insults that can lead to cell stress and DNA damage.

Moreover, WWOX tumor suppressive function was questioned in different research articles. For example, in a publication by Watanabe et al., it was found that WWOX is expressed in 48 cell lines out of 49. Moreover, it was shown that, using immunohistochemistry staining, WWOX is not down regulated in cancer tissue (32). However, a careful inspection of the data presented by Watanabe et al. showed the following: first, there was no normal control cells to which the expression of WWOX was compared and second, the expression of WWOX in the tested cell lines varied and there were at least 16 cell lines that showed a very low WWOX expression when compared to, for example, MCF7 cell line. These results indicate that WWOX loss in cancer is not a black and white phenomenon, but like many other tumor suppressor genes, WWOX expression is very heterogeneous in cancer samples. In addition, the expression of a tumor suppressor gene in a cancer sample or cell line does not preclude it from being a tumor suppressor gene, since a tumor suppressor gene might be expressed in a cell line but is not functional. This might be due to a posttranslational modification, mislocalization or the lack of its partner in a specific tumor type. Another paper showed that WWOX might have a role in cancer progression toward a pre metastatic state *in vivo* (33). In this article, Chang et al. showed that WWOX1 and 2 expression upregulation and Tyr33 phosphorylation correlates with the progression of breast cancer to a pre-metastatic state. Although the authors showed evidence that WWOX is overexpressed in pre-metastatic cancer, the conclusion that WWOX may play function in tumor progression might not be accurate for the

following reasons. First, the authors tested the expression of WWOX on different tumor grades obtained from different patients and not on different stages from the same patient, which means that the authors didn't compare the staining pattern to the baseline of the same patient. Second, the fact that WWOX expression is induced in pre-metastatic stage of breast cancer does not exclude the possibility that WWOX expression is induced in order to suppress tumor progression rather than promoting it, analogous to p53 stabilization after stress. These controversial findings indicate that the molecular mechanisms that regulate WWOX function as well as the molecular functions mediated by WWOX should be investigated in more depth since at least WWOX partners can have dual and controversial functions. For example, ITCH, a WWOX partner and a WW-domain protein, was found to play an important role in DDR and genomic stability (anti-tumorigenic effect) (23, 24). On the other hand ITCH in the context of the Hippo pathway was shown to have a pro-tumorigenic function (34, 35). This raises the question; How does WWOX affect ITCH pro-tumorigenic function? Does it antagonize ITCH function and suppresses tumor growth? Which is the expected scenario, or does it work synergistically with ITCH like under DDR and thus promotes tumorigenesis?

A very interesting aspect of WWOX tumor suppressive function is changing the signaling pathway of different proteins to achieve the same outcome. For example, p73 is a tumor suppressor gene that induces apoptosis through its transcriptional transactivation function. When WWOX interacts with p73, paradoxically after WWOX activation with the Src oncogene, it sequesters p73 in the cytoplasm and inhibits its transactivation function. Although this has a negative effect on p73 nuclear function, it enhances p73-WWOX interaction in the cytoplasm leading to apoptosis. This raises the following questions; why does WWOX interfere with p73 nuclear pro-apoptotic function to induce apoptosis via a different mechanism? Is cell context the only reason for this? Or is it because WWOX's pro-apoptotic function is superior to p73 pro-apoptotic function?

Although the controversy about WWOX function, it will be exciting to try to test how would peptides that resemble WWOX WW domains affect cancer cell behavior to evaluate their therapeutic functions.

HIPPO PATHWAY AND BREAST CANCER

The Hippo pathway is a highly conserved pathway that regulates many cellular functions including cell proliferation, growth, differentiation and apoptosis (2). The pathway is composed of mainly a core cascade of kinases that include Macrophage-stimulating protein 1/2 (MST1/2) and Large tumor suppressor kinase 1/2 (LATS1/2). These kinases, when activated lead to the phosphorylation, sequestration and inactivation of two WW domain downstream effector proteins; yes-associated protein (YAP) and TAZ (**Figure 4**). The pathway is enriched with WW domain mediated interactions on the level of upstream modulators, core components, as well as downstream effectors (**Figure 4**) (1, 36, 37).The pathway is either inactivated or harbors a mutation in one of its components in many types of cancer; including breast cancer.

Animal Models and Clinical Data as Evidence for YAP/TAZ Roles in Breast Cancer

In different animal models, YAP and TAZ functions supported tumor initiation and progression (38, 39). In the clinical context, TAZ was shown to have a negative prognostic effect that correlates with shorter disease-free survival (DFS) of breast cancer patients (40) and negatively correlates with treatment outcome (41). In luminal A breast cancer samples, YAP1 expression levels negatively correlates with Estrogen positive (ER+) samples, and positively correlates with proliferation in ER- samples. Moreover, in the same study, low YAP1 levels correlated with impaired response to tamoxifen treatment (42). In a different study YAP nuclear levels were higher in metaplastic breast cancer tumors when compared to triple negative tumors (43). In another study that examined the expression of YAP in different molecular types of breast cancer, cytoplasmic and phospho-YAP levels were elevated in HER-2 breast cancer type. In this molecular subtype, cytoplasmic YAP expression concurred with poorer disease free survival. In addition, nuclear YAP staining was associated with shorter overall survival (44). In contrast to all findings that revealed that YAP expression correlates with a more aggressive breast cancer and with a poor survival and disease free rates, a recent study by Cao et al. demonstrated that YAP expression is associated with the low grade type of breast cancer luminal A. In addition, the study revealed that YAP expression correlates with favorable DFS and overall survival in patients with luminal A breast cancer and with favorable DFS association among patients with invasive ductal carcinoma, luminal B (HER2-), and luminal B (HER2+) breast cancers (45). These results indicate that there is still some controversy about YAP expression and its correlation with clinical outcome in breast cancer clinical samples. Of course these controversies can be related to different reasons starting from very technical issues related the staining techniques, the antibodies used in each study to data scoring and data analysis. While these can still be possibilities, other biological reasons can still be an explanation. For example, other genetic factors and expression profiles of many other different proteins, in addition to YAP status, that were not tested in most of the studies can provide explanations.

YAP/TAZ Roles in Breast Cancer Cell Transformation and Tumorigenesis

In addition clinical data, it is obvious that YAP and TAZ play critical roles in breast tumorigenesis. For example, YAP over expression in MCF10A, non-transformed mammary gland cells, increases cell proliferation, anchorage independent growth and inhibits apoptosis (38). On the other hand, YAP knockdown results in cell growth inhibition *in vitro* and *ex vivo* tumor formation (46). In different studies, YAP oncogenic function was related to Hippo pathway malfunction. For example, it was demonstrated that LATS1degradation mediated by ITCH releases YAP function and enhances breast cell tumorigenicity

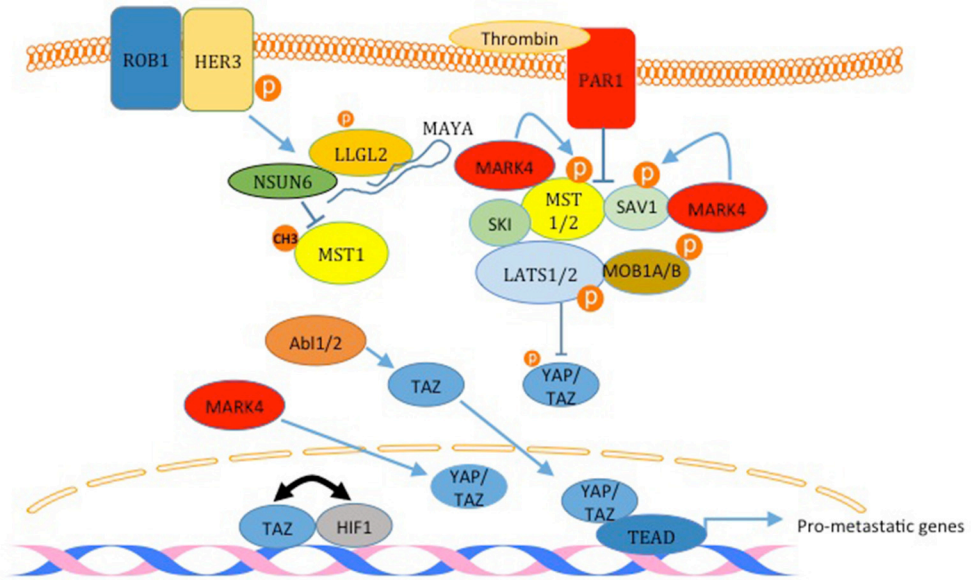

FIGURE 4 | Inactivation of the Hippo pathway induces breast cancer metastasis. The factors labeled in red are examples of proteins that inactivates the Hippo pathway and induces metastasis. PAR1 activation and the kinase MARK4 destabilize the core complex and inhibits its activity. MARK4 can increase nuclear YAP and TAZ too. The activation of HER3 by ROB1 leads to the assembly of a complex between MAYA ncLRNA, LLGL2 and the methyl transferase NSUN6 that methylates and inactivates MST1. Abl kinases can also increase nuclear TAZ. Finally, TAZ and HIF1 and reciprocally act as transcription co activators for each other. The only Hippo pathway activator shown here is SKI which binds LATS2 and MST1 and strengthen the core complex.

in vivo and *in vitro* (34, 35). Similar to YAP, TAZ was shown to be involved in breast tumorigenesis. While overexpression of TAZ in MCF10A cells causes transformation morphologic changes, TAZ knockdown reduces their tumorigenicity *in vitro* and *in vivo* (47). Similar to YAP, uncoupling of TAZ from the inhibitory effect of the Hippo pathway, upon LATS1 knock down, enhances TAZ-mediated phenotypes (48). Moreover, TAZ transforming activity was shown to be dependent on Transcriptional enhancer factor TEF-1 (TEAD) transcriptional activity (49). On the mechanism level, it was demonstrated that coordination between YAP, TAZ and Transforming growth factor beta (TGFβ executes a specific pro-tumorigenic transcriptional program that is important for overcoming the anti-tumorigenic effect of TGFβ. In addition, TAZ was shown to stabilize Krüppel-like factor 5 (KLF5) which promotes breast cell proliferation and tumorigenesis by competing with the E3 ubiquitin ligase WW-domain protein1(WWP1) on KLF5 (48).

YAP/TAZ Roles in Breast Cancer Metastasis

The link between the Hippo pathway and YAP/TAZ mediated breast cancer metastasis was proved in different publications. For example, Ski interacts with LATS2, Sav, Mob, and Mer and facilitates the phosphorylation of YAP and TAZ, which leads to inhibition of YAP/TAZ induced transformation and epithelial to mesenchymal transition (EMT) (**Figure 4**) (50). In another study, activation of human epidermal growth factor receptor 3 (HER3) by ROR1 was shown to recruits the adaptor protein LLGL2, lncRNA MAYA (MST1/2-Antagonizing for YAP Activation), and methyltransferase NSUN6 (**Figure 4**). This leads to methylation and inactivation of MST1, which liberates YAP and increases breast cancer cell metastasis to the bone (51). Recently, it was demonstrated that TWIST, via the activation of thrombin receptor PAR1, inactivates the Hippo pathway and induces EMT and promotes breast cancer cell invasion (**Figure 4**) (52). Also, it was shown that TAZ and HIF1α interaction progresses breast cancer metastasis (**Figure 4**) (53, 54). Different kinases were also shown to promote breast cancer cell migration through inactivating the Hippo pathway. These kinases include ABL and MARK4 kinases (55, 56). In addition to these studies, different other studies have shown that YAP and TAZ lead to breast cancer migration, invasion and metastasis (38, 40, 47, 57, 58).

Hippo Pathway and BC Stem Cells

The link between BCSC and the Hippo pathway was initially established in a study that demonstrated that TAZ is required to sustain self-renewal and tumor-initiation capacities in BCSCs (59). TAZ was also shown to be overexpressed in tumors derived from BCSC compared to tumors derived from non-BCSC (40). In addition, overexpression of TAZ in non-transformed basal cells (MCF10A) confers cancer stem cell phenotype in these cells (60). Moreover, TAZ is indirectly tied to breast cancer stemness after the finding that revealed that miRNA125, by targeting the Hippo pathway and activator leukemia inhibitory factor receptor (LIFR), induces TAZ activity and breast cancer stemness (**Figure 5**) (61). On the mechanism level, TAZ was found to be involved in a positive feedback loop that activates the expression of α5 subunit of laminin (LM) 511 and the formation of a LM511 matrix. Reciprocally, LM511 activates

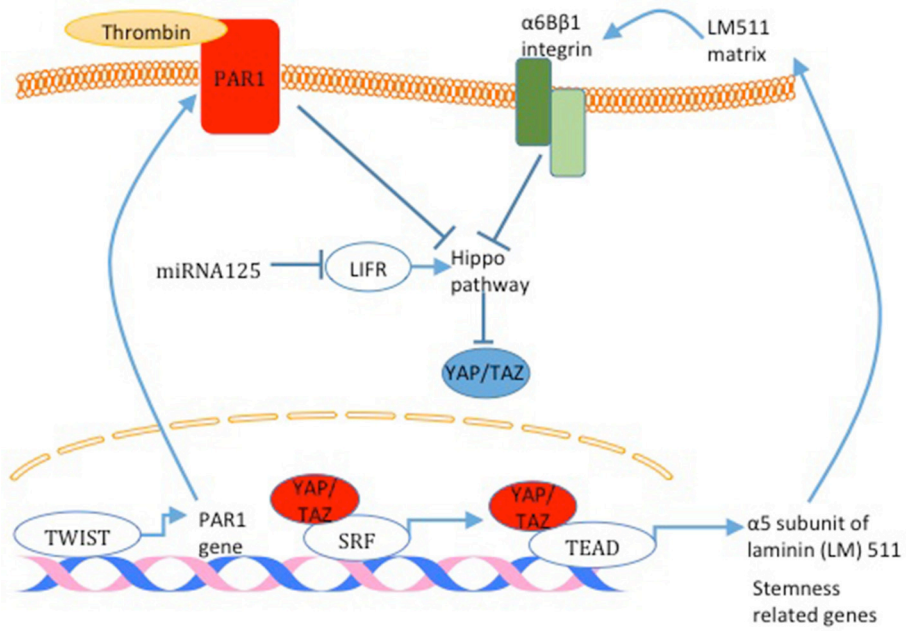

FIGURE 5 | Hippo pathway activity and breast cancer cell stemness. Different mechanisms responsible for Hippo pathway inactivation are illustrated in this figure. miRNA125 targets the Hippo pathway activator LIFR and induces TAZ activity. Also, TAZ was found to activate the expression of α5 subunit of laminin (LM) 511 and the formation of a LM511 matrix. Reciprocally, LM511 activates α6Bβ1 integrin that activated TAZ. Finally, TWIST increases PAR1 expression which consequently inhibits the Hippo pathway and induces TAZ function. TAZ activation through all these mechanisms increases the expression of breast cancer cell stemness related genes.

α6Bβ1 integrin, which activates TAZ and promotes breast cancer stemness (**Figure 5**) (62). Moreover, TWIST increases PAR1, which was shown to inhibit the Hippo pathway and induce breast cancer stemness phenotype by activating TAZ (**Figure 5**) (52). YAP was also connected to breast cancer cell stemness. YAP overexpression upregulates mammary stem cell (MaSC) signature genes (63) (**Figure 5**). Moreover, it was concluded that YAP is more important in cancer stemness in basal-like breast cancer compared with luminal-type breast cancer and that YAP correlates with poor relapse-free survival specifically in basal-like breast cancer compared to luminal-type breast cancer (63).

Recently, in breast cancer patient samples, high YAP expression profiles correlated with EMT and stemness gene signature enrichment (64). Finally, in an attempt to study the role of Hippo pathway in mammary gland development and tumor formation using gland reconstitution, it was found that Hippo signaling is involved in mammary tumor formation but not essential for mammary gland development (65).

Immune Evasion

Immune evasion, or escape from immune system control is one of Cancer "Hallmarks" (21). Paradoxically, it is documented that the presence of intact and functional immune system can prevent or promote carcinogenesis by a process known as "Cancer Immuno editing" (66–68). Accordingly, immunotherapy gained interest and is seen promising in curing cancer. Recently, it has been published that either deletion of LATS1/2 or overexpression of YAP/TAZ increases tumor immunogenecity and thus enhance its destruction by the immune system *in vivo* (69). These findings, to some extent, contradict previous work that showed that LATS1/2 are tumor suppressor genes and YAP/TAZ are oncogenes. In contrast to these findings about the immune activating function of YAP, it was shown that YAP overexpression induces an immunosuppressive environment (70, 71). In breast cancer, a recent work revealed that while LATS1/2 and MST1/2 reduce the expression of the immune checkpoint molecule PD-L1 in cancer cells, YAP/TAZ have an opposite effect (72). Since PD-L1 has an immunosuppressive function, these results are in concordance with the previous results that YAP/TAZ are immunosuppressive (70, 71) rather than being more immunogenic (69). Since PD/PD-L1 interaction is currently targeted for cancer immunotherapy, these findings suggest that YAP/TAZ might be used as a predictive factors for PD/PD-L1 based cancer immunotherapy.

Therapeutic Potential of the Hippo Pathway

Based on various roles played by the Hippo pathway and its downstream effectors YAP and TAZ in breast cancer, it seems that modulating the activity of the Hippo pathway holds a hope of being therapeutic target in breast cancer. In fact there are different compounds that were used and showed an effect on YAP/TAZ functions. For example, Verteporfin treatment inhibits proliferation, invasion and migration of the breast cancer cell line MDA MB231 (73). This effect of Verteporfin was observed with other types of cancer (74–77). Other molecules that have anti-tumorigenic effect by attenuating YAP/TAZ function are statins,

which inhibit the synthesis of geranylgeranyl pyrophosphate produced by the mevalonate cascade. This product stimulates Rho GTPases and in turn activates YAP/TAZ by inhibiting their phosphorylation. Thus, by inhibiting mevalonate cascade, statins inhibit YAP/TAZ activity (78). In breast cancer, simvastatin was shown to down regulate the expression of the downstream gene RHAMM. This attenuates breast cancer cell invasion and motility induced by signal–regulated kinase (ERK). Of note, these outcomes were revealed to be independent of MST and LATS kinase activities (79). In another study, combinations of Dasatinib and statins (which induce YAP/TAZ phosphorylation) and pazopanib (which induces proteasomal degradation of YAP/TAZ), with other anti-cancer drugs, like doxorubicin and paclitaxel, inhibited YAP/TAZ-dependent breast cancer cell proliferation. For example, in MDA MB231 cell line, which is YAP/TAZ dependent, these combinations synergistically reduced cell viability and tumorigenicity *in vitro*. On the other hand, in MCF7 cells, which are YAP/TAZ independent, these combinations didn't show the effects observed in MDA MB231 (80). The fact that different cells respond differently to YAP/TAZ inhibition indicates that other biomarkers play a role in cell response to therapy and not only the presence or absence of a single molecule like YAP and TAZ. Thus, future studies should address the effect of targeting YAP/TAZ, and concentrate on wider analysis and profiles of other proteins related to the functions of YAP/TAZ. Also, as discussed below, there is no consensus about YAP functions in breast carcinogenesis. In addition, it is always mandatory to perform drug screening experiments in a model that recapitulates what happens in tumors *in vivo*. In relation to this, while different studies revealed that Hippo pathway activation or YAP/TAZ inactivation results in an anti-tumorigenic outcome, It was found that inactivation of the Hippo pathway (by LATS1/2 knockdown or YAP/TAZ overexpression) increases tumor immunogenecity and thus enhances its destruction by the immune system *in vivo* (69). Over all, although the results from Hippo pathway manipulation in cell lines and animal models makes it a potential therapeutic target in cancer, it seems that a lot is still needed to be done to prove the reproducibility of these results in breast cancer patients.

Controversies About YAP Oncogenic Functions

As discussed above, compelling evidence proved that TAZ has only pro-tumorigenic functions. On the other hand, research regarding YAP function in cancer still holds some controversies. For example, YAP interacts with p73 and increases its transcriptional activity (81). This YAP-p73 interaction is important in driving p73 gene-target specificity in response to DNA damage (82). Moreover, under DNA damage YAP1 protects p73 from ITCH mediated degradation and thus induces p73-dependent apoptotic response (83). In breast cancer too, YAP has tumor suppressive functions. For example, it was demonstrated that YAP locus undergoes loss of heterozygosity, which might indicate that the YAP locus (11q22–23) harbors a tumor suppressor (84, 85). In addition, it was demonstrated that YAP has a pro-apoptotic function that can be inhibited by AKT oncogene (86). Moreover, knockdown of YAP in breast cancer cell lines suppressed anoikis, increased migration and invasiveness, inhibited response to taxol and enhanced tumor growth in nude mice (87). Finally, it was revealed that MicroRNA-200a promotes anoikis resistance and metastasis by targeting YAP1 in human breast cancer (88).

This controversy about YAP tumor suppressor and oncogenic functions can be explained, at least in part, by the fact that YAP has different isoforms which are differentially expressed in tissues as a result of differential splicing (89). These isoforms, as one might speculate, have different functions under different cell physiological contexts or between different cell types. In fact, most of the publications that studied YAP function in cancer did not acknowledge which YAP isoform was used or investigated, which makes it difficult to compare findings obtained from these different studies. For example, overexpression of hYAP1-2γ in mammary gland cells promotes oncogenic phenotypes including protection from apoptosis (89). On the other hand, overexpression of hYAP1-2α in squamous carcinoma cells induced apoptosis (90). Moreover, in a recent paper that studied the transcriptional potencies of YAP isoforms, it was shown that splice variant insertions in the C-terminus, which lead to the disruption of YAP leucine zipper, decreased YAP transcriptional activity (91). In conclusion, the discrepancy about YAP function in breast cancer and the presence of different YAP isoforms that seem to have different effects on the Hippo pathway signaling, ensures the need for better understanding of the role of the different YAP isoforms in breast cancer. This will help in the development of better YAP/TAZ based potential breast cancer treatments.

E3 UBIQUITIN LIGASES AND BREAST CANCER

Ubiquitination is an important post translation modification that alters protein function, either by destabilizing it, or through changing its subcellular localization. The fate of the ubiquitinated protein seems to depend on the length and architecture of the ubiquitin chain. For example, while K48-linked polyubiquitin chains mediate protein degradation in the proteasome, K63-linked polyubiquitin chains change protein subcellular localization under different cell physiological situations (92). E3s can be generally classified into three subfamilies: (1) The homologous to E6-AP carboxyl terminus (HECT) domain-containing E3s; (2) Finger domain-containing E3s and; (3) and the U box E3s (2). Ubiquitination involves a cascade of reactions catalyzed by 3 different enzymes; E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligating) enzymes. The specificity of this reaction is determined by the E3 ligase, which binds in a specific manner to a specific substrate. Ubiquitination is involved in many biological processes including DNA damage response, cell proliferation, apoptosis, cell cycle, transcription, and immune response (93–96). Deteriorations in the ubiquitination system are connected to the development of different diseases including; autoimmunity, and inflammatory diseases (97), neurodegeneration (98) cardiac

diseases (99), and cancer (1, 2, 93, 100). In cancer, the ubiquitin system regulates different cellular processes and targets involved in carcinogenesis including cell cycle, p53, transcription, DNA repair, cell signaling and apoptosis.

NEDD4 or NEDD4-like E3s interacts with their target proteins via their WW domains. These E3s are involved in different processes in different types of cancer including breast cancer as discussed below.

E3s Role in Breast Cancer Growth and Survival

Estrogen receptor alpha (ERα) is expressed in almost 70% of breast cancers and promotes estrogen-dependent cancer cell proliferation and tumor progression. Recently, it was shown that Secondary Metabolite Unique Regions Finder 1 (SMURF1), via its HECT domain, interacts with and stabilizes ER, and that depletion of SMURF1 decreases ERα-positive cell proliferation *in vitro* and *in vivo* (101). This pro-proliferative and pro-oncogenic effect of SMURF1 in breast cancer was found to be the case with SMURF2 too (**Figure 6**). SMURF2 silencing in human breast cancer cells results in a low tumorigenicity of the cells *in vitro*, and also, arrests cells in G0/G1 phase of cell cycle (102). Analysis of the mechanism that results in these phenotypes revealed that SMURF knockdown destabilizes CNKSR2 (connector Enhancer Of Kinase Suppressor Of Ras 2) protein in the cell. Of note, this effect is mediated by WW domain protein-protein interaction (102). Another E3 that was connected to breast cancer cell proliferation is WWP1. WWP1 enhances cell proliferation after polyubiquitination and proteosomal degradation of LATS1 (**Figure 6**) (103, 104). In this context, while over-expression of WWP1 enhances cell proliferation in LATS1-positive MCF10A mammary epithelial cells, knockdown of WWP1 in MCF7 breast cancer cells reduces their proliferation (103, 104). Also, WWP1 supports cell proliferation and survival by targeting ErbB4 for proteosomal degradation (105, 106). This pro-proliferative effect of WWP1 on breast cancer is in concordance with other results that showed that WWP1 stabilizes ER (**Figure 6**), which supports cell proliferation in ER+ cells. When WWP1 is knocked down, cells become more sensitive to tamoxifen treatment (103). While all the studies discussed above point out to the fact that WWP1 is a pro-proliferative factor, two independent studies published by the same group showed that WWP1 might have an anti-proliferative role through targeting KLF5 (**Figure 6**), which is a transcription factor that promotes breast cell proliferation and survival (107, 108). In these studies, both YAP (107) and TAZ (48) prevented the E3 ubiquitin ligase WWP1 from ubiquitinating and sending KLF5 for degradation (**Figure 6**). Recently, Lim et al. demonstrated that ITCH overexpression reverses breast cancer progression mediated by Wnt signaling. The authors showed that wnt signaling blocks ITCH mediated degradation of YAP/TAZ transcriptional coactivator WBP2 (**Figure 6**), and thus promotes breast cancer cell proliferation (109). On the same principle, but utilizing a different mechanism, in a different cellular context, Amot130 and ITCH were shown to promote the ubiquitination, degradation and inhibition of YAP function in response to serum starvation (110) (**Figure 6**).

E3s Modulate Breast Cancer Cell Migration, Invasion, and Metastasis

WW domain-containing E3s function in tumor metastasis was also established. Analysis of the expression level of SMURF2 protein revealed that it is elevated in 30% of mammary ductal carcinomas as well as in aggressive and metastatic breast cancer cell line MDA MB231 (111). In this article, it was disclosed that, while SMURF2 knockdown lowers aggressiveness and motility of breast cancer cells, its overexpression promotes metastasis *in vivo* and *in vitro* (111). These findings are supported by results obtained by David et al. (102), who showed a high expression level of SMURF2 in the aggressive MDA-MB-231 cell line compared to other less aggressive cancer cell lines. Moreover, they showed that SMURF2 silencing in human breast cancer cells decreases cell migration/invasion *in vitro* (102). Also, in tissue samples, SMURF2 protein levels were high in infiltrating ductal carcinoma when compared to normal tissue. These findings support the notion that SMURF2 supports invasiveness and metastasis in breast cancer. In fact these findings are in controversy with a publication by Liu (112), which demonstrated that SMURF2 expression is downregulated in triple negative metastatic tumors in comparison with either benign lesions or ductal carcinoma *in situ*. Also, human triple-negative breast cancer cell lines such as BT549, MDA-MB-436, DU-4475 and MDA-MB-468 cells showed significantly lower expression of SMURF2 protein, compared to ER + or HER2+ cell lines (112), indicating that SMURF2 might play an inhibitory function in the context of breast cancer invasion and metastasis. Indeed, two other studies supported this notion. in these studies, SMURF2 knockdown was shown to lead to a more aggressive and more migratory metastatic phenotypes *in vitro* and *in vivo* (113, 114). Interestingly, in one of these studies, SMURF2 inhibited cell invasiveness by reducing SMURF1 levels (113). In line with these finding, SMURF1 was shown to be important for EGF mediated cell migration and invasion (114). In addition, SMURF1 expression is elevated and required for MDA-MB-231 breast cancer cells motility. In these cells, Ubiquitin Specific Peptidase 9 X-Linked (USP9X) stabilizes endogenous SMURF1, and depletion of USP9X leads to down-regulation of SMURF1 and significantly impaired cellular migration (**Figure 7**) (115). Another WW domain containing E3 ligase that was shown to play a role in breast cancer migration and metastasis is WWP1. WWP1 knockdown in MDA-MB-231 breast cancer cells results in more osteolytic lesions and increases tumor area in bone marrow of mice when injected into the left ventricle of the heart (116). In this study, WWP1 knockdown reduced CXCL12-induced CXCR4 lysosomal trafficking and degradation (**Figure 7**) (116). These results proved that WWP1 inhibits breast cancer cell metastasis to the bone. ITCH E3 ligase was also connected to tumor invasion and metastasis. It was shown that in order to mediate TGF-β-induced breast cancer invasion, ITCH must degrade Ras association domain family 1 isoform A (RASSF1A) (**Figure 7**). As a consequence, Hippo pathway effector YAP1 associates with SMADs and results in their nuclear translocation (117). The pro-tumorigenic activities of ITCH were shown to be mediated also through LATS1. ITCH was shown to interact with LAST1 and send it to proteosomal degradation. As a

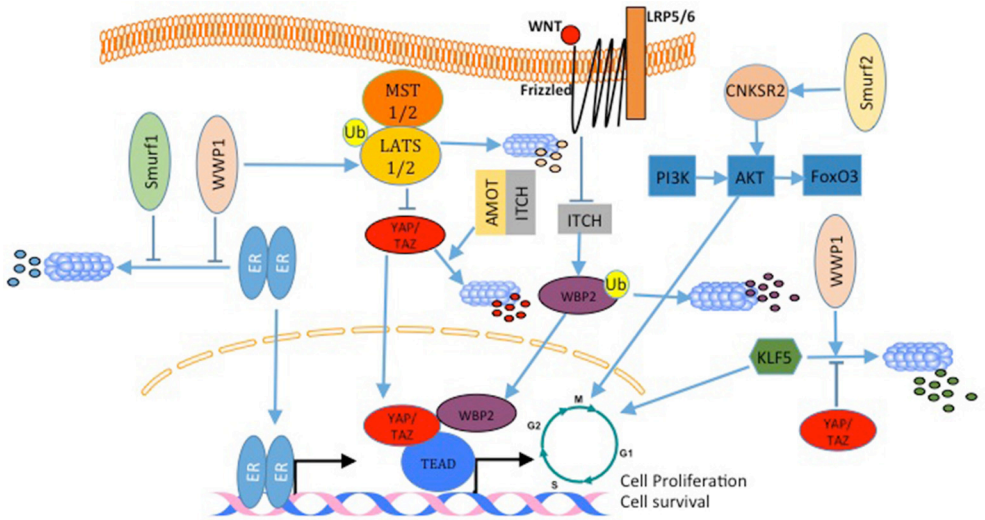

FIGURE 6 | E3 ligases regulate breast cancer cell proliferation. In breast cancer, both, WWP1 and SMURF1 stabilize ER and enhances its activation. WWP1 can also ubiquitinate and degrade LATS1 and liberates YAP/TAZ transcriptional activities. In contrast to this WWP1 can also target the pro-proliferative factor KLF5 and inhibit cell growth. This WWP1 activity can be inhibited by YAP and TAZ. SMURF2 supports cell proliferation by activating the PI3K-A-AKT-FoxO3 pathway. ITCH with angiomotin lead to YAP degradation and inhibits YAP proliferative activities. Wnt signaling inhibits WBP2 degradation by ITCH, and increases its transcription transactivation function. All these are mechanisms that activate cell cycle and transcription of pro-proliferative genes.

consequence, LATS1 degradation mediates YAP translocation into the nucleus and induces its transcriptional activities. This YAP activation induces EMT and breast cancer invasion and metastasis *in vitro* and *in vivo* (34, 35).

Apoptosis

Previous studies that investigated WWP1 expression level in breast cancer revealed that WWP1 knockdown significantly induces cell growth arrest and apoptosis in breast cancer cell lines by activating different caspases. In addition, it was found that overexpression of WWP1 in immortalized breast epithelial cell lines MCF10A and 184B5 promotes cell proliferation (118). In concordance with these findings, it was found that WWP1 depletion activates the TNF-related apoptosis-inducing ligand (TRAIL) extrinsic apoptotic pathway (**Figure 1**). Moreover, a correlation was found between the expression levels of WWP1 in four breast cancer cell lines and TRAIL resistance, but not tumor necrosis factor alpha (TNFα) and doxorubicin resistance (108). Another E3 ligase that plays a role in apoptosis is ITCH, which interacts with and destabilizes the tumor suppressor gene Ras association domain family Member 5 (RASSF5). This interaction inhibits RASSF5-mediated G1 phase transition of cell cycle as well as apoptosis (119) (**Figure 1**).

E3s and Genomic Stability

SMURF2$^{-/-}$ aging mice develop different types of tumors including breast cancer. In an attempt to explain this phenotype, it was discovered that SMURF2 deletion leads to the stabilization of the RNF20/hBre1A (**Figure 2**), the major ubH2B-specific E3. This upregulation of RNF20 leads to changes in chromatin landscape and genomic instability (120). Furthermore, the same group elucidated that SMURF2, by stabilizing Topoisomerase IIa (Topo IIa) (**Figure 2**), protects cells from DNA damage and genomic instability. They also found that SMURF2-depletion leads to reduced cell ability to resolve DNA catenanes and to

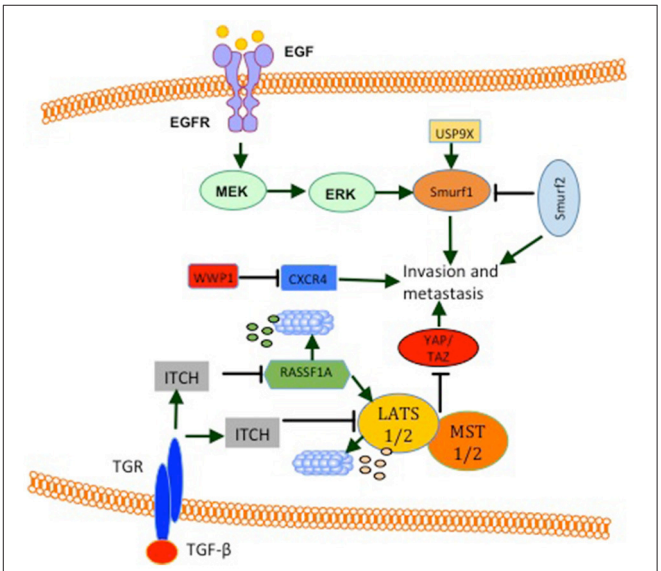

FIGURE 7 | E3 modulate breast cancer cell invasion and metastasis. EGF induces the activity of ERK signaling pathway that increases SMURF1 pro-invasive activities. SMURF1 is also stabilized by USP9X. SMURF2 has both pro- and anti- invasive and metastatic functions. By inhibiting SMURF1, SMURF2 inhibits cell invasion and metastasis. On the other hand SMURF2 was shown to induce cell invasion and metastasis. WWP1 reduces bone metastasis by inhibiting CXCR4. ITCH induction by TGF-β or other unknown mechanisms promotes cell invasion and metastasis by sending RASSF1 and LATS1 (Hippo pathway activators) for proteosomal degradation.

pathological chromatin bridges formed during mitosis, which are traits that were observed in Topo II deficient cells and are also a hallmark of chromosome instability (121). ITCH was also connected to genomic stability. In response to DNA damage, ITCH activity leads to nuclear accumulation of WWOX through its K63-linked ubiquitination at lysine residue 274. Nuclear WWOX then interacts with ATM or ATR (under different types of insults) and enhances their activation and thus enhances cellular DNA damage response and genomic stability (**Figure 2**) (23, 24).

E3s and Breast Cancer Therapy

As described above, E3s are involved in many cancer related processes. Thus, they might be good targets for cancer treatment. However, some facts might limit the potential of E3s to be targets for cancer therapy. For instance, E3s can play antagonistic effects under different cellular contexts. For example, ITCH was shown to have both, anti-tumorigenic as well as pro-tumorigenic activities by targeting different proteins. More than that, some E3s can target related proteins that have antagonistic functions. For example p63 is a WWP1 target. p63 has different isoforms that are differentially expressed in different tissues and can have even opposite functions. For example, while TAp63 is believed to sensitize cells to apoptosis, DNp63 has an opposite function. It was found that WWP1 ubiquitinates, and destructs both DNp63α and TAp63alpha. While knockdown of WWP1 increases the DeltaNp63α levels in the MCF10A and 184B5 immortalized breast epithelial cell lines and conferred resistance to doxorubicin-induced apoptosis, knockdown of WWP1 increases TAp63α level, induces apoptosis, and increases sensitivity to doxorubicin and cisplatin in the HCT116 colon cancer cell line (122). In addition to these limitations, it is well documented that a specific E3 ligase can target different substrates, which to a certain extent reduces the approach specificity and may predict the presence of side effects for E3s based therapies. Moreover, different types of ubiquitinations can result in different effects. For example while K48 type of ubiquitination is usually connected to protein degradation, K63 usually changes protein function and localization. The way the type of the ubiquitination is being selected under different cellular contexts is another issue that might impede the development of E3s based therapies. Another issue that needs to be addressed when trying to look for therapeutic targets related to ubiquitination is the fact that different E3s can have different isoforms. In breast cancer cell line T47D, six isoforms of WWP1 have been identified (123). These isoforms were shown to have different domain structures. Some of these isoforms contain or lack an N-terminal C2 domain. The distribution of these isoforms is tissue specific (123). Consequently, Flasza et al. raised the possibility that alternative forms are targeted to different locations in the cell, and thus may possibly regulate target protein selection. This means that an E3 might have different targets in different tissues or under different cellular contexts.

Chemo resistance is one important mechanism that impedes successful cancer treatment. E3s were also tied to chemo resistance (124). For example, WWP1 was shown to inhibit TRAIL induced apoptosis in breast cancer cell lines (108). Moreover, WWP1 was shown to stabilize p53 and leads to its exportation to the cytoplasm and thus inhibiting its transcriptional activity (108). These findings suggest that WWP1manipulation in p53 wild type breast cancer tumors can sensitize them to anti-cancer treatment (125).

Another example on the link between E3s and response to therapy is ITCH. In a recent publication, ITCH was shown to sensitize ER+ breast cancer cells, who acquired resistance to endocrine treatment (126). In this study, the transition from an endocrine therapy sensitive to a resistant state was accompanied with c-Jun N-terminal kinases (JNK) activation. This JNK activation seemingly resulted in ITCH phosphorylation and activation and thus c-FLIP degradation (126). In another study, in an attempt to characterize the mechanism of action of the neuregulin-non-competitive anti-HER3 therapeutic antibody 9F7-F11 that blocks the PI3K/AKT pathway and induces cell cycle arrest and apoptosis of breast cancer, it was found that 9F7-F11 activates JNK and consequently ITCH. This ITCH activation was shown to induce rapid HER3 down-regulation (127). Also, several ITCH inhibitors were identified in high throughput screening for putative ITCH modulators. One of these identified ITCH inhibitors was clomipramine—a clinically useful antidepressant drug. Treatment of different cancer cell lines including breast cancer cell lines with clomipramine, or its homologs, reduced cancer cell growth, and synergized cancer cell killing by gemcitabine or mitomycin by blocking autophagy (128). In conclusion, these findings and others indicate that E3s themselves can be targets for cancer therapies, or can be biomarkers for the prediction of a specific therapy response.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In conclusion, studies reviewed here demonstrate that WW domain-containing proteins are critical players in breast cancer initiation and progression. As discussed above, WW domain-containing proteins can act as singles, or involved in the assembly of signaling complexes like in the case of the Hippo pathway. They can act as oncogenes or tumor suppressors by altering different cancer related processes. However, some of WW domain-containing proteins could act as either tumor suppressors or oncogenes with some controversies about the identity of some of these proteins.

As we discussed above, these controversies about the functions of some of WW domain-containing proteins could be related not only to cell context, but also to their interactions and their partners. Moreover, we explained that these controversies could stem from the presence of different isoforms of the same protein. These isoforms can have different cell and tissue distribution patterns and thus may have different partners and targets. Thus, in future research, it might be very important and necessary to mention or identify the isoform studied in a specific context. For example the current literature does not specifically mention which YAP or WWP1 isoform(s) are addressed in a specific study, which makes it difficult compare and judge controversial results obtained. These controversies and different functions of

these proteins can be resolved also by future studies that will be based on more global approaches that will study the proteome or even the interactomes of these proteins in different cell contexts and in relation to different cancer related processes regulated by WW domain-containing proteins. Such studies, in addition to illustrating the mechanism(s) of action of the different WW domain-containing proteins, they will identify other biomarkers involved in the execution of phenotypes related to the different WW domain-containing proteins. In fact the identification of such biomarkers and elucidation of exact mechanism of action can be also beneficial in the prediction of therapeutic outcomes related to the manipulation of WW domain-containing proteins in the future.

Another future direction that should be followed in the context of WW domain-containing proteins and breast cancer is the identification of new such proteins. It is predicted that there are many other different WW domain-containing proteins that will have impact on breast tumorigenesis. The fishing for such proteins can be based on pulling down WW proteins using known PY rich domains, or by developing and using different softwares to help predict the presence of WW proteins in the human genome.

The use of WW domain-containing proteins as drug targets is still a premature idea that needs intensive future research. In this regard, one can raise the question of what to target in these proteins? Shall a drug target the catalytic activity of these proteins? Or their interactions by targeting their WW domains? Or target even their partners and targets? The answer to these questions will never be easy with our current knowledge about these proteins. However, we can speculate about the complexity in choosing which proposed strategy would be a successful one. For example, upon targeting the catalytic domain of different WW domain-containing proteins, a drug can target different proteins that have antagonistic functions in the context of cancer. For instance, if drugs were developed to target the evolutionary-conserved catalytic HECT domain, these drugs will target for example SMURF1 and SMURF2, which were shown to have antagonistic functions in breast carcinogenesis, and this, theoretically, might make such a strategy a failing one. In choosing to target WW domains in order to disrupt protein-protein interaction is also complex. Although there are different types of WW domains, there is still redundancy regarding the presence of a specific type of WW domain in different proteins, and thus it is expected that upon targeting the interaction of a specific WW domain-containing protein, the interactions of different other WW domain-containing proteins will be affected. In this context, here, another issue is present, which is the presence of usually more than one WW domain in one protein. These domains do not seem to be redundant in their interactions. On the contrary, they seem to be very specific which raises another question; Which domain in a specific protein to target? Regarding the last possibility of targeting WW domain-containing protein partners as means of therapeutic intervention, it might seem to be the best option. Although the fact that different WW domain-containing proteins can partner with common and shared partners, there are different examples for the presence of specific targets for different WW domain-containing proteins. These specific targets might act as biomarkers to predict specific treatment success or even be a therapeutic target.

Finally, it is obvious that WW domain-containing proteins are critical factors in breast carcinogenesis and that a better understanding of their roles in breast cancer will likely lead to the identification and development of biomarkers and drug targets for cancer treatment.

AUTHOR CONTRIBUTIONS

AJ prepared figures and bibliography. ZS wrote the manuscript.

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Delineating WWOX Protein Interactome by Tandem Affinity Purification-Mass Spectrometry: Identification of Top Interactors and Key Metabolic Pathways Involved

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It has become clear from multiple studies that WWOX (WW domain-containing oxidoreductase) operates as a "non-classical" tumor suppressor of significant relevance in cancer progression. Additionally, WWOX has been recognized for its role in a much wider array of human pathologies including metabolic conditions and central nervous system related syndromes. A myriad of putative functional roles has been attributed to WWOX mostly through the identification of various binding proteins. However, the reality is that much remains to be learned on the key relevant functions of WWOX in the normal cell. Here we employed a Tandem Affinity Purification-Mass Spectrometry (TAP-MS) approach in order to better define direct WWOX protein interactors and by extension interaction with multiprotein complexes under physiological conditions on a proteomic scale. This work led to the identification of both well-known, but more importantly novel high confidence WWOX interactors, suggesting the involvement of WWOX in specific biological and molecular processes while delineating a comprehensive portrait of WWOX protein interactome. Of particular relevance is WWOX interaction with key proteins from the endoplasmic reticulum (ER), Golgi, late endosomes, protein transport, and lysosomes networks such as SEC23IP, SCAMP3, and VOPP1. These binding partners harbor specific PPXY motifs which directly interact with the amino-terminal WW1 domain of WWOX. Pathway analysis of WWOX interactors identified a significant enrichment of metabolic pathways associated with proteins, carbohydrates, and lipids breakdown. Thus, suggesting that WWOX likely plays relevant roles in glycolysis, fatty acid degradation and other pathways that converge primarily in Acetyl-CoA generation, a fundamental molecule not only as the entry point to the tricarboxylic acid (TCA) cycle for energy production, but also as the key building block for *de novo* synthesis of lipids and amino acids. Our results provide a significant lead on subsets of protein partners and enzymatic complexes with which full-length WWOX protein interacts with in order to carry out its metabolic and other biological functions while also becoming a valuable resource for further mechanistic studies.

Keywords: WWOX, TAP-MS, interactome, WW domains, protein transport, metabolic pathways

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INTRODUCTION

The *WWOX (WW domain-containing OXidoreductase)* gene, spans 1.1 Mb, and contains common chromosomal fragile site FRA16D at ch16q23.1-23.2 (1, 2). It encodes a 414-amino acid, 46-kDa protein composed of two WW domains in tandem (designated WW1 and WW2) located N-terminal to the short-chain dehydrogenase/reductase (SDR) domain (1). *WWOX* was originally discovered by our laboratory and described by us and others as a putative tumor suppressor protein mostly associated with tumor progression, and therapy resistance in multiple cancer types (3–6). It has become clear by experimental evidence from multiple studies and human data that WWOX operates as a "non-classical" tumor suppressor [Reviewed in (5, 7)], likely of more relevance for affecting tumor progression rather than cancer initiation. Importantly, over the years *WWOX* has become recognized for its role in a much wider array of human pathologies including metabolic conditions and central nervous system (CNS) related syndromes (5, 8–10). WWOX is ubiquitously expressed in various tissues and cell types (11) and has been suggested to play roles in multiple cellular processes including but not limited to: apoptosis (12, 13); regulating the availability of various transcription factors, cofactors, and signaling molecules (14–19), cell adhesion (20, 21), metabolic functions (5, 10, 22, 23), and maintenance of genomic stability (6, 24). In most of the described cellular processes, WWOX was proposed to exert its regulatory/homeostatic roles *via* direct protein-protein interactions. Therefore, the apparent versatile nature of WWOX can be attributed in part to its ability to interact with different proteins in multiple cellular pathways. However, it is noteworthy that the significance of most of the reported interactions to the most relevant biological functions of WWOX in the normal cell still remains to be settled.

In early studies, we defined the characteristics of the prime candidate domains within the WWOX protein structure that drive protein-protein interactions, i.e., the WW domains. WW domains are small protein modules named for their unique structure: two conserved tryptophan (W) residues spaced ∼20–22 amino acids apart (25). These domains are typically 35–40 amino acids in length and fold into a three-stranded, antiparallel β-sheet with two ligand-binding grooves (25, 26). WW domains were originally classified into four classes depending on their binding affinity to a diverse set of proline-rich ligand consensus motifs: Group I binding preferentially PPXY (27), Group II binding PPLP (28) (where P is proline, Y is tyrosine, L is leucine, and *X* is any amino acid), Group III binding poly-proline sequences flanked by Arg or Lys (29), and Group IV binding phospho-Ser-Pro or phospho-Thr-Pro (30). We determined that WWOX WW1 domain, conformed by tryptophan (W) residues at positions 22 and 44, belongs to Group I due to its predilection for binding proteins harboring PPXY motifs (31). WWOX WW2 domain is not a classical WW domain due to the replacement of the second signature tryptophan by a tyrosine at position 85 and no binding motif was identified (31). More recently, it was further confirmed that the WW2 domain does not bind to any consensus proline-rich motif, but does augment the ability of WW1 to do so (32).

The vast majority of studies utilized low throughput protein-protein interaction assays to identify potential WWOX interacting partners, such as affinity capture-western, reconstituted complex, and yeast two-hybrid systems. Only two high throughput scale studies were reported where GST-fusion WWOX WW1 domain protein constructs were used as bait and its interacting partners were precipitated through GST-pulldown approaches followed by identification using mass spectrometry (MS) (33, 34). The report of Ingham et al. was done *in-vitro* on cell extracts (33) while the study of Abu-Odeh et al. was done after ectopically expressing a GST-WW1 domain fusion construct in HEK293 cells followed by GST-pulldown and MS (34). Although important in their own way, some limitations are intrinsic to the mentioned approaches and this include: (i) the use of only a very small portion of the WWOX protein (i.e., WW1 domain); (ii) the large size of the fusion GST tag (221 amino acids, 22-kDa) with potential non-physiological folding of the ectopically expressed fusion protein (i.e., WW1 domain + GST tag); and (iii) lack of the functional enzymatic SDR domain known to significantly affect intracellular localization (5, 35).

The key question regarding the myriad of reported WWOX interacting proteins identified both by low and high throughput approaches is how many are truly relevant and really reflect the most important biological functions of WWOX in the normal cell. It is important to stress that WWOX is also an enzyme predicted to carry out NAD(H) or NADP(H)-dependent dehydrogenase reactions with yet to be identified substrate/s, and undoubtedly this predicted metabolic role of WWOX is likely to be of much relevance to ultimately answer the aforementioned question.

The development of high-throughput Tandem Affinity Purification-Mass Spectrometry (TAP-MS) methods has revolutionized proteomics research (36, 37). As an unbiased approach, TAP-MS technology is ideal for the identification of not only the individual bait interacting proteins but also larger protein complexes "associated" with specific bait-protein partner pairs. In this article, we report using a TAP-MS approach with full-length WWOX protein as bait in order to identify WWOX-protein/complex interactions under physiological conditions on a proteomic scale. This work led to the identification of both well-known and novel WWOX interacting partners, suggesting the involvement of WWOX in multiple specific biological and molecular processes while delineating a comprehensive portrait of WWOX's protein interaction network (i.e., WWOX interactome) and becoming a valuable resource for further mechanistic studies on the key WWOX biological functions.

MATERIALS AND METHODS

Vector Construct, Cell Culture, Transfection and Clone Selection for TAP-MS

HEK293T cells stably expressing SBP-S-FLAG (SFB) triple-tagged WWOX were generated as described previously (38). Briefly, the cDNA WWOX full reading frame construct (1) was subcloned into a pDONOR201 vector using Gateway Technology

(Invitrogen, Carlsbad, CA, United States) as the entry clone. Next, a lentiviral-gateway-compatible destination vector was used to recombine WWOX entry clone for expression of a C-terminal triple (S tag-Flag tag-SBP tag, i.e., SFB) tagged fusion protein. All constructs were sequence verified. HEK293T cell line was obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Using polyethylenimines the SFB-tagged WWOX encoding vector construct was transfected into HEK293T cells. Cells were selected with puromycin, 12 single clones were picked and examined for WWOX expression by Western blotting using anti-FLAG antibody (MilliporeSigma, Burlington, MA, United States).

Tandem Affinity Purification of WWOX Interacting Protein/Complexes and MS Analysis

The TAP-MS procedure has been previously described in detail (38–41). Briefly, HEK293T cells stably expressing SFB-tagged WWOX were lysed with NETN lysis buffer. For the first affinity purification step, crude lysate was subjected to centrifugation and the supernatant was incubated with streptavidin-conjugated beads (GE Healthcare, Pittsburg, PA, United States). The beads were washed three times with NETN buffer, and the bound proteins were eluted with NETN buffer containing 2 mg/ml biotin (MilliporeSigma, Burlington, MA, United States). For the second purification step, eluate obtained in step 1 was incubated with S protein beads (Novagen, Kenilworth, NJ, United States). The beads were washed again with NETN buffer thrice and subjected to SDS-PAGE. The protein band containing the entire sample was excised, chopped, and gel pieces were subjected to in-gel trypsin digestion followed by drying. Dried sample was reconstituted in 5 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5 μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter x ∼20 cm length) with a flame-drawn tip. After equilibrating the column, the sample was loaded *via* a Famos autosampler (LC Packings, San Francisco, CA, United States) onto the column. A gradient was created and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). As the peptides eluted, they were subjected to electrospray ionization and then entered into an LTQ Velos ion trap mass spectrometer (Thermo Fisher, San Jose, CA, United States).

Peptide sequences (protein identity) were determined by matching the acquired fragmentation pattern with protein databases by the software program, SEQUEST (ver. 28) (Thermo Fisher) and spectral counts were generated. To evaluate potential protein-protein interactions, assign probabilistic scores to individual interactions and eliminate non-specific interactions, the Minkowski distance-based unified probabilistic scoring environment (MUSE) statistical model was applied. Next, to remove background noise contaminants the data was analyzed using the CRAPome database (https://www.crapome.org). The detailed procedure for CRAPome proteomic data analyses is described elsewhere (42). Identification of WWOX WW1 domain putative binding motifs in interacting partners was done using the ExPASy-PROSITE web resource that offers tools for protein sequence analysis and motif detection (43). Biological pathway analysis was done using the Innate Database (http://www.innatedb.com)(44).

Co-immunoprecipitation

Full open reading frame, sequenced verified Myc-DDK-VOPP1 (Catalog # RC221464), Myc-DDK-SCAMP3 (Catalog # RC201633), and Myc-DDK-SEC23IP (Catalog # RC209056) expression construct plasmids were purchased from OriGene (Rockville, MD, United States). Amino-terminal GFP-WWOX plasmid construction was previously described (3). Anti-Myc and anti-GFP antibodies were purchased from Cell Signaling (Danvers, MA, United States) and Invitrogen (Waltham, MA, United States), respectively. The anti-WWOX rabbit polyclonal monospecific primary antibody was developed in our laboratory (31). For co-immunoprecipitation, HEK293T cells were co-transfected with either 1 μg of Myc-DDK-VOPP1, Myc-DDK-SCAMP3, or Myc-DDK-SEC23IP along with 1 μg of GFP-WWOX plasmid. After 36 h cells were lysed with lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 2 mM Na_3VO_4, and 10% glycerol) containing 1 × complete protease inhibitor mixture (Roche, Indianapolis, IN). After centrifugation (12,000 g at 4°C for 10 min), total cell lysate supernatant fractions were collected and ∼600 μg of total protein was incubated with respective antibodies at a 1:200 dilution for 2 h followed by incubation with Protein A/G PLUS-Agarose (Santa Cruz, Dallas, TX, United States) for 16 h at 4°C on a rotary shaker. Corresponding IgG (MilliporeSigma, Burlington, MA, United States) was used as a negative control. Antibody-bound beads were washed three times and bound protein complexes precipated. A 3.3% of the total cell lysate was used as input and 50% of immuno-precipitated protein sample was used for western blotting using 1:2,000 dilution of anti-Myc, anti-GFP, or anti WWOX antibodies.

GST Pull-Down Assay

GST-fusion protein constructs: (i) wild-type WW domains, i.e., WW1 + WW2 (GST-WW1-2); (ii) mutant WW1 domain (GST-Mut-WW1), i.e., WW1 W44F/P47A + WW2 WT; (iii) mutant WW2 domain (GST-Mut-WW2), i.e., WW1 WT + WW2 Y85A/P88A; (iv) WW1 WT (GST-WW1); (v) WW2 WT (GST-WW2); (vi) and full length wild-type WWOX (GST-WWOX) were constructed and purified as previously described (31). GST-fusion proteins were expressed in *Escherichia coli* strain BL21 and purified as described by the manufacturer (MilliporeSigma, Burlington, MA, United States). Cleared bacterial lysates from 100 ml cultures were made by sonication in PBS containing 1x complete protease inhibitor mixture (Roche, Indianapolis, IN, United States). GST-fusion proteins were purified using Glutathione Sepharose 4B (MilliporeSigma, Burlington, MA, United States). NP-40 to a final concentration of 0.1%. was added to the bacterial lysates. Cleared total cell lysates from HEK293T cells were prepared using lysis buffer (50 mM Tris–Cl, pH 7.5,

150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). GST pull-down assays were performed by addition of 10 μg of purified GST-fusion protein and 20 μl of Glutathione Sepharose 4B (50% slurry) to 600 μg of total cell lysate from HEK293T cells. After incubation, overnight at 4°C with mixing, the beads were washed three times with cold lysis buffer and once with cold PBS. Twenty micrograms of bound proteins were separated by SDS/PAGE and analyzed with Western blotting using 1:2,000 anti-Myc antibody.

Statistical Methods

The comparison of interaction scores between WWOX binding proteins with- and without-motif was done using statistical software GraphPad PRISM 7 and the Student's two-tailed unpaired *t*-test was used for comparisons. Statistical values of the enriched pathways were obtained through analysis with InnateDB. $P < 0.05$ were considered significant.

RESULTS

TAP-MS Based Proteomic Profiling of WWOX Interactome

To obtain a comprehensive view of the human WWOX protein interactome, we performed TAP-MS analyses as illustrated in the stepwise summary shown in **Figure 1**. We used HEK293T cells stably expressing SBP-S-FLAG (SFB) triple-tagged WWOX as previously described by expression construct transfections followed by puromycin selection (38). Twelve cell clones were isolated and examined for expression levels of tagged WWOX by immunoblotting and selected those with similar expression level as the endogenous WWOX protein to proceed with the TAP steps. Total cell lysates from cells stably expressing SFB-tagged WWOX were subjected to two rounds of affinity purifications using streptavidin beads and S-beads, the final eluate was analyzed by LC-MS/MS. We identified a total of 7,589 peptides from two replicates, corresponding to a total of 795 proteins in replicate-1 and 594 proteins in replicate-2 (**Supplementary File 1**). A total of 1,006 unique interacting proteins were identified from the sum of both replicates. Next, to increase the probability of identifying true binding partners and protein complexes we focused and annotated a total of 383 proteins that were common between replicates 1 and 2 for further analysis (**Figure 1**).

In order to refine the list of WWOX interactors, we further analyzed our dataset using the Contaminant Repository for Affinity Purification database (https://www.crapome.org). CRAPome is a web-accessible resource that stores annotated negative controls generated using 411 affinity purification based MS experiments performed by the proteomics research community, and enables analyzing affinity purification based MS data for ultimately providing a CRAPome frequency score as described in detail by Mellacheruvu et al. (42). Briefly, a lower CRAPome frequency score indicates a higher specificity of the prey protein. From the identified 383 proteins, we narrowed down the list to 253 proteins with a CRAPome frequency < 0.2. The data was further shortlisted based on the MUSE algorithm generated interaction scores (40) thus generating a final list of 216 WWOX prey proteins provided in **Supplementary File 2**.

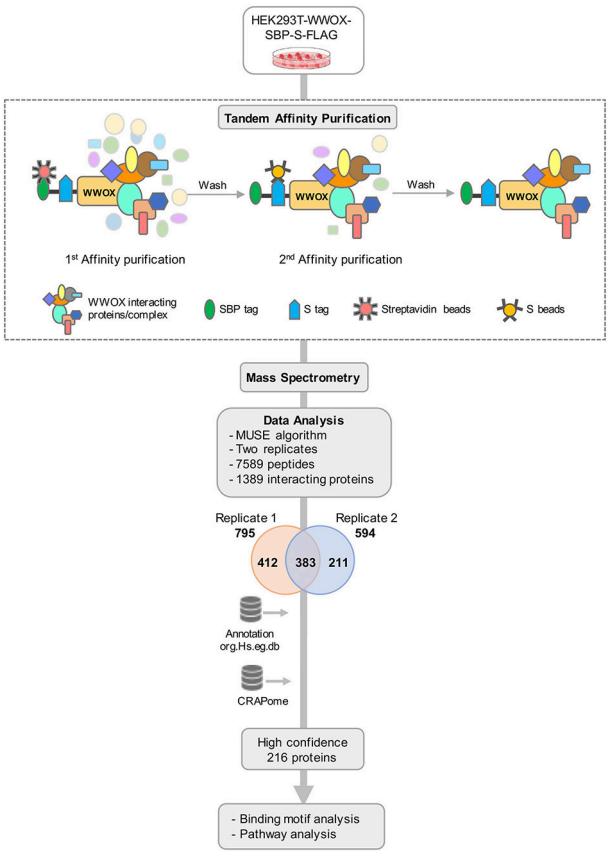

FIGURE 1 | TAP-MS based Proteomic Profiling of WWOX Interactome. Schematic diagram showing the major steps involved in the TAP-MS procedure and data analysis. HEK293T cells stably expressing WWOX with C-terminal SFB-tag (Streptavidin binding protein (SBP)-tag, S-tag, and Flag-tag) were generated by stable transfection and puromycin selection. Following the described tandem affinity purification steps, purified protein complexes were identified by mass spectrometry analysis (LC-MS/MS), and final interacting proteins were identified after proper annotation and MUSE algorithm-based analysis to define interaction scores. Data annotation was done using Bioconductor. To remove the non-specific bindings or contaminants data was analyzed using CRAPome database to obtain a list of high confidence 216 binding partners. Finally, data were subjected to motif and pathway analysis.

High Confidence WWOX Protein Interactors

As per analysis with the MUSE algorithm (40), we identified 14 proteins with TAP-MS interaction scores (IS) > 0.3. These targets have a very high likelihood of being direct WWOX physical binding partners (**Table 1** and **Figure 2**). An additional set of 18 good candidate WWOX binding proteins with IS < 0.3 and > 0.2 are also shown in **Table 1** and **Figure 2**. These proteins have varied functions including signal transduction molecules, enzymes, nucleic acid binders, or transporters and exhibit diverse sub-cellular localizations including, cellular membrane,

TABLE 1 | Candidate WWOX interacting proteins with highest confidence interaction scores.

Symbol	Protein name	Subcellular localization	Interaction score	Candidate binding motifs
DVL2	Disheveled 2	Cytosol/Nucleus/Plasma membrane	1.07	^{133}PPSF^{136}, ^{565}PPPY^{568}, ^{78}LPCF^{81}, ^{548}LPTF^{551}
WBP2	WW domain-binding protein 2	Cytoplasm/Nucleus	0.70	^{167}PPGY^{170}, ^{176}PPEF^{179}, ^{197}PPPY^{200}, ^{249}PPPY^{252}, ^{250}PPYY^{253}
DHRS13	Dehydrogenase/ reductase SDR family member 13	Cytoplasm/Secreted	0.65	–
HIRIP3	HIRA-interacting protein 3	Nucleus	0.62	–
SEC23IP	SEC23-interacting protein	Endoplasmic reticulum	0.56	^{164}PPSY^{167}
VOPP1	Vesicular, Overexpressed in Cancer, Pro-survival Protein 1	Cytoplasmic vesicle membrane	0.51	^{116}PPYY^{119}, ^{154}PPAY^{157}, ^{162}PPPY^{165}
CATSPERE	Catsper Channel Auxiliary Subunit Epsilon	Plasma membrane	0.45	–
AMOT	Angiomotin	Cell junction/Tight junction	0.42	^{239}PPEY^{242}, ^{284}PPEY^{287}, ^{106}LPTY^{109}
DVL1	Disheveled 1	Cytosol/Plasma membrane/Cytoplasmic vesicle	0.42	^{117}PPSF^{120}, ^{546}PPCF^{549}, ^{550}PPAY^{553}, ^{70}LPCF^{73}, ^{374}LPRY^{377}
VARS2	Valyl-tRNA synthetase	Mitochondrion	0.39	^{101}PPAY^{104}, ^{22}LPRF^{25}
HPF1	Histone PARylation Factor 1	Nucleus	0.37	^{112}PPEF^{115}
USP24	Ubiquitin carboxyl-terminal hydrolase 24	Nucleus	0.33	^{98}PPAY^{101}
SCAMP3	Secretory Carrier Membrane Protein 3	Endosome/Exosome/Secreted	0.31	^{50}PPAY^{53}, ^{138}LPSF^{141}
DAZAP1	DAZ Associated Protein 1	Nucleus	0.30	^{259}PPPF^{262}, ^{287}PPQF^{290}, ^{370}PPSY^{373}
DNM1L	Dynamin 1 Like protein	Cytosol/Peroxisome/Mitochondrion/Golgi apparatus/	0.27	–
STIP1	Stress Induced Phosphoprotein 1	Nucleus	0.26	–
SPART	Spartin	Cytoplasm	0.26	^{171}PPAY^{174}, ^{265}PPGF^{268}
PEF1	Peflin	Endoplasmic reticulum	0.26	–
TMF1	TATA element modulatory factor 1	Nucleus/ Golgi apparatus	0.25	–
RBM22	RNA Binding Motif Protein 22	Nucleus	0.25	^{376}PPGF^{379}, ^{390}PPPF^{393}
LRRCC1	Leucine Rich Repeat and Coiled-Coil Centrosomal Protein 1	Cytoskeleton	0.24	^{180}LPGY^{183}
GFPT1	Glutamine-fructose-6-phosphate aminotransferase 1	Cytosol/Secreted	0.24	–
UPF1	Regulator of non-sense transcripts 1	Cytoplasm	0.24	^{1005}PPGY^{1008}
HNRL1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	Nucleus	0.23	^{714}PPSY^{717}, ^{781}PPAY^{784}, ^{834}PPYY^{837}, ^{394}LPGF^{397}
DIS3	Ribosomal RNA-Processing Protein 44	Nucleus	0.23	–

(Continued)

TABLE 1 | Continued

Symbol	Protein name	Subcellular localization	Interaction score	Candidate binding motifs
MTCH2	Mitochondrial Carrier 2	Mitochondrion	0.22	–
ATXN10	Ataxin 10	Cytosol/ Secreted/ Plasma membrane	0.22	–
BCKDHA	Branched Chain Keto Acid Dehydrogenase E1, Alpha Polypeptide	Mitochondrion	0.21	–
ASNS	Asparagine synthetase	Cytosol	0.21	–
NPEPPS	Puromycin-Sensitive Aminopeptidase	Cytosol/ Nucleus	0.20	^{289}LPFY^{292}
FDFT1	Farnesyl-Diphosphate Farnesyltransferase 1	Endoplasmic reticulum	0.20	–
TARS	Threonyl-tRNA Synthetase	Cytoplasm	0.20	–

cytoplasm, nucleus, Golgi apparatus, endoplasmic reticulum, mitochondria, and cell junctions. Not unexpectedly, among the highest confidence group, we find previously described WWOX binding proteins such as DVL2, DVL1, and AMOT (17, 34), with DVL2 displaying by far the strongest interaction score with WWOX. Other previously described WWOX interactors can be found in this high confidence dataset such as WBP2 (45), and as will be described below some of these top candidates were identified as well by a previous high throughput study (34). However, the vast majority of the 216 proteins we identified using TAP-MS shown in **Supplementary File 2** have not been previously reported as WWOX interactors. It is worth noting that among the 14 highest confidence protein interactors (IS $>$ 0.3) 80% (i.e., 11 of 14 proteins) contain candidate WWOX WW1 domain binding motifs (**Table 1**, green bars in the graph- **Figure 2**). This observation reinforces the notion that this dataset can help us identify novel and relevant direct WWOX binding proteins *via* WW domains. On the other hand, among the list of top interactors with IS $>$ 0.2, 15 of 32 proteins do not display canonical WW domain binding motifs suggesting that physical interactions may occur *via* other WWOX protein regions or some of these prey proteins could be members of larger protein complexes.

WWOX Physical Interaction With Members of Protein Complexes Related to Protein Trafficking From ERES to Golgi and From Late Endosomes to Lysosomes

We have previously shown that WWOX is predominantly a cytoplasmic protein localizing to the perinuclear compartment significantly overlapping with the Golgi region (3, 31). Interestingly, among the most likely direct WWOX interactors identified by TAP-MS, at least three proteins are related to the endoplasmic reticulum (ER), Golgi, late endosomes, protein transport, and lysosomes networks: SEC23IP (SEC23-interacting protein), SCAMP3 (Secretory Carrier Membrane Protein 3), and VOPP1 (Vesicular, Overexpressed in Cancer, Pro-survival Protein 1). All these WWOX interacting proteins have an IS $>$ 0.3 and based on PROSITE analyses, all of them contain canonical WW domain binding motifs (**Table 1**). We validated the direct physical interaction of WWOX with these three proteins by means of co-immunoprecipitation (co-IP) in human cells. Myc-tagged expression constructs for each protein were co-transfected with a GFP-tagged WWOX expression vector in HEK-293T cells. Cell lysates were immunoprecipitated with anti-Myc or anti-GFP antibodies and immunostained with anti-Myc or anti-WWOX antibodies. Indeed, co-IP of WWOX with SEC23IP, SCAMP3, and VOPP1 proteins demonstrated a strong physical interaction between WWOX and each of these proteins. Furthermore, reciprocal co-IP with all of the partners for each interaction was observed (**Figure 3A**). Very recently, Bonin et al. (46) have also identified VOPP1 as a WWOX interactor in human cells by means of yeast-two hybrids and demonstrated that the WW1 region is the interacting domain mostly with the ^{162}PPPY^{165} motif of VOPP1.

In order to determine whether the observed interaction of WWOX with SEC23IP and SCAMP3 is also dependent on WWOX WW1 domain, GST-pull down experiments were performed. We observed that SEC23IP and SCAMP3 from HEK-293T whole cell lysates readily bind to GST-fusion constructs of full-length wild-type WWOX (GST-WWOX), wild-type GST-WW1-2, and wild-type GST-WW1 (**Figure 3B**). On the other hand, no binding was observed to GST-Mut-WW1 (WW1 W44F/P47A + WW2 WT) or wild-type GST-WW2. Thus, the interaction with SEC23IP and SCAMP3 is lost when key functional residues of the WW1 domain are mutated, while the mutation in the WW2 domain (GST-Mut-WW2) does not affect the overall binding (**Figure 3B**). In summary, these results demonstrate that SEC23IP and SCAMP3 are indeed novel direct interacting partners of WWOX *via* WW1 domain.

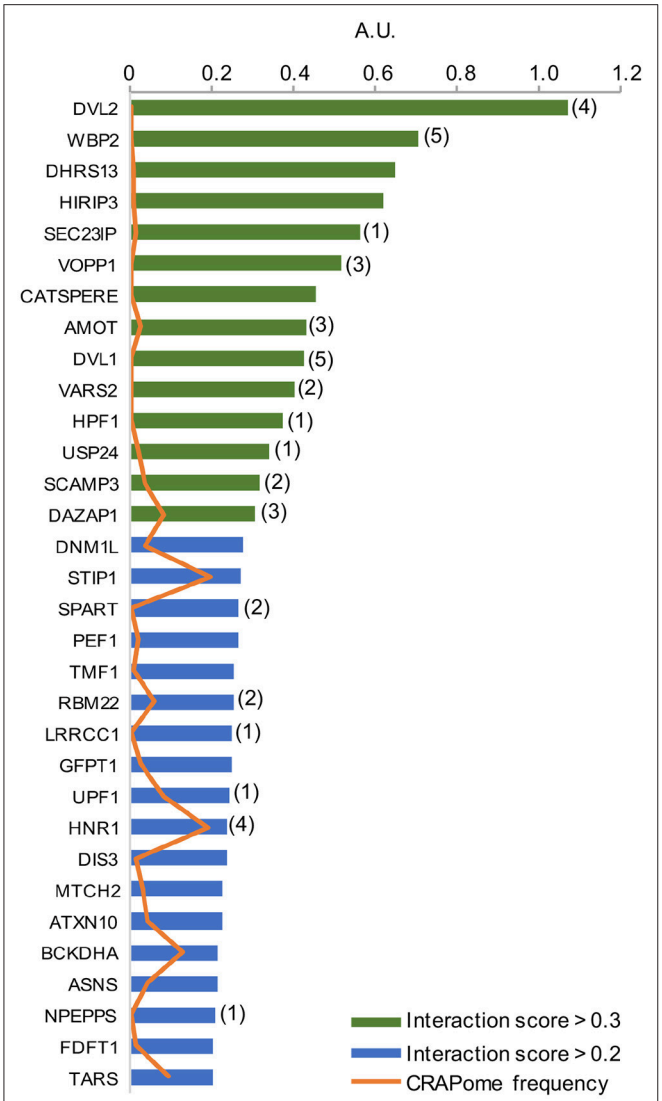

FIGURE 2 | High confidence WWOX protein interactors. Graph showing candidate WWOX interacting partners with interaction score (IS) > 0.2. Bars in green represent the set of 14 proteins with an extremely high likelihood of being direct physical WWOX binding proteins with IS > 0.3. Bars in blue represent good candidate WWOX direct binding proteins with IS < 0.3 and > 0.2. Numbers in parenthesis next to bars represent the number of potential WWOX binding motifs for each respective interacting protein. The orange line graph represents CRAPome frequency for each WWOX binding protein.

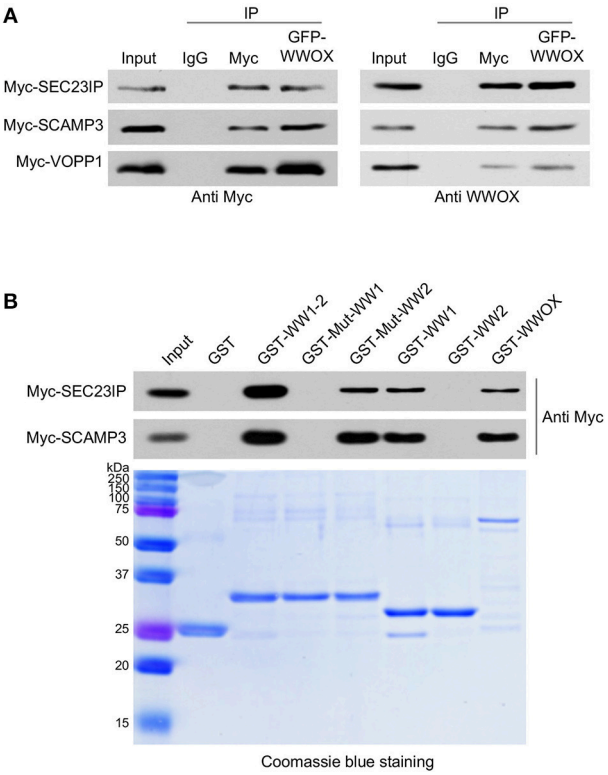

FIGURE 3 | (A) Direct physical interaction between WWOX and SEC23IP, SCAMP3, and VOPP1 proteins. Reciprocal co-immunoprecipitation of GFP-WWOX and Myc-SEC23IP, Myc-SCAMP3 or Myc-VOPP1 in HEK293T cells. Cells were co-transfected with either 1 μg of Myc-DDK-VOPP1, Myc-DDK-SCAMP3, or Myc-DDK-SEC23IP along with 1 μg of GFP-WWOX plasmid. After 36 h transfected cells were lysed and were immunoprecipitated with either rabbit IgG (control), anti-Myc, or anti-GFP antibodies. The immunoprecipitates were immunoblotted with anti-Myc or anti-WWOX antibodies as indicated. **(B)** Western blot and corresponding Coomassie blue staining of GST pull-down assay. HEK293T cells were transfected with either Myc-SEC23IP or Myc-SCAMP3 expression vectors. Cell lysates were subjected to pulldowns with the indicated GST fusion proteins: either wild-type WW domains, i.e., WW1 + WW2 (GST-WW1-2); mutant WW1 domain (GST-Mut-WW1), i.e., WW1 W44F/P47A + WW2 WT; mutant WW2 domain (GST-Mut-WW2), i.e., WW1 WT + WW2 Y85A/P88A; WW1 WT (GST-WW1), WW2 WT (GST-WW2), full length wild-type WWOX (GST-WWOX), or GST alone. Bound protein was detected by anti-Myc immunoblot.

WWOX WW1 Binding Motifs in TAP-MS Dataset

A previous study by Abu-Odeh et al. (34) employed an MS-based screen utilizing GST-WWOX WW1 domain constructs as bait which confirmed the predilection of the WW1 domain to bind proteins with PPXY motifs. However, they also observed that many of WWOX WW1 interacting proteins did not contain PPXY motifs but exhibited instead PPXF, LPXY, or LPXF motifs suggesting that the WW1 domain of WWOX binds also non-canonical class 1 WW proline-containing motifs. We examined the prevalence of potential WW domain binding motifs as defined in the aforementioned study among the 216 proteins identified in our TAP-MS experiment. Analysis of these candidate WWOX interacting partners using PROSITE showed that 31% (67 out of 216) proteins contained canonical WW1 domain putative PY binding motifs as well as other proline-containing motifs (**Figure 4A**), as previously described (34). Of a total of 67 proteins, 12 candidate WWOX binding partners displayed both PPX(Y/F) and LPX(Y/F) motifs (**Figure 4A**). Thirty-nine proteins displayed PPX(Y/F) motifs at 56 sites and 40 proteins displayed LPX(Y/F) motifs at 49 sites (**Figure 4B**

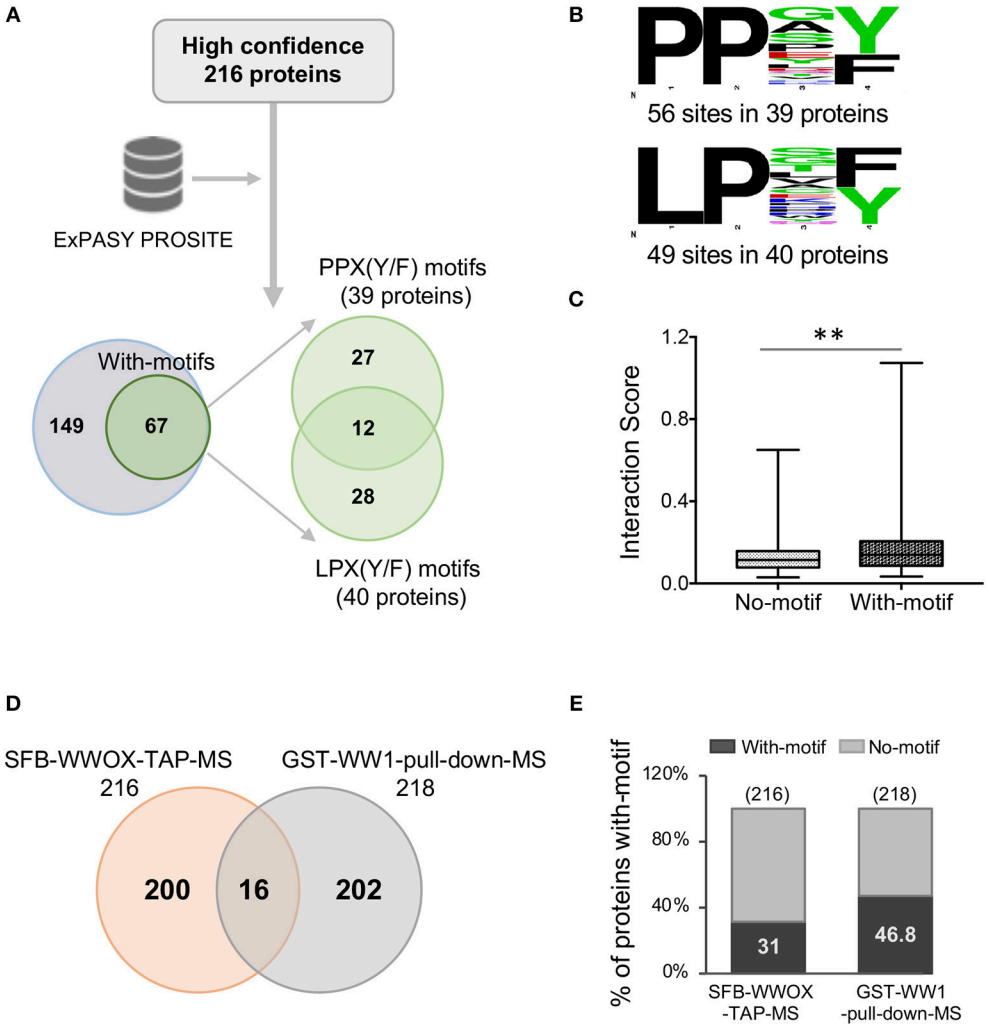

FIGURE 4 | WW domain binding motifs in WWOX interacting proteins. **(A)** ExPASY PROSITE analysis was performed on the list of 216 WWOX interactors (**Supplementary File 2**). Candidate WWOX WW1 domain binding motifs were identified in 31% (67 out of 216) proteins. Out of 67 proteins with putative WW1 binding motifs, 12 candidate WWOX binding interactors displayed both PPX(Y/F) and LPX(Y/F) motifs. **(B)** Amino acid sequence of candidate WWOX WW1 domain binding motifs. The probability of the residue at each position is proportional to the size of the letters. The image was generated using the Weblogo 3.4 program. Thirty-nine proteins displayed PPX(Y/F) motifs at 56 sites and 40 proteins displayed LPX(Y/F) motif at 49 sites. **(C)** Box and whisker plot showing the comparison of median interaction scores between proteins with- and without-motifs. Proteins with-motifs display higher interaction scores (mean interaction score = 0.1875) vs. proteins with No-motifs (mean interaction score = 0.1287). Statistical analysis was done using two-tailed unpaired Student's *t*-test, $^{**}p < 0.001$. **(D)** Comparison of SFB-WWOX-TAP-MS data with GST-WW1-pull-down-MS data from Abu-Odeh (34). Only 16 common WWOX interactors were identified overlapping both datasets. **(E)** Bar graph showing a comparison of the percentage of proteins with WWOX binding motifs between the aforementioned datasets. Thirty-one percent of proteins from the SFB-WWOX-TAP-MS dataset (this study) displayed candidate WW1 binding motifs vs. 46.8% of proteins in the GST-WW1-pull-down-MS dataset (34).

and **Supplementary File 3**). Notably, 69% (149 out of 216) WWOX candidate binding proteins were devoid of any putative WW1 domain binding motifs. We compared the interaction scores of proteins with- and without-motifs. Proteins with-motifs displayed a significantly higher interaction score (mean interaction score = 0.1875, $p < 0.001$) as compared to proteins with no-motifs (mean interaction score = 0.1287, **Figure 4C**), thus suggesting as expected a higher affinity for direct physical WWOX binding.

We also compared our full-length WWOX TAP-MS dataset with the previous GST-WW1 pull-down report (34). In the Abu-Odeh et al. study 218 WWOX interactors were identified as reported in BioGRID (https://thebiogrid.org/) (34). Surprisingly, we identified only 16 common interactors (**Table 2** and **Figure 4D**) comparing both studies. Eight of these 16 proteins were also found among our high-to-good confidence candidate WWOX binding proteins with interaction scores >0.2. Ten of these 16 proteins harbored candidate proline-containing binding motifs (**Table 2**). We also compared the prevalence of candidate

TABLE 2 | Common WWOX interactors identified in TAP-MS (this study) and Abu-Odeh et al. (34) datasets.

Symbol	Protein name	Interaction score	Binding motifs
DVL2	Disheveled 2	1.07	Yes
WBP2	WW domain-binding protein 2	0.70	Yes
SEC23IP	SEC23-interacting protein	0.56	Yes
DVL1	Disheveled 1	0.42	Yes
AMOT	Angiomotin	0.42	Yes
DAZAP1	DAZ Associated Protein 1	0.30	Yes
SPART/ SPG20	Spartin	0.26	Yes
HNRL1/ HNRNPUL1	Heterogeneous nuclear Ribonucleoprotein U-like protein 1	0.23	Yes
FARSA	Phenylalanine-tRNA ligase alpha subunit	0.16	–
ATP2A1	Endoplasmic reticulum calcium ATPase 1	0.15	–
HSD17B10	3-hydroxyacyl-CoA dehydrogenase type-2	0.13	–
RAPGEF2	Rap guanine nucleotide exchange factor 2	0.12	Yes
TARDBP	TAR DNA-binding protein 43	0.09	–
ATAD3A	ATPase family AAA domain-containing protein 3A	0.08	–
HADHA	Trifunctional enzyme subunit alpha, mitochondrial	0.06	–
CYFIP1	Cytoplasmic FMR1-interacting protein 1	0.04	Yes

WWOX WW1 binding motifs among the putative interactors between the two datasets (**Figure 4E**).

Metabolic Pathways Associated With WWOX Protein Interactome

In order to investigate if any biological pathways are significantly over-represented within the list of 216 TAP-MS identified WWOX binding proteins, we used the Innate Database (*http://www.innatedb.com)* (44). Among the most significantly associated pathways we identified key metabolic pathways including: "Valine, Leucine, and Isoleucine degradation" (KEGG pathway hsa00280), "Glycolysis/Gluconeogenesis" (KEGG pathway hsa00010), "Pyruvate metabolism" (KEGG pathway hsa00620), and "Fatty acid degradation" (KEGG pathway hsa00071) (**Figure 5A** and **Supplementary File 4**). Interestingly, all these highly significant pathways that derive from proteins, carbohydrates, and lipids breakdown converge primarily in Acetyl-CoA generation, the key molecule that delivers its acetyl group for oxidation and energy production to the tricarboxylic acid (TCA) cycle (**Figure 5B**) and notably at least 22 of the 216 high confidence proteins (**Supplementary File 2**), play key enzymatic roles in the identified pathways, mostly as members of larger enzymatic complexes (marked in red in **Figure 5B**).

DISCUSSION

TAP-MS utilizing full-length WWOX as a bait under physiological conditions was employed in order to better define specific WWOX protein interactors and by extension interaction with multiprotein complexes. This work led to the identification of both well-known, but more importantly novel high confidence WWOX interactors, suggesting the involvement of WWOX in specific biological and molecular processes while delineating a comprehensive portrait of WWOX protein interactome.

We identified endoplasmic reticulum (ER), Golgi, late endosomes, protein transport, and lysosomes networks related proteins SEC23IP, SCAMP3, and VOPP1 as direct binding partners of WWOX with high interaction scores > 0.3 (**Figure 2** and **Table 1**). SEC23IP is localized to ER exit sites (ERES) as part of the multimeric protein complex II (COPII) that coats protein transport vesicles and plays a critical function in ER to Golgi protein transport (47, 48). PROSITE analysis identified a candidate WWOX WW1 domain binding motif at ^{164}PPSY^{167} residues within a larger proline-rich region (AA 135–259) of SEC23IP and importantly this same protein region was shown to be responsible for the binding to SEC23P (47). In early reports, we demonstrated that indeed WWOX predominantly localizes to the perinuclear Golgi region (3, 31). We now demonstrated that WWOX directly binds to SEC23IP *via* WW1 domain. Thus, it is possible to speculate that the WWOX-SEC23IP interaction might modulate binding of SEC23IP to the COPII complex and play an important role in ERES assembly or cargo transport.

SCAMPs are present in all post-Golgi cycling membranes and colocalize with endosomal markers thus functioning in vesicular transport. It was shown that SCAMP3 interacts with endosomal sorting complexes required for transport (ESCRT) components and plays a role in the biogenesis of multivesicular endosomes (49) and as a regulator of endosomal morphology and composition (50). SCAMP3 was suggested to be involved in negatively regulating epidermal growth factor receptor (EGFR) degradation and promoting its recycling (51). Here we showed that WWOX binds to SCAMP3 *via* WW1 domain, likely binding

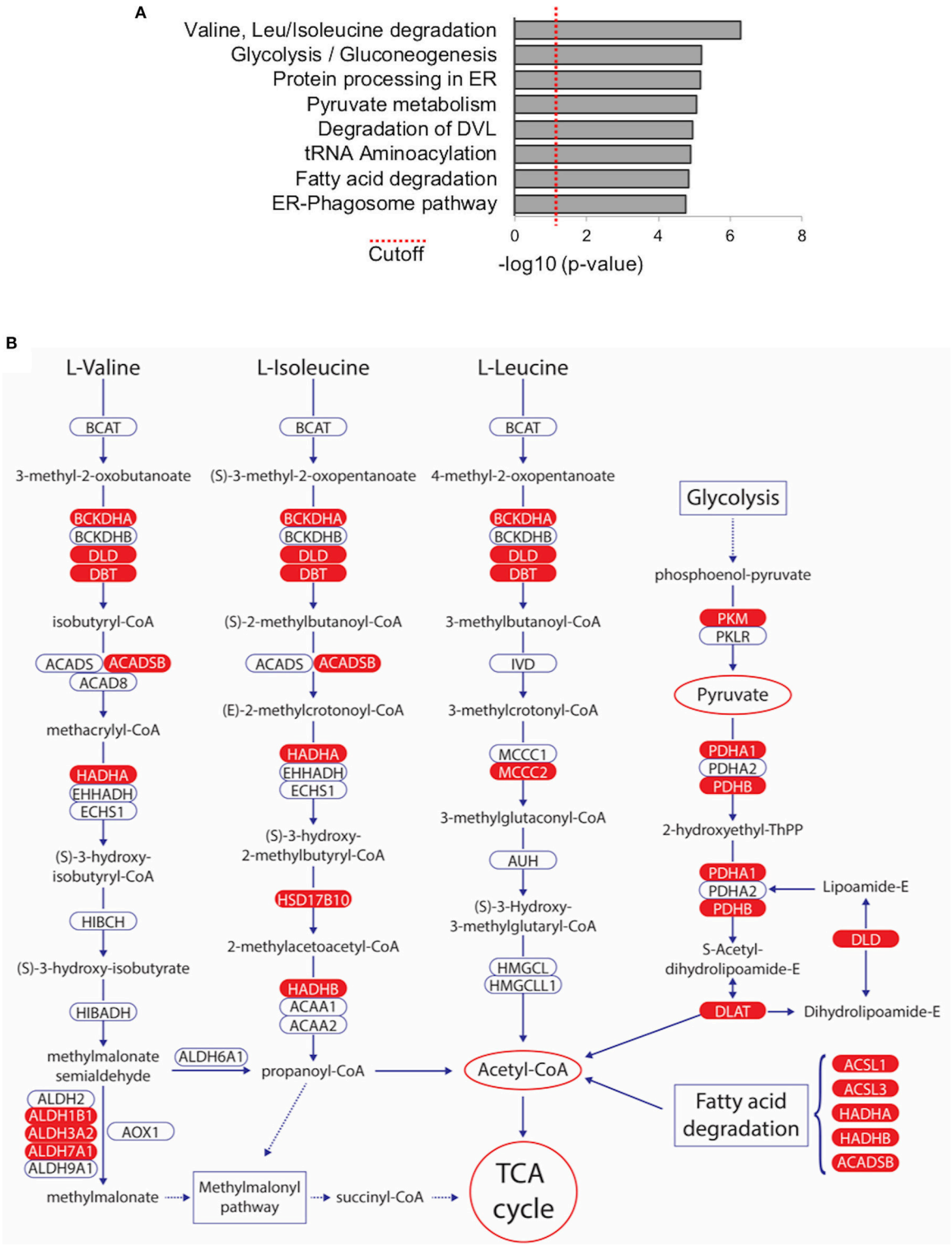

FIGURE 5 | Metabolic Pathways significantly associated with WWOX protein interactome. **(A)** Bar graph represents biological pathways that are significantly over-represented within the list of 216 WWOX interactors. See **Supplementary File 4** for detail of interactors and *p*-values of pathway shown **(B)** Among the most significantly associated pathways we identified key metabolic pathways including: "Valine, Leucine, and Isoleucine degradation" (KEGG pathway hsa00280), "Glycolysis/Gluconeogenesis" (KEGG pathway hsa00010), "Pyruvate metabolism" (KEGG pathway hsa00620), and "Fatty acid degradation" (KEGG pathway hsa00071). All pathways converge to Acetyl-CoA generation the key molecule that delivers its acetyl group for oxidation and energy production to the tricarboxylic acid (TCA) cycle. WWOX interactors identified in our TAP-MS dataset are highlighted in red.

one of two candidate SCAMP3 WW1 binding motifs at residues ^{50}PPAY^{53} or ^{138}LPSF^{141}.

VOPP1 previously also known as ECOP (EGFR-co-amplified overexpressed protein) was found amplified and overexpressed in multiple malignancies and thus considered to be pro-oncogenic (52, 53). VOPP1 protein structure includes a transmembrane domain and it was reported to partially co-localize with perinuclear lysosomes, suggesting that VOPP1 containing vesicles enter and participate in final lysosomal pathways (54). Very recently, Bonin et al. (46). using a yeast-two-hybrid system and co-IP reported that VOPP1 physically interacts with WWOX. They further described that upon binding, WWOX translocates to the VOPP1-containing lysosomal compartment and proposed that VOPP1 behaves as a negative regulator of WWOX tumor suppressor activity *via* this protein-protein sequestration mechanism (46). Independently, our TAP-MS studies confirmed the observations of Bonin and colleagues and validated as well the interaction by means of reciprocal co-IP assays. Of the three candidate VOPP1 PY motifs found at residues ^{116}PPYY^{119}, ^{154}PPAY^{157} and ^{162}PPPY^{165} it was concluded that the latter one was the most relevant for the interaction between these two proteins (46).

Interestingly, we have also previously shown an interaction of endogenously expressed WWOX with SIMPLE (small membrane protein of the lysosome/late endosome), which also plays a role in endosomal protein trafficking and in targeting proteins for lysosomal degradation (31). This in conjunction with the aforementioned observations involving SEC23IP, SCAMP3, and VOPP1 suggest that indeed WWOX is a prime candidate to play a relevant role in the homeostasis and regulation of perinuclear protein complexes related to protein trafficking between ERES to Golgi and from late endosomes to lysosomes.

It is noteworthy that only 16 common WWOX interactors were identified between the high throughput screen of Abu-Odeh et al. (34) and our study. Most of the identified interactors do not overlap between both datasets (**Figure 4D**). The causes that may account for such lack of overlap between both datasets could be due to differences in the methodological approach to identify WWOX binding partners. Abu-Odeh et al. used ectopic expression of a GST-WW1 domain fusion construct (i.e., using only a small portion of WWOX) followed by GST-pulldown and MS, while we used a full-length WWOX protein construct for our TAP-MS approach. The latter approach likely assures proper protein folding and very importantly counts with the presence of the active SDR domain which can be highly significant for proper WWOX intracellular localization (5, 35).

We believe that since TAP-MS was performed under *in-cellulo* physiological conditions, much relevant information can be gained from analyzing the data *in toto* as a portrait for identifying protein networks, pathways and cellular compartments in which WWOX likely plays a functional role. Indeed, when performing pathway analyses of WWOX interactors we identified a significant enrichment of metabolic pathways associated with proteins, carbohydrates and lipids breakdown. Thus, suggesting that WWOX likely participates in glycolysis, fatty acid degradation and other pathways that converge primarily in Acetyl-CoA generation

It is particularly intriguing that practically all members of the Pyruvate Dehydrogenase Complex (PDC), in charge of the enzymatic steps that catalyze the conversion of pyruvate (generated from glycolysis) into acetyl-CoA, can be found among the 216 proteins (**Supplementary File 2**), these include the PDHA1 and PDHB subunits of pyruvate dehydrogenase (i.e., PDC component E1), DLAT (dihydrolipoamide acetyltransferase, i.e., PDC component E2), and DLD (dihydrolipoamide dehydrogenase, i.e., PDC component E3). Furthermore, PDHA2 the only missing component of PDC E1, although it did not make the list of 216 proteins it was detected in replicate-1 of the TAP-MS analysis (**Supplementary File 1**). Additionally, PDK3 (pyruvate dehydrogenase kinase 3, not shown in **Figure 5B**) can also be found within our dataset (**Supplementary File 2**).

Wwox KO mice display abnormalities associated to metabolic processes including severe hypoglycemia, metabolic acidosis and additional metabolic abnormalities that lead to early death at 3–4 weeks of age, however although informative, these mouse models have been of limited used to fully understand the normal biological role of WWOX (16, 55). Importantly, evidence suggesting a direct link between WWOX loss of function and alterations in cellular respiration has been argued (7, 56). Strikingly similar to our TAP-MS findings, O'Keefe et al. (57) by using *D. melanogaster* (DMel) *Wwox* mutant models, observed that a significant number of Wwox interactors identified by a limited proteomic screen are either directly or indirectly related to metabolic pathways that precisely converge in the TCA cycle as in our findings (**Figure 5B**). These include many enzymes associated with glucose metabolism, lipid metabolism, ethanol metabolism, and oxidation/reduction, thus suggesting a contributing function of Wwox associated with the maintenance of aerobic metabolism (57). Furthermore, from analyzing the candidate Wwox protein interactors reported by O'Keefe et al. several orthologs and potential orthologs can be identified that closely match several of the targets identified by TAP-MS in our study. Of particular relevance is the finding of interactor DMel protein CG7430 which is the ortholog of human DLD (dihydrolipoamide dehydrogenase) precisely the E3 component of the Pyruvate Dehydrogenase Complex found in our dataset and discussed in a previous paragraph. The Drosophila proteomic screen also identified Wwox interactor CG7470 (57) ortholog of human ALDH18A1 (aldehyde dehydrogenase 18 family member A1) also found in our dataset. Various other aldehyde dehydrogenases can be found in both datasets, enzymes of much relevance in pyruvate metabolism, alcohol metabolism, fatty acids degradation and amino acids degradation (**Figure 5B**). Examples include Drosophila Wwox interactors DMel CG3752 and CG31075 with significant similarity to ALDH1B1 a member of enzymatic complex IUBMB/KEGG EC 1.2.1.3 also known as aldehyde dehydrogenase (NAD). Two other members of EC 1.2.13, ALDH3A2 and ADH7A1 are also found in our dataset (**Supplementary File 2** and **Figure 5B**). This enzymatic complex plays roles in all the metabolic pathways described and shown in **Figure 5B** and in multiple other metabolic reactions. In conjunction, studies in human cells and Drosophila indicate that WWOX appears to play critical roles in glucose,

lipid metabolism and other pathways that converge primarily in acetyl-CoA generation, which is not only the entry point to the TCA cycle for energy production, but also the key building block for the *de novo* synthesis of lipids and amino acids.

In agreement with the suggestion of WWOX playing a role or modulating cellular respiration, Abu-Remaileh and Aqeilan reported that *Wwox KO* mouse embryo fibroblasts displayed increase glucose uptake, enhanced glycolysis and reduced mitochondrial respiration resembling a "Warburg effect" like condition. It was also found that WWOX deficiency correlates with enhanced levels and activity of HIF1α over specific transcriptional targets related to glycolysis and that WWOX physically interacts with HIF1α. Based on these observations it was proposed that WWOX, *via* modulating HIF1α availability, might regulate glucose metabolism and that WWOX loss leads to activation of anaerobic glycolysis (Warburg effect) (22, 56). However, the exact mechanisms on how WWOX could affect HIF1α activity and in turn modulate cellular respiration are not clear. The association of WWOX and the described metabolic pathways also appear consistent with studies that have shown a link between WWOX and metabolic-syndrome related human traits (5, 10, 58). We also observed that gene expression profiles on samples from liver-specific *Wwox* conditional KO mice displayed significantly altered lipid metabolic profiles and increased plasma triglyceride levels, suggesting a significant role for WWOX in modulating lipid metabolism (10).

In summary, our results provide a significant lead on subsets of protein partners and enzymatic complexes with which WWOX might interact with in order to carry out its metabolic functions and other significant biological roles.

AUTHOR CONTRIBUTIONS

CA and JC designed research, TH, CA, and MA analyzed the data, JL performed experiments, TH and CA wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00591/full#supplementary-material

Supplementary File 1 | List of WWOX interacting peptides identified by TAP-MS in replicate-1 and replicate-2.

Supplementary File 2 | List of 216 WWOX protein interactors shortlisted based on MUSE and CRAPome analyses.

Supplementary File 3 | List of WWOX protein interactors containing PPX(Y/F) and LPX(Y/F) motifs and motif mapping within each protein target.

Supplementary File 4 | List of most significantly enriched biological pathways with list of participating WWOX interacting proteins and corresponding *p*-values.

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REVIEW
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Gears-In-Motion: The Interplay of WW and PPIase Domains in Pin1

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Pin1 belongs to the family of the peptidyl-prolyl *cis-trans* isomerase (PPIase), which is a class of enzymes that catalyze the *cis*/*trans* isomerization of the Proline residue. Pin1 is unique and only catalyzes the phosphorylated Serine/Threonine-Proline (S/T-P) motifs of a subset of proteins. Since the discovery of Pin1 as a key protein in cell cycle regulation, it has been implicated in numerous diseases, ranging from cancer to neurodegenerative diseases. The main features of Pin1 lies in its two main domains: the WW (two conserved tryptophan) domain and the PPIase domain. Despite extensive studies trying to understand the mechanisms of Pin1 functions, how these two domains contribute to the biological roles of Pin1 in cellular signaling requires more investigations. The WW domain of Pin1 is known to have a higher affinity to its substrate than that of the PPIase domain. Yet, the WW domain seems to prefer the *trans* configuration of phosphorylated S/T-P motif, while the PPIase catalyzes the *cis* to *trans* isomerasion. Such contradicting information has generated much confusion as to the actual mechanism of Pin1 function. In addition, dynamic allostery has been suggested to be important for Pin1 function. Henceforth, in this review, we will be looking at the progress made in understanding the function of Pin1, and how these understandings can aid us in overcoming the diseases implicated by Pin1 such as cancer during drug development.

Keywords: Pin1, WW domain, peptidyl-prolyl *cis*/*trans* isomerase (PPIase), phosphorylation, interdomain communication, cancer target, drug development

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INTRODUCTION

Posttranslational modifications (PTMs) introduce diversity to the functions of many proteins in the cellular proteome. This allows the cells to exert more biological functions with lesser number of proteins. There exist many types of PTMs, of which, the reversible phosphorylation is widely studied for its role in regulating many signaling cascade (1). Initially, phosphorylation of a protein was thought to be the final step in activating or inhibiting signaling cascades until the discovery of the group IV WW domain proteins, notably the Pin1 protein (2, 3). Pin1 possesses two major domains, namely the WW domain and the peptidyl-prolyl *cis*/*trans* isomerase (PPIase) domain (3). The WW domain consists of two highly conserved tryptophan amino acids separated by ∼20–22 residues, allowing Pin1 to bind to the phosphorylated consensus site ser/thr-pro (pS/T-P) motif (3–5). This allows Pin1 to exert its molecular function as an isomerase *via* the PPIase domain, leading to the *cis*/*trans* conversion of its substrate to elicit the intended biological outcomes (2, 6). This additional modification on the phosphorylation sites of multiple Pin1 substrates has provided an alternate view on how signaling cascades could be regulated under different cellular conditions (7, 8).

So far, Pin1 has been extensively studied for its role in various cellular functions, particularly in cell cycle regulation (2, 6, 9–11). Besides its importance in cell cycle progression, Pin1 has been further implicated in many biological processes such as embryonic development, cell motility, immune responses, gene transcription, and apoptosis (12–17). Due to its diverse role, perturbation to Pin1 expression levels has been implicated in many diseases such as cancer, and neurodegenerative diseases like Parkinson's and Alzheimer's disease (10, 15). Especially in the case of cancer, many functional substrates of Pin1 have been identified to potentially contribute to the manifestation of cancer via various biological processes as summarized in **Figure 1**. As such, Pin1 has been identified as an important target for therapeutic intervention for these diseases (48–50). Indeed, many Pin1 inhibitors have been identified, with the most recent ones being API-1, KPT-6566, compound 1 and 8 (51–54). Unfortunately, there remains much to do before any breakthrough in targeting Pin1 for the treatment of various diseases is achieved. This stems from our limited understanding of Pin1 mechanism in these biological processes, and how the unique WW and PPIase domains of Pin1 work to elicit its function.

In addition, there have been many structural and protein dynamic studies to understand how Pin1 could target its biological substrates to exert its intrinsic *cis*/*trans* isomeric activity. This would then trigger its intended downstream cellular signals and effects. Many models have been introduced to explain the molecular mechanism of how Pin1 exert its catalytic activity. However, there has not been a model in agreement to truly explain how Pin1 acts on its biological substrates. Therefore, in this review, we will highlight the progress of Pin1 research in elucidating the actual mechanism of Pin1, with an emphasis on the structural importance of Pin1 on its function, and how the perturbation to this fundamental structure could explain for its roles in diseases such as cancer. We will also discuss how these structural features could be used in the drug development of Pin1 inhibitors.

DETAILED STRUCTURAL FEATURES OF HUMAN PIN1

The human Pin1 consists of a total of 163 amino acid residues that forms two distinct domains, the WW domain and the PPIase domain as mentioned previously (**Figure 2**). The WW domain spans the first 39 amino acid residues of the Pin1 protein, while the PPIase domain spans residues 50–163. Both domains are known to be able to bind to the pS/T-P motifs, with the WW domain binding being noncatalytic in nature, while the PPIase domain possesses the sole catalytic site of the *cis*/*trans* isomerase activity in Pin1 (55–57).

Apart from the two major domains, the Pin1 protein also consists of a flexible linker region consisting of approximately 17 amino acid residues spanning from residue E35 to A53 (58). In addition, within the WW domain, there exist two loops. Loop I is situated at residue S16 to R21, and Loop II at residue H27 to N30 (58). As for the PPIase domain, it also displays two main components. They are the substrate recognition segment (residue K63 to R80) where residues K63, R68, and R69 create a positive charged phosphate-binding loop to facilitate binding to the pS/T-P motif (58, 59). The other important segment of the PPIase domain is the catalytic active site that consists of amino acid residues such as H59, S115, C113, L122, M130, F134, T152, and H157 (58, 60, 61).

Besides these characteristics of Pin1, there exist the presence of a domain interface within Pin1, consisting of amino acid residues I28, the WW domain Loop II (H27 to N30), and part of the PPIase domain (S138 to R142). This domain interface has been suggested to play an important role in interdomain communication that regulates the function of Pin1 upon substrate binding (60–62). These minor features of Pin1 suggest the complexity of Pin1 function upon substrate binding and are a potential reason why Pin1 can interact with a large and diverse number of biological substrates to regulate cellular functions. However, how these characteristics can work hand-in-hand to confer the functions of Pin1 on its substrates remain a largely unfinished work.

THE PROPOSED MODELS OF THE WW DOMAIN AND PPIASE DOMAIN IN PIN1 SUBSTRATE INTERACTIONS

As the two main domains of Pin1 protein, much research has been carried out on both WW and PPIase domains to understand their role in the interaction of Pin1 with its biological substrates. It has been reported in the early stages of Pin1 research that the WW domain has a ten-fold higher binding affinity with phosphorylated peptides *in vitro* as compared to the PPIase domain (3, 63). This property of the WW domain in Pin1 has led to a proposition that its major role is to aid Pin1 in specific targeting of its substrate, as well as to increase local concentration of Pin1 substrate to exert its catalytic function (7, 64). This scenario gave rise to a few proposed models of Pin1 substrate interactions. The first proposed model is the sequential binding model of Pin1 (**Figure 3A**) and remains widely accepted. In this model, the WW domain would first bind to the pS/T-P motif on its target substrate. This allows the PPIase domain to bind with another pS/T-P motif that is present on the same target substrate. Alternatively, the binding of the WW domain would lead to structural conformation change that allows the PPIase domain to displace the WW domain to bind to the same pS/T-P motif to exert its isomeric activity.

In the multimeric binding model (**Figure 3B**), it is applied in multi-protein complexes containing a Pin1 biological substrate and an active kinase that is present to phosphorylate the S/T-P motif of the Pin1 substrate (66). In this configuration, Pin1 first binds to the kinase at the pS/T-P motif *via* its WW domain. Subsequently, the active kinase would phosphorylate the S/T-P motif on the target substrate of Pin1. As Pin1 is already near its substrate, the PPIase domain would recognize and bind to the pS/T-P motif on the target substrate to exert its isomeric activity. This model is further supported by the identification of Pin1

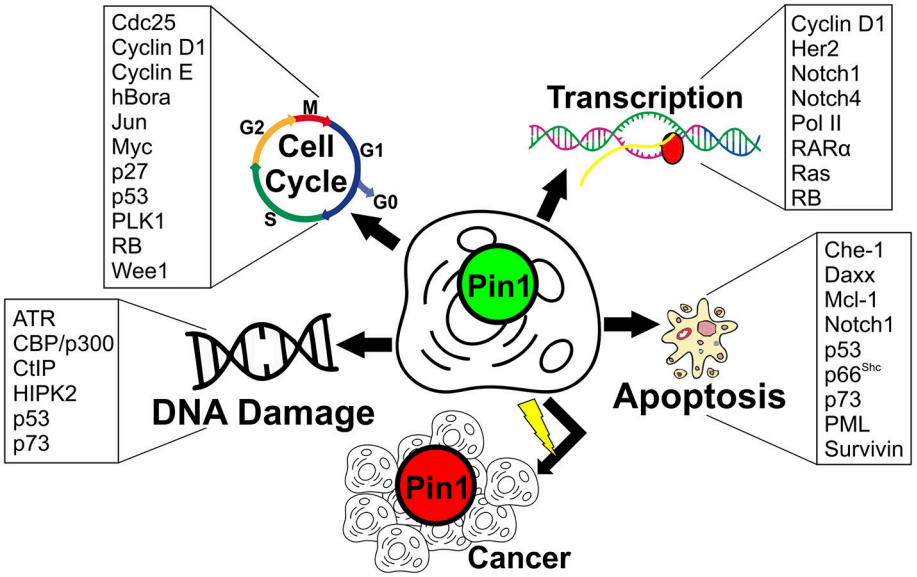

FIGURE 1 | Pin1 substrates belonging to various cellular processes linked to cancer. Cell cycle – Cdc25 (18): Cell division cycle 25; Cyclin D1 (19–21); Cyclin E (22); hBora (23): Human protein aurora borealis; Jun (19, 24): Transcription factor AP-1; Myc (25): Myc proto-oncogene protein; p27 (26); p53 (27, 28); PLK1 (6, 29): Polo-like kinase 1; RB (30): Retinoblastoma-associated protein; Wee1 (31). Transcription – Cyclin D1 (19); Her2 (32, 33): Human epidermal growth factor receptor 2; Notch1 (34, 35): Neurogenic locus notch homolog protein 1; Notch4 (35): Neurogenic locus notch homolog protein 4; Pol II (36): RNA polymerase II; RARα (37): Retinoic acid receptor alpha; Ras (38); RB (30). DNA damage-ATR (39): Ataxia telangiectasia and Rad3-related protein; CBP/p300 (24): CREB-binding protein/p300; CtIP (40): C-terminal-binding protein (CtBP)-interacting protein; HIPK2 (41): Homeodomain-interacting protein kinase 2; p53 (27, 28); p73 (42). Apoptosis-Che-1 (43): Apoptosis-antagonizing transcription factor (AATF); Daxx (44): Death domain-associated protein 6; Mcl-1 (45): Induced myeloid leukemia cell differentiation protein; Notch1 (34, 35); p53 (27, 28); $p66^{Shc;}$ (46):66 kDa proto-oncogene Src homologous-collagen homolog (Shc) adaptor protein; p73 (42); PML (47): Promyelocytic leukemia protein; Survivin (47).

interactors which are known kinases themselves as reflected in the IntAct Molecular Interaction database (67).

In another model termed the catalysis-first binding model (**Figure 3C**), it is believed that the phosphorylation of S/T-P motif on the target substrate for WW domain binding requires the PPIase function of Pin1 (57). This proposition was put forth by the observation that in all the known structures of the WW domain bound substrate peptides, the identified binding site is present in a *trans* configuration state (56, 57, 68). Moreover, a study conducted by Verdecia et al. (56) seems to suggest the strict preference of the wild type WW domain of Pin1 to bind to the *trans* configuration of its substrate peptide. Of interest, Pin1 also enhances the dephosphorylation activity of protein phosphatase such as PP2A, which too requires the pS/T-P motif to be in the *trans* configuration state. Therefore, the binding of the WW domain of Pin1 might be a stabilizing action for the Pin1 substrate to remain in its *trans* configuration state for dephosphorylation to occur (69, 70). In addition, a study by Namanja et al. (60) found the preference of WW domain binding to the *trans* but not the *cis* configuration of pS/T-P motif. Thus, if the WW domain prefers binding to a *trans* configured substrate peptide, the PPIase domain of Pin1 would first bind to the *cis* configured pS/T-P motif to catalyze the *cis*/*trans* isomerization to the *trans* configuration. This would lead to WW domain binding to the now *trans* configured pS/T-P motif to prevent the reverse isomerization to occur. This process would then allow the local concentration of the Pin1 PPIase to initiate further *cis*/*trans* isomerization to fast forward the propagation of downstream cellular signaling of the stabilized Pin1 substrate.

Indeed, previous studies have shown that the PPIase domain of Pin1 is able to bind to pS/T-P motif instead of just its WW domain (56, 58, 65). As mentioned earlier, this binding occurs at the substrate recognition segment, in particular, the phosphate-binding loop created by the three amino acid residues of K63, R68, and R69. Furthermore, a study done by Innes et al. (65) demonstrated the importance of this phosphate-binding loop in the target binding of Pin1 to initiate its catalytic isomerase function. This is despite the reported higher binding affinity of the WW domain of Pin1 and reinforced by the presence of PPIase domain-specific Pin1 inhibitors that do not interact with the WW domain (71). As such, the authors suggest that Pin1 could interact with its biological substrate *via* the simultaneous binding model (**Figure 3D**). In their study, they noticed that pS/T-P motifs that have an addition P residue in the +1 position, pS/T-P-P, seem to be targeted by the WW domain but not the PPIase domain of Pin1. This led them to believe that a substrate with multiple phosphate binding sites could allow for the simultaneous binding of Pin1 to its substrate. They observed this in the binding of Pin1 to Cdc25 and Serine/Threonine-protein kinase (PLK1). On the other hand, they identified that Pin1 binding *via* the PPIase domain is sufficient in proteins with only a single pS/T-P motif such as non-POU domain-containing octamer-binding (NONO)

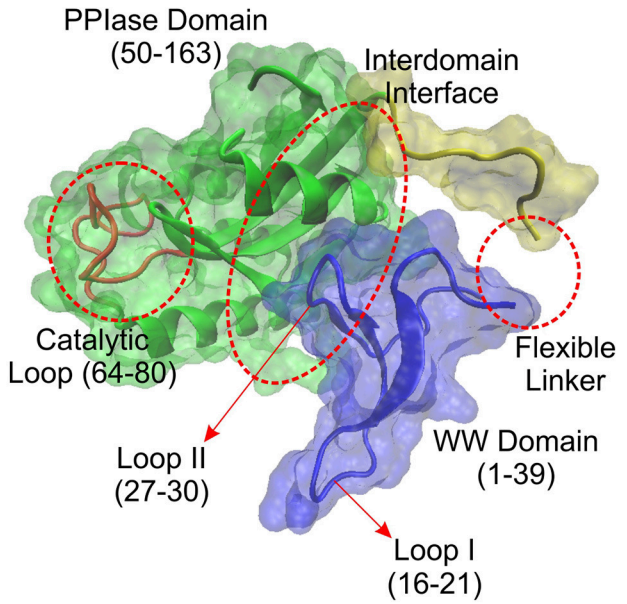

FIGURE 2 | Molecular structure of human Pin1 (PDB: 1PIN) without ligand binding. Pin1 is predominantly made up of two major domains: WW domain and PPIase domain. Within the WW domain, there exist two loops, namely Loop I and II. As for the PPIase domain, there is a catalytic loop at the catalytic site. There also exists PPIase binding domain where it could bind to the pS/T-P motif apart from the WW domain. In addition, existing between the two domains, there lies an interdomain interface that has been found to be important in Pin1 allosteric regulation.

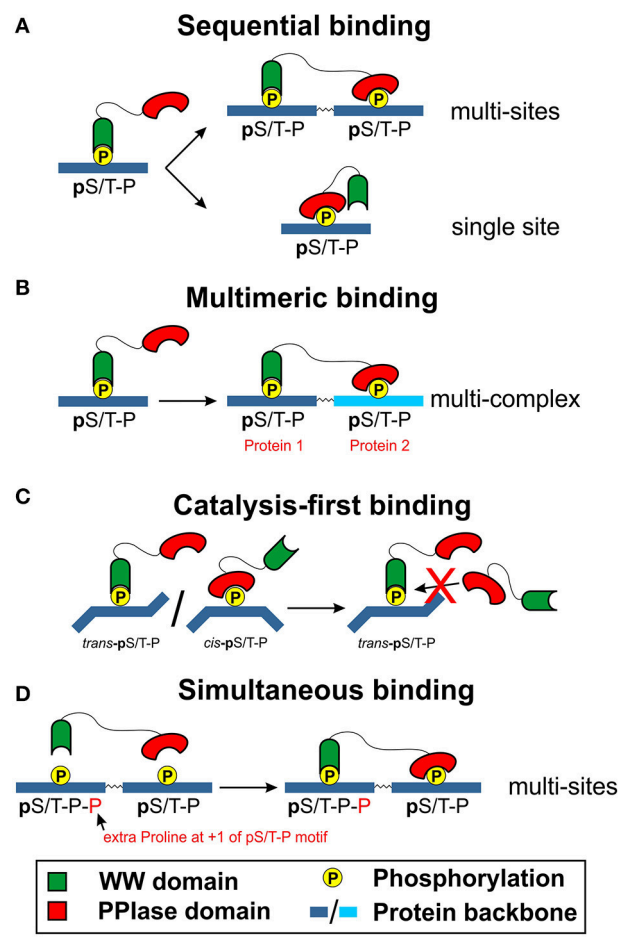

FIGURE 3 | Proposed models of WW domain and PPIase domain interaction with the substrate of Pin1 [first highlighted by Innes et al. (65)]. Two decades of research has shed light on the potential mechanism as to how the WW and PPIase domains could be involved in substrate binding of Pin1. **(A)** Sequential binding model. The sequential binding model involves the initial binding of the WW domain of Pin1 before the PPIase domain could bind to the same pS/T-P motif or another pS/T-P motif on the same protein. **(B)** Multimeric binding model. The WW domain first binds to an active kinase with a pS/T-P motif before being brought close to a substrate of Pin1. The active kinase then phosphorylates S/T-P motif to allow the PPIase domain to bind to it to initiate the isomeric reaction. **(C)** Catalysis-first binding model. Existing *trans* pS/T-P motif will be bound to the WW domain of Pin1, while the PPIase domain could bind to the *cis* pS/T-P motif to catalyze the isomeric reaction to the *trans* configuration. Subsequently, the WW domain would then bind to the new *trans* pS/T-P motif to prevent the reverse conversion. **(D)** Simultaneous binding model. Both WW domain and PPIase domain can bind simultaneously, with the WW domain binding to pS/T-P-P motif and PPIase domain binding to the pS/T-P motif.

protein and splicing factor, proline-, and glutamine-rich (SFPQ) protein for its activity. As such, there seems to be two types of Pin1 interactors that requires either the binding of both WW and PPIase domains, or just the WW/PPIase domain alone.

These proposed four models of Pin1 interaction with its biological substrates for functional regulation might suggest two aspects. Firstly, there is still lack of concrete evidences to highlight which model(s) Pin1 may deploy for its interaction with its biological substrates. Secondly, these models demonstrate the potentially diverse interaction that Pin1 has on its already diverse biological interactors to play a role in the regulation of various biological functions. All these models do have their merits as well as potential doubts as to its suitability. For instant, the sequential binding model does fulfill the characteristics of the WW domain having a higher affinity of peptide binding to the pS/T-P motif than the PPIase domain. In proteins with multiple sites of pS/T-P motifs, this model could explain the use of the WW domain to localize the concentration of the PPIase domain for isomerization. However, for substrate with a single pS/T-P motif, the potential release of the WW domain from this motif, followed by the binding of the PPIase domain for catalytic activity does not seem to be energy favored. This is also highlighted by studies that we have mention previously (56, 57, 60, 68–70), where the WW domain favors the *trans* over *cis* configuration. As such, the PPIase isomerization would not have occurred with this sequential binding.

For multimeric binding to an active kinase *via* the WW domain of Pin1, the increased local concentration of Pin1 would allow the binding to its biological substrate in the vicinity to elicit its downstream biological processes. Furthermore, the pS/T-P motif of the kinase could be in a *trans* configuration state to allow for WW domain binding. In addition, the catalysis-first binding model could also be associated with the multimeric binding model where Pin1, bound to the active kinase *via* the

WW domain, is brought to its target substrate by the active kinase. Subsequently, the active kinase would phosphorylate the Pin1 substrate, allowing for the PPIase domain of the Pin1 to bind and catalyze the *cis-trans* conformational change. Furthermore, the suggested simultaneous binding model could also fit the multimeric binding model, although this would occur on a single protein, instead of the proposed protein complex in the multimeric binding. Of interest, the binding of the WW domain in the *trans* configured pS/T-P motif could lead to conformational changes to the PPIase domain, increasing its binding capacity to the *cis* configured p-S/T-P motif and subsequently its catalytic efficiency. The potential role of interdomain communications between the WW domain and the PPIase domain of Pin1 has indeed been studied in recent years as discussed in the next section.

INTERDOMAIN COMMUNICATIONS BETWEEN WW DOMAIN AND PPIASE DOMAIN OF PIN1

As highlighted earlier, Pin1 is made up of two distinct major domains in WW and PPIase domains. Besides these two domains, there exist an internal conduit of hydrophobic residues cluster that connects the interdomain interface and the catalytic site of the PPIase domain (60, 62). This has led to the suggestion of an allosteric mechanism present in Pin1. Classic allostery stems from the binding of a ligand to a part of a protein where it is distal to the active site. This binding would then lead to global conformation change of the protein, or localized changes to its active site, resulting in increased binding affinity of the active site or its enzymatic activities (72, 73). Recently, the concept of allostery, termed dynamic allostery, has been looked into to explain for the absence of structural conformation changes under allosteric effect of ligand binding on the main protein (74–76) since its first introduction more than three decades ago (77). This absence of structural change is replaced by the use of the protein's interdomain communications as demonstrated by computational studies (78, 79) and reviewed elsewhere (80). Indeed, a study by Behrsin et al. (59) showed that the WW domain is essential for the function of Pin1 isomeric activity *in vivo*, suggesting the presence of interdomain communication. In addition, studies conducted on Pin1 and tau interactions highlighted the need of WW domain interaction with the pS/T-P motif for the effective function of the PPIase activity (63, 81). Therefore, understanding the interdomain communication in Pin1, and how this could lead to the dynamic allostery of Pin1 has been of much focus in recent times.

Much work in understanding the presence of interdomain communication and dynamic allostery of Pin1 has been well studied using nuclear magnetic resonance (NMR) studies (82). As highlighted in a review by Sudol and Hunter (83), and studied by Verdecia et al. (56), both Cdc25c and FFpSPR [artificial substrate derived from peptide libraries screening for optimal PPIase efficiency (84)] have higher affinity to the WW domain of Pin1. This is also reflected in other Pin1 substrates that have been identified (66). This higher affinity of the WW domain

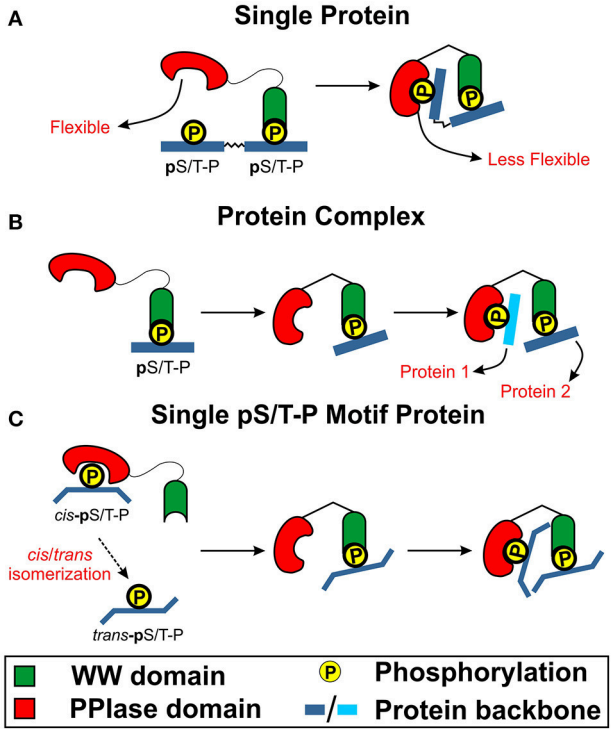

FIGURE 4 | The role of interdomain communication in Pin1 function. Based on recent findings, WW domain could be required for initial binding to initiate conformation changes to the PPIase domain for its binding and catalytic function. **(A)** WW domain binds first to pS/T-P motif that leads to interdomain communication to trigger conformation change (decreased flexibility) that lead to PPIase binding to a distal pS/T-P motif on the same protein to initiate catalytic function. **(B)** WW domain first binds to the pS/T-P motif to initiate interdomain communication to trigger PPIase domain conformation change to bind a pS/T-P motif on another protein to induce catalytic function. **(C)** PPIase domain could first catalyze a few pS/T-P motifs from the *cis* to *trans* configuration. This would lead to WW domain binding and initiate interdomain communication to trigger PPIase conformation change. This would then increase the activity of PPIase domain activity to lead to more cis to trans isomerization of the pS/T-P motif of the same protein in the vicinity.

for Pin1 substrate is contributed by the Loop I structure of this domain, where modification of the Loop I sequence led to the reduction of substrate binding affinity to Pin1 as demonstrated by Peng et al. (85) using the pCDC25C and FFpSPR peptides. Yet, by using a *trans*-locked inhibitor and a *cis*-locked inhibitor synthesized by Wang et al. (86, 87), Namanja et al. (60) found that the isolated PPIase domain has 2–4 times higher affinity to the *cis*-locked inhibitor than the full-length Pin1, while the *trans*-locked inhibitor can be bound by both domains as mentioned previously. This higher binding affinity of the PPIase domain without the WW domain suggests that the removal of the WW domain seems to remove the restriction imposed by the unbound WW domain on the PPIase domain for the *cis* configured pS/T-P motif. This observation clearly suggests a potential form of allostery present in Pin1.

Indeed, data obtained from studies by Namanja et al. (60, 62) showed that the changes in side chain dynamics instead of

large structural changes are a means of allosteric communication within the PPIase domain (82). This was further supported using molecular dynamics (MD) simulations (79, 88–90). These changes in side chain dynamics could dictate the flexibility of the PPIase domain to allow for greater substrate binding or enzymatic activity. Base on this side chain dynamics study of Pin1, Peng and co-workers identified the I28 residue as an important amino acid within a conserved hydrophobic conduit of amino acid residues that is affected by substrate binding of Pin1 (62). The subsequent study based on the mutation of I28 to I28A by Wilson et al. (61) led to the discovery of interdomain communications governed by I28, the WW domain Loop II, and the PPIase domain residues S138 to R142. The authors highlighted the influence of this interdomain communications on the side chain dynamics of the conserved hydrophobic conduit region that would affect PPIase domain binding and catalytic activity. This study is further supported by a recent MD study from Barman and Hamelberg (91). They highlighted that the binding of the substrate compacts the WW domain closer to the catalytic domain *via* a hinge-like movement, an observation echoed by a more recent study done by Campitelli et al. (92) using their dynamic flexibility index analysis.

Collectively, these studies highlight the fact that the binding of WW domain could be crucial in transmitting an intra-protein signal to the PPIase domain and the catalytic site *via* the interdomain contact as highlighted previously. This transmission of signal could be the result of side chain dynamics of the conserved hydrophobic conduit region. This could subsequently lead to subtle hinge-shift mechanism induced by the bound WW domain that leads to improved PPIase domain binding and catalytic activity. Based on these evidences as well as the four binding models of Pin1 to its substrate, the multimeric and simultaneous binding models are the potential model of how Pin1 works in biological signaling cascades. This is due to the potential need of a bound WW domain for Pin1 binding and catalytic activity. Hence, having the WW domain bound to an active kinase or a simultaneous binding of a WW domain followed by the binding of the PPIase domain would support the presence of Pin1 interdomain allostery (**Figure 4**).

In contrast, there are known examples of Pin1 that only contains one half of the two domains structure. For instant, Pin1At, identified in the plant *Arabidopsis thaliana*, only contains the PPIase domain but not the WW domain as opposed to Pin1 (93). In addition, another single-domain Pin1, known as TbPin1, was identified from the parasite *Trypanosoma brucei* (94). TbPin1 is also known to only contain the PPIase domain. Despite the absence of the WW domain, Pin1At was enough to rescue the temperature-sensitive mutation of Pin1 homolog gene (*ESS1*/*PTF1*) in *Saccharomyces cerevisiae* from death (95). Furthermore, our lab previously showed that TbPin1 alone was also able to rescue the same temperature sensitive *S. cerevisiae* mutant from death (96). These studies highlight that the PPIase domain alone is enough to recover the loss of Pin1 function, brining into question the importance of WW domain in Pin1 function. In hindsight, the WW domain of Pin1 could possess an alternative role in maintaining Pin1 function and could be seen in a complementary role to accelerate the effect of Pin1 function. As discussed earlier in the review, the preference of WW domain for the *trans*-configured substrate of Pin1 potentially suggest that the WW domain of Pin1 seeks to stabilize the *trans* population of the target substrates, leading to increased concentration of the PPIase domain in the vicinity and accelerating the effect of Pin1. Nonetheless, the highlighted studies of TbPin1 and PinAt needs to be replicated in mammals before conclusions could be made to obsolete the role of WW domain in Pin1 function.

IMPLICATIONS OF PIN1 AS A THERAPEUTIC TARGET IN CANCER

As mentioned previously, Pin1 has been extensively studied due to its diverse involvement in many signaling pathways that has many implications in various diseases, with great emphasis on cancer and neurodegenerative diseases. Despite many studies done on understanding the mechanism of Pin1 interactions with its biological substrates, there remains a gap as to how the understanding of Pin1 mechanism could lead to clinical translation. The most obvious application would be to identify or synthesize inhibitors that could block the functions of Pin1. However, the fact that Pin1 has so many interactors brings the problem of off-target effects. Indeed, the regulation of Pin1 in cancer against that in neurodegenerative diseases is inversely related (97). Therefore, targeting just Pin1 might not be specific enough to reduce the pathogenesis of these diseases.

The development of Pin1 inhibitors has remained a challenge in the field of cancer studies as many known inhibitors of Pin1 remains unspecific enough to only block the effects of Pin1 despite a number of Pin1 inhibitors already found (49, 98, 99). Yet, we cannot rule out the fact that evidences point to the potential key role of Pin1 in tumourigenesis (49, 99, 100). As such, there are continued efforts to identify new and novel Pin1 inhibitors. This is observed from recent efforts to identify various types of Pin1 inhibitors that targets the PPIase catalytic domain (51–53, 101, 102). For instant, a study by Pu et al. (51) identified a chemically synthesized small molecule API-1 that has an Pin1 inhibition concentration (IC_{50}) of 72.3 nM, which is 100× less than the IC_{50} of the first Pin1 inhibitor juglone. API-1 was found to bind to the PPIase domain of Pin1. The authors went on to show that API-1 was able to suppress the proliferation of hepatocellular carcinoma cells as well as inhibiting the tumor growth of xenografted mice. These evidences demonstrated the therapeutic value of API-1 as a cancer drug candidate. Another study by Cui et al. (102) demonstrated the use of a novel strategy to develop anticancer agents by inhibiting Pin1 activity *via* the synthesis of various pyrimidine derivatives. The authors were able to identify four potential Pin1 inhibitors with IC_{50} of $<3\,\mu M$. Furthermore, this type of drug is only the second covalent binding Pin1 inhibitors to the phosphate-binding loop in the PPIase domain besides juglone. These studies clearly highlight the potential therapeutic value of Pin1 as a cancer drug target. Unfortunately, no drugs thus far has reached clinical trials (49, 98).

Of interest, most drugs identified thus far are mainly targeting the PPIase domain, especially the catalytic domain, with few drug

candidates found to target the WW domain (98) as summarized in the list of known Pin1 inhibitors discovered thus far in **Table 1**. For instant, a series of studies conducted by Murray's group (107, 108, 115) set out to identify novel Pin1 inhibitors that are non-phosphorylated small molecule inhibitors that target the Pin1 active site at the PPIase domain using a structure-based design approach. They focused on finding a Pin1 inhibitor that binds to the phosphate-binding loop and were successful in first identifying benzothiophene with sub-micromolar inhibitory activity on Pin1 (107, 108). Unfortunately, this class of inhibitor showed low binding affinity to Pin1 when tested with whole cell assay. This led to the authors to suggest that its low cellular permeability could be the cause of such poor *in vivo* binding affinity to Pin1. The authors went on to improve the permeability of the benzothiophene class of drug to eventually yield dihydrothiazoles as a potent Pin1 inhibitor with low micromolar inhibitory activity toward cancer cell proliferation (115). However, the improvement of permeability led to reduced Pin1 inhibitory potency. This brings a question whether cellular permeability could be major limitation in their development

TABLE 1 | Selected known Pin1 inhibitors shown to block Pin1 function by targeting various part of Pin1 protein [adapted from Zhou and Lu (49)].

Inhibition Site	Pin1 inhibitor	Mode of discovery	IC_{50}	Mechanism of action	System tested	Limitation(s)
Pin1 active site	Juglone (103)	Low-throughput enzymatic (PPIase) assay	-	Covalent modification of Cys in the active site	*in vitro*	Low specificity
	PiB (104)	Low-throughput enzymatic (PPIase) assay	1.5 μM	-	*in vitro*; cell lines	Little evidence of Pin1 binding; insoluble in DMSO
	Pepticinnamin analogs (104)	Combinatorial synthesis	600 nM	-	*in vitro*	Inconclusive evidence of Pin1 binding
	Cis-locked alkene peptidomimetics (87)	Structure-based design	1.5 μM	Bind to Pin1 active site via substrate mimicking	*in vitro*; cell lines	-
	D-peptide inhibitor such as Ac-Phe-D-Thr(PO_3H_2)-Pip-Nal-Gln-NH_2 (68, 105)	Solid phase peptide library synthesis	As low as 1 nM	Competitive inhibitor of Pin1 active site	*in vitro*; cell lines	Inactive in cell lines
	Aryl indanyl ketones (106)	Structure-based design	As low as 200 nM	Reversible inhibitor of Pin1 active site undergoing "twisted-amide" transition state	*in vitro*; cell lines	Binding to Pin1 not as well as hypothesized
	Benzothiophene (107, 108)	Structure-based design	6 nM	Binds to Pin1 active site with high specificity	*in vitro*; cell lines	Potential low permeability; inactive in cell lines
	Phenyl imidazoles (109, 110)	Structure-based design	830 nM	Binds to Pin1 active site	*in vitro*; cell lines	Some variant inactive in cell lines
	ATRA (all trans retinoic acid) (111)	High-throughput mechanism-based screening	800 nM	Binds to Pin1 active site via substrate mimicking	*in vitro*; cell lines; mouse models; APL human patients	Moderate efficacy in humans; short half-life in humans
	KPT-6566 (52)	High-throughput structural- and mechanism-based screening	1.2 μM	Covalent binding to Pin1 active site at C113 specifically	*in vitro*; cell lines	-
	Pyrimidine derivatives (102)	In-house library screening	As low as 1.68 μM	Covalent binding to the binding pocket of Pin1 active site	*in vitro*; cell lines	-
PPIase domain	Dipentamethylene thiauram monosulfide (112)	Protease coupled enzymatic (PPIase) assay	50 nM	Competitive inhibitor of Pin1 PPIase domain	*in vitro*; cell lines	Possible low specificity
	Halogenated phenyl-isothiazolone TME-001 (113)	Real-time fluorescence detection method	6.1 μM	Competitive inhibitor of Pin1 PPIase domain	*in vitro*; cell lines	Possible low specificity
	Cyclic peptide inhibitor Cys-Arg-Tyr-Pro-Glu-Val-Glu-Ile-Cys (113)	Phage display screening	500 nM	Competitive inhibitor of Pin1 PPIase domain	*in vitro*	Cannot be used to inhibit intracellular Pin1 activity
	API-1 (51)	Computer-aided high-throughput virtual screening	72.3 nM	Binds to Pin1 PPIase domain specifically	*in vitro*; cell lines; mouse models	-
WW domain	EGCG (epigallo-catechin-3-gallate) (114)	Phenotypic association	20 μM	Bind to both WW and PPIase domains	*in vitro*; cell lines; mouse models	No reports of inactivation on isolated PPIase domain

of these structure-based Pin1 inhibitors targeting the PPIase domain.

Of importance, these studies only made use of the PPIase domain as the main consideration in designing of their structure-based Pin1 inhibitor. The authors did highlight some important points supporting intra-structure dynamics of the whole Pin1. Firstly, they noticed that despite the rich hydrogen bonding potential within the phosphate-binding loop in the PPIase domain of Pin1, hydrophobic interactions were central to benzothiophene binding to Pin1. Secondly, they also highlighted the presence of H-bond formation beyond the phosphate-binding loop that could be important for the Pin1 inhibitor binding infinity. Lastly, they highlighted the high degree of flexibility in Pin1 active site interaction with the inhibitors. All these observations do align with our previous discussion where interdomain communications between WW and PPIase domains could affect the side chain dynamics on the conserved hydrophobic conduit region and alters PPIase domain binding affinity (61, 91, 92). Moreover, we previously mentioned that the removal of the WW domain could alleviate the restriction imposed by the unbound WW domain on the PPIase domain for the *cis* configured pS/T-P motif (60). Henceforth, in line with these observations, much remains to be done to utilize the information from the studies of the actual roles by the WW and PPIase domains of Pin1, including the potential impact of interdomain communication could play in future drug screening for Pin1 inhibitors.

As suggested from all the studies on the two domains of Pin1 covered in this review, the WW domain binding displays a potentially important role in driving the signaling cascade triggered by Pin1 catalytic function. To advance our development of promising Pin1 inhibitors for treatment of diseases such as cancer, there are important considerations to be taken note of. Much interest has been put forth on identifying Pin1 inhibitors that target the PPIase domain as well as its active site. As suggested in this review, the binding affinity of Pin1 inhibitors could be affected by the substrate that is bound to the WW domain. Therefore, binding affinity studies for Pin1 inhibitors screening should consider the substrate effect upon its binding to the WW domain, and how this effect could alter the Pin1 inhibitors binding to the PPIase domain or its active site. Of interest, a very recent study by Momin et al. (116) using MD analysis suggests that substrate sequence can influence the eventual outcome of Pin1 function by differential triggering of Pin1 allosteric changes after substrate binding. This is explained by the substrate sequence-dependent allostery that affects the type of residue to residue contact within the interdomain interface. This led to the authors to suggest that drugs targeting the specific geometry of the interdomain interface at a specific Pin1 substrate binding could lead to better treatments of specific diseases. Whether these MD simulations could be translated to *in vivo* circumstances are yet to be determined and could be explored in future drug development for Pin1 inhibitor.

This potential substrate-dependent Pin1 activity could be important in future studies. This is highlighted in the lack of depth in the field of Pin1 research thus far. Most studies done on Pin1 mechanism focuses on just a few biological substrates of Pin1. As suggested in this review, the vast repertoire of Pin1 functional substrates and the different biological processes it could affect do point to the fact that Pin1 might behave differently in the presence of different substrate. This difference in behavior could affect its structural dynamics that makes identifying a functional Pin1 inhibitor difficult for different types of diseases. Therefore, to advance the research of the role of Pin1 in the pathogenesis of various diseases, more emphasis on the diversity of Pin1 substrates being tested must be done. As mentioned previously, Pin1 inhibitor screening could be done in the presence of its effector substrate to evaluate the efficacy of the Pin1 inhibitor in affecting the phenotype of the disease being studied. In addition, studies looking into how Pin1 cooperation with kinase in exerting its isomeric activity could be conducted to investigate if the proposed multimeric model holds true, and how the subsequent findings could affect the considerations when developing Pin1 inhibitors. Indeed, studies thus far do suggest that Pin1 is not a lone ranger, and it always needs a helping hand to exert its function. To advance the development of drugs to highlight the impact of Pin1 as a cancer therapy target, both domains should be considered during the drug development process for the field to realize the potential of Pin1 as a cancer therapeutic target.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Modeling WWOX Loss of Function *in vivo*: What Have We Learned?

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The WW domain–containing oxidoreductase (*WWOX)* gene encompasses a common fragile sites (CFS) known as *FRA16D*, and is implicated in cancer. *WWOX* encodes a 46kDa adaptor protein, which contains two N-terminal WW–domains and a catalytic domain at its C–terminus homologous to short–chain dehydrogenase/reductase (SDR) family proteins. A high sequence conservation of *WWOX* orthologues from insects to rodents and ultimately humans suggest its significant role in physiology and homeostasis. Indeed, data obtained from several animal models including flies, fish, and rodents demonstrate WWOX *in vivo* requirement and that its deregulation results in severe pathological consequences including growth retardation, post–natal lethality, neuropathy, metabolic disorders, and tumorigenesis. Altogether, these findings set WWOX as an essential protein that is necessary to maintain normal cellular/physiological homeostasis. Here, we review and discuss lessons and outcomes learned from modeling loss of WWOX expression *in vivo*.

Keywords: model organisms, knockout, mouse, *Drosophila*, zebrafish, cancer, metabolism, epilepsy

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INTRODUCTION

As a result of perturbed DNA synthesis, chromosomal fragile sites display genomic perturbations in the form of gaps, breaks, constrictions and rearrangements at specific DNA loci on metaphase chromosomes (1–6). The first human chromosomal fragile site was presented in 1965 by Dekaban that was found in an irradiated female patient who received X–rays for the treatment of eczematous dermatitis. These sites harbored two types of chromosomal abnormalities; (a) frequent breaks in the long arm of the chromosome number 9 and (b) moderately increased rate of chromosome–type aberrations occurring at random in all chromosomes (7). Later, in 1970, the name "fragile site" was used to explain recurrent chromosomal breaks on chromosome 16, which followed Mendelian segregation within a large family in a dominant fashion showing linkage of the heptoglobin alpha locus (8). Subsequently, chromosomal fragile sites associated with X–linked mental retardation within different families were reported (9–12). These preliminary reports led to the classification of chromosomal fragile sites as common fragile sites (CFSs) and rare fragile sites, depending on their frequency in the population as well as their inheritance pattern and further subdivided these sites based on the type of inducing chemical (5, 9–14). Ultimately, these early cytogenetic findings intrigued the geneticist community to further explore fragile sites and study their relevance in human diseases.

CFSs are the largest class of chromosomal fragile sites, which represent a component of normal chromosome structure that are observed in all individuals. CFSs are late replicating regions and are known as preferential hotspots for metaphase chromosome breaks and chromosome

rearrangements due to partial DNA replication stress (4, 5). In addition, CFSs are reported to be targets of replication stress in preneoplasia as well as sensors for DNA damage (15–18). Normally, CFSs are stable in cell culture settings but are highly susceptible to form chromosome breaks when DNA synthesis is impeded by using low doses of aphidicolin (APH) or hydroxurea (HU) (2, 4). As a consequence, chromosome breaks at 20 CFSs were revealed in lymphocytes treated with low doses of APH causing over 80% of the cytogenetic lesions. *FRA16D*, a genomic region where the *WWOX* gene resides, was noted as one of the most highly expressed CFSs amongst the 20 CFSs identified in human lymphocytes (4). Today, we know that there are about 90 CFSs that are tissue–specific and are induced at a stressor-specific manner [reviewed in (19)]. Based on these findings and others, CFSs were proposed to be involved in chromosome rearrangements observed in cancer (20–23).

WWOX, encompassing *FRA16D* at 16q23.2, was cloned and mapped in early 2000 by two research groups simultaneously explaining the significance of this archetypal fragile gene in human cancer (24, 25). The *WWOX* gene spans over 1 Mb of the genomic locus with nine exons separated by large introns encoding 2.2kb mRNA and 1.245kb ORF, which then translates into a 414–amino acid protein product (26). Following these studies, murine *Wwox* (also known as *Wox1)* was cloned suggesting the presence of *WWOX* orthologues in rodents (27). The WWOX protein, as the name suggests, contains two N-terminal WW domains and catalytic domain at its C-terminus homologous to short chain dehydrogenase/reductase (SDR) family proteins. Through its WW1 domain, WWOX interacts with partner proteins harboring proline–tyrosine (PY) motifs and acts as an adaptor protein regulating their localization transactivation, and stability (28, 29). Interestingly, the region of homozygous deletions reported in various malignancies coincides with *FRA16D* where intron eight spans ∼750 kb of the *WWOX* gene (25, 30, 31).

Loss of WWOX expression has been described in various tumors including breast, colon, esophageal, gastric, lung, ovarian, and prostate cancers [reviewed in (32)]. Besides its location in a fragile site, WWOX altered expression was also attributed to hypermethylation of its promoter, regulation by microRNAs and posttranslational modifications [reviewed in (26, 33)]. As a result of its dyregulation, several lines of evidence revealed that WWOX affects DNA damage response (DNA repair and apoptosis) (6, 27, 34, 35), cellular metabolism (36–42) and numerous signaling pathways (26, 29, 38, 43–46). Altogether, these observations have led to the generation of animal models mimicking WWOX loss of function *in vivo* to further investigate its physiological significance.

Phylogenetic analysis of the amino acid sequences of WWOX protein reveals that WWOX is an evolutionary conserved protein as a discrete orthologue between insects to humans. The conserved sequences of WW domains and SDR domain in *WWOX* imply its tight regulation across species. All species exhibit distinct sequence relationships of WWOX orthologues through evolution except the closest organism *Caenorhabditis elegans*, which lacks the two N–terminal WW domains but have sequence homology to WWOX SDR domain suggesting common substrate of this putative enzymatic domain independent of its WW domain interactions (47). Indeed, there is a remarkable conservation in sequence, for example, between human *FRA16D*/*WWOX* and mouse *FRA8E1*/*Wwox* both at exon as well as intron levels exhibiting 94% identity along with 96% similarity at protein level (27, 48). Additionally, *FRA16D/WWOX* shares 49% identity and 66% similarity with *Drosophila melanogaste*r homologous protein CG7221/DmWWOX (49).

Several lessons were learned from modeling WWOX perturbations *in vivo* using different animal models facilitating dissecting its significance in physiology and disease states. For example, after a thorough scrutiny of *Wwox* mutant mice, it was proposed that WWOX behaves as a tumor suppressor gene (50–52). Apart from its role in cancer, many reports revealed direct involvement of WWOX in neurological as well as metabolic disorders. Homozygous mutations in *WWOX* are reported in patients with epilepsy and mental retardation (53, 54). Many reports also indicate the role of WWOX in different metabolic disorders including lipid metabolism (55, 56), obesity (57), and type 2 diabetes (58). Increasing lines of evidence are, therefore, suggesting the significant contribution of *WWOX* in human maladies and requiring deeper understanding of WWOX function both at the molecular and cellular levels. In this review article, we describe the knowledge and conclusions learned by modeling loss of WWOX expression *in vivo* using various animal models including mice, rats, *Drosophila* and zebrafish.

ANIMAL MODELS OF *WWOX* ABLATION

Mice

Considering the notion that WWOX is frequently lost in human cancers led to generation and characterization of mouse models that depict this ablation to further deepen our understanding of WWOX function in physiology and in pathophysiology. Several mouse models were established and studied.

Phenotypic Analysis of *Wwox* Null Mice

The first knockout (KO) mouse model for *Wwox* was established by Aqeilan et al. (50). In this article, the authors altered ∼6 kb of DNA sequences within the genomic locus of murine *Wwox* using homologous recombination. This was achieved by replacing exons 2–4 of *Wwox* with a targeting cassette thus generating a *Wwox* mutant allele and later homozygous *Wwox* KO (*Wwox* $^{-/-}$) mice. These mice were monitored very carefully and phenotyped. While wild type (WT), heterozygous (*Wwox* $^{+/-}$), and *Wwox* $^{-/-}$ mice appeared normal in size and were indistinguishable at birth, *Wwox* $^{-/-}$ pups showed retarded post–natal growth from day 3 and eventually 100% of mice died at 3–4 weeks of age. These KO pups did not exhibit any histological lesions in the main organs but displayed severe metabolic defects and analysis of blood serum chemistry revealed altered levels of carbohydrates, proteins, and lipids hence associating WWOX with cellular metabolism (59). For example, *Wwox* $^{-/-}$ mice display reduced serum lipid

levels (50, 60) as well as impaired expression of steroidogenic enzymes (61).

Bone phenotype of Wwox null mice

Severe bone metabolic disease is one of the major and prominent phenotypes observed in *Wwox* $^{-/-}$ mice. Although no abnormalities in the skeletal patterning was observed, *Wwox* $^{-/-}$ mice experienced impaired bone metabolism as there was a decrease in the density of trabeculae bone along with thinning of the inner cortex from postnatal day 7 leading to eventual development of smaller limbs in proportion to the body weight of KO pups. This delay in bone formation was the result of cell–autonomous defect in osteoblast differentiation beginning at the mineralisation stage. Reduced trabecular member connectivity and bone surface area in both *Wwox* $^{+/-}$ and *Wwox* $^{-/-}$ pups was seen at day 15 in three–dimensional μCT images but the mineral content of trabecular bone in *Wwox* $^{+/-}$ mice was not significantly changed in comparison to WT. Serum calcium levels were 50% reduced and serum phosphate levels were increased by 20% in *Wwox* $^{-/-}$ pups indicating a metabolic bone disease. In contrast, *Wwox* $^{+/-}$ pups did show any altered levels of serum calcium and phosphorous (59)

A physical, functional and molecular link between WWOX and RUNX2, the master regulator of bone differentiation, was later reported. WWOX suppresses transactivation of RUNX2 resulting in repression of many RUNX2 targets. This intimate relationship between WWOX and RUNX2 was also documented in cancer (62–65). In addition to impaired osteoblast differentiation, *Wwox* $^{-/-}$ mice also exhibited enhanced osteoclast activity (59), though the molecular mechanism of the later is poorly studied. Therefore, it is plausible to conclude that WWOX loss contributes to an osteopenic phenotype and bone metabolic disease. Although this phenotype is very prominent in mice, supporting evidence linking WWOX with osteopenia or osteoporosis in human patients is still lacking. This could be due to the fact that WWOX loss of function due to germline mutations in human patients is postnatal lethal or that patients were not carefully assessed for bone density.

Steroidogenesis defect in Wwox null mice

Detailed analysis of the phenotypic abnormalities of *Wwox* $^{-/-}$ mice also revealed that these pups are born with gonadal abnormalities displaying impaired spermatogenesis (61). A comprehensive study focusing on the defects in the reproductive system of both male and female *Wwox*$^{-/-}$ mice revealed an altered expression of several steroidogenesis related enzymes responsible for this phenotype. Lack of Leydig cells and remarkable reduction of serum testosterone was noted in *Wwox*$^{-/-}$ male mice likely led to the observed testicular hypoplasia. Likewise, *Wwox*$^{-/-}$ female mice have also displayed abnormalities in their reproductive system manifested by impaired theca cells proliferation and small primary follicles resulting in reduced ovary size. Additionally, expression of numerous genes encoding steroid biosynthesis enzymes, such as *Hsd3b6* and *Cyp11a1*, is downregulated in the reproductive organs of *Wwox* $^{-/-}$ pups when compared with WT and *Wwox* $^{+/-}$ littermates (61). Altogether, these observations suggest that WWOX has an important role in steroidogenesis and its loss could be associated with infertility though future studies are required to dissect this interesting phenotype.

Lessons Learnt on WWOX Tumor Suppressor Function From Studying *Wwox* Mutant Mice

Wwox null mice

As introduced, altered WWOX expression has been reported in several human malignancies (32). Hence, *Wwox* ablation in mice has been predicted to associate with either tumor initiation or progression. The first *in vivo* evidence associating WWOX with tumor suppression was revealed when juvenile *Wwox* $^{-/-}$ mice developed femoral neoplastic focal lesions resembling early osteosarcoma (50, 63). These findings were based on histology sectioning through paraffin–embedded bones as well as screening by micro (μ)–CT imaging of intact limbs. These observations paved the way to hypothesize that WWOX deficiency can contribute to osteosarcoma development. Indeed, several reports have shown that WWOX deletion or altered expression is a frequent event in osteosarcoma (63, 64, 66, 67). Since *Wwox* $^{-/-}$ mice die prematurely, osteosarcoma development could not be followed in adult mice. Nevertheless, a more recent report revealed that somatic ablation of *Wwox* in committed osteoblasts accelerate osteoblastic osteosarcoma formation in *Trp53f/f* mice (68) (also see below).

Wwox–heterozygous (Wwox$^{+/-}$*) mice*

The fact that *Wwox* ablation in mice leads to early postnatal lethality (50) prompted studying WWOX tumor suppressor function using *Wwox*–heterozygous (*Wwox*$^{+/-}$) mice. The incidence of spontaneous tumor formation in *Wwox*$^{+/-}$ mice was significantly higher than that of WT littermates. Tumors formed in *Wwox*$^{+/-}$ mice included development of lung papillary carcinomas and lymphoblastic leukemia, but not osteosarcoma (50). Interestingly, several reports have documented altered WWOX expression in lung cancer (33, 69–71) and lymphomas (72–76) suggesting WWOX as a potential tumor suppressor in these malignancies.

As introduced earlier, perturbations in WWOX is commonly observed in various tumor kinds, other than lung cancer and lymphoma (32). One of the prominent cancer types that display absent or reduced WWOX expression is breast cancer (77–82). Monitoring of *Wwox*$^{+/-}$ mice for mammary tumor development in B6/129 mixed genetic background revealed very low incidence (52). These observations led the Aqeilan group to transfer the *Wwox*$^{+/-}$ allele onto C3H, a mammary tumor susceptible genetic background. Heterozygous *Wwox*$^{C3H+/-}$ mice were generated by backcrossing the *Wwox*$^{+/-}$ allele onto C3H pure genetic background mice, for at least 4 generations (51). Monitoring of these mice demonstrated that inactivation of a single *Wwox* allele is associated with increased incidence of mammary tumors. These tumors very much resemble those observed in human basal-like breast cancer (BLBC) with WWOX alteration (83). Poorly differentiated invasive ductal carcinomas and squamous cell carcinomas were observed. Immunohistochemistry staining of hormone receptors: estrogen receptor (ER) and progesterone receptor (PR)

were absent/reduced in $Wwox^{C3H+/-}$ mammary tumors. These results, therefore, suggest that heterozygous *Wwox* loss could be sufficient for developing mammary tumors of BLBC-like nature (51). These results were further confirmed in a subsequent recent study by Abdeen and colleagues using a tissue-specific knockout mouse model of *Wwox* (84) (also see below).

Chemical–induced tumorigenesis in Wwox–heterozygous (Wwox$^{+/-}$) mice

To further investigate WWOX tumor suppressor function, a number of studies tested susceptibility of $Wwox^{+/-}$ mice to chemical–induced tumorigenesis. In one study, $Wwox^{+/-}$ mice were treated with ethyl nitrosourea (ENU)–a mutagen that is commonly used to study tumor spectrum in a given mouse strain. As expected, the incidence of tumor formation, especially lung cancer and lymphoblastic lymphoma, was significantly higher in ENU–treated $Wwox^{+/-}$ mice in comparison with ENU–treated WT mice (50). In addition, various types of epithelial tumors like liver hemangiomas, chondrosarcomas, fibroadenoma, and squamous cell carcinomas were observed in ENU–treated $Wwox^{+/-}$ mice. It was also noted that multiplicity of tumors is significantly higher in ENU–treated $Wwox^{+/-}$mice in comparison with ENU–treated WT mice. Of note, signs of lymphoma aggressiveness were also higher in ENU-treated $Wwox^{+/-}$ mice suggesting that WWOX loss could lead to more advanced tumors (50). These results could suggest that $Wwox^{+/-}$ mice might be a good model system to study the effects of carcinogens and chemoprevention studies.

In a subsequent study, the susceptibility of loss of one *Wwox* allele on *N* – nitrosomethylbenzylamine (NMBA)–induced forestomach tumors (modeling human esophageal cancer) was assessed. Cohorts of $Wwox^{+/-}$ and WT mice were treated with NMBA, an environmental carcinogen that is extensively utilized to induce esophageal and forestomach tumors in rodents, and monitored for tumor formation. Consistent with WWOX tumor suppressor function, it was observed that tumor incidence and multiplicity were higher in NMBA–treated $Wwox^{+/-}$ mice in comparison to NMBA–treated WT littermates (52). These results confirm that perturbations in *WWOX* could be associated with esophegeal tumor formation (85) and further suggest its important tumor suppressor function.

Wwox hypomorphic mice

This oncosuppressor function of WWOX was again strengthened by the phenotypes observed in a mouse model generated by the Aldaz group. In late 2007, Ludes–Meyers and co-workers reported the generation of a hypomorphic mouse strain containing mutated *Wwox* alleles using a gene trap approach (86). This mutagenesis resulted in the expression of a *Wwox* splice variant containing intact WW domains but no SDR domain, thus, generating homozygous *Wwox* mutant mouse named as gene trap alleles: $Wwox^{gt/gt}$. In these mice, WWOX expression was undetectable in most organs but low levels were found in a minority of tissues, hence, concluding that $Wwox^{gt/gt}$ mice are WWOX hypomorphs. Unlike $Wwox^{-/-}$ mice (50), $Wwox^{gt/gt}$ mice were viable for up to two years but had decreased survival rate when compared to WT mice. This increased viability phenotype was presumed to be a result of low expression of WWOX in some tissues, which might have been sufficient for postnatal survival of $Wwox^{gt/gt}$ mice (86). Consistent with the Aqeilan $Wwox^{-/-}$ mouse model (50), higher incidence of tumor formation was noted in $Wwox^{gt/gt}$ mice. $Wwox^{gt/gt}$ female mice developed spontaneous B–cell lymphomas that was noted to be invasive in nature with a high degree of infiltration to lymph nodes, pancreas, kidneys, and liver. On the contrary, $Wwox^{gt/gt}$ male mice developed lung adenomas as a characteristic of its genetic background. In addition, poorer reproductive capabilities were observed in both young and old $Wwox^{gt/gt}$ male mice. The reduced fertility phenotype was predicted to be a consequence of severe degeneration of numerous seminiferous tubules causing premature testicular degeneration, therefore, indicating the important role of WWOX in the development and function of the reproductive system (86). These reports ultimately explained that loss of WWOX expression strongly correlates with tumor formation and aggression, impaired metabolism and defective steroidogenesis, as reported in (50).

Conditional *Wwox*–Knockout Models

Wwox-null mice suffer from post–natal lethality, hence restricting studying WWOX functions in adult mice (50). This certainly limits our understanding of WWOX comprehensive function in physiology and homeostasis as well as tumorigenesis. To overcome this limitation, the Aldaz group was the first to generate mice carrying a conditional mutant allele of *Wwox* by targeting exon 1 with LoxP–recombination sites (87). $Wwox^{lox/lox}$ mice were then bred with a general deleter (*EIIA–Cre*) line to develop total *Wwox* KO mice. In agreement with conventional $Wwox^{-/-}$ mice results (50), these conditional *Wwox* KO mice displayed growth retardation and eventually post–natal lethality in comparison to HET and WT mice. The relative weights of multiple organs were significantly reduced whereas brain weight was increased in conditional *Wwox* KO pups consistent with previous reports on *Wwox*–null mice (59). Histology studies of spleen in these conditional *Wwox* KO mice revealed signs of splenic atrophy showing reduced cellularity of red pulp and lymphoid aggregates of white pulp. These mice also displayed signs of leukopenia with lower white blood cell count suggesting impaired hematopoiesis. Interestingly, conditional *Wwox* KO mice had significantly reduced bicarbonate levels in blood, a condition similar to metabolic acidosis. Blood chemistry results of these mice also displayed hypoglycaemia, hypocalcemia as well as had 2–fold higher blood urea nitrogen (BUN) causing kidney failure (87).

In parallel, the Aqeilan group also generated a conditional *Wwox* knockout model (named $Wwox^{f/f}$) by flanking exon 1 of *Wwox* with loxp sites (88). $Wwox^{f/f}$ mice were also crossed with *EIIA–Cre* general deleter mice to generate *Wwox* KO ($Wwox^{\Delta/\Delta}$) mice in all tissues. As expected, $Wwox^{\Delta/\Delta}$ mice displayed significant growth impairments and 100% of mice died by 3–4 weeks of age as a consequence of hypoglycemia and a severe metabolic disorder. Femurs of these mice displayed osteopenic phenotype, defected bone mineralisation process and presence of osteosarcoma–like cells, as observed in previously generated $Wwox^{-/-}$ mice (59). Altogether, both conditional

mouse models (87, 88) were very much alike and recapitulated the null phenotype [*Wwox* $^{-/-}$ mice] (50). Nevertheless, there have been also some inconsistencies in phenotyping these models that shall be further investigated in future studies. Importantly, these models will be beneficial in investigating WWOX function in adult stages (see below).

Conditional Wwox ablation in mammary tissue

As mentioned earlier, perturbations in *WWOX* is a common event in breast tumors (32). Therefore, there has been tremendous efforts to uncover whether *WWOX* somatic deletion leads to mammary tumor formation. The Aldaz group generated a mammary-specific knockout of *Wwox* in a mixed 129SV/C57B1/6 genetic background using *BK5–Cre* and *MMTV–Cre* transgenic strains. Unlike the *MMTV–Cre* model which had no effect on mice survival, WWOX homozygous deletion using *BK5–Cre* mice resulted in premature death for unidentified reasons, as reported by the authors (89). Interestingly, both models (*BK5* and *MMTV*) showed aberrant mammary branching morphogenesis displaying defects in branching development, impairment in ductal invasion, and expansion as well as abnormal mammary epithelium development with few ducts having no or reduced number of branching (89). Similar results on mammary development were observed in $Wwox^{MGE-/-}$ mice in B6/129 mixed genetic background, generated by the Aqeilan group using their $Wwox^{f/f}$ mice upon breeding with *MMTV–Cre* transgenic mice (90). Surprisingly, none of these models developed mammary tumors (89, 90) hence questioning initiation role of tumor suppressor WWOX. This observation also led to propose that WWOX is a non–classical tumor suppressor, however this still remains to be proven as it depends on various factors, among which is dependency on cell of origin, combination with other genetic changes or even genetic background of the mouse model utilized as shown previously (51).

Recently, the Aqeilan group reported the first mouse model of somatic deletion of *Wwox* in mammary epithelium with mammary tumors resembling basal–like breast cancer (BLBC) (84). The authors back–crossed $Wwox^{MGE-/-}$ mice onto a mammary tumor susceptible background, *C3H/HeJ* mice, for seven rounds to generate $Wwox^{\Delta MMTV}$. These mice were monitored for the incidence of tumor formation and indeed the majority (∼76%) developed mammary tumors with median latency of 270 days whereas no tumors were obtained in WT control littermates. Histological and pathological characterization of these tumors revealed their Grade III invasive ductal carcinoma nature with occasional lung metastasis. The expression of ER and PR was negative in these tumors but ∼60% were positive for CK14 hence resembling human BLBC. Further molecular analysis of $Wwox^{\Delta MMTV}$ tumors revealed that somatic loss of WWOX in mammary epithelium results in reduced expression and activity of p53. Interestingly when comparing $Wwox^{\Delta MMTV}$ tumors to those of $Trp53^{\Delta MMTV}$ they cluster very closely indicating that WWOX and p53 cooperate to antagonize breast cancer development. Furthermore, perturbations in *WWOX* and *TP53* co-occur and are correlated with poor survival of breast cancer patients. Altogether, these findings reveal WWOX as an important breast cancer tumor suppressor that regulates p53 function and activity (84)

Conditional ablation of Wwox in hepatocytes

Since *Wwox* $^{-/-}$ mice displayed reduced levels of serum lipids (50) and low expression of steroidogenic enzymes (61), a detailed analysis regarding role of WWOX in lipid metabolism was performed. Microarray analysis of total *Wwox* null mice (*Wwox* $^{-/-}$) (50) and liver tissue–specific *Wwox* KO (*Wwox* $^{hep-/-}$) mice (60) revealed altered levels of key regulators of high density lipoprotein (HDL) metabolism. Additionally, reduced levels of crucial biomolecules, ApoA−1 and AbcA1, necessary to reverse cholesterol transport and generate nascent HDL particles, were observed in both *Wwox* null and *Wwox* $^{hep-/-}$ mice. Interestingly, *Wwox* $^{hep-/-}$ male mice showed a small decrease in mRNA levels of *AbcA1* but a significant reduction at protein levels. Conversely, unchanged mRNA and protein levels of ABCA1 were observed in *Wwox* $^{hep-/-}$ female mice. This discrepancy of ABCA1 expression at transcriptional as well as translational levels in both the sexes of *Wwox* $^{hep-/-}$ mice was considered to be a gender specific regulation of HDL metabolism. On the contrary, protein levels of ApoA–I were reduced in both *Wwox* $^{hep-/-}$ mice genders. These results were consistent with the observations reported in human disorders of HDL biogenesis suggesting a key role of WWOX in regulating HDL physiology (55). Additionally, older *Wwox* $^{hep-/-}$ female mice exhibited an increase in triglyceride (TG) levels and in very low–density lipoprotein (VLDL)–TG content within serum lipoproteins confirming previously reported association of *Wwox* and TG levels (50). Irrespective of the significant and prominent deregulation of lipid metabolism observed in *Wwox* $^{hep-/-}$ mice, microarray analysis also identified differential expression of several lipid–related canonical pathways specifically revealing the genes involved in cholesterol homeostasis, hydrolysis and biosynthesis of TG and fatty acid between WT and *Wwox* $^{hep-/-}$ mice. Further, network analysis of the microarray data demonstrated decreased HDL metabolism as well as upregulation of *Angptl4*, *Fasn*, *Pltp*, *Gpam*, *Lipg*, and downregulation of *ApoA-I*, *Lpl*, *Insig2*; the key genes involved in several lipid metabolic pathways, hence shedding light on the global and important effects of *Wwox* ablation in liver. Thus, this comprehensive examination of WWOX loss in hepatocytes demonstrated overall deregulation of lipid metabolic pathways along with gender specific regulation of HDL and TG metabolism indicating vital implications of WWOX in atherosclerosis and cardiovascular diseases (60).

In a more recent report, the effect of *Wwox* deletion in hepatocytes was assessed on development of liver cancer and liver regeneration (39). Given that WWOX is frequently altered in liver cancer, Abu–Remaileh and colleagues generated a mouse model with specific targeted deletion of murine *Wwox* alleles in hepatocytes *($Wwox^{\Delta Hep}$)* and studied consequences on liver biology and development of hepatocellular carcinoma (HCC). Interestingly, $Wwox^{\Delta Hep}$ mice exhibited more potent liver regeneration upon partial hepatectomy. This effect was accompanied with elevated Ki67 staining, a marker of proliferation, and higher levels of c–Myc transcripts, one of the

major regulators of liver regeneration. Monitoring $Wwox^{\Delta Hep}$ mice for 2–years didn't reveal increase in spontaneous liver cancer incidence in B6–129 background. Nevertheless, combined ablation of WWOX in hepatocytes and treatment with diethylnitrosamine (DEN), a known liver carcinogen, increased HCC tumor burden and load (39). This outcome was associated with increased levels of HIF1α, WWOX partner that drives aerobic glycolysis (36). WWOX deficiency also resulted in upregulation of HIF1α glycolytic target genes feeding enhanced proliferation. Inhibition of HIF1α activity in DEN–treated $Wwox^{\Delta Hep}$ mice through systemic treatment with digoxin significantly attenuated tumor development suggesting that accelerated HCC development is indeed mediated by increased HIF1α activity. It has been also shown that feeding DEN–treated $Wwox^{\Delta Hep}$ mice with high fat diet synergize to result in enhanced HCC formation. Altogether, these findings indicate that perturbations in *WWOX* could increase the risk of HCC development likely, but not only, through alteration in glucose metabolism (39).

Conditional ablation of Wwox in osteoblasts

Osteosarcoma is a highly aggressive and metastatic form of bone cancer that is frequent in adolescents and young adults. As mentioned before, WWOX loss has been associated with human osteosarcoma (63, 64, 66) and some animal models provide support for murine osteosarcoma development (50, 62, 88). Furthermore, WWOX protein is inversely associated with expression and function of RUNX2 (62, 63, 91), which is highly expressed in osteosarcoma and various metastatic cancers (64, 92, 93). To better clarify the role of WWOX in osteosarcoma, the Aqeilan group generated two osteoblast-specific knockout mouse models in which WWOX is ablated in either pre–osteoblasts ($Wwox^{\Delta Osx1}$) or in fully mature osteoblasts ($Wwox^{\Delta Oc}$) (68). Analysis of these mice revealed that $Wwox^{\Delta Osx1}$ mice exhibit a severe defect in osteogenesis, which was associated with induction of p53. Deletion of *Trp53* in $Wwox^{\Delta Osx1}$ mice rescued the osteogenic defect and resulted in the development of high penetrance of poorly differentiated osteosarcomas. The murine phenotype very much resembled human osteosarcoma in different aspects including histology, gene expression resistance to chemotherapy and metastatic behavior (68). Strikingly, it was demonstrated that WWOX might undergo loss of heterozygosity and that it promotes p53 loss of heterozygosity supporting a the emerging role of WWOX in DNA damage response and genomic stability (35, 94) ultimately confirming an intimate relationship between the two tumor suppressors. Importantly, co–occurrence of WWOX and p53 inactivation has been demonstrated as a common event in osteosarcoma (68). Altogether these findings provided the first *in vivo* evidence that WWOX suppresses osteosarcomagenesis through regulating p53 activity.

Significance of WWOX in Neurological Disorders

The high levels of WWOX in the different parts of the brain (95, 96), suggests an indispensable role of WWOX in central nervous system (CNS) homeostasis. Initially, low WWOX expression was reported in the hippocampus of Alzheimer's disease (AD) patients (97) suggesting a plausible role of WWOX in the biology of AD. The Chang group and collaborators have subsequently generated a *Wwox* null mouse by targeting exons 1, 2, 3, and 4 to evaluate physiological significance of WWOX in this neurodegenerative disease. In agreement with previously published data (50), KO mice survived only for about a month (98). Interestingly, it was revealed that homozygous loss of *Wwox* leads to the aggregation of Tau and TPC6AΔ (known to be involved in AD), in brain cortex of juvenile *Wwox* null mice, prior to their death (98). This suggests that WWOX plays a crucial role in inhibiting the aggregation of these plaque forming proteins which cause neurodegenerative diseases (98). Whether specific deletion of *Wwox* in hippocampal or cortical neurons would be associated with or contribute to AD in mice is still unknown.

Numerous subsequent reports documented a number of *WWOX* mutations in different neurological disorders. Indeed, *WWOX* germline mutations were found associated with developmental retardation, ataxia, early onset of epilepsy and intellectual deficiency (53, 54, 99–103). WWOX-mutant patients display a wide range of neurological behaviors ranging from progressive microcephaly, global developmental delay, seizure disorders, bilateral optic atrophy, and spastic quadriplegia in very young infants (∼1.5 months) (known as WOREE phenotype, for WWOX–related epileptic encephalopathy), to non–progressive microcephaly and less severe phenotype in adolescence–adult with later onset at 9–12 months (associated with spinocerebellar ataxia type 12 (SCAR12)) (101). This wide range of phenotypic abnormalities could be due to the nature of these mutations. Relatively milder phenotypes seem to originate from missense point mutations (P47T and P47R, G372R), whereas severe manifestations were observed in nonsense mutations (R54*, K297*, and W335*) or partial/complete deletions [reviewed in (38)]. Therefore, WWOX genotypes might correlate with the reported phenotypes, although analysis of more patients would be required to further support this relationship.

WWOX involvement in epilepsy was also documented in animal models. Indeed, *Wwox* gene mutations were first associated with epilepsy and ataxia in mice. Mallaret *et al.* showed that the short–lived *Wwox* KO mouse displays spontaneous and audiogenic seizures (53). This phenotype was also observed in a spontaneous homozygous rat mutation of *Wwox*, (lethal dwarfism, ataxia, and epilepsy) presenting similar phenotype as the *Wwox* KO mice and symptoms similar to mutant *WWOX* patients (104). The molecular function and importance of WWOX in epilepsy and ataxia is largely unknown and is currently under investigation by several labs.

RATS

The role of WWOX in biology was also documented in rats as an animal model. The phenotype of spontaneously mutated–Lethal dwarfism with epilepsy (*lde*) in rats display severe dwarfism, early post–natal lethality, a high incidence of epileptic seizures, male hypogonadism and the presence of numerous extracellular vacuoles of different sizes in the hippocampus and amygdala of mutant brain (105). To define the genes responsible for

the *lde* phenotype, the authors backcrossed *lde/lde* rats with Brown Norway rats to generate *lde/lde* rats with an altered genetic background displaying all the pleotropic phenotypes. Mapping of 1.5–Mbp region of chromosome 19 within *lde* locus revealed a 13 base pair deletion in exon 9 of *Wwox* transcript that caused a frame–shift mutation resulting in the translation of aberrant amino acid sequences at WWOX C–terminus. Considering epileptogenesis as congenital in *lde/lde* rats, these rats were exposed to different sound stimulus resulting in audiogenic seizures that had a pattern beginning with wild running followed by tonic–clonic convolusions. There was no correlation between the age of rat and the incidence of audiogenic seizures. Overall, these results further suggest an important role for WWOX in the CNS as WWOX loss leads to neuronal excitation (104).

WWOX was also described to be a putative target to alleviate symptoms for patients who experience a neuronal injury (106). Here, the authors studied the functional significance of rat *Wwox* and other transcription factors necessary to decide injured neuronal cell fate. Nuclear WWOX levels were rapidly increased in the neurons of sciatic nerve transection model in rats. This induction was accompanied with activation of other known genes involved in neuronal injury such as NF–κB activation and phosphorylation of CREB and JNK1. Eventually, the authors concluded that the elevation of WWOX levels signals the injured neurons to undergo apoptosis through activation of pro-apoptotic pathways (106).

Altogether, *Wwox* manipulation in the rat is associated with important phenotypes highlighting WWOX physiological functions, nevertheless the early death of these rats precludes analysis of adult phenotypes. It is of note that some of the phenotypes reported in the mouse models were not reported or observed in the rat model. This could be related to the approach used in genetic manipulation and/or genetic background.

DROSOPHILA MELANOGASTER

A high amino acid sequence conservation along with distinct phylogenetic relationship of WWOX across species proposes retention of the major biological functions governed by the *WWOX* gene. The *Drosophila* orthologue of *WWOX* (*DmWwox*) was investigated with an aim to study its involvement in WWOX biological functions. *DmWwox* mutants ($DmWwox^1$) were therefore generated and characterized (49). The $DmWwox^1$ mutants were surprisingly viable and exhibited no obvious phenotype. Neverthless, a higher sensitivity and a decrease in the survival rate of $DmWwox^1$ mutant third instar larvae was observed following dose–dependent irradiation when compared with WT. Protective role of WWOX in *Drosophila* was confirmed when $DmWwox^1$ mutants rescued the WT phenotype with both *DmWwox* and human *WWOX* constructs. Therefore, based on these results, authors concluded that CFSs and the genes within its vicinity would be involved in protective mechanisms against environmental perturbations (49). Later on, it was reported that background mutation as a result of insertion of *piggyBag* transposon into the second intron of *DmWwox* was responsible for this phenotype. Hence, the authors proposed that a thorough screening of alleles generated by targeted mutagenesis via homologous recombination is mandatory to control the genetic background in *Drosophila* (107). To overcome this drawback and to determine the biological functions of WWOX, a distinct combinatorial approach by using biochemical screening, such as proteomic and microarray screens, as well as genetic analysis to explain functionality of the interactions was performed in *Drosophila*. It was surprising that proteomic analysis did not show any physical interactions of WWOX through its WW domain with PPxY motifs containing proteins. However, functional partners of DmWWOX protein were identified by mass spectrometry and shed light on novel

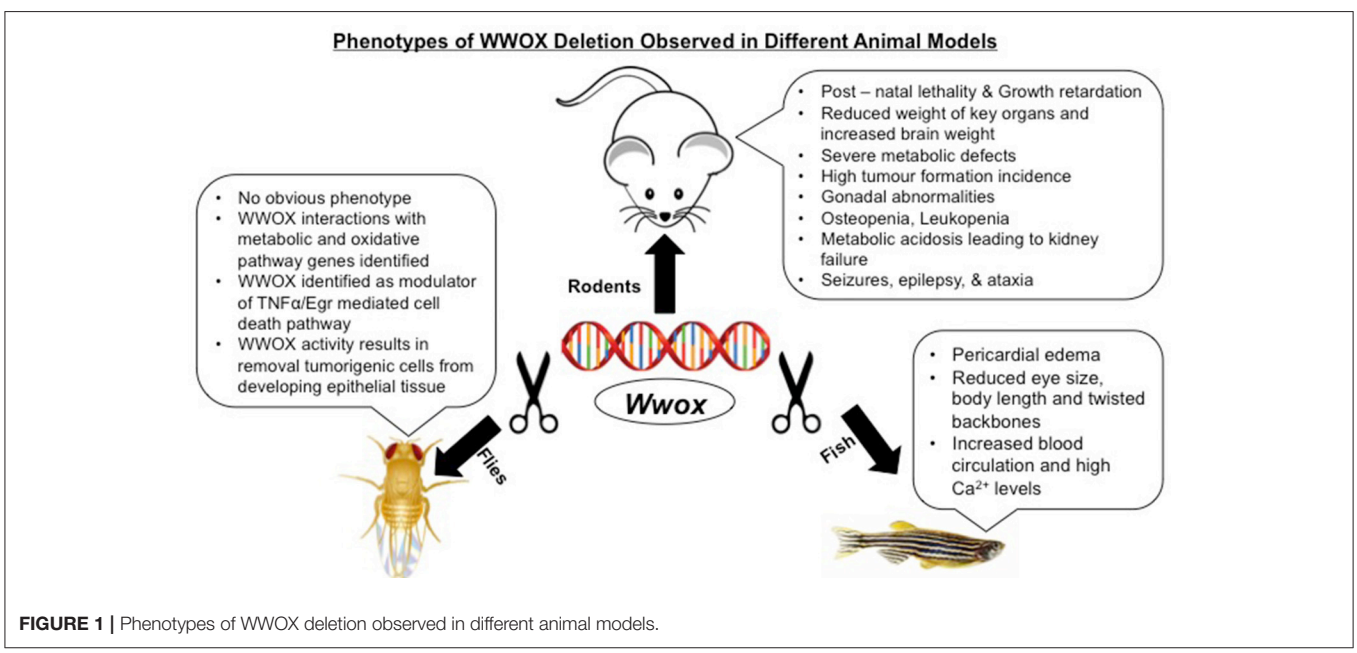

FIGURE 1 | Phenotypes of WWOX deletion observed in different animal models.

functional characteristics of WWOX contributing to cellular metabolism. The analysis identified significantly altered 13 candidate proteins in *DmWwox* mutants as well as 16 proteins in WWOX ectopically over–expressed adult flies most of which were known to be involved in metabolic pathways. It was striking that two candidate interactors, catalytic enzymes–isocitrate dehydrogenase (*CG6439*) and malate dehydrogenase (*CG7998*) involved in Tri–Carboxylic Acid (TCA) cycle were identified indicating an important contribution of WWOX toward aerobic metabolism. Also several candidates involved in glucose and lipid metabolism were identified. Additionally, enzymes invovled in oxidative stress pathways, such as superoxide dismutase (*Sod*) enzymes were identified as candidate WWOX interactors. This later finding could propose a direct role of the oxidoreductase domain of WWOX in biology of reactive oxygen species (ROS), a known byproduct of oxidative phosphorylation (41).

Using *Drosophila* as a model system, WWOX was also reported to be a modulator of apoptosis via modulation of Caspase-3 and regulation of ROS activity. During *Drosophila* development, ectopic over–expression of low level expression construct for TNFα/Egr caused disruption of repeated ommatidial unit patterning on the eye surface and reduced overall eye size. Introduction of *DmWwox* knock down construct rescued this TNFα/Egr mediated rough eye phenotype as ommatidial patterning across the surface of the eye was restored and the size of the eye was increased. Further, it was shown that WWOX activity is essential for the removal of tumorigenic cells from a developing epithelial tissue (42). Altogether, it is obvious that modeling WWOX loss in *Drosophila* identified conserved WWOX functions in cellular metabolism and apoptosis that has also been reported, at least in part, in mammalian cells (37, 40).

ZEBRAFISH

The biology of WWOX was also studied in zebrafish as an animal model. Tsuruwaka and co–workers generated antisense morpholinos (MO) and siRNA oligos against *wwox* gene sequence in *Danio rerio* and studied their phenotypes. Embryos

TABLE 1 | Common and unique phenotypes for WWOX animal models.

Phenotype / Animal model	Post–natal lethality	Growth retardation	Tumors observed	Metabolic defects	Reproductive defects	Neurological disorders	References
Mice							
Wwox $^{-/-}$	√	√	√ (focal lesions - femurs)	√	√	√	(50)
Wwox$^{+/-}$	–	–	√ (Chemical +spontaneous)	–	–	–	(50, 52)
Wwox$^{C3H+/-}$	–	–	√ (mammary)	–	–	–	(51)
WWOX$^{gt/gt}$	↓survival	–	√	–	√	–	(86)
EIIA–Cre, Wwox$^{flox/flox}$	√	√	–	√	√	√	(87)
Wwox$^{\Delta/\Delta}$	√	√	√ (focal lesions – femurs)	√	√	√	(88)
Wwox$^{flox/flox(Ex1-4)}$	√	NR	NR	NR	NR	√	(98)
BK5–Cre, Wwox$^{flox/flox}$	√	Mammary gland defect	–	–	–	–	(89)
MMTV–Cre, Wwox$^{flox/flox}$		Mammary gland defect	–	–	–	–	(89, 90)
Alb – Cre, Wwox$^{flox/flox}$	–	–	–	√	–	–	(60)
Wwox $^{\Delta Hep}$	–	–	√ +DEN (Liver)	√	–	–	(39)
Wwox $^{\Delta OSX1}$	–	–	√ +*p53*$^{f/f}$ (osteosarcoma)	–	–	–	(68)
Wwox $^{\Delta MMTV}$	–	–	√	–	–	–	(84)
Rats							
Spontaneously mutated *lde/lde* rat	√	√	–	–	–	√	(104)
Drosophila melanogaster							
Mutations generated by homologous recombination	–	–	√	√	–	–	(41, 42)
Zebrafish							
Antisense MO and siRNA mediated knockdown	√	√	–	–	–	–	(108)

NR, stands for not reprorted; -, no phenotypes.

harboring genetic manipulated *wwox* were characterized by pericardial edema causing post–natal lethality within a week of age. *Wwox*-deficient zebrafish embryos display growth retardation, as revealed by small eye and head size as well as short body lengths combined with impaired bone development and intracellular Ca^{2+} levels (108). Altogether, *wwox*–mutant zebrafish embryos recapitulate many of the phenotypes of *Wwox*–null rodent models further implicating the conservation of WWOX function across species.

SUMMARY AND FUTURE PERSPECTIVE

Modeling of *WWOX* gene in mice, rats, *Drosophila*, and zebrafish have not only advanced our understanding of its tumor suppressor functions but also have identified its emerging physiological roles in different human pathologies (**Figure 1**). Targeted ablation of *WWOX* in these established animal models facilitated the characterization of cellular pathways controlled by WWOX and that its deregulation exhibited severe pathological conditions like cancer, CNS related pathologies, metabolic syndromes, and reproductive defects (**Table 1**).

The phenotypes of *Wwox* null mice include early post–natal lethality, significant growth retardation and dwarfism, severe metabolic defects, osteopenia, and gonadal abnormalities (62, 83, 109). Along with the above mentioned complex phenotypes, *Wwox* null mice also displayed spontaneous and audiogenic seizures as well as showed aggregation of plaque forming protein in the brain cortex indicating that WWOX has an importnat role in the CNS and its loss could lead to epilepsy, ataxia and AD (45, 83). Similar epileptogenic and ataxic phenotype was observed in spontaneously mutated *lde* rats along with male rat hypogonadism (104).

Despite of early post–natal lethality, juvenile *Wwox* null mice phenotype demonstrated the occurrence of lesions resembling osteosarcomas, the first direct evidence of tumor suppressor functions of WWOX (50, 63). Although, this later finding was not detected in all null models but a conserved anti-oncosuppressor function was documented in heterozygous or hypomorph mutant mice (50, 52, 86). Moreover, carcinogen experiments in *Wwox*-heterozygous mutant mice showed higher tumor incidence and multiplicity, compared with control mice, of different tumor types implying WWOX haploinsufficiency (50, 52), a hallamrk of many known tumor suppressors. Conditional *Wwox* KO mouse models recapitulated many of the *Wwox* null phenotypes (87, 88). These conditional mouse models facilitated and will be instrumental in studying the distinct physiological functions of WWOX in a tissue-specific manner. Conditional deletion of *Wwox* in murine mammary epithelium revealed mammary developmental defects suggesting that WWOX expression is indispensable for proper normal breast development (89, 90). More recently, somatic deletion of *Wwox* in mammary epithelium of C3H mice resulted in high penetrance of mammry tumorigenesis that is associated with p53 perturbations (84). Liver tissue-specific conditional *Wwox* KO mouse model revealed WWOX involvement in the complex network of cholesterol homeostasis, HDL and lipoprotein metabolism highlighting a role of WWOX in regulating lipid metabolic pathways and underscoring a therapeutic use of WWOX for atherosclerosis and cardiovascular diseases (60). Somatic *Wwox* deletion in hepatocytes combined with DEN–treatment and/or western diet have been also reported to result in increased HCC incidence further elucidating the tumor suppressor role of WWOX (39).

Drosophila models of *WWOX* orthologues identified candidate interactors of WWOX in metabolic as well as oxidative pathways (47). Also, WWOX function for the removal of tumorigenic cells from developing epithelial cells was also reported in the *Drosophila* model (42). Lastly, WWOX ablation within zebrafish embryos displayed a non–cell–autonomous phenotype characterized by the symptoms of pericardial edema, reduced eye size and body length as well as twisted backbones along with high blood circulation and Ca^{2+} levels at ventral–dorsal–posterior regions (108).

Taken together, modeling WWOX in different animal models established WWOX functions in cancer as well as revealed its contribution in metabolic syndromes and neuropathy. These studies identified numerous interacting protein partners of WWOX and indicated new and possible physiological functions (110). The emerging roles of WWOX in DNA damage response (34, 35, 111) and in cellular metabolism (36, 41, 46) could have far reaching effects on the disorders identified in animal models and human patients. Complete understanding of the physiological functions of WWOX is yet under explored and much still remains to be learned about the enzymatic functions of SDR domain. Further characterization of these phenotypic defects, observed both at molecular and cellular levels in WWOX animal models, would be essential for designing novel diagnostic, prognostic, and therapeutic tools.

AUTHOR CONTRIBUTIONS

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

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Cancerous Protein Network That Inhibits the Tumor Suppressor Function of WW Domain-Containing Oxidoreductase (WWOX) by Aberrantly Expressed Molecules

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Recent findings indicate that the WW domain-containing oxidoreductase (WWOX) is a tumor suppressor protein that contains two N-terminal WW domains and a central short-chain dehydrogenase/reductase domain. WWOX protein mediates multiple signaling networks that suppress carcinogenesis through binding of its first WW domain to various cancer-associated proteins, i.e., p73, AP-2γ, and others. Although the tumor suppressor property of WWOX is inarguable, WWOX is not inactivated in the manner characteristic of the canonical Knudson hypothesis. Impairment of both alleles of *WWOX* is thought to be a rare event, only occurring in a few cancer cell lines. How is the tumor suppressor function of WWOX impaired in cancer cells? Recent advances highlight that a small transmembrane protein possessing a PPxY motif, called TMEM207, and its relatives are aberrantly expressed in various cancer cells and hinder the tumor suppressor function of WWOX through inhibiting its WW domain. Here, we review the recent findings related to the pathobiological properties of TMEM207 and its relatives based on clinicopathological and experimental pathological studies.

Keywords: WW domain, WWOX, PPxY motif, cancer, aerobic glycolysis

INTRODUCTION

WW domains are small protein modules with two conserved tryptophan (W) residues spaced at typically 35–40 amino acids in length (1). WW domain appeared to be slightly curved with an antiparallel β sheet to form a groove-like structure for ligand binding (2).

The WW-domain containing oxidoreductase (WWOX) is composed of two WW domains for signaling and a central short-chain dehydrogenase/reductase (SDR) domain for metabolism (3). The first N-terminal WW domain of WWOX binds protein ligands harboring motifs with core proline-rich sequences, not only PPXY (amino acid single-letter code; X is any amino acid) (PY) but also LPXY and LPXF motifs (where F is phenylalanine and L is leucine) (4–7). In contrast, the second WW domain contains two distinct amino acid residues within the WW binding pocket, compared to R25/W44 (R is Arginine/W is tryptophan) of the first N-terminal side WW domain, and exhibits no binding activity to PPXY ligands (8) (**Figure 1A**).

WWOX is believed to play a tumor suppressor role in carcinogenesis (10–13). WWOX interacts with various carcinogenesis-related molecules through its first N-terminal WW domain, probably

interacting with growth regulatory proteins p73 (14), AP-2 γ (15), and others (16), thereby blocking cancer cell growth. Notably, WWOX suppresses p73 and AP-2γ oncogenic activity by sequestering them in the cytoplasm (14, 15). These findings indicate the important role of the WW domain in the tumor suppressor function of WWOX (**Figure 1A**).

WWOX is not inactivated in the manner characteristic of the classical Knudson hypothesis (17). *WWOX* is located on the common fragile site FRA16D, which has been linked to cancer-causing deletions and translocations (14, 18). In addition, loss of heterozygosity (15, 19) and promoter hypermethylation of *WWOX* (16, 17, 20, 21) may also be responsible for loss of the WWOX protein. However, impairment of both WWOX-encoding alleles is rare, occurring only in few cell lines (17). In mice, the susceptibility to N-nitrosomethylbenzylamine-induced forestomach tumorigenesis is higher in $Wwox^{+/-}$ mice compared to that in wild type mice (22). However, many cancer cells escape loss of WWOX expression and express WWOX at various degrees, even robustly (23), as found in the data of The Human Protein Atlas (https://www.proteinatlas.org/ENSG00000186153-WWOX/pathology). WWOX is related to tumor progression rather than to initiation, and may not work as a highly penetrate tumor suppressor molecule (24).

The authors agree with the opinion that "despite many lines of evidence that suggest a role for loss of WWOX in the progression of cancer, our understanding of WWOX tumor-suppressive function is incomplete, as found in a recent remarkable report from Schrock et al. (25).

Recent advances suggest another molecular mechanism that could abrogate the tumor suppressor function of WWOX. A small transmembrane protein, TMEM207, appears to be aberrantly expressed and blocks the tumor suppressor function of WWOX through its PPxY motif (23, 26). TMEM207 competes with WWOX-interacting oncogenic molecules for binding to the WW domain of WWOX, thereby inhibiting the tumor suppressor function of WWOX. In addition, there are several cancer-promoting proteins with a molecular structure similar to that of TMEM207, such as VOPP1 (23, 27) (**Figures 1B**). There may be a tumor-associated family of transmembrane proteins, cancerous expression of which abrogates the tumor suppressor function of WWOX.

There are several outstanding reviews which highlight the protein networking, with which WWOX work as tumor suppressor molecule (28, 29).

In this review, we have summarized the recent findings of pathobiological protein networks that abolish the tumor suppressor function of WWOX in cancer cells, especially focusing on a small transmembrane protein, TMEM207.

MECHANISMS OF WWOX INACTIVATION IN CANCER CELLS

It is believed that the tumor suppressor function of WWOX is impaired by various molecular events in cancer cells. First, *WWOX* spans in the second most common human chromosomal fragile site, *FRA16D* at 16q23 (18). Chromosomal fragile sites

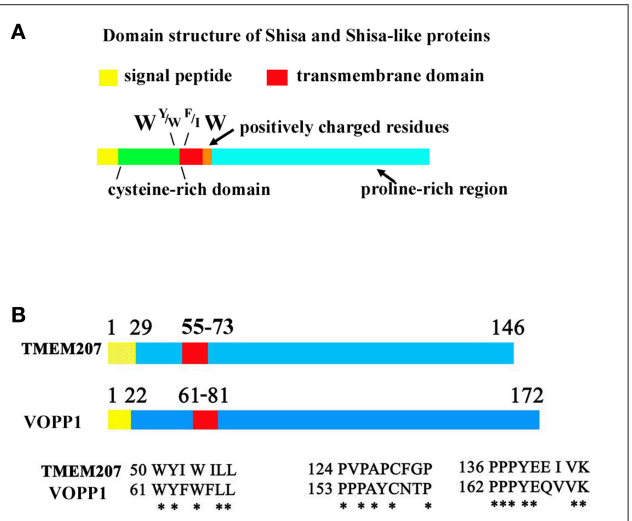

FIGURE 1 | Protein sequence of Shisa/Shisa like family proteins and TMEM207. **(A)** Fundamental protein structure of Shisa/Shisa-like family proteins proposed by Pei and Grishin (9). In addition, we note the W, Y or W, F or I, W protein sequence at juxta-transmembrane domain. **(B)** Graphical depiction of homologous regions of TMEM207 and VOPP1. Note the PPxY motif, which binds WW-domain, at the C-terminus.

are specific loci that preferentially exhibit gaps and breaks on metaphase chromosomes. Replication stress at *FRA16D* may participate in loss of WWOX protein expression. Second, the promoter region of *WWOX* can be hypermethylated, leading to gene silencing in several cancer cell types, i.e., *Helicobacter pylori* infection-related gastric cancer, intraductal papillary mucinous neoplasms of the pancreas (30, 31). Third, microRNAs (miRNAs), which can control gene expression, are also reported to be responsible for *WWOX* silencing. miR-134 expression contributes to head and neck carcinogenesis by targeting the WWOX (32). miR-153 regulates β-catenin activation through suppression of WWOX in hepatocellular carcinoma (33).

However, many cancer cells do express WWOX at various degrees, even robustly (23). Thus, our understanding of the tumor-suppressive function of WWOX remains inadequate (25).

Recent advances have shed light on the fourth molecular mechanism, which represses WWOX function through binding of undesirably expressed proteins to WWOX.

TMEM207 AND RELATIVES AS UNDESIRABLE BINDING MOLECULES TO WWOX IN CANCER CELLS

In 2012, Pei and Grishin referred to several proteins, Shisa/Shisa-like, WBP1 (WW domain-binding protein 1), WBP1L (also known as OPAL1 (outcome predictor in acute leukemia 1), VOPP1 (vesicular over-expressed in cancer prosurvival protein 1), and more as STMC6 proteins (single-transmembrane proteins with conserved 6 cysteines) (9). STMC6 proteins contain a proline-rich C-terminal region. Although TMEM207 has no canonical cysteine-rich domain,

TMEM207 is a single-transmembrane protein, which shares a $W^{Y}/_{W}^{F}/_{I}W$ amino acid motif in the extracellular portion adjacent to the cell membrane and C-terminal proline-rich domain, as found in STMC6 members (**Figure 1**). Percentage homology of representative STMC6 proteins and TMEM207 also suggests the close relation of these proteins (**Supplementary Table 1**).

TMEM207 and several STMC6 proteins bind to the WWOX protein. For example, WBP1, VOPP1, and TMEM207 bind to WWOX through its C-terminal PPxY motif (4, 23, 25, 27). Artificial mutation of PPxY motif abolishes the binding of WBP1 or TMEM207 to WWOX. VOPP1, also known as ECop (EGFR-coamplified and overexpressed protein), is overexpressed in esophageal squamous cell and gastric adenocarcinoma.

Recently, Bunai et al. reported that overexpressed TMEM207 co-localized and bound to WWOX in oral squamous cell carcinoma, especially glycogen-rich cancer cells, by using an *in situ* proximal ligation assay (26). They also demonstrated that TMEM207 promoted aerobic glycolysis of oral squamous cell carcinoma by abrogating the WWOX-mediating regulation of HIF1α protein. Since the *in situ* proximity ligation assay is well accepted to detect sub-cellular spatial molecular protein-protein interactions (34), this finding again verified the interaction of TMEM207 and WWOX in cancer cells.

VOPP1 promotes cell proliferation and migration and thus might serve as a putative oncogene. Very recently, Lallemand et al. (27) reported that VOPP1 physically interacts with WWOX. Upon binding, WWOX is recruited to the VOPP1-containing lysosomal compartment. This recruitment inhibits WWOX-mediated apoptosis at least in part by preventing WWOX-p73 interaction (27). Although further studies are required, TMEM207 and other members of the STMC6 family may constitute a novel transmembrane protein family that hinder the WWOX tumor suppressor function.

PHYSIOLOGICAL CELLULAR AND TISSUE EXPRESSION AND BIOLOGICAL PROPERTIES OF TMEM207

It was in 2003 that "The secreted protein discovery initiative" first identified TMEM207 as a novel transmembrane protein (35). Later, TMEM207 appeared to be also localized in the endoplasmic reticulum by bioinformatic analysis (36). TMEM207 expression is strictly regulated in human tissues and cells. *TMEM207* mRNA is found in human kidney (36) and brain microvascular endothelial cells (37); low levels are found in other cells and tissues, except intestinal goblet cells (23, 38).

Maeda et al. demonstrated the binding and co-localization of intelectin-1 and TMEM207 in cytoplasm by an *in situ* proximal ligation assay (38). Notably, siRNA-mediated down-regulation of TMEM207 increases polyubiquitination and proteasome degradation of intelectin-1, subsequently decreasing intelectin-1 secretion (38). These data indicate that TMEM207 may participate in the quality and quantity control of intelectin-1 at the endoplasmic reticulum.

Human intelectin-1 recognizes multiple glycan epitopes found exclusively on microbes and plays a role in intestinal pathogen-host interaction through assisting phagocytic clearance of microorganisms (39, 40). TMEM207 may participate in intestinal innate immunity through appropriate secretion of intelectin-1 (**Figure 2**). Since intelectin-1 is also called omentin, which appears to be an adipokine with insulin-sensitizing properties (41), TMEM207 may also play metabolic roles through proper processing and secretion of intelectin-1 (42).

PATHOLOGICAL ASPECTS RELATED TO ABERRANT TMEM207 EXPRESSION IN MICE

Relation to Cutaneous Adnexal Tumors

Kito et al. generated several transgenic mice lines in which murine TMEM207 was highly expressed under a truncated (by ∼200 bp) proximal promoter of the murine intestinal trefoil factor (ITF) gene (also known as Tff3) (43). One of these TMEM207-overexpressing transgenic mouse lines spontaneously exhibited a cutaneous adnexal tumor as found in human Brooke-Spiegler syndrome (a genetic condition associated with predisposition to cutaneous adnexal tumors) (44). In this line, the ITF-TMEM207 construct was inserted into a major satellite repeat sequence in chromosome 2, in which no definite coding molecule was found. In addition, cutaneous adnexal tumors were found, although less frequently, in three other transgenic mouse lines. Notably, hair follicle bulge cells exhibit overexpression of TMEM207 in this transgenic mouse line. It is well established that hair follicle bulge cells are multipotent stem cells that support hair follicle cycling and repopulation (45). Taken together, the data suggest that the origin of cutaneous adnexal tumors, which has long been uncertain, may be a transformed hair follicle bulge cell. Further study exploring whether loss of WWOX participates in cutaneous adnexal tumorigenesis is needed.

Relation to Renal Cystogenesis

Kito et al. also reported renal cystogenesis in a TMEM207-Tg mouse line, in which the transgene (ITF-TMEM207) was inserted into a basic helix-loop-helix leucine zipper lesion of the microphthalmia-associated transcription factor (*Mitf*) gene, thereby disrupting the expression of MITF proteins (46). MITF protein regulates key transcriptions for survival and differentiation of pigmented cells (47). Mutational studies in mice have shown that *Mitf* is essential for melanocyte and eye development (48) However, renal cystogenesis has not been reported in mice with MITF mutations. In addition, the 13 other strains harboring the same transgene (ITF-TMEM207) do not exhibit renal cystogenesis. A combination of loss of MITF and overexpression of TMEM207 may be important to renal cystogenesis in this novel transgenic mouse line.

WWOX is also strongly expressed in in the distal convoluted tubules and proximal collector ducts (49). Ludes-Meyers et al. reported that WWOX-knockout mice suffered from severe metabolic defects and suggested a role of WWOX in acid/base balance (50). However, the exact physiological role of WWOX in

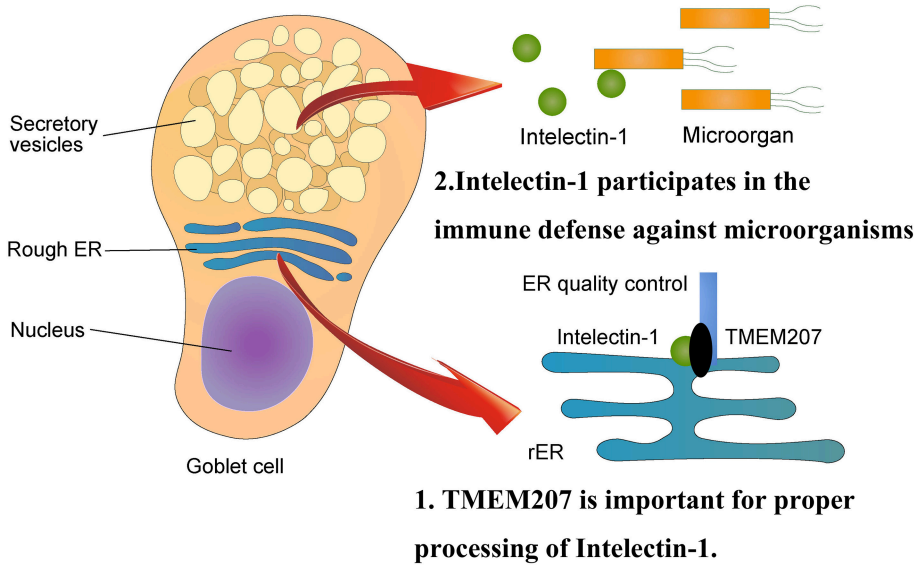

FIGURE 2 | Physiological aspects of TMEM207. At the endoplasmic reticulum, TMEM207 participates in the quality control of intelectin-1, which recognizes glycan epitopes found exclusively on microbes and plays a role in intestinal innate immunity through assisting phagocytic clearance of microorganisms. TMEM207 may participate in intestinal innate immunity by promoting the proper secretion of intelectin-1. TMEM207 and its relatives harbor a canonical PPxY motif to bind the WW domain of WWOX.

the kidney remains unclear. It should be determined whether loss of WWOX function leads to kidney cystogenesis.

According to "The Human Protein Atlas," human kidney tissue expresses several WW domain-containing proteins, i.e., BCL2 associated athanogene 3 (BAG3), WW Domain Containing Transcription Regulator 1 (WWTR1), and IQ motif containing GTPase activating protein 2 (IQGAP2). Further studies, which aim to examine the relation between TMEM207 and these WW domain-containing proteins expressed in the kidney, are needed to unravel the physiological property of TMEM207 in the kidney.

Relation to Gastrointestinal Carcinogenesis

Until now, no reports describing TMEM207 transgenic mice that develop gastrointestinal cancer were available. We speculate that overexpression of TMEM207 may not be enough to cause gastrointestinal carcinogenesis, but it might promote gastrointestinal cancer progression by enhancing the cancer cell invasion activity, as described in Gastric and Colonic Carcinogenesis section.

PATHOLOGICAL ASPECTS RELATED TO ABERRANT TMEM207 EXPRESSION IN HUMANS

Gastric and Colonic Carcinogenesis

In 2004, Aqeilan RI et al. reported that loss of WWOX protein expression was found in 65% of primary gastric adenocarcinoma specimens. They also reported that loss of heterozygosity at the WWOX locus was found in 31% of gastric adenocarcinoma specimens. Interestingly, Helicobacter pylori infection, a major risk factor for gastric cancer, increased hypermethylation of WWOX (29). These findings indicate that impaired expression of WWOX leads to gastric carcinogenesis.

In 2012, we identified TMEM207 as selectively expressed in collagen gel-invading gastric signet-ring cell carcinoma cells (SRCCs) (23). A subsequent study unraveled that TMEM207 promotes the invasion of gastric SRCCs in a manner dependent on its C-terminal PPxP motif. Interestingly, TMEM207 bound to WWOX and impair the WWOX-mediated repression of Matrigel invasion activity of gastric SRCCs.

As described in section TMEM207 and Relatives as Undesirable Binding Molecules to WWOX in Cancer Cells, TMEM207 is expressed in intestinal goblet cells for the precise quality control of intelectin-1. TMEM207 is not expressed in intact gastric epithelium, whereas TMEM207 is strongly expressed in intestinal metaplastic gastric epithelium with well-formed goblet cells. Gastric intestinal metaplasia is well characterized as an intermediate precancerous gastric lesion in the gastric cancer cascade of *Helicobacter pylori H.*-associated chronic active gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and adenocarcinoma. During this gastric cancer cascade, gastric epithelium may gain TMEM207 expression.

Maeda et al. also found that TMEM207 is strongly expressed in mucinous colon cancer, which harbors abundant mucin-rich cytoplasm, similar to gastric SRCCs and the intestinal metaplastic epithelium (38). We propose that highly expressed TMEM207 may competitively bind to the WW

domain of WWOX, thus inhibiting the tumor suppressor function of WWOX during carcinogenesis in digestive tract cancers.

Oral Squamous Cell Carcinogenesis With Relation to Aerobic Glycolysis

Another example of an oncogenic property of highly expressed TMEM207 is found in invasive oral squamous cell carcinoma (OSCC). Very recently, Bunai et al. found that TMEM207 was highly expressed in 40 of 90 OSCC samples but not in neighboring non-tumorous epithelial tissues (26). Overexpression of TMEM207 is significantly associated with nodal metastasis and poor prognosis of OSCC patients. Notably, co-localization of TMEM207 and WWOX in invasive OSCC cells, especially glycogen-rich ones, was demonstrated by an *in situ* proximal ligation assay. *In situ* proximal ligation assay, originally developed by Fredriksson and colleagues in 2002, is now well accepted to be a dependable technique to see the spatial cellular protein-protein binding. Combined together with the finding of co-immunoprecipitation assay, which showed the binding of TMEM207 to WWOX as its PPxY motif dependent manner, pathobiological link of TMEM207 and WWOX may be occurred in various cancer cells.

Bunai et al. further demonstrated that TMEM207 contributes to tumor progression in OSCC, possibly via promoting aerobic glycolysis (26). Currently, WWOX is believed to modulate cancer metabolism (51). WWOX is downregulated under hypoxic conditions, while WWOX decreases HIF1α protein levels without affecting transcription of HIF1α under normal oxygen conditions. WWOX directly binds to HIF1α in a WW domain-dependent manner and increases HIF1α hydroxylation, which is known to lead to the degradation of HIF1α protein under normal oxygen conditions. It is well characterized that cancer cells preferably undergo aerobic glycolysis, the 'Warburg effect'. In other words, glycolysis is markedly upregulated in cancer cells even in the presence of oxygen. HIF1α plays a critical role in aerobic glycolysis through activating its downstream factor Glut1. Therefore, impaired WWOX function may participate in the "Warburg effect" through HIF1α stabilization, which increases the expression of Glut1 and other aerobic glycolytic metabolism-related molecules in cancer invasion microenvironments.

Overexpression of TMEM207 may participate in cancer metabolism to promote cancer growth.

PROSPECTS OF REGULATING TMEM207 EXPRESSION IN CANCER CELLS

Notably, when examining TMEM207 in cBioPortal for Cancer Genomics (http://www.cbioportal.org/), it was revealed that TMEM207 is amplified in most cancers, especially in lung squamous cell carcinoma, esophageal, ovary, and head and neck cancers. Moreover, Kaplan Meier plotter analysis, http://kmplot.com/analysis/, indicated that high expression of TMEM207 correlates with worse prognosis in gastric cancer.

The recent findings described above indicate that highly expressed TMEM207 could be a therapeutic target for patients with TMEM207-expressing cancers. Since TMEM207 is also expressed in the cell surface membrane of dysplastic and cancer cells, we are now developing antibody-based approaches targeting several cancers. Furthermore, the PPxY motif of TMEM207 using small molecules, which targets the neighboring lesion of the PPxY motif, might be promising.

CONCLUDING COMMENTS

Recent advances demonstrate that several cancer cell types gain ectopic overexpression of TMEM207 during carcinogenesis, and then abolish the tumor suppressor function of WWOX through competitively binding with its WW domain. We propose that this inhibitory mechanism of TMEM207 to WWOX might lead to carcinogenesis if cancer cells continue to express the WWOX protein. Three important points have yet to be revealed. First, detailed molecular mechanisms that occur upon binding of TMEM207 to WWOX should be investigated. What cancer-related pathway molecule(s) would be affected by this phenomenon? Is it the p73 signaling pathway? Next, TMEM207 expression should be rigorously examined in most cancers or malignant tumors, including rare malignant tumors, i.e., types of sarcoma. Last, the exact molecular mechanism that is responsible for aberrant TMEM207 expression remains unclear. To our knowledge, the promoter or enhancer regions of *TMEM207* also remain undetermined.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00350/full#supplementary-material

Supplementary Table 1 | % identity of deduced amino acid sequence of human Shisa/Shisa-like family proteins, VOPP1, WBP1, Shisa-like-1, TMEM92, and TMEM207 determined by SIM program, https://web.expasy.org/sim/ with comparison matrix: BLOSUM30, Number of alignments computed: 30, Gap open penalty: 25, and Gap extension penalty: 0.

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REVIEW
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WWOX Tumor Suppressor Gene in Breast Cancer, a Historical Perspective and Future Directions

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The *WWOX* tumor suppressor gene is located at 16q23. 1–23.2, which covers the region of FRA16D—a common fragile sites. Deletions within the *WWOX* coding sequence are observed in up to 80% of breast cancer cases, which makes it one of the most common genetic alterations in this tumor type. The *WWOX* gene is known to play a role in breast cancer: increased expression of *WWOX* inhibits cell proliferation in suspension, reduces tumor growth rates in xenographic transplants, but also enhances cell migration through the basal membrane and contributes to morphological changes in 3D matrix-based cell cultures. The WWOX protein may act in several ways, as it has three functional domains—two WW domains, responsible for protein-protein interactions and an SDR domain (short dehydrogenase/reductase domain) which catalyzes conversions of low molecular weight ligands, most likely steroids. In epithelial cells, WWOX modulates gene transcription through interaction with p73, AP-2γ, and ERBB4 proteins. In steroid hormone-regulated tissues like mammary gland epithelium, the WWOX SDR domain acts as a steroid dehydrogenase. The relationship between WWOX and hormone receptors was shown in an animal model, where WWOX(C3H)+/–mice exhibited loss of both ER and PR receptors. Moreover, in breast cancer specimens, a positive correlation was observed between *WWOX* expression and ER status. On the other hand, decreased *WWOX* expression was associated with worse prognosis, namely higher relapse and mortality rates in BC patients. Recently, it was shown that genomic instability might be driven by the loss of WWOX expression. It was reported that *WWOX* plays role in DNA damage response (DDR) and DNA repair by regulating ATM activation through physical interaction. A genome caretaker function has also been proposed for *WWOX*, as it was found that WWOX sufficiency decreases homology directed repair (HDR) and supports non-homologous end-joining (NHEJ) repair as the dominant DSB repair pathway by Brca1-Wwox interaction. In breast cancer cells, *WWOX* was also found to modulate the expression of glycolysis pathway genes, through hypoxia-inducible transcription factor 1α (HIF1α) regulation. The paper presents the current state of knowledge regarding the WWOX tumor suppressor gene in breast cancer, as well as future research perspectives.

Keywords: WWOX, breast cancer, tumor suppressor, carcinogenesis, cancer progression

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WWOX TUMOR SUPPRESSOR GENE—DISCOVERY AND CHARACTERISTICS

The *WWOX* tumor suppressor gene was identified and cloned in the year 2,000 following research on the 23.1–23.2—region of long arm of chromosome 16. This region was also identified as FRA16D: one of the common chromosomal fragile sites (1). For a long time this region was of particular interest, because of the high incidence of loss of heterozygosity (LOH); this was first observed in prostate cancer (2, 3), and later in several other tumor types, including liver cancer (40% LOH) (4–7), ovarian cancer (8), ductal breast cancer *in situ* (DCIS) (about 80% LOH) (9), sporadic breast cancer (10), extrahepatic bile ducts (11), esophageal squamous cell carcinoma (12, 13), non-small lung (14), pancreas (15, 16), multiple myeloma (17), thyroid (18), glioblastoma multiforme (19), and Wilms tumors (20–30% LOH) (20).

A significant reduction or lack of expression of WWOX gene was observed mainly in breast cancer, but also (amongst other) in esophageal squamous cell carcinomas (12), non-small lung cancers (14, 21), pancreatic tumors (15, 16, 22) prostate, (23), gastric, ovarian (24), thyroid (18, 25), and bladder (26) cancers.

The highest expression of *WWOX* was observed in normal testis, prostate, and ovary tissues, while considerably lower expression was observed in the colon, small intestine, thymus, and spleen. The fact that that highest expression was observed in hormone-regulated tissues also characterized by the presence of an enzymatic dehydrogenase/reductase domain indicated that its protein product may be involved in the metabolism of steroid hormones (27). Nunez MI et al. carried out a wide range of tissue array analysis of *WWOX* expression in human normal tissues, derived from more than 30 organs. Highest expression was found in the fallopian tubes, ovaries, mammary gland epithelial cells, endometrial, prostate, testes, liver, stomach, salivary glands, adrenal gland, thyroid, parathyroid, pituitary, cerebellum, and brain cells. In contrast, no expression was observed in fatty tissues, connective tissues, lymphoid tissues, myelin structures or blood vessels. Several recent RNASeq analyses of normal human tissues correlate previous findings, revealing highest *WWOX* expression in thyroid tissue, testis and brain–particularly the cerebellum (28–30).

WWOX PROTEIN VERSATILE FUNCTIONS

Further studies revealed that *WWOX* gene encodes a protein of 414-amino acids (46 kDa) localized mainly within the Golgi system and cytoplasm. At that time, it was the only known protein containing both a short-chain dehydrogenase/reductase (SDR) central domain and two WW domains at the NH_2 (1, 31). Studies of *WWOX* expression identified several alternative transcripts. Such aberrant transcripts encoding truncated proteins (predominantly devoid of oxidoreductase-coding sequence) were found not only in the breast cancer cell lines and tumor samples (31), but also in various cancerous tissues; however, no other form of WWOX truncated protein was found (31–34).

Interestingly, it was shown that only the first WW domain binds to PPXY motifs in a physiologically relevant manner, whereas the second does not exhibit affinity toward any WW-domain targets. This has been attributed to the double substitution of chemically distinct amino acids located within the binding pocket of the WW2 domain. Also while the first WW domain was found to be unfolded just until ligand binding, the second adopts a fully structured conformation and was also found to help in the stabilization and binding of the ligand to the WW1 domain. All these results suggest that the WW2 domain of WWOX protein serves as a chaperone to augment binding of WW1 domain within WWOX to PPXY motifs of WBP1 and WBP2 proteins (35–37), along with other partner proteins, such as ERBB4 (38).

In Eukaryotes, proteins with a WW domains are involved in various processes associated with cellular signaling, transport of proteins, transcription and RNA processing. Based on pull-down assay, Abu-Odeh et al. published the list of above 200 potential WWOX protein partners (37), which included several transcription factors, such as P73 (37, 39), AP2-γ (40), c-Jun (41), DVL-2 (37, 42), RUNX2 (43), SMAD3 (44), and GLI-1 (45). Binding with WWOX causes their sequestration in the cytoplasm (or in the nucleus, in the case of RUNX2), thereby inhibiting their trans-activating function in the nucleus. WWOX protein also has the ability to bind to the cytoplasmic intracellular part of the ErbB4 receptor, which results in the inhibition of Yap protein-mediated transcription co-activation in the nucleus (46). In addition, studies have found the WWOX protein to bind to ezrin (47)—an actin-binding protein responsible for driving cell migration which may affect remodeling of the actin cytoskeleton (48). Recently, it was also confirmed that WWOX physically interacts with the ITCH protein—an E3 ubiquitin ligase which ubiquitinates Lys-63 of WWOX leading to its nuclear translocation. Lys-63-linked poly-ubiquitination is known to play a role in the DNA damage response; therefore, WWOX translocation to the nucleus might be associated with increased cell death. WWOX nuclear translocation was also formerly described for RUNX2 (43) and p53 (49) -WWOX protein interactions. Although several WWOX interactions have been identified, the exact function of the WWOX protein is not yet fully understood.

WWOX amino-acid sequence analysis identified two most conserved features of SDR proteins: the coenzyme NAD(H)/NADP(H) binding site and the potential substrate binding site (50, 51). Sequence similarity studies have classified WWOX protein as steroid dehydrogenase (1, 52), more precisely, 17β-hydroxysteroid dehydrogenase (53, 54). However, attempts at characterizing WWOX protein enzymatic activity have proved to be unsuccessful due to inability to purify the protein without loss of activity. This may suggest that *in vivo*, the WWOX protein is only present in liaison with other cell proteins which should be substituted in bacterial expression systems (55).

Animal-model based studies strongly support the hypothesis that the WWOX protein indeed participates in sex-steroid metabolism (56). Aqeilan et al. confirmed the importance of

WWOX in steroidogenesis and proper gonadal function in knock-out mice (KO). The reduction or absence of WWOX was associated with Leidig cell formation failure, untraceable status of testosterone in serum, decreased theca cell proliferation and tinier ovarian follicles in KO mice. Additionally, the absence of WWOX was found to lead to differential expression of 15 steroidogenesis-associated genes (57). Other studies have also established the importance of WWOX in the alteration of HDL and lipid metabolism (58, 59).

WWOX IN DNA DAMAGE RESPONSE, AND GENOMIC STABILITY

The relationship between the variable expression of DNA damage response proteins (p53, BRCA1, γH2AX, pChk2), DNA damage-sensitive tumor suppressors (WWOX, FHIT), WWOX-interacting proteins (Ap2- α and γ, ERBB4), cancer subtype, and clinical factors was tested in a tissue microarray analysis of 479 cases of breast cancer samples. BRCA1 nuclear expression was associated with FHIT and WWOX, and the absence of WWOX was strongly associated with loss of FHIT expression and cytoplasmic ERBB3. Additionally, a strong correlation was observed between FHIT expression and two WWOX partner proteins: cytoplasmic ERBB4 and Ap2α. In the multifactor model, triple negative breast tumors showed significantly reduced expression of WWOX. Furthermore, the expression of WWOX and ERBB4 was significantly lower in tissues derived from lymph node matched metastases than primary breast cancer tissues. The authors suggest that the loss of the signal pathway in which WWOX is involved contributes to lymph node metastasis by allowing detached cancer cells to survive without contact with the basal membrane (60).

An analysis of the significance of WWOX in DNA damage response (DDR) and DNA repair in MCF7 breast cancer cells revealed that induction of DNA double-strand breaks (DSBs) by ionizing radiation resulted in transient twofold elevation of WWOX mRNA level after 10 min exposure, which subsequently returned to baseline after 1–2 h. To exclude line specificity, this was further confirmed at the protein level, not only for MCF7 but also for primary mouse embryonic fibroblast (MEF), human embryonic kidney cells (HEK293), and osteosarcoma LM7 cells, both employing IR, as well as radiomimetic drug neocarzinostatin (NCS). Moreover, loss of WWOX expression in MCF7 breast cancer cells was found to be associated with the increased DSB level following DNA damage, highlighting the role of WWOX in genomic stability. The absence of WWOX leads to diminished activation of checkpoint kinase ATM, inefficient induction and maintenance of γ-H2AX foci, and defective DNA repair (61). Upon DNA damage, ATM stimulates the activity of ITCH (E3 ubiquitin ligase) (62), which in turn enables WWOX Lys-63 ubiquitination, thus promoting WWOX protein translocation to the nucleus. WWOX in the nucleus was found to interact with ATM, enhancing its monomerization and activation in a positive feed-forward loop manner (61).

The same research group underlined the importance of WWOX upon DNA single-strand breaks (SSBs) checkpoint activation. It turned out that introducing SSBs resulted in raised WWOX protein level and its accumulation in the nucleus, whereas WWOX depletion was associated with reduced activation of ATR checkpoint proteins and increased chromosomal breaks. The molecular mechanism of WWOX regulation of ATM activation was observed as described previously (61). On the other hand, the correlation between the inhibition of ATM and reduction in activity of ATR checkpoint kinases indicates that the effect of WWOX on ATR is influenced by ATM (63).

Recently Schrock et al. employed human embryonic kidney 293T cells and MDA-MB-231 breast cancer cells to describe interaction of Wwox and Brca1 proteins. The latter is known to mediate homology directed repair (HDR) of DSBs. Unlike for Wwox-ATM, the interaction of WWOX with Brca1 did not seem DNA-damage dependent; it was therefore suggested that at sufficient levels, Wwox might compete with various Brca1-interacting proteins important for HDR. In a model based on Rad50 protein, which is a component of the MRN complex interacting with Brca1, Wwox competes with it for binding to Brca1 and consequently impairs end resection. Thus in cancerous cells exhibiting depletion of Wwox, an important inhibitory step would be absent, which might result in enhanced end resection and HDR repair, finally allowing the cells to survive DNA damage-inducing cytotoxic treatments. Indeed it was found that loss of Wwox protein expression contributed to radiation and cisplatin resistance in mouse embryonic fibroblasts (MEFs) and human MDA-MB-231 breast cancer cells, which in turn might be associated with cancer recurrence and poor clinical outcome. Both human breast MCF10A and mouse MEF cells lacking Wwox exhibited enhanced survival upon DSBs inducement by means of ionizing radiation and bleomycin treatment. MDA-MB-231 cancer cells, which survived IR recurred faster in a xenograft model of irradiated breast cancer cells. Also Wwox-deficient MDA-MB-231 cells revealed shorter tumor latencies than the cells expressing Wwox. In a group of brain cancer patients treated with radiation, Wwox deficiency significantly correlated with shorter overall survival times: data obtained from Repository of Molecular Brain Neoplasia Data (REMBRANDT).

Thus according to the model proposed, Wwox might influence the choice of DNA DSB repair pathway by suppressing HDR by interacting with Brca1, and at the same time enhancing non-homologous end-joining (NHEJ) repair. It was also suggested that WWOX might serve as a genome caretaker, with its function based on Brca1-Wwox interaction in turn supporting NHEJ as the principal DSB repair pathway in Wwox-expressing cells (64).

BIOLOGICAL AND CLINICAL IMPLICATIONS OF WWOX IN BREAST CANCER

WWOX Genetic Changes—*in vivo* and *in vitro* Studies

Suppressive properties of *WWOX* gene were widely studied and confirmed by various research groups. *In vivo* studies showed

TABLE 1 | Historical and contextual advances in breast cancer *WWOX* research.

WWOX role or association		References
DNA damage response and genome instability	"Fhit and Wwox loss-associated genome instability: A genome caretaker one-two punch"	Schrock et al. (99)
	"Wwox-Brca1 interaction: role in DNA repair pathway choice"	Schrock et al. (64)
	WWOX modulates the ATR-mediated DNA damage checkpoint response	Abu-Odeh et al. (63)
	"WWOX, the common fragile site FRA16D gene product, regulates ATM activation and the DNA damage response"	Abu-Odeh et al. (61)
	"Aberrant expression of DNA damage response proteins is associated with breast cancer subtype and clinical features"	Guler et al. (60)
Bone metastasis from breast carcinoma	"Epigenetic regulation of HGF/Met receptor axis is critical for the outgrowth of bone metastasis from breast carcinoma"	Bendinelli et al. (100)
	"Functions and epigenetic regulation of Wwox in bone metastasis from breast Carcinoma: Comparison with primary tumors"	Maroni et al. (95)
	"HGF and TGFβ1 differently influenced Wwox regulatory function on Twist program for mesenchymal-epithelial transition in bone metastatic vs. parental breast carcinoma cells"	Bendinelli et al. (94)
	"Hypoxia induced E-cadherin involving regulators of Hippo pathway due to HIF-1α stabilization/nuclear translocation in bone metastasis from breast carcinoma"	Maroni et al. (93)
	"Hypoxia inducible factor-1 is activated by transcriptional co-activator with PDZ-binding motif (TAZ) vs. WWdomain-containing oxidoreductase (WWOX) in hypoxic microenvironment of bone metastasis from breast cancer"	Bendinelli et al. (101)
	"Bone metastatic process of breast cancer involves methylation state affecting E-cadherin expression through TAZ and WWOX nuclear effectors"	Matteucci et al. (92)
Glucose metabolism	"Tumor suppressor WWOX regulates glucose metabolism via HIF1α modulation"	Abu-Remaileh et al. (96)
	"WWOX loss activates aerobic glycolysis"	Abu-Remaileh et al. (102)
Mammary branching morphogenesis	"Characterization of WWOX inactivation in murine mammary gland development"	Abdeen et al. (103)
	"Conditional Wwox deletion in mouse mammary gland by means of two Cre recombinase approaches"	Ferguson et al.(72)
	"WWOX, the tumor suppressor gene affected in multiple cancers"	Lewandowska et al. (68)
WNT signaling pathways	"Conditional Wwox deletion in mouse mammary gland by means of two Cre recombinase approaches"	Ferguson et al. (72)
	"Inhibition of the Wnt/beta-catenin pathway by the WWOX tumor suppressor protein"	Bouteille et al. (42)
Transcription regulation	"The cancer gene WWOX behaves as an inhibitor of SMAD3 transcriptional activity via direct binding"	Ferguson et al. (44)
	"WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB4 and modulate its transcriptional function"	Aqeilan et al. (46)
	"Physical and functional interactions between the Wwox tumor suppressor protein and the AP-2gamma transcription factor"	Aqeilan et al. (40)
Tamoxifen resistance in BC patients	"Wwox expression may predict benefit from adjuvant tamoxifen in randomized breast cancer patients"	Göthlin et al. (91)
	"Wwox inactivation enhances mammary tumorigenesis"	Abdeen et al. (71)
	"Wwox and Ap2γ expression levels predict tamoxifen response"	Guler et al. (90)
Lymph node metastasis in BC patients	"Aberrant expression of DNA damage response proteins is associated with breast cancer subtype and clinical features"	Guler et al. (60)
Prognostic marker in BC patients	"The prognostic significance of WWOX expression in patients with breast cancer and its association with the basal-like phenotype"	Wang et al. (104)
	"Breast cancer relapse prediction based on multi-gene RT-PCR algorithm"	Pluciennik et al. (87)
	"Association of Wwox with ErbB4 in breast cancer"	Aqeilan et al. (105)
	"WWOX–the FRA16D cancer gene: expression correlation with breast cancer progression and prognosis"	Pluciennik et al. (88)

(Continued)

TABLE 1 | Continued

WWOX role or association		References
ER correlation in breast carcinomas	"Fragile histidine triad protein, WW domain-containing oxidoreductase protein Wwox, and activator protein2γ expression levels correlate with basal phenotype in breast cancer"	Guler et al. (89)
	"Frequent loss of WWOX expression in breast cancer: correlation with estrogen receptor status"	Nunez et al. (85)
	"The fragile genes FHIT and WWOX are inactivated coordinately in invasive breast carcinoma"	Guler et al. (84)
Hypermethylation	"Association between CpG island methylation of the WWOX gene and its expression in breast cancers"	Wang et al. (75)
	"Inhibition of breast cancer cell growth *in vitro* and *in vivo*: effect of restoration of Wwox expression"	Iliopoulos et al. (69)
	"Fragile genes as biomarkers: epigenetic control of WWOX and FHIT in lung, breast and bladder cancer"	Iliopoulos et al. (74)
Loss of expression	"Inhibition of breast cancer cell growth *in vitro* and *in vivo*: effect of restoration of Wwox expression"	Iliopoulos et al. (69)
	"The fragile genes FHIT and WWOX are inactivated coordinately in invasive breast carcinoma"	Guler et al. (84)
	"WWOX, the FRA16D gene, behaves as a suppressor of tumor growth"	Bednarek et al. (31)
	"Deletion map of chromosome 16q in ductal carcinoma *in situ* of the breast: refining a putative tumor suppressor gene region"	Chen et al. (9)
LOH	"WWOX: a candidate tumor suppressor gene involved in multiple tumor types"	Paige et al. (34)
	"WWOX, the FRA16D gene, behaves as a suppressor of tumor growth"	Bednarek et al. (31)
	"WWOX, a novel WW domain-containing protein mapping to human chromosome 16q23.3–24.1, a region frequently affected in breast cancer"	Bednarek et al. (1)
	"Construction of a high-resolution physical and transcription map of chromosome 16q24.3: a region of frequent loss of heterozygosity in sporadic breast cancer"	Whitmore et al. (10)
	"Deletion map of chromosome 16q in ductal carcinoma *in situ* of the breast: refining a putative tumor suppressor gene region"	Chen et al. (9)

that transduced MDA-MB-435 breast cancer cells exhibiting elevated WWOX expression injected into mammary gland of athymic mice delayed and inhibited tumorigenesis and slowed tumor growth rates compared to xenografts formed after control injections with unmodified MDA-MB-435 cells. Moreover, the tumors formed by MDA-MB-435/vector transduced cells were found to be about 10-fold larger than tumors formed by cells transduced with the *WWOX* gene (31). A similar inhibition of tumor growth in athymic mice after injection of *WWOX* transduced cells was also observed for other cancer cell lines, including pancreatic cancer cell line- Panc-1 (15), lung cancer - A549, H1299 H460 (65), prostate cancer–DU-145 (66), and ovarian cancer–PEO1 (67).

In vitro studies confirmed the suppressive characteristics of the WWOX gene. Soft agar growth assay showed the restoration of *WWOX* gene expression in breast cancer cell lines MDA-MB-453, T47D (31), BT-474, MDA-MB-231 (68, 69) and HCC1937 (69) significantly reduced their ability to anchor independent growth. Similar observations were reported for other *WWOX*-transduced cell lines from other cancers: pancreatic–AsPc1 and Panc1 (15, 16), prostate–DU145 (66), lung–A549, H460 and H1299 (65), and ovarian–A2780 (70).

Additionally, induction of apoptosis was observed through activation of the internal caspase cascade in the ectopically *WWOX*-expressing breast cancer (Ad-WWOX MDA-MB- 231) and lung cancer cell lines (Ad-WWOX A549, Ad-WWOX H460) (65, 69).

Another cancer-specific feature is the migration of cancer cells through a basal membrane, which is a basis of tumor aggressiveness. Surprisingly, contrary to animal studies, where WWOX inhibits tumorigenesis, elevated transcription of *WWOX* in MDA-MB-231 breast cancer cells was found to escalate migration through the basal membrane (Matrigel test), suggesting increased invasiveness (68). On the other hand, a morphogenesis test in Matrigel revealed MDA-MB-231 cells transduced with *WWOX* to display branched structures, compared with the spherical tumor-like structures of control cells. This results suggest that the *WWOX* gene might be involved in normal mammary tissue development (68). In ovarian cancer cell lines it was also observed that the restoration of WWOX modulated interaction of tumor cells with the extracellular matrix (67).

Gene function can be effectively investigated using gene knock-out experiments. A 2011 study based on

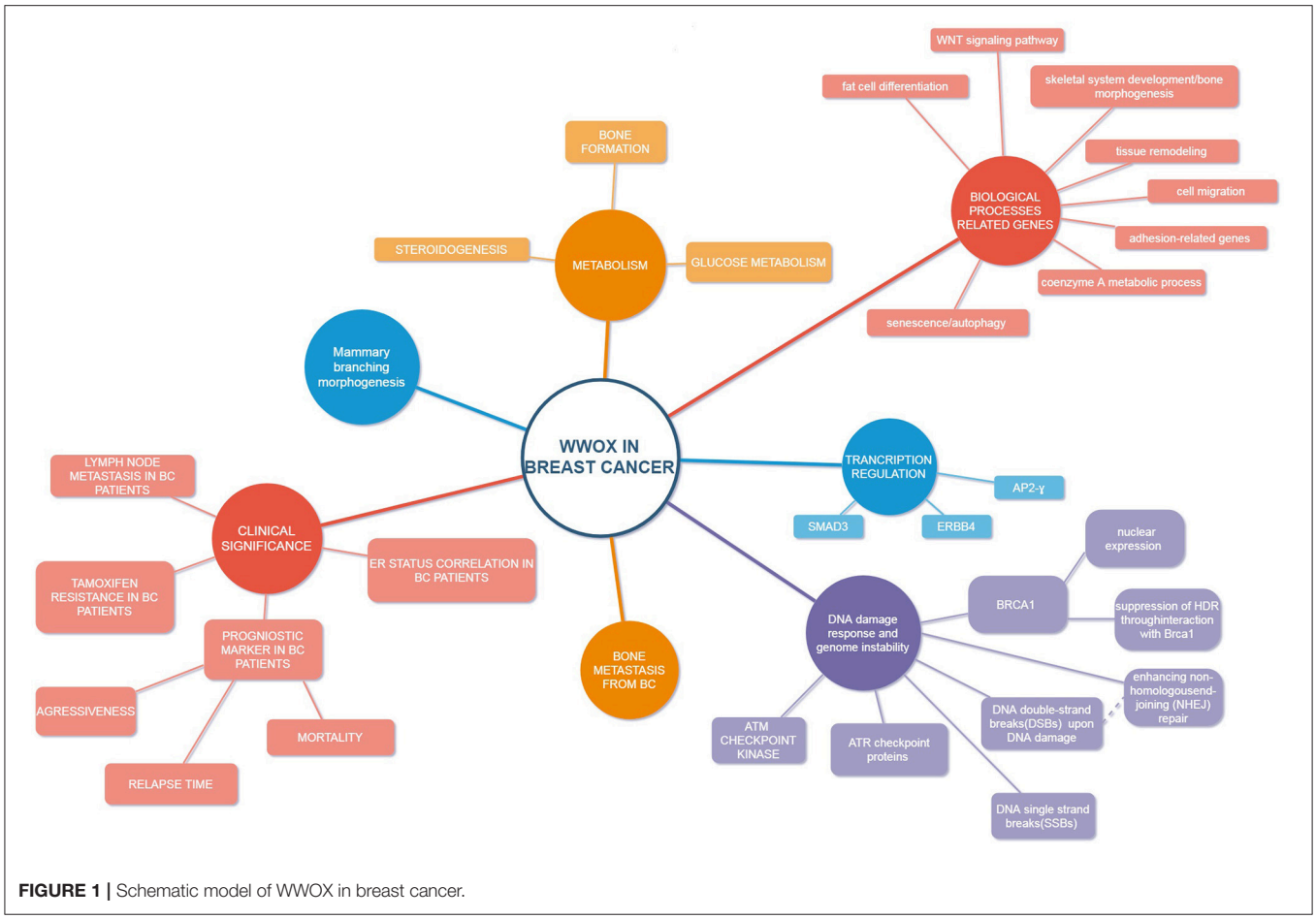

FIGURE 1 | Schematic model of WWOX in breast cancer.

WWOX(C3H)$^{+/-}$, a WWOX-heterozygous C3H mammary tumor-susceptible genetic background mouse strain which resembles possible malignant transformation in humans, found that 50% of female mice formed breast cancer compared to 7% in the control WWOX(C3H)$^{+/+}$. In most of the WWOX(C3H)$^{+/-}$ tumors, loss of estrogen and progesterone receptors was also observed. Furthermore, cDNA array expression analysis of normal murine breast tissue and heterozygous WWOX(C3H)$^{+/-}$ tumors was carried out, and identified 292 genes of significantly variable expression. Most identified genes turned out to be involved in cellular movement, signaling and interactions, cellular development, growth, proliferation, and cell death (71). Initial attempts of developing adult WWOX$^{-/-}$ knock-out mice turned out unsuccessful, as animals were smaller and died within 4 weeks. Moreover, WWOX$^{-/-}$ mice developed spontaneous osteosarcomas, whereas WWOX$^{-/+}$ mice squamous lung carcinomas. Compared to wild-type animals (WWOX$^{+/+}$), neoplasms were five times more frequent. In addition, the presence of WWOX protein in tumors of WWOX$^{-/+}$ animals, suggests a predisposition to malignancy as an effect of haploinsufficiency (57). WWOX$^{-/-}$ mice showed also severe metabolic defects, which affected proper bone formation (43).

Interestingly, in 2012 Ferguson et al. for the first time described tissue specific targeted ablation of *Wwox* gene in adult mouse tissues. Their MMTV-Cre mouse model exhibited significant down-expression of *Wwox* in the mammary epithelium without any adverse effect on survival. What is more, *Wwox* deletion did not affect tumorigenicity, nor did haploinsufficiency affect the mammary gland phenotype: It was concluded that *Wwox* might not be a classical tumor suppressor gene, but that rather loss of *Wwox* expression is associated with tumor progression. Nevertheless, *Wwox* knockdown in a mouse model resulted in impaired mammary branching morphogenesis (72). Similar observations, were made on a breast cancer cell line, where ectopical overexpression of *WWOX* gene in MDA-MB-231 breast cancer cells changed cell growth in Matrigel from tumor-like to branched structures, which resembled normal mammary duct formation (68).

Great efforts have been made to reveal the biological functions of *WWOX* and identify the signaling pathways associated with its expression, not limited to breast cancer. For *Wwox* KO MMTV-Cre mice, significant deregulation of the genes involved in various cellular processes was observed in the mammary epithelium. Gene ontology enrichment analysis of the Biological Processes GO category identified WWOX-associated expression of Wnt signaling pathway genes, including

significant upregulation of *Wnt5a*, which is also transcriptional target of the TGFβ/SMAD signaling pathway, skeletal system development/bone morphogenesis, genes associated with tissue remodeling, and cell migration as well as adhesion-related genes (for instance *Timp2* and *Timp3* upregulation) (72). An interesting strategy for elucidating WWOX function was employed by Aldaz et al. Their study used a Multiexperiment Matrix bioinformatics tool to identify the top 100 genes positively-correlated with WWOX and the top 100 negatively-correlated genes based on approximately 4,800 samples of both normal and tumor tissues, as well as breast cancer cell lines, obtained from breast datasets. Among the top enriched biofunctions, the following were identified: "regulation of mammary gland morphogenesis and branching," "coenzyme A metabolic process," "WNT signaling pathway," "senescence/autophagy" and "fat cell differentiation" (73); these results are consistent with those of previous studies.

Clinical Significance of WWOX in Breast Cancer

As mentioned above, breast cancer deletions within the common chromosomal fragile site FRA16D are observed in more than 80% cases, which makes changes in the WWOX coding region the most common genetic alternation in breast cancer (9). However, LOH is not the only mechanism responsible for *WWOX* downregulation: hypermethylation (69, 74, 75) and some infrequent point mutations within the coding region of *WWOX* (76) have also been found to be involved in breast carcinogenesis and cancer progression. In addition, rare homozygous deletions have also been observed for lung, ovarian, pancreatic (34) and colon cancers (77), WWOX protein degradation has been observed as a result of ubiquitination in prostate cancer (78), and hypermethylation in gastric (6, 79), pancreatic (16, 80), bladder (74), lung (74, 81, 82), and prostate (66) tumors.

The first cell line-based *WWOX* gene expression study related to breast cancer found HME-87 expression to be elevated in all eight tested cell lines compared to normal epithelial breast cells. Interestingly, the studied cell lines demonstrated wide variations in expression, ranging from relatively low levels in T47D, MDA-MB-435, and MDA-MB-231 to significant over-expression in ZR75-1, MCF-7, and MDA-MB-361 (1, 31, 83). This diversity in WWOX expression in different breast cancer cell lines was confirmed by Driouch et al. who also showed decreased expression in breast cancer tumors (32).

Immunohistochemical studies also found 63.2% of 97 invasive breast cancers to demonstrate a reduction in WWOX protein level. In addition, in 32.9% of these cases also demonstrated reduced expression in normal breast tissue, and correlation between reduced expression and a higher degree of tumor stage was shown ($P = 0.033$) (84). Similarly, Nunez et al. report high WWOX protein levels in normal human epithelial breast tissues, but very low or no expression in 33% of DCIS and 59.6% of invasive breast cancer cases (85). Reduced expression of WWOX was also observed in immunohistochemical study of 44 DCIS tumors (68.2%), 31 DCIS tumors adjacent to invasive breast cancer (54.8%), 30 cases of invasive breast cancer (61.3%), 39 normal tissues adjacent to DCIS tumors (56.4%), and 30 healthy tissues surrounding DCIS tumors adjacent to invasive breast cancers (29%). Moreover, reduced expression was observed more frequently in tissue adjacent to invasive breast cancer of a higher DCIS tumor stage ($P = 0.004$) (86). Another analysis of 267 breast cancer cases revealed an association between lower WWOX expression, the basal breast cancer subtype ($P = 0.01$) and shorter relapse-free survival (DFS), although its level did not correspond to patient overall survival (OS) (86).

WWOX was also proved to be a potent prognostic marker in breast cancer. In 2010 Pluciennik et al. assessed *WWOX* and three other genes (*ESR1, CDH1, BAX*) as a markers of favorable prognosis in nearly 120 BC patients. Along with *KTR5, KRT14, KRT17, CCNE1, BCL2,* and *BIRC5* genes, found to be associated with unfavorable prognosis, those 10 genes constituted an algorithm dividing patients into statistically significant groups of diverse disease-free survival times ($p = 0.0056$). Additionally, after division of the patients according to ER status, the algorithm was only well diversified in the group of ER-patients ($p = 0.0039$) (87).

The prognostic value of WWOX expression in breast carcinomas, was also assessed by Aldaz et al. (73), when he and his co-workers employed publicly available gene expression microarray data sets and Kaplan–Meier Plotter tool, in order to stratify breast cancer patients according to high and low WWOX expression. The findings revealed low WWOX expression to be associated with shorter relapse-free survival times in breast cancer patients ($n = 3259$). Additionally, while this trend was confirmed in all breast cancer subtypes, it was definitely more evident in luminal B and basal-like subtypes than luminal A (73).

Association of WWOX and ER Status and Chemotherapy Effectiveness in Breast Cancer

The first report of a correlation between WWOX and estrogen receptor status obtained by a immunohistochemical study of WWOX in 97 archived breast carcinoma specimens in relation to various patient and tumor characteristics by Guler et al. It was found that reduced WWOX staining was associated with less favorable ER status ($P = 0.033$). Lowered WWOX expression was observed in 56.3% ER-negative tumors and <25% ER-positive tumors, while normal WWOX staining correlated with negative or nearly negative ER status only in 30.4% of cancer cases (84).

Nunez et al. identified a correlation between WWOX expression and ER status. They compared WWOX protein level with patient clinico-pathological profile in a group of 16 human normal breast epithelium samples, 15 DCIS tumors and 203 invasive breast cancer cases. They observed that 27% of estrogen-positive breast carcinomas were negative for *WWOX* expression, compared with 46% for ER– cancers ($p = 0.0054$). In addition, when combining *WWOX*–deficient and nearly-negative cases, the difference became even more substantial, with 51% of ER+ cases and 73% of ER– cases being recognized ($p = 0.003$) (85). Similar correlations were also observed in other studies. The level of *WWOX* expression correlated with ER and PR status in a quantitative real-time RT-PCR study of 132 breast cancer

cases: significantly higher WWOX expression was observed in ER+ tumors compared to ER– tumors, as well as in PR positive cancers compared to PR negatives, and in ER+PR+ tumors compared to ER–PR– cases (88). A strong positive relationship was found between WWOX expression and ER ($p < 0.001$) or PR ($p = 0.001$) by immunostaining of tissue microarrays constructed from 837 breast cancer blocks (89).

WWOX has been also described as a promising marker of chemotherapy effectiveness. Scientists observed 4.6-times greater probability of tamoxifen resistance in patients with reduced expression of WWOX ($n = 89$ breast cancer patients). In addition, the expression of WWOX was found to be a better marker of tamoxifen resistance in high-risk patients than progesterone receptor level (90). Also, an *in vitro* study showed that a reduction of WWOX resulted in diminished ER levels and diminished tamoxifen sensitivity (71). The association between WWOX expression and sensitivity of tamoxifen treatment was finally confirmed on tissue microarrays employing 912 randomized breast cancer tissues. In patients treated with tamoxifen, there was a significant correlation between high WWOX expression and a lowered risk of recurrence, indicating that WWOX might be a potential marker of tamoxifen effectiveness (91).

WWOX in Bone Metastases of Breast Cancer and Metabolism

In 2013, Matteucci et al. investigated WWOX, E-cadherin and TAZ protein levels, with the intention of deciphering the contribution of Hippo-related pathways in bone metastasis from breast cancer. Interestingly, the findings indicated elevated WWOX levels in bone metastases prevalently at plasma membrane/nuclei level and in cytosolic-perinuclear areas ($n = 15$), while being almost absent in primary invasive ductal breast carcinoma tissues ($n = 6$; two specimens matched) (92–94). Moreover, the adjacent mammary tissues showed a WWOX signal (93, 94). *In vitro* studies showed that WWOX and E-cadherin were higher in bone-metastatic 1,833 cells when compared with parental-MDA-MB-231 cells, while being elevated in non-invasive MCF-7 breast carcinoma cells. Knocking-down endogenous WWOX reduced invasion and E-cadherin expression in 1,833 cells, whilst its overexpression enhanced E-cadherin transactivation and protein level in the 1,833, but not in MDA-MB-231 cells, increasing also metastatic-cell migration. It was concluded that in 1833 cells, WWOX expression varies with DNA-methylation state and hypoxic conditions (92, 93), as endogenous levels of WWOX protein in the 1833 cells was regulated by DNA methyltransferases. Long-term exposure to the inhibitor 5-azacytidine caused WWOX downregulation and treatment of 1,833-xenograft mice with another inhibitor of DNA methyltransferases, 5-aza-2′-deoxycytidine, prolonged mouse survival and increased WWOX expression both in cytosol and nuclei. WWOX and TAZ were found to activate Hypoxia inducible factor-1 (HIF-1) binding to E-cadherin promoter, while PPARγ receptor (Peroxisome proliferator-activated receptor γ) mediated in E-cadherin transactivation supporting WWOX and preventing TAZ functions (92–94). Further research revealed that by influencing E-cadherin expression, Wwox contributes to mesenchymal-epithelial transition (MET) and colonization of bone metastasis from breast carcinoma (94).

HIF-1 was found to be a major regulator of oxygen homeostasis and of aerobic glycolysis in cancer (Warburg effect), and HIF-1α its inducible subunit (95). It was found that WWOX regulates the expression of glycolysis-related genes through HIF-1α (96).

At this point it is scarcely possible not to mention the emerging role of WWOX in the metabolic state of the cells, which is of particular importance given the fact that reprogramming of energy metabolism was recently added to the list of hallmarks of cancer cells (97). During glycolysis, normal cells convert glucose to pyruvate in the cytosol, which is followed by complete oxidation of pyruvate to CO_2 through the tricarboxylic acid (TCA) cycle and then oxidative phosphorylation in the mitochondria, under aerobic conditions; under anaerobic conditions, the pyruvate is metabolized to lactate. However, cancer cells convert most glucose to lactate even in aerobic conditions, diverting glucose metabolites from energy production to anabolic process to accelerate cell proliferation. This state has been termed the Warburg effect, after the first researcher to describe the phenomenon, or as aerobic glycolysis (98).

As mentioned above, Abu-Remaileh and Aqeilan (96) reported that WWOX, physically and functionally interacts with HIF1α and regulates its transactivation function both *in vitro* and *in vivo*: *WWOX* KO mice exhibit elevated levels of serum lactic acid. The loss of *WWOX* resulted in activation of glycolysis in mouse embryonic fibroblasts (MEF) from KO embryos, compared with fibroblasts from *WWOX*-wild type embryos. Importantly, genetic or pharmacologic depletion of HIF1α was able to reverse WWOX-mediated phenotypes associated with its loss, including tumorigenesis. In addition, breast cancer tissue microarrays have shown that in breast cancer samples, WWOX expression is inversely correlated with levels of the glucose transporter GLUT1, which is known to be a direct target of HIF1α. This again highlights the modulatory role of WWOX in cancer metabolism (96). Major research on WWOX in breast cancer are listed in **Table 1**. **Figure 1** depicts crucial nodes of differential WWOX molecular functions and its clinical implications.

SUMMARY AND FUTURE PERSPECTIVES

Initially, when the *WWOX* gene was discovered, it seemed almost certain that it was related to the classical tumor suppressor gene. Downregulation of WWOX expression was found to be associated with more advanced tumors, higher aggressiveness, and poorer disease-free and overall patient survival, not only in breast cancer but in the majority of investigated neoplasms. However, despite evidence from clinical outcomes and *in vivo* and *in vitro* models supporting its tumor suppressor function, it was found that WWOX does not act as a standard tumor suppressor gene. In various cancer types, it is more common to observe low levels of Wwox than none at all, which does not fit Knudson's two-hit hypothesis. In fact its haploinsufficiency

is prevalent for WWOX inactivation, and inactivation of both alleles or missense mutations are very rare.

WWOX possessing a classical SDR domain turned out to take part in steroid and bone metabolism. The *WWOX* gene is in fact highly expressed in hormone-dependent tissues, where it might be involved in the regulation of metabolic steroids. Reduced or lost expression of WWOX resulted in the development of metabolic diseases, and Wwox KO mice were found to die early prematurely from multiple physiological defects before any tumors developed.

Two WW domains of Wwox are responsible for its interactions. Although several transcription factors have been identified amongst WWOX partners, various Wwox interactors have been found to play roles in aerobic metabolism. Indeed, the contribution of Wwox to pathways involving aerobic metabolism and oxidative stress are well-documented, which provides further evidence for the non-classical tumor suppressor characteristics of WWOX. Its ability to facilitate the circumvention of mitochondrial damage-induced glycolysis (Warburg effect) was proposed as a possible mechanism for its tumor suppressor activity.

Loss or dysregulation of WWOX expression leads not only to tumorigenesis and cancer progression, but also genomic instability and resistance to therapy. This way, WWOX can be used as a potent marker of prognostic and clinical outcome. Wwox expression might also serve as a significant predictor of response to radiotherapy and chemotherapy.

WWOX is involved in many signaling pathways for regulating cell apoptosis, autophagy, differentiation and metabolism. Mutations and deletions completely silencing *WWOX* expression are very rare and most often we observe abrogation of its function through lowered WWOX protein synthesis and haploinsufficiency. Therefore, due to multifunctional nature of WWOX there is very difficult to justify prevalent dysfunction in a specific tumor type. Final result of *WWOX* gene silencing depends also on abrogation of function of its partner proteins and appropriate protein network. That suggests that while in breast cancer we observe around 70% loss of heterozygosity of WWOX gene and significant expression reduction final effect of WWOX abrogation depends on topology of multiprotein network associated with WWOX protein-protein interaction that may be differentiated in tumor subtypes.

AUTHOR CONTRIBUTIONS

KP, EP, and AB contributed to the design and to the writing of the manuscript.

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REVIEW
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WWOX Phosphorylation, Signaling, and Role in Neurodegeneration

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Homozygous null mutation of tumor suppressor *WWOX/Wwox* gene leads to severe neural diseases, metabolic disorders and early death in the newborns of humans, mice and rats. WWOX is frequently downregulated in the hippocampi of patients with Alzheimer's disease (AD). *In vitro* analysis revealed that knockdown of WWOX protein in neuroblastoma cells results in aggregation of TRAPPC6AΔ, TIAF1, amyloid β, and Tau in a sequential manner. Indeed, TRAPPC6AΔ and TIAF1, but not tau and amyloid β, aggregates are present in the brains of healthy mid-aged individuals. It is reasonable to assume that very slow activation of a protein aggregation cascade starts sequentially with TRAPPC6AΔ and TIAF1 aggregation at mid-ages, then caspase activation and APP de-phosphorylation and degradation, and final accumulation of amyloid β and Tau aggregates in the brains at greater than 70 years old. WWOX binds Tau-hyperphosphorylating enzymes (e.g., GSK-3β) and blocks their functions, thereby supporting neuronal survival and differentiation. As a neuronal protective hormone, 17β-estradiol (E2) binds WWOX at an NSYK motif in the *C*-terminal SDR (short-chain alcohol dehydrogenase/reductase) domain. In this review, we discuss how WWOX and E2 block protein aggregation during neurodegeneration, and how a 31-amino-acid zinc finger-like Zfra peptide restores memory loss in mice.

Keywords: WWOX, TRAPPC6AΔ, TIAF1, 17β-estradiol, sex steroid hormone receptor, neurodegeneration, Zfra

INTRODUCTION

Human and mouse WW domain-containing oxidoreductase, designated WWOX, FOR, or WOX1, has been generally regarded as a tumor suppressor since its discovery in 2000 (Bednarek et al., 2000; Ried et al., 2000; Chang et al., 2001). Human *WWOX* gene is located on a common fragile site *FRA16D*, spanning 1.1 million bases on chromosome ch16q23.3-24.1. *WWOX* is composed of 9 exons with an open reading frame (ORF) of 1245 base pairs long, encoding a 414-amino-acid (46 kDa) protein. *WWOX* gene possesses multiple long non-coding RNA *PARTICLE*

(Gene *PARTICL*- '*P*romoter of *MAT2A*-*A*ntisense *R*adia*T*ion *I*nduced *C*irculating *L*ncRNA) triplex clusters, suggesting its control of gene expression in the genome (O'Leary et al., 2017). WWOX protein possesses two *N*-terminal WW domains, a nuclear localization signal between the WW domains, a *C*-terminal short-chain alcohol dehydrogenase/reductase (SDR) domain, and a proapoptotic *C*-terminal tail termed D3 (Bednarek et al., 2000; Ried et al., 2000; Chang et al., 2001; Hong et al., 2007; Lin et al., 2011; Abu-Remaileh and Aqeilan, 2015, Abu-Remaileh et al., 2015; Huang and Chang, 2018).

The WW domain participates in protein/protein interactions for transducing signals (Chang et al., 2007; McDonald et al., 2012; Reuven et al., 2015). The first WW domain of WWOX binds PPxY or PPPY-containing proteins (e.g., p73, ErbB-4, SIMPLE, WWBP1, WWBP2, Ezrin, AP-2g, Runx-2, and many others) (Ludes-Meyers et al., 2004; Jin et al., 2006; Chang et al., 2007; McDonald et al., 2012; Reuven et al., 2015). When Tyr33 in the first WW domain is phosphorylated, activated WWOX acquires an enhanced capability in binding a broad spectrum of proteins (Chang et al., 2007; Reuven et al., 2015), including p53 (Chang et al., 2001, 2003a,b, 2005a,b, 2007), c-Jun *N*-terminal kinase (JNK) (Chang et al., 2003a), Zinc finger-like protein that regulates apoptosis (Zfra) (Hong et al., 2007), c-Jun and cAMP response element binding protein (CREB) (Li et al., 2009) and others. The second tandem WW domain assists synergistically with the first WW domain in enhancing the protein/protein binding (Farooq, 2015). Transiently overexpressed WWOX frequently sequesters transcription factors in the cytoplasm, and thereby blocks their transcription for prosurvival proteins in the nucleus in cancer cells *in vitro* (Gaudio et al., 2006). In contrast, endogenous WWOX binds and co-translates with many transcription factors to relocate to the nucleus to enhance or block neuronal survival under sciatic nerve dissection (Li et al., 2009). Endogenous trafficking protein particle complex 6A (TRAPPC6A) acts as a carrier for WWOX to undergo nuclear translocation (Chang et al., 2015). Indeed, WWOX works together with many transcription factors to either support neuronal survival or death under physiological or pathological conditions.

TYR33-PHOSPHORYLATED WWOX IN APOPTOSIS AND IN TEMPERATURE-RELATED BUBBLING CELL DEATH (BCD)

The proapoptotic function of WWOX has been previously reviewed (Chang, 2002, 2015; Chang et al., 2003b, 2007; Huang and Chang, 2018). Briefly, UV irradiation activates cytosolic WWOX via Tyr33 phosphorylation (pY33-WWOX), followed by binding Ser46-phosphorylated p53. Both proteins relocate to the mitochondria or nuclei to induce cell death (Chang et al., 2003a, 2005a). As an inhibitor, JNK or Zfra suppresses WWOX in inducing apoptosis (Chang et al., 2003a; Hong et al., 2007; Aderca et al., 2008). Zfra acts by reducing Tyr33 phosphorylation in WWOX (Hong et al., 2007). Zfra binds the *N*-terminal WW domain and *C*-terminal SDR domain of WWOX. This binding interferes with Tyr33 phosphorylation by tyrosine kinase Src (Aqeilan et al., 2004).

At temperatures lower than 37°C, WWOX is needed for a recently described type of cell death, designated bubbling cell death (Chen et al., 2015; Chang, 2016). BCD is not apoptosis, necroptosis, or necrosis. When cells are subjected to UV irradiation and cold shock followed by culturing at 37°C, the cells undergo apoptosis (e.g., caspase activation, whole cell and nuclear condensation, DNA fragmentation, etc.). However, if the UV/cold shock-treated cells are incubated at a lower temperature (e.g., 4, 10, or 22°C), they generate, in most cases, a nuclear nitric oxide (NO)-containing bubble per cell. Some cells may generate 2–3 bubbles. The bubble continues to inflate and finally is released from the cell membrane. The cells die later on. Membrane phosphatidylserine flip over, caspase activation and DNA fragmentation, which are found in apoptosis, are not involved in BCD. Raising the temperature back to 37°C resumes the event to apoptosis. If cells are devoid of WWOX (e.g., $Wwox^{-/-}$ MEF), cell death is retarded (Chen et al., 2015; Chang, 2016). Overall, UV energy is absorbed by the nucleus, and cold shock assists the rapid relocation of cytosolic p53, WWOX, and NOS2 to the nucleus. Nitric oxide synthase NOS2 is responsible for the bubble generation that leads to cell death (Chen et al., 2015; Chang, 2016).

ACTIVATED WWOX INDUCES CELL DEATH FROM THE MITOCHONDRIA AND NUCLEI

WWOX in Neuronal Injury

Constant light-induced retinal neural degeneration involves WWOX activation and pY33-WWOX accumulation in the mitochondria and nuclei to cause damage and death (Chen et al., 2005). Neurotoxin MPP^+ (1-methyl-4-phenylpyridinium) also induces pY33-WWOX upregulation and nuclear accumulation to cause neuronal death in rats (Lo et al., 2008). During the acute phase of sciatic nerve dissection, pY33-WWOX, along with its interacting transcription factors, becomes accumulated in the nucleus that leads to the rapid death of the large-sized neurons *in vivo* (Li et al., 2009). WWOX blocks the prosurvival function of CREB-, CRE-, and AP-1-mediated promoter activation *in vitro* (Li et al., 2009). In stark contrast, WWOX enhances the promoter activation governed by c-Jun, Elk-1 and NF-κB (Li et al., 2009). Apparently, a balance in the protein levels for WWOX and transcription factors is critical in determining the fate of dissected neurons.

Hyal-2/WWOX Signaling in Traumatic Brain Injury (TBI) Links to BCD

WWOX localizes in many subcellular compartments, including cell membrane, mitochondrion, lysosome, nucleus, and others. WWOX does not have a membrane localization signal. It is anchored, in part, to the membrane/cytoskeleton area by hyaluronidase Hyal-2 (Hsu et al., 2009, 2016, 2017) and Ezrin (Jin

et al., 2006). WWOX acts as a transducer in many stress-related signal pathways induced by tumor necrosis factor (TNF), chemotherapeutic drugs, UV irradiation (Chang et al., 2007, 2010, 2014, 2015; Abu-Remaileh and Aqeilan, 2015), Wnt/β-catenin (Bouteille et al., 2009), transforming growth factor-β (TGF-β) (Hsu et al., 2009; Chang et al., 2010), complement C1q (Hong et al., 2009), hyaluronan and Hyal-2 (Chang et al., 2010; Hsu et al., 2016, 2017), sex steroid hormones (Su et al., 2012), T cell differentiation reagents (Huang et al., 2016), and others.

During TBI, activation of the Hyal-2/WWOX/Smad4 signaling complex causes neuronal death (Hsu et al., 2017) (**Figure 1A**). Hyal-2 and WWOX are accumulated in the nuclei of damaged neurons in rat brain (Hsu et al., 2017) (**Figure 1A**). Hyal-2 is a cognate receptor for hyaluronan and TGF-β1 (Hsu et al., 2009, 2016, 2017). Both hyaluronan and TGF-β1 may utilize the Hyal-2/WWOX/Smad4 signaling to enhance the cell survival or death. It has been shown that long-term overexpression of TGF-β1 causes neurodegeneration in mice (Ueberham et al., 2005).

Bubbling cell death can also occur at 37°C. For example, when cells are transiently overexpressed with hyaluronidase Hyal-2 and WWOX followed by treating with high-molecular-weight hyaluronan, BCD occurs at 37°C (Hsu et al., 2017) (**Figure 1B**). Hyaluronan binds membrane Hyal-2 to initiate the Hyal-2/WWOX signaling, and that both Hyal-2 and WWOX are accumulated in the nuclei. It is reasonable to assume that during TBI, the nuclear Hyal-2 and WWOX may exert BCD due to the production of NO. Formation of nuclear bubbles in the dying neurons *in vivo* is unknown. However, bubble formation *in vivo* is difficult to detect, because it is technically impossible to fix bubbles for microscopic examination. Reactive oxygens species (ROS) are rapidly upregulated during TBI (Bains and Hall, 2012). WWOX, via its *C*-terminal SDR domain, controls the generation of ROS in *Drosophila* (O'Keefe et al., 2011) and mammalian cells (Dayan et al., 2013). Also, the SDR domain of WWOX controls the cellular outgrowths caused by genetic deficiencies of the components of the mitochondrial respiratory complexes in *Drosophila* (Choo et al., 2015). Under physiologic conditions, oxidative phosphorylation sustains WWOX expression (Dayan et al., 2013). However, when glycolysis (or Warburg metabolism) goes up in aberrant cells, WWOX expression is downregulated (Dayan et al., 2013). Reduced WWOX levels in *Drosophila* allow cellular outgrowths to various extent caused by genetic deficiencies of components of the mitochondrial respiratory complexes and aberrant ROS production (Choo et al., 2015). Together, WWOX participates in TBI and this is related with ROS generation and brain tissue repair.

WWOX IN NEURODEGENERATION *IN VIVO*

Pathological Features in Neurodegeneration

Neurodegenerative diseases (NDs) encompass a heterogeneous group of chronic progressive diseases, each affecting specific central nervous system (CNS) compartment. The pathologies of NDs are not specific for each individual disease. Neurofibrillary tangles and Lewy bodies, for example, may appear in non-demented and non-idiopathic Parkinson disease patients. Also, the pathological or clinical features may overlap. Over the past decades, many animal models have been established to seek potential propagation mechanisms and associated risk factors for NDs (Martin, 2012; Niccoli and Partridge, 2012; Dugger and Dickson, 2016; Hartl, 2017; Chételat, 2018). Aging-related stress, oxidative stress, reduced mitochondrial function, altered subcellular transport, and activation of the ER stress and unfolded protein response (UPR) pathways are considered important during neurodegeneration (Martin, 2012; Hartl, 2017).

In the aging processes, chaperones may become dysregulated and the degradation machineries stop working properly, which leads to protein misfolding, aggregation, and accumulation for neuronal damage. Among these, UPR exists in the mitochondria and the endoplasmic reticulum, along with disordered cytosolic heat shock response, ubiquitin-proteasome system, and autophagy (Taylor and Dillin, 2013; Hartl, 2017). Presence of aberrant protein aggregates, inclusion bodies and/or tangled fibrous proteins in the aging neurons, glial cells, and brain matrix is the pathological hallmarks of neurodegeneration (Ross and Poirier, 2005; Richter-Landsberg and Leyk, 2013; Higuchi-Sanabria et al., 2018). Furthermore, formation and spread of prion-like Aβ aggregates occur during AD progression, and this is not due to overexpression of APP (amyloid precursor protein) (Ruiz-Riquelme et al., 2018). Prion protein in the exosomes facilitates the spreading and aggregation of neurotoxic Aβ (Hartmann et al., 2017).

WWOX Deficiency Leads to Severe Neural Damage and Metabolic Disorders

The WWOX protein is heterogeneously expressed in the central nervous system. WWOX-positive stains are found in the human cerebrum, specifically in the pyramidal neurons and astrocytes from the frontal and occipital cortices, and in the nucleus caudate, pons and nuclei olivaris of medulla. Neuropils and small neurons are also immunoreactive to WWOX antibody. However, parietal, limbic and temporal cortices and substantia nigra are minimal or negative for WWOX immunoreactivity (Nunez et al., 2005). In the developing mouse brain, WWOX protein expression is essentially present in every brain region and the expression level is reduced in the newborns (Chen et al., 2004). In the adult brain, WWOX is abundant in the epithelial cells of the choroid plexus and ependymal cells, while a low to moderate level of WWOX is observed within white matter tracts, such as axonal profiles of the corpus callosum, striatum, optic tract, and cerebral peduncle (Chen et al., 2004).

Despite its role in cell death, WWOX is essential in homeostasis *in vivo*. *WWOX*/*Wwox gene* deficiency severely affects normal physiological functions, especially in embryonic neural development (Chen et al., 2004; Aldaz et al., 2014; Chang et al., 2014, 2015; Tabarki et al., 2015). Deficiency of *WWOX*/*Wwox* gene due to point mutations or homozygous nonsense mutation may result in childhood onset autosomal

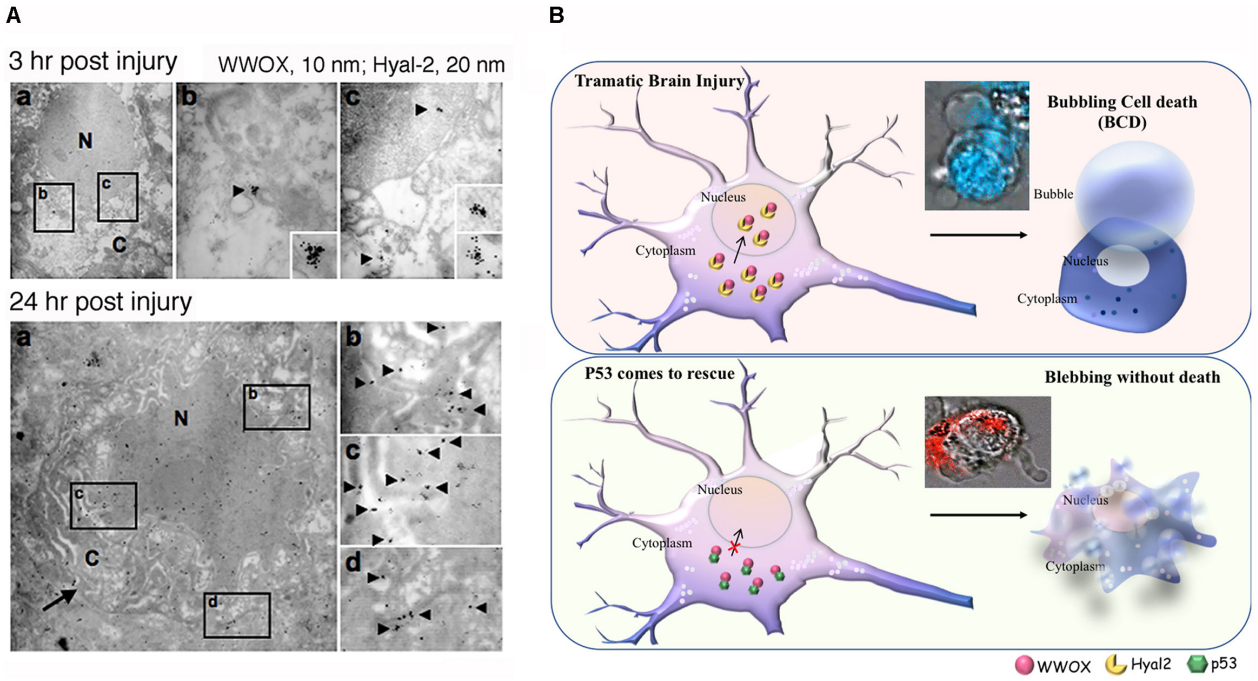

FIGURE 1 | Potential role of WWOX and BCD in neuronal death during traumatic injury. **(A)** Needle insult to the brain was carried out in rats. Post injury for 3 and 24 h, the animals were sacrificed. By immunoelectron microscopy, accumulation of Hyal-2 and WWOX is found in the nuclei of dying neurons in the brain cortex (Hsu et al., 2017). **(B)** Nuclear accumulation of Hyal-2 and WWOX leads to BCD (Chen et al., 2015). Both schematic graphs and a real-time image are shown. If p53 competes with Hyal-2 to complex with WWOX, both p53/WWOX proteins are retained in the cytoplasm and the extent of Hyal-2/WWOX complex is reduced, no BCD occurs (Hsu et al., 2017). (Data in **A** is adapted from Hsu et al., 2017, republishing according to the guideline of *Oncotarget*).

recessive cerebellar ataxia and epilepsy, growth retardation, microcephaly with seizure, retinal degeneration, and early death at 16 months of age (Valduga et al., 2015; Alkhateeb et al., 2016; Elsaadany et al., 2016). Similar observations are shown in rats (Suzuki et al., 2009).

WWOX gene is involved in the regulation of lipid homeostasis and metabolism (Ludes-Meyers et al., 2004, 2009; Lee et al., 2008; Yang et al., 2012; Dayan et al., 2013; Iatan et al., 2014; Li et al., 2014; Abu-Remaileh and Aqeilan, 2015; Abu-Remaileh et al., 2015). *WWOX* gene alteration is associated with the low plasma high-density lipoprotein cholesterol (HDL-C) levels and aberrant HDL-C and triglyceride levels (Lee et al., 2008; Sáez et al., 2010). Furthermore, whole body and liver conditional *Wwox* knockout mice revealed a significant role for *Wwox* in regulating HDL and lipid metabolism (Iatan et al., 2014).

Interference in lipid metabolism may be a critical contributor in the pathogenesis of neurological diseases. For example, WWOX is not expressed in the lipid-rich myelin sheath in the normal neurons, but activated pY33-WWOX is accumulated in the myelin sheath during neurotoxin MPP^+-induced neuronal death (Lo et al., 2008). While both apolipoprotein E (Apo E) and WWOX are involved in AD and TBI, the functional relationship between these two proteins (e.g., binding) needs further elucidation. Taken together, WWOX plays a crucial role in neural development and lipid metabolism. Without WWOX, severe neural diseases, metabolic disorders and early death occur in humans and animals.

WWOX Gene Expression in the Brain

By analyzing the database in the Allen Brain Atlas[1], *WWOX* gene expression levels are shown to be significantly downregulated in the postmortem normal hippocampus, compared to those in the pons and white matter (n = 6; age 42.5 ± 13.4; 3 Caucasians, 2 blacks, 1 Hispanic) (**Figure 2A**). There were only six normal brain samples exhibiting detectable signals for *WWOX* gene expression (as shown in the **Supplementary Table S1**). *WWOX* gene expression is upregulated in the cingulum bundle of the white matter by 2.31-fold, and the central glial substance of the myelencephalon by 2.78-fold.

In the "Possible AD" group (77 to 100+ years old), *WWOX* gene expression levels are barely changed in the hippocampus (**Figure 2B**). Also, compared to the hippocampus, *WWOX* gene expression is significantly downregulated in the parietal and temporal neocortex, but is significantly upregulated in the white matter of the forebrain (**Figure 2B**). Interestingly, similar expression profiles are observed in the "Traumatic Brain Injury (TBI)" group (77 to 100+ years old; **Figure 2C**).

Also, in other gene databases (GTEx, Illumina, BioGPS, and CGAP SAGE, as summarized in the GeneCard[2]), *WWOX* gene expression levels in the brain, cerebellum, cortex, spinal cord and tibial nerve are similar to those from other tissues and organs in normal humans. However, WWOX protein expression

[1]http://www.brain-map.org

[2]https://www.genecards.org/cgi-bin/carddisp.pl?gene=WWOX

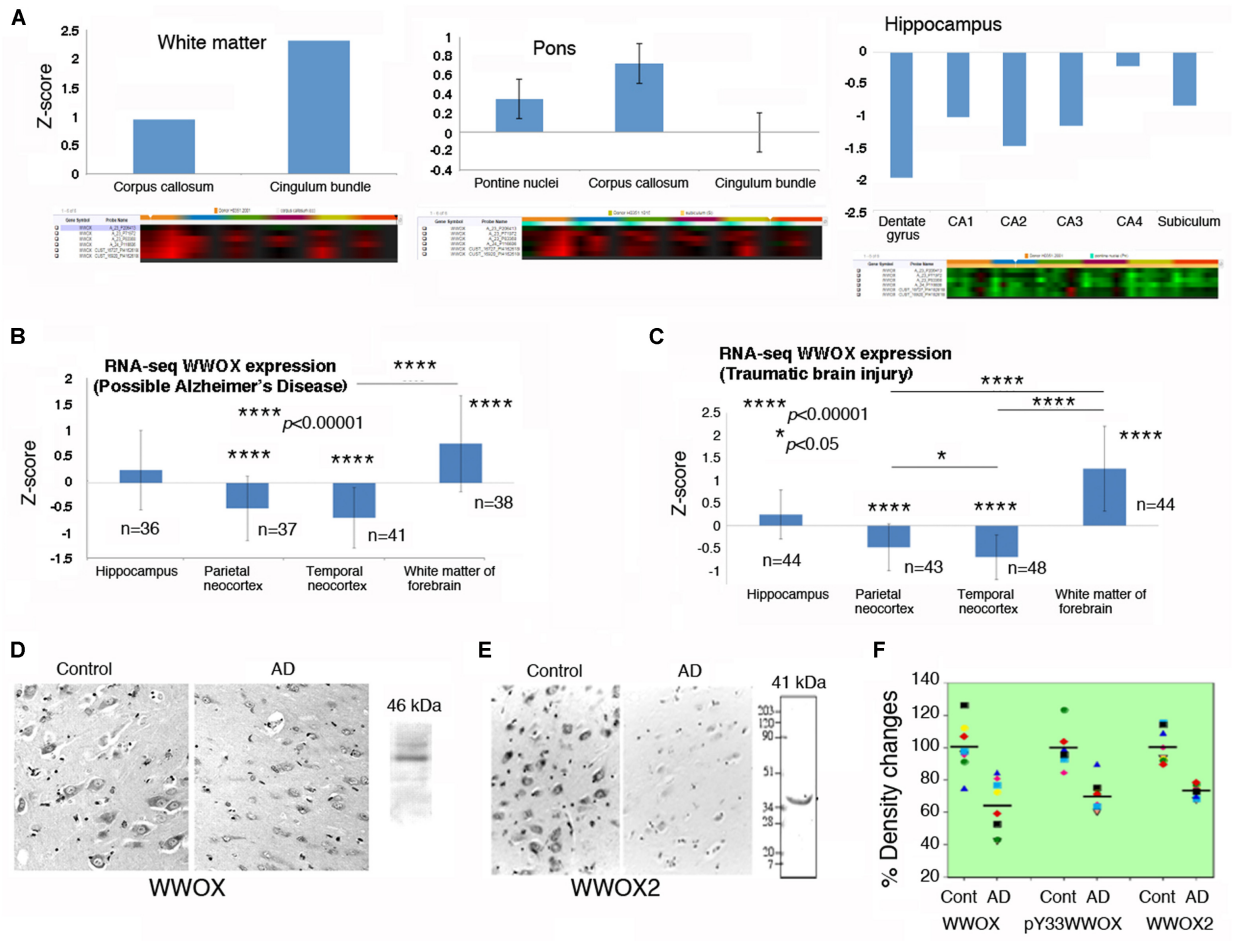

FIGURE 2 | *WWOX* gene and protein expression in human brain. **(A)** *WWOX* gene expression was analyzed using the database in the Allen Brain Atlas (http://www.brain-map.org). Detectable signals for *WWOX* gene expression were found in six postmortem normal individuals (age 42.5 ± 13.4; 3 Caucasians, 2 blacks, 1 Hispanic). Representative *WWOX* gene expression levels in the brain white matter, pons and hippocampus are shown. Also, see the **Supplementary Table S1** for *WWOX* gene expression in the normal brains (around one-fold changes for all indicated regions). **(B,C)** In the "Possible AD" and "Traumatic Brain Injury" groups (77–100+ years old), *WWOX* gene expression levels are shown in the indicated brain areas. **(D,E)** Expression of wild type WWOX (46 kDa) and isoform WWOX2 (41 kDa) is downregulated in the neurons of AD hippocampi compared with normal controls (a representative set from five immunostains; magnification, 200×; data from Sze et al., 2004). **(F)** In AD patients, the protein levels for WWOX (n = 8), isoform WWOX2 (n = 8), and pY33-WWOX (n = 6) are significantly downregulated in the hippocampi as determined by Western blotting, compared to age-matched controls (~32 ± 5% reduction, $p < 0.005$; data with minor revisions for the art work are adapted from Sze et al., 2004; republishing according to the guideline of the *Journal of Biological Chemistry*).

levels are significantly increased in the human fetal brains (GeneCard database shown above). This is in agreement with our observations using mouse fetal brains (Chen et al., 2004).

WWOX Protein Downregulation in Alzheimer's Disease (AD)

It is generally agreed that gene expression cannot always correlate with protein expression. The aforementioned *WWOX* gene expression levels do not correlate positively with the extent of WWOX protein expression. For example, downregulation of *WWOX* gene occurs in the hippocampi of young adults (**Figure 2A**) and many other areas (**Supplementary Table S1**). However, WWOX protein expression levels are detectable in neurons of many regions in the brain (Nunez et al., 2005).

Indeed, significant downregulation of the protein level for WWOX, isoform WWOX2, and pY33-WWOX has been shown in the hippocampi of AD patients, compared to age-matched controls (Sze et al., 2004) (**Figures 2D–F**). However, during sciatic nerve injury, rapid upregulation of *Wwox* gene expression occurs in less than 30 min in the neurons of dorsal root ganglion, followed by significant upregulation of WWOX protein and its Tyr33 phosphorylation in the damaged neurons in 24 h (Li et al., 2009). Activated WWOX is needed to initiate neuronal death in the damaged tissue.

There is no positive correlation between *WWOX*/*Wwox* mRNA expression and protein expression. For example, translational blockade of *Wwox* mRNA has been shown in the development of skin squamous cell carcinoma (SCC) in hairless mice (Lai et al., 2005). During the acute exposure of

hairless mice to UVB, both WWOX and pY33-WWOX proteins are upregulated in epidermal cells in 24 h. SCCs then start to develop in 3 months. There are significant reductions in WWOX and pY33-WWOX proteins in the SCC cells. However, no downregulation of *Wwox* mRNA occurs (Lai et al., 2005). In SCC patients, significant reduction of WWOX and pY33-WWOX proteins are observed in non-metastatic and metastatic cutaneous SCCs, whereas no downregulation of *WWOX* mRNA occurs (Lai et al., 2005). Together, *WWOX/Wwox* mRNA is subjected to translational blockade in the skin and probably other tissues and organs under pathological conditions.

WWOX Control of Neuronal Survival via Binding and Suppressing Tau and Tau Hyperphosphorylating Enzymes

Compelling evidence reveals that WWOX is likely to slow down neurodegeneration such as in AD. For example, the *C*-terminal SDR domain of WWOX binds and limits the enzymatic activity of glycogen synthase kinase 3β (GSK-3β) (Sze et al., 2004; Wang et al., 2012) (**Figure 3**). GSK-3β is known to hyperphosphorylate Tau which leads to restriction of neurite outgrowth, and prevention of neuronal differentiation resulting in formation of neurofibrillary tangles (NFTs) and senile plaques (SPs) in AD (Augustinack et al., 2002; Avila et al., 2010; Chang et al., 2014; Llorens-Martin et al., 2014; Sze et al., 2004). WWOX binds Tau via its SDR domain (Sze et al., 2004) (**Figure 3**), and prevents enzyme-dependent Tau hyperphosphorylation (Sze et al., 2004). WWOX contains two potential GSK-3β-binding FXXXLI/VXRLE motifs (Wang et al., 2012). Precisely, the α-helical segment (amino acids #388 to 407) at the SDR domain of WWOX interacts physically with the GSK3β binding-pocket α-helix (amino acid #262 to 273) (Wang et al., 2012). Transiently overexpressed WWOX inhibits GSK-3β-stimulated S396 and S404 phosphorylation within the microtubule domains of Tau. Consequently, WWOX represses GSK-3β activity in hyperphosphorylating tau, restores tau's ability to assemble the microtubule network, and promotes neurite outgrowth in neuroblastoma SH-SY5Y cells. When WWOX is knocked down by small interfering RNA, retinoic acid is not able to mediate differentiation of SH-SY5Y cells (e.g., neurite outgrowth) (Wang et al., 2012).

The WWOX also binds JNK via its Tyr33-phosphorylated first WW domain, and the binding results in neutralization of the functions of both proteins in a reciprocal manner (Chang et al., 2003a) (**Figure 3**). Additionally, the first WW domain of WWOX physically interacts with ERK (extracellular signal-regulated kinase) (Huang and Chang, 2018). ERK has been implicated in Tau hyperphosphorylation (Augustinack et al., 2002) (**Figure 3**). Cyclin dependent kinase 5 (Cdk5) hyperphosphorylates many substrates such as amyloid precursor protein, tau and many other proteins in the brain (Shah and Lahiri, 2015); however, functional interaction between WWOX and CDK5 has never been documented.

TIAF1 and TRAPPC6AΔ Protein Aggregates in the Hippocampi of Mid-Aged Normal Individuals

In an inducible transgenic mouse model, neuron-specific expression of TGF-β in the neocortex, hippocampus and striatum for a long term results in deposition of amyloid fibrils in these brain areas (Ueberham et al., 2005). Deposits of apolipoprotein E (ApoE) are also found in perivascular areas (Ueberham et al., 2005). When TGF-β induction stops, the amyloid and ApoE aggregates stably remain in the brain and vascular lesions. We have discovered a few novel proteins, whose aggregation is found in the brain hippocampal and cortical areas of both non-demented healthy individuals and demented AD patients. TGF-β1-induced antiapoptotic factor 1 (TIAF1; 12 kDa) is involved in the pathogenesis of AD and cancer, as well as in allograft rejection by activated T helper cells (van der Leij et al., 2003; Lee et al., 2010; Hong et al., 2013; Chang and Chang, 2015). Presence of aggregated TIAF1 protein in the dead neurons is shown in the hippocampi of middle-aged normal humans (Lee et al., 2010; Chang and Chang, 2015). Notably, little or no Aβ aggregates are found in the TIAF1 plaques in the mid-aged humans (Lee et al., 2010) (**Figure 4A**). For example, TIAF1 aggregation is detected in 59% of non-demented control hippocampi (age 59.0 ± 17.0, n = 41), and only 15% of the total samples have Aβ aggregates, as determined by filter retardation assay (Lee et al., 2010). However, 54% of TIAF1 aggregation is shown in the hippocampi of older postmortem AD patients (age 80.0 ± 8.8, n = 97), in which 48% of the total AD samples possess Aβ aggregates. Presence of a representative TIAF1-containing plaque from the hippocampus of a 9-month-old APP/PS1 transgenic mouse is shown (**Figure 4B**). A minimal amount of Aβ aggregates is found within the center of the plaque. The observations imply that TIAF1 aggregates are difficult to remove with age by the ubiquitination/proteasomal degradation system. *In vitro* analysis revealed that TIAF1 undergoes self-polymerization and this leads to amyloid β formation (Lee et al., 2010). Together, TIAF1 aggregation occurs in the middle age and this may result in slow formation of amyloid β in humans (Lee et al., 2010).

TGF-B-induced TIAF1 self-aggregation leading to the formation of Aβ aggregates probably occurs via a signaling pathway independently of the type II TGF-β receptor (Lee et al., 2010; Hong et al., 2013; Chang and Chang, 2015). TGF-β binds membrane Hyal-2 to initiate a non-canonical Hyal-2/WWOX/Smad4 pathway (Hsu et al., 2009, 2016, 2017). TIAF1 physically binds Smad4 and strongly suppresses SMAD-regulated promoter activation, and Smad4 blocks TIAF1 aggregation caused by TGF-β (Lee et al., 2010). In addition, p53 binds TIAF1. In the absence of TIAF1, pS15-p53 fails to undergo nuclear translocation (Schultz et al., 2004). Interestingly, without p53, self-aggregating TIAF1 spontaneously activates the SMAD-regulated promoter to cause cell death (Chang et al., 2012).

Under physiologic conditions, TGF-β1 promotes binding of TIAF1 with Smad4, and the TIAF1/Smad4 complex co-relocates to the nucleus and modulates gene transcription (Lee et al., 2010; Chang et al., 2012; Hong et al., 2013). This normal

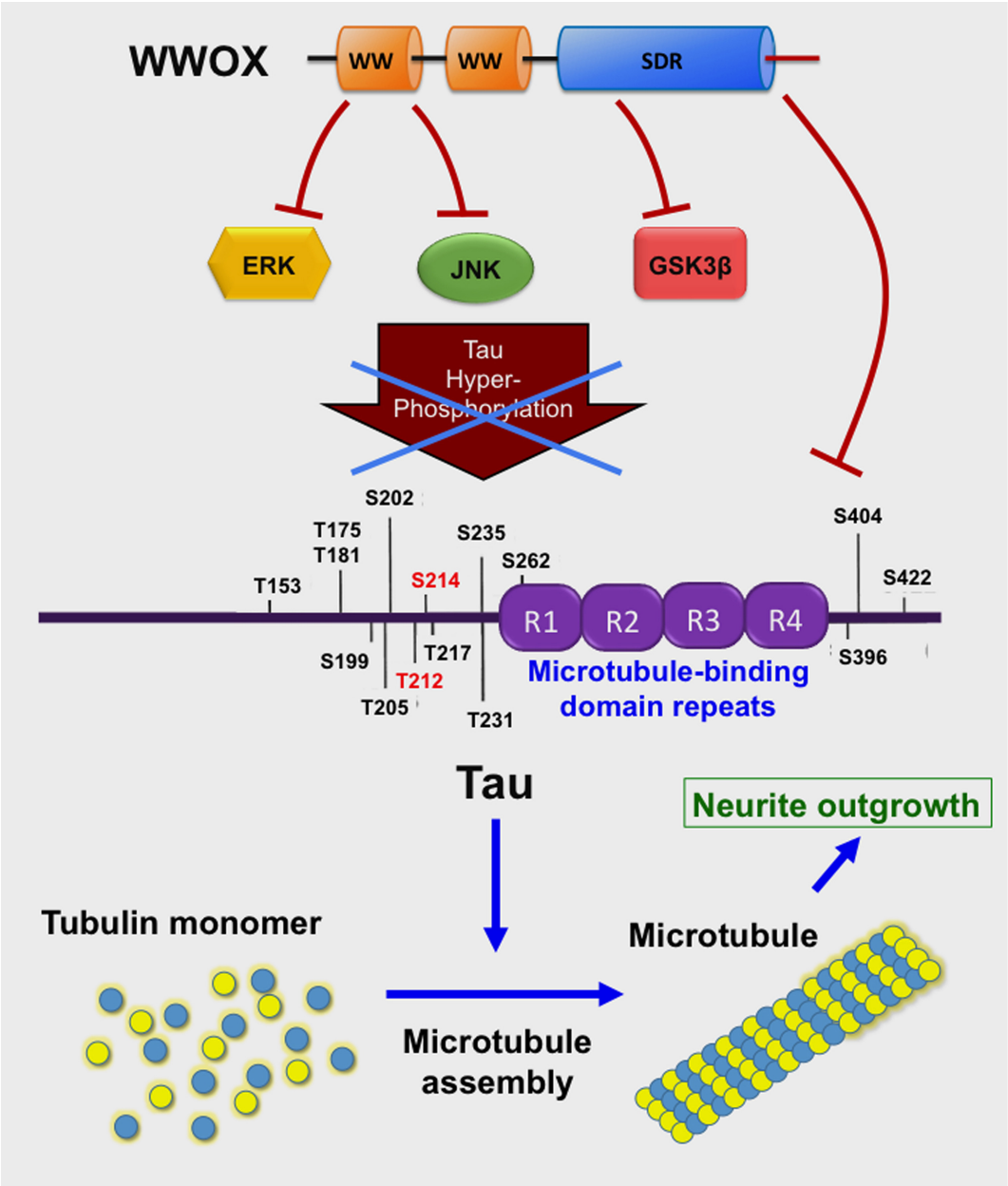

FIGURE 3 | WWOX limits Tau hyperphosphorylation and aggregation. The *C*-terminal SDR domain of WWOX physically binds GSK3β preventing hyperphosphorylation of Tau (Sze et al., 2004; Wang et al., 2012). Also, the first WW domain binds JNK, thereby preventing Tau hyperphosphorylation (Sze et al., 2004). The first WW domain binds ERK (Huang et al., 2016). Tau protein supports polymerization of tubulin monomers to assemble microtubules, which are needed for neurite outgrowth (Wang et al., 2012).

signaling event does not cause protein aggregation. However, under aberrant signaling, TGF-β1 causes TIAF1 aggregation and reduces its binding with membrane APP, thus leading to APP de-phosphorylation at Thr688 and then degradation and production of amyloid β monomer, intracellular domain of the APP intracellular domain (AICD), and amyloid fibrils (Henriques et al., 2009; Lee et al., 2010; Chang et al., 2012; Hong et al., 2013). Presence of aggregated TIAF1 in the peritumor coats of metastatic brain tumor cells does not cause cancer cell death (Lee et al., 2010; Chang et al., 2012; Hong et al., 2013). However, the coat-associated TIAF1 aggregates are cytotoxic to neurons (Lee et al., 2010).

TRAPPC6AΔ Protein Aggregation Is Upstream of TIAF1

We have identified a TGF-β-induced trafficking protein particle complex 6A (TRAPPC6A or TPC6A) (Chang and Chang, 2015;

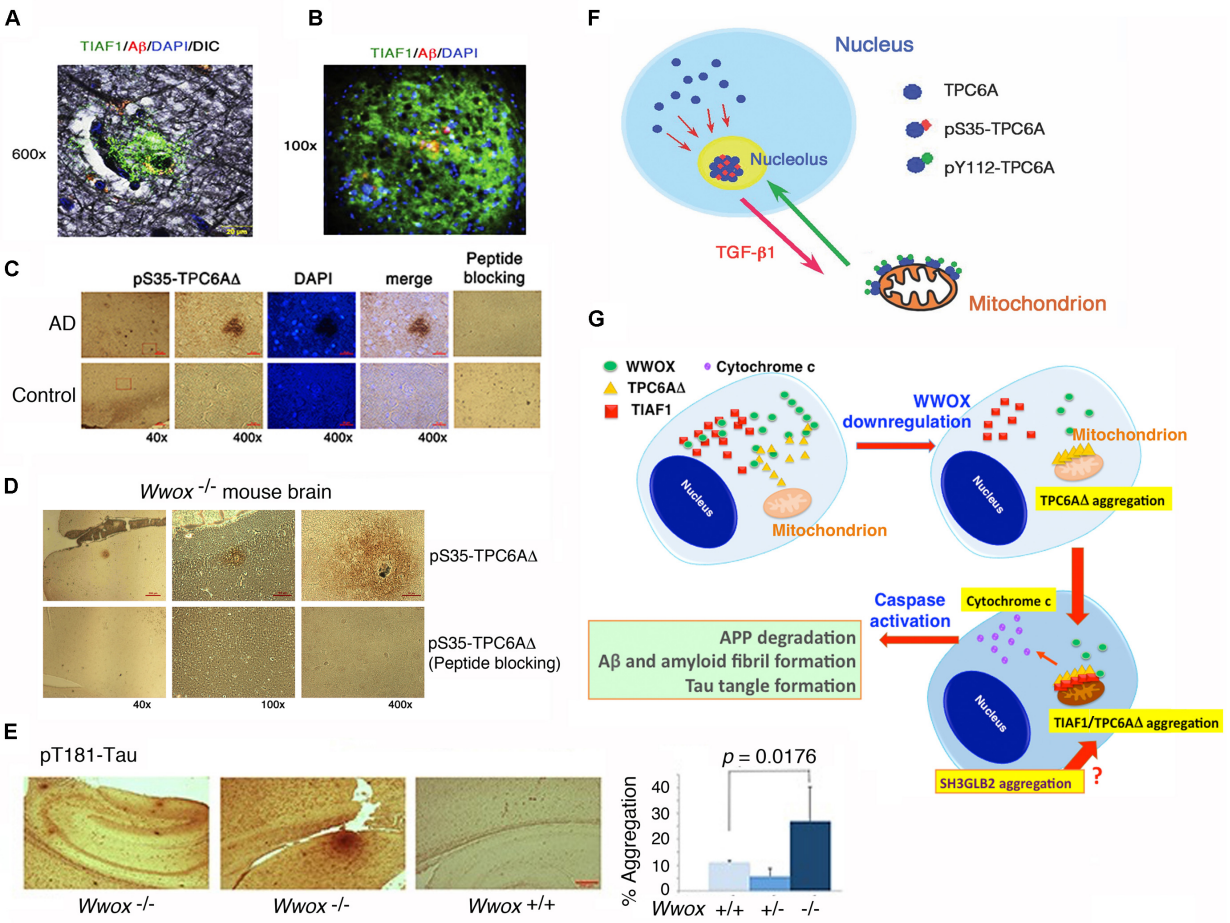

FIGURE 4 | TPC6AΔ and TIAF1 in a cascade of protein aggregation and WWOX blocks the aggregation. **(A)** Representative human AD hippocampal tissue sections were pre-stained with Bielschowsky stain, followed by staining with specific antibody against TIAF1 (green), and Aβ (red) and DAPI for nuclei. A representative confocal image of a plaque is shown (Lee et al., 2010). **(B)** Shown is a TIAF1-containing plaque from a hippocampal section of a 9-month-old APP/PS1 transgenic mouse, containing Aβ aggregates in the center (Lee et al., 2010). **(C)** In representative human brain cortical tissue sections from AD patients and age-matched controls, a pS35-TPC6AΔ-containing plaque is shown. In negative controls, the immunizing peptide blocks the immunoreactivity (Chang et al., 2015). **(D,E)** Presence of pS35-TPC6AΔ and pT181-Tau aggregates is shown in the cortex and hippocampus of 3-week-old *Wwox* knockout mice (Chang et al., 2015). **(F)** Endogenous TPC6A and TPC6AΔ shuttle between nucleoli and mitochondria. Ser35 phosphorylation supports shuttling from the nucleus to the nucleolus, and Tyr112 phosphorylation is needed for translocation from the nucleolus to the mitochondrion (Chang and Chang, 2015). **(G)** Upon WWOX downregulation, a sequential protein aggregation cascade occurs. When WWOX level is reduced, pS35-TPC6AΔ starts to polymerize and recruit pS37-TIAF1 for further polymerization and accumulation in the outer membrane of mitochondria (Chang and Chang, 2015; Chang et al., 2015). The aggregated pS35-TPC6AΔ and pS37-TIAF1 cause caspase 3 activation and cytochrome c release. The activated caspase 3 leads to APP degradation and formation of Aβ and amyloid fibrils and Tau tangles. SH3GLB2 aggregation (Lee et al., 2017) occurs probably right after that of pS37-TIAF1. (All data are adapted with revisions in art work from Lee et al., 2010; Chang and Chang, 2015; Chang et al., 2015, under the guidelines of the publishers).

Chang et al., 2015). *TRAPPC6A*/*Trappc6a* gene is associated with skin pigment formation in mice (Gwynn et al., 2006), AD in humans (Hamilton et al., 2011), and other neural diseases (Mohamoud et al., 2018). An intra-*N*-terminal deletion isoform of TRAPPC6A, designated TRAPPC6AΔ or TPC6AΔ, tends to spontaneously form aggregates or plaques in the extracellular matrix of the hippocampi of postmortem middle-aged normal humans and older AD patients (**Figure 4C**) and 3-week-old *Wwox* gene knockout mice (**Figure 4D**) (Chang and Chang, 2015; Chang et al., 2015). Presence of pT181-Tau, a marker for tau phosphorylation and aggregation in mice, is also shown in the cortex of *Wwox* knockout mice, but is barely detectable in the wild type and heterozygous *Wwox* mice (**Figure 4D**). Conceivably, without WWOX, cellular proteins tend to undergo aggregation.

TPC6A aggregates are also present in the human brain cortex and hippocampus, which are ∼50 and 40% positive, respectively, for both control (59 ± 17 years old; n = 42) and AD (80 ± 8.8 years old; n = 96) groups (Chang et al., 2015), suggesting that the aggregated proteins are stable and hard to undergo degradation with age. In comparison, protein aggregates for pY33-WWOX are significantly reduced by ∼40% in the AD samples, compared to non-demented controls (Chang et al., 2015). Again, compared with the non-demented controls, tangled

tau and Aβ aggregates are significantly increased in the AD samples (Chang et al., 2015). If our observations hold true, TPC6AΔ/TIAF1 starts polymerization in the middle age, and takes at least 10–40 years to generate significant amounts of tau and amyloid β protein aggregates for clinically defined AD symptoms.

We have recently determined that endogenous TPC6A undergoes a novel mitochondrion-nucleolus shuttling (**Figure 4F**) (Chang and Chang, 2015). TGF-β1 causes nuclear TPC6A to undergo Ser35 phosphorylation, followed by entering the nucleoli and then relocating to the mitochondria as a dimer, which probably requires phosphorylation at Tyr112. The mitochondrial TPC6A shuttles back to the nucleolus. TPC6A carries WWOX to the nucleus.

TPC6AΔ protein possesses an internal frame deletion of amino acids #29–42 at the *N*-terminus. Wild type TPC6A is less likely to undergo aggregation. Both TPC6A and TPC6AΔ proteins are able to shuttle between nuclei and mitochondria (Chang and Chang, 2015; Chang et al., 2015). Under aberrant signaling, TPC6AΔ molecules are accumulated as aggregates in the mitochondria, where TIAF1 binds TPC6AΔ. Both proteins induce caspase activation and apoptosis (**Figure 4G**) (Chang and Chang, 2015; Chang et al., 2015). A BAR domain-containing SH3GLB2 (SH3 Domain Containing GRB2 Like, Endophilin B2) is a potential downstream protein for aggregation via direct binding with TIAF1 (Pierrat et al., 2001) (**Figure 4G**). Aggregation of SH3GLB2 can be found in the brain cortex and hippocampus (Lee et al., 2017).

WWOX Controls TRAPPC6AΔ, TIAF1, and Tau Aggregation *in vivo*: Effect of TGF-β

The WWOX physically binds TPC6AΔ, TIAF1, and Tau and prevents their aggregation (Lee et al., 2010; Chang and Chang, 2015; Chang et al., 2015; Sze et al., 2015). TGF-β induces the dissociation between WWOX and TIAF1, or TPC6AΔ. The dissociated TPC6AΔ/TIAF1 aggregates cause caspase activation, APP degradation, and ultimately formation of amyloid β (Lee et al., 2010; Chang and Chang, 2015; Chang et al., 2015). Similarly, when WWOX protein expression is downregulated, TPC6AΔ polymerizes first and then binds TIAF1 to induce further polymerization (Chang and Chang, 2015; Chang et al., 2015; Sze et al., 2015) (**Figure 4G**).

Also, knockdown of WWOX by small interfering RNA (siRNA) induces spontaneous aggregation of TPC6AΔ and TIAF1 *in vitro*. Knockdown of TPC6AΔ fails to cause TIAF1 aggregation (Chang and Chang, 2015), suggesting that TPC6AΔ aggregates first, followed by TIAF1 aggregation. Collectively, when WWOX is significantly downregulated, TPC6AΔ becomes phosphorylated at Ser35 and forms aggregates in the nucleus, followed by relocating to the mitochondria to bind TIAF1 and both proteins become aggregated (Chang and Chang, 2015; Sze et al., 2015) (**Figure 4G**). Thus, one line of *in vitro* evidence reveals that without WWOX, the TPC6AΔ/TIAF1 aggregates cause formation of extracellular amyloid β and intracellular Tau aggregates (Lee et al., 2010; Chang and Chang, 2015; Chang et al., 2015). Further, *in vivo* evidence revealed that when *Wwox* gene is knocked out in mice, aggregation of TIAF1, TPC6AΔ, amyloid β, Tau, and many other proteins occurs in the brains in less than 3 weeks (Chang and Chang, 2015; Chang et al., 2015) (**Figures 4A–E**). Taken together, WWOX plays a role in limiting protein aggregation *in vivo*.

WWOX Phosphorylation at Ser14 and Its Potential Role in Neurodegeneration

Site-specific WWOX phosphorylation is associated with cell differentiation and many other events (Huang et al., 2016; Huang and Chang, 2018). During forced cell differentiation, WWOX rapidly undergoes phosphorylation at Ser14 in leukemia cells (Huang et al., 2016; Huang and Chang, 2018) and in diseased organs (Lee et al., 2017). pS14-WWOX does not cause apoptosis. In contrast, overly expressed pY33-WWOX induces apoptosis (Chang et al., 2007). It suggests that the levels of pS14-WWOX and pY33-WWOX must be in a good balance *in vivo*. Under stress conditions, WWOX is phosphorylated at Tyr33 to induce apoptosis. During cell differentiation or disease progression (e.g., AD), WWOX is phosphorylated at Ser14 (Huang and Chang, 2018).

Ten-month-old triple transgenic (3xTg) mice for AD develop memory loss probably due, in part, to accumulated aggregates of TPC6AΔ, SH3GLB2, tau and Aβ, along with inflammatory NF-κB activation, in the hippocampal and cortical areas (Lee et al., 2017). Notably, significantly increased phosphorylation of WWOX at Ser14, but not Tyr33, is shown in their brain lesions (Lee et al., 2017). Zfra blocks Ser14 phosphorylation in WWOX, significantly reduces accumulation of TPC6AΔ, SH3GLB2, tau and Aβ aggregates, suppresses NF-κB activation, and restores memory in these mice (Lee et al., 2017). *In vitro* analysis showed that Zfra binds cytosolic proteins for accelerating their degradation in ubiquitin/proteasome-independent manner (Lee et al., 2017).

B16F10 melanoma-growing nude mice develop neuronal death in the hippocampus, amyloid plaque formation in the cortex, and melanoma infiltration in the lung in less than 2 months (Lee et al., 2017). Zfra inhibits pS14-WWOX expression in the lung and brain lesions, clears up cortical plaques, and thereby suppresses cancer growth and neuronal death (Lee et al., 2017). Together, WWOX phosphorylation at Ser14 supports the progression of neurodegeneration in the hippocampus and plaque formation in the cortex, as well as cancer progression (Huang and Chang, 2018).

Is WWOX a Molecular Chaperone?

WWOX retards neurodegeneration pathology by binding and blocking GSK-3β, ERK, JNK and probably other kinases and enhancing neurite outgrowth and neuronal differentiation (Sze et al., 2004; Wang et al., 2012). WWOX probably functions as a protein chaperone to prevent protein misfolding and degradation by the ubiquitin/proteasome system. Under stress conditions, activated WWOX with Tyr33 phosphorylation binds p53, and both proteins work synergistically to induce apoptosis (Chang et al., 2005a). Without binding, p53 relocates to the cytoplasm

and undergoes degradation (Chang et al., 2005a). It has been proposed that the second WW domain of WWOX is an orphan module devoid of ligand binding function but is a chaperone necessary to stabilize the first WW domain in conducting protein/protein interactions (Farooq, 2015).

SEX STEROID HORMONES IN NEUROPROTECTION

Sex steroid hormones are decreased in menopause women and aged men. Deficiency of 17-β-estradiol (E2), a major form of estrogens, is implicated in age-related cognitive decline in human and non-human primates. Estrogens modulate hippocampal synaptic spine growth, structural plasticity, and neuronal excitability, which affect long-term potentiation in learning and memory (Teyler et al., 1980; Brinton, 1993; Warren et al., 1995; Engler-Chiurazzi et al., 2016; Muñoz-Mayorga et al., 2018).

Decreased serum sex steroid hormone levels in postmenopausal women or in aged men increase the risk for developing NDs. Participation of steroid sex hormones in neuroprotection through the interaction of E2 and estrogen receptors (ER) during brain injury and neurodegeneration has been extensively investigated and very well reviewed (Brann et al., 2007; Arevalo et al., 2015; Engler-Chiurazzi et al., 2016).

There are two classes of ERs, namely nuclear and membrane receptors. Upon stimulation with estrogens, ERα and ERβ translocate to the nucleus, bind chromosomal DNA, and function as transcription factors (Shang et al., 2000; Safe and Kim, 2008; Carroll, 2016). Membrane estrogen receptors (mERs) are mostly G protein-coupled receptors and are responsible for transducing signals upon stimulating with an estrogen. Known mERs are GPR30, ER-X, and G_q-mER. During signaling, ERα and ERβ translocate to the nucleus and bind estrogen-responsive elements (EREs) in the promoter regions of specific genes to recruit transcriptional co-activators and co-repressors to control gene transcription (Shang et al., 2000; Safe and Kim, 2008; Carroll, 2016). Alternatively, ERs act as transcriptional partners at non-ERE sites. ERs are also associated with plasma membrane lipid rafts to bind neurotransmitters and proteins, which drives the growth factor receptor signaling to interact with other neuroprotective signaling pathways or elicit redundant neuroprotection signaling (e.g., PI3K-AKT, ERK1-ERK2, and JAK-STAT3) (Ramírez et al., 2009; Arevalo et al., 2015).

ER Protective Signaling Pathways

Regarding the protective signaling pathways, ERs activate the ERK and PI3K signaling cascades, which leads to the inhibition of pro-apoptotic JNK signaling and thereby protects neural tissues from damages (Mannella and Brinton, 2006; Jover-Mengual et al., 2010; Tang et al., 2014). E2/ER signaling suppresses apoptosis by upregulating antiapoptotic Bcl-2 and family proteins, and downregulating proapoptotic Bcl-2 family members (**Figure 5**) (Koski et al., 2004; Yao et al., 2007). E2 activates PI3K via ERα and mERs. Phosphorylated PI3K activates Akt, followed by Akt phosphorylating GSK-3β at Ser9 to decrease GSK-3β activity (**Figure 5**) (Cardona-Gomez et al., 2004; Ruiz-Palmero et al., 2013). The inhibition of GSK-3β activity is a common mechanism of neuroprotection by several factors including WWOX (Cardona-Gomez et al., 2004; Sze et al., 2004; de Paula et al., 2009; Perez-Alvarez et al., 2012; Wang et al., 2012). GSK-3β inhibition-induced neuroprotection also involves β-catenin, which is regulated by E2 through the ERα/PI3K/AKT/GSK3β signaling pathway (**Figure 5**) (de Paula et al., 2009; Perez-Alvarez et al., 2012). Together, these observations suggest that E2/ERs-mediated neuroprotection is mediated through the interactions of ERs with the pathways induced by different neuroprotective signaling pathways or the protective effects of growth factors.

WWOX AS A RECEPTOR FOR SEX STEROID HORMONES FOR SIGNALING

The WWOX is a potential cytosolic or membrane receptor for sex steroid hormones (Chang et al., 2005b; Su et al., 2012). WWOX is highly expressed in hormone- or enzyme-secreting organs. WWOX is most abundant in the ductal epithelial cells such as in the breast and prostate. WWOX controls the growth and progression of breast and prostate cancers (Bednarek et al., 2000; Chang et al., 2005b; Nunez et al., 2005; O'Keefe et al., 2011). Loss of WWOX accelerates cancer growth and metastasis. The SDR domain of WWOX is associated with aerobic metabolism and control of the generation of reactive oxygen species (O'Keefe et al., 2011; Choo et al., 2015), which is crucial in limiting the progression of neurodegeneration (Su et al., 2012; Chang et al., 2014).

Estrogens and Androgens Bind the SDR Domain of WWOX

Estrogens or androgens bind the NSYK (Asn-Ser-Tyr-Lys) motif in the *C*-terminal SDR domain of WWOX (Chang et al., 2005b; Su et al., 2012). This binding causes nuclear accumulation of activated or Tyr33-phosphorylated WWOX (pY33-WWOX) (Chang et al., 2005b; Su et al., 2012). Excessive accumulation of pY33-WWOX in the nucleus induces apoptosis. Notably, estrogen or androgen-mediated WWOX activation is independent of ERs or androgen receptor (AR), suggesting that WWOX by itself acts as a receptor (Chang et al., 2005b; Su et al., 2012). WWOX expression is significantly upregulated during the early stage of normal prostate and breast tissue progression toward hyperplasia and cancerous stages (Chang et al., 2005b). Upon reaching metastatic stage, cancer cells do not express WWOX due, in part, to hypermethylation at the promoter region.

Indeed, the expression levels of WWOX positively correlate with the hormone receptor status, but negatively correlate with the clinical stages of breast and ovarian cancers (Chang et al., 2005b; Guler et al., 2011). Loss of WWOX confers resistance to tamoxifen due to upregulation of ER and human epidermal growth factor receptor 2 (Her2) and their transcriptional activities (Guler et al., 2007; Salah et al., 2010). Tamoxifen is one of the estrogen receptor modulators, which regulates hormone-secreting tissue activities for treatment and prevention

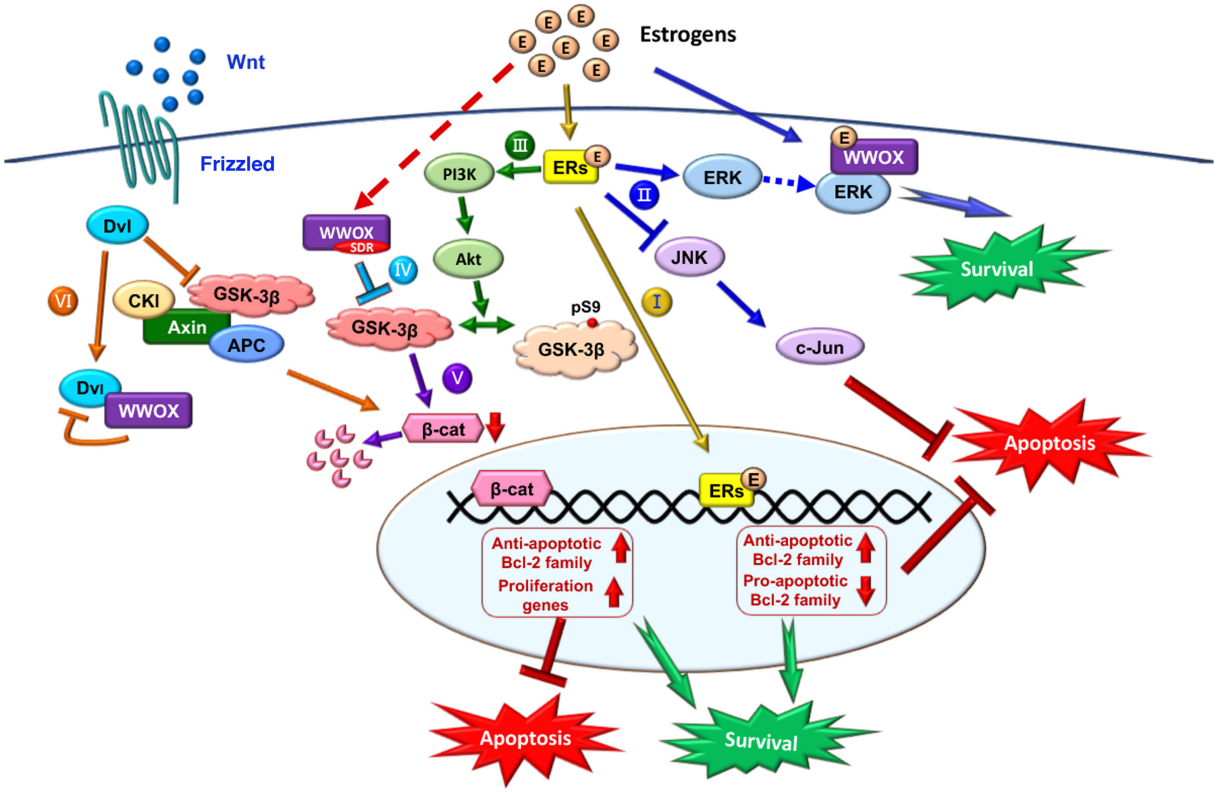

FIGURE 5 | Role of E2/ER/WWOX in initiating protective pathways. The pathways include: Route I, E2/ER-mediated upregulation of antiapoptotic Bcl-2 family proteins, and downregulation of proapoptotic Bcl-2 family members (Yao et al., 2007) (see the route in yellow line). Route II, Activation of the pro-survival ERK/WWOX and PI3K/Akt signaling cascades to block the pro-apoptotic JNK signaling and protect the neural tissues from damages (Tang et al., 2014) (route in blue). Route III, E2 activates PI3K via ERα and mERs, followed by activating Akt to phosphorylate GSK-3β at Ser9 for functional inactivation (Ruiz-Palmero et al., 2013) (route in green). Route IV, The SDR domain of WWOX binds and limits GSK-3β activity for neuroprotection (Wang et al., 2012) (route in light blue). Route V, Suppression of GSK-3β (e.g., by WWOX) leads to a reduced β-catenin degradation, which is regulated by E2 through the ERα/PI3K/AKT/GSK-3β signaling pathway (Perez-Alvarez et al., 2012) (route in purple). Route VI, In the Wnt/Frizzled signaling pathway, Wnt protein induces the activation of Dvl to block the activity of GSK-3β. Without Wnt, β-catenin is subjected to destruction by the complex of axin, APC, CK1α, and GSK-3β (Bouteille et al., 2009). Transiently overexpressed WWOX binds Dvl to suppress the Wnt signaling (Bouteille et al., 2009) (route in orange).

of ER-positive cancers. Together, these observations suggest WWOX functions as an enzyme or a receptor involved in sex steroid metabolism to modulate disease progression.

Crosstalk of ERs, WWOX, and Wnt Signaling

Shown in the **Table 1** is a comparison between WWOX and ERs/mERs regarding their molecular structures, actions and potential mechanisms. WWOX is involved in many signal pathways (Chang et al., 2007, 2010, 2014; Chang, 2015; Huang and Chang, 2018), and this allows its crosstalk with the signaling from ERs and mERs. For example, ERs activate the ERK1/2 and PI3K signaling cascades (Mannella and Brinton, 2006; Jover-Mengual et al., 2010; Tang et al., 2014), and that WWOX physically binds ERK1/2 for supporting cell survival (Lin et al., 2011) (**Figure 5**) and lymphocyte differentiation (Huang et al., 2016).

During the signaling, E2/ER upregulates the antiapoptotic Bcl-2 family proteins and downregulates the proapoptotic Bcl-2 family members (Yao et al., 2007) (Route I, **Figure 5**). Also, E2/ER-mediated activation of the ERK and PI3K/Akt signaling cascades suppresses the JNK signaling for neuroprotection (Tang et al., 2014) (Route II, **Figure 5**).

Also, E2/ER activates PI3K and Akt to inactivate GSK-3β by phosphorylating GSK-3β at Ser9 (Ruiz-Palmero et al., 2013) (Route III, **Figure 5**). WWOX via its SDR domain binds and limits GSK-3β activity (Wang et al., 2012) (Route IV, **Figure 5**), and this leads to a reduced β-catenin degradation regulated by E2 via the ERα/PI3K/AKT/GSK-3β signaling pathway (Perez-Alvarez et al., 2012) (Route V, **Figure 5**). GSK-3β initiates the proteasomal degradation of β-catenin by phosphorylating β-catenin on key residues (**Figure 5**). In addition, nuclear GSK-3β binds β-catenin, without causing β-catenin phosphorylation and degradation, but reduces the activity of β-catenin/TCF-dependent transcription via GSK-3β-Axin binding (Caspi et al., 2008). GSK-3β inhibits competitive phosphorylation of β-catenin and hence facilitates the function of β-catenin, thus enabling cells with phosphorylated Tau to escape apoptosis (Li et al., 2007).

TABLE 1 | Functional comparison between ER and WWOX in neurodegeneration.

		Estrogen receptor	WWOX
Gene		ERα-6q25.1 (Hattori et al., 2016); ERβ-14q23.2 (Al-Nakhle et al., 2013)	16q23 (Bednarek et al., 2000; Ried et al., 2000)
Localization in cell		Plasma membrane, cytosol, mitochondria, nucleus. (Shang et al., 2000; Safe and Kim, 2008; Tang et al., 2014)	Plasma membrane, cytosol, mitochondria, nucleus. (Chang et al., 2007; Hsu et al., 2009, 2016)
Role in embryonic development		ER impact on the development of reproductive system, liver, muscle, pancreas, blood, and brain. (Bondesson et al., 2015)	Loss of WWOX related to defects in bone formation and steroidogenesis, and abnormal neural and sexual development. (Ludes-Meyers et al., 2009; Abu-Remaileh and Aqeilan, 2015)
Role in neural system	Alzheimer's disease	Inhibition of Tau hyperphosphorylation by activating PI3K/Akt to reduce GSK3β activity. (Cardona-Gomez et al., 2004; Ramírez et al., 2009; Arevalo et al., 2015)	Down-regulation of Tau phosphorylation through interacting with GSK-3β. (Sze et al., 2004; Chang et al., 2007; Wang et al., 2012)
	Injury	As a pro-survival factor by up-regulating Wnt/β-catenin signaling, PI3 kinase/Akt signaling, anti-apoptotic Bcl-2, and inhibiting microglia activation. (Dubal et al., 1999; Quesada et al., 2008; Zhang et al., 2008; Perez-Alvarez et al., 2012)	As a pro-apoptotic factor through interacting with CREB, JNK, NF-κB, p53. (Chang et al., 2007; Li et al., 2009)
Metabolic syndromes		Aberrant ERs related to imbalance of energy metabolism, homeostasis of lipid and glucose, distribution of fat, and type II diabetes. (Brann et al., 2007; Hevener et al., 2015; Zeng et al., 2016)	Deficiency in WWOX associated with hypertension, hypoglycemia, hypocalcemia, metabolic acidosis, type II diabetes, and homeostasis of lipid and steroids. (Chang et al., 2005b, 2007; Nunez et al., 2005; Ludes-Meyers et al., 2009; O'Keefe et al., 2011; Chang et al., 2014)

The Wnt signaling pathway modulates amyloid β peptide-mediated neuropathology in AD by inactivating GSK-3β, which in turn prevents Tau phosphorylation (Bhat and Budd, 2002; Boonen et al., 2009) (Route VI, **Figure 5**). Wnt protein induces the activation of Dvl in the Wnt/β-catenin signaling to block the activity of GSK-3β. In the absence of Wnt ligand, β-catenin is destructed by the complex of axin, APC, CK1α, and GSK-3β (Bhat and Budd, 2002; Boonen et al., 2009). Ectopic WWOX sequesters cytosolic disheveled family protein (Dvl) and thus inhibits the Wnt/β-catenin pathway (Bouteille et al., 2009; Huang and Chang, 2018). WWOX suppresses the c-Jun transcriptional activity and cAMP response element-binding protein (CREB) function, which is associated with amyloid depositions (Savage et al., 2002; Li et al., 2009).

Estrogen in the pY33-WWOX/pS15-p53 Complex

Catechol estrogens have been shown to covalently conjugate with serum proteins from diabetic patients – the so-called "estrogenization" (Ku et al., 2016). When insulin is estrogenized, its receptor-binding pocket is blocked, thus resulting in functional blockade (Ku et al., 2016). The conserved NSYK motif in the SDR domain of WWOX is capable of interacting with androgens and estrogens and other proteins (Chang et al., 2007; Su et al., 2012). At micromolar levels, exogenous E2 binds WWOX and induces activation of both WWOX and p53 via phosphorylation at Tyr33 and Ser15, respectively, in COS7 fibroblasts (Chang et al., 2007; Su et al., 2012). Excessive accumulation of the E2/pY33-WWOX/pS15-p53 complex in the nucleus results in cell death (Chang et al., 2007; Su et al., 2012). JNK1 blocks the apoptotic function of overly expressed WWOX (Chang et al., 2003a).

PERSPECTIVES

A Focus on WWOX and Protein Aggregation in Middle Age

Both aggregated tau and Aβ are considered as the key pathological markers of AD, and have been the center of focus for drug development over the past several decades. Aggregated tau and Aβ are usually found in the brain of AD patients over 70 years old while normal individuals from 40–70 years old possess very low amounts of aggregated tau and Aβ. We have determined the presence of aggregated proteins such as TPC6AD and TIAF1 in approximately 50% of the brains of mid-aged normal humans (Chang and Chang, 2015; Chang et al., 2015; Sze et al., 2015). Indeed, WWOX downregulation causes self-aggregation of TPC6AD and TIAF1 *in vitro* (Lee et al., 2010; Chang and Chang, 2015; Chang et al., 2015). *Wwox* gene knockout mice rapidly exhibit aggregation of many proteins in the brains just in 15 days after birth. These proteins include TPC6AD, TIAF1, and SH3GLB2, tau and Aβ (Lee et al., 2017). Notably, human newborns with *WWOX* deficiency rapidly develop severe neural diseases, metabolic disorders, retarded growth and early death. While TRAPPC6AΔ and TIAF1 are starters for protein aggregation, these proteins are indeed potential targets for drug development. Development of therapeutic peptides and humanized monoclonal antibodies is under way.

Zfra Initiates a Novel Immune Response to Block Protein Aggregation and Restores Memory Loss

Zfra restores memory deficits in Alzheimer's disease triple-transgenic mice by blocking the aggregation of TPC6AΔ, SH3GLB2, Tau, and amyloid β, and reducing inflammatory

NF-κB activation (Lee et al., 2017). As a WWOX-binding protein, exogenous Zfra peptide, when introduced in the circulation in mice, is mainly deposited in the spleen. Zfra binds membrane hyaluronidase Hyal-2 in non-T/non-B Z lymphocytes. Z cells then become activated to suppress cancer growth (Lee et al., 2015). Intriguingly, Z cells exhibit a memory function in killing cancer cells, even though these cells have never exposed to the cancer cells. Autologous Z cells, once activated by Zfra, are of great therapeutic use in treating cancer and probably neurodegeneration such as AD. Both full-length Zfra and a truncated 7-amino-acid Zfra4-10 are effective in suppressing cancer growth (Lee et al., 2015) and restoring memory loss (Lee et al., 2017). Since Zfra is stably retained on the Z cell surface, Zfra activates the Hyal-2/WWOX/Smad4 signaling in Z cells. Peptides or monoclonal antibodies are being developed to target membrane Hyal-2 as well as WWOX and to activate Z cells in blocking cancer and neurodegeneration.

A pTyr33-WWOX Peptide as an Agent for Blocking Neuronal Injury and Death

Finally, an 11-amino-acid phospho-Try33 WWOX peptide was developed to block neurotoxin MPP+-induced neuronal death in the brain (Lo et al., 2008). This phospho-peptide effectively suppresses neuronal death via inhibition of JNK1 activation. In controls, non-phospho-WWOX peptide has no effect. The phospho-Try33 WWOX peptide is now being tested for its efficacy in blocking neuronal death in AD and traumatic brain injury.

AUTHOR CONTRIBUTIONS

C-CL and C-CT carried out the literature review. Y-AC, C-CL, P-CH, and N-SC prepared schematic graphs. C-HC reviewed and revised the manuscript. C-IS and N-SC wrote the manuscript. N-SC completed the final version and provided rebuttal letters to all reviewers. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00563/full#supplementary-material

TABLE S1 | Functional comparison between ER and WWOX in neurodegeneration.

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REVIEW
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Smurfs in Protein Homeostasis, Signaling, and Cancer

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Protein ubiquitination is an evolutionary conserved highly-orchestrated enzymatic cascade essential for normal cellular functions and homeostasis maintenance. This pathway relies on a defined set of cellular enzymes, among them, substrate-specific E3 ubiquitin ligases (E3s). These ligases are the most critical players, as they define the spatiotemporal nature of ubiquitination and confer specificity to this cascade. Smurf1 and Smurf2 (Smurfs) are the C2-WW-HECT-domain E3 ubiquitin ligases, which recently emerged as important determinants of pivotal cellular processes. These processes include cell proliferation and differentiation, chromatin organization and dynamics, DNA damage response and genomic integrity maintenance, gene expression, cell stemness, migration, and invasion. All these processes are intimately connected and profoundly altered in cancer. Initially, Smurf proteins were identified as negative regulators of the bone morphogenetic protein (BMP) and the transforming growth factor beta (TGF-β) signaling pathways. However, recent studies have extended the scope of Smurfs' biological functions beyond the BMP/TGF-β signaling regulation. Here, we provide a critical literature overview and updates on the regulatory roles of Smurfs in molecular and cell biology, with an emphasis on cancer. We also highlight the studies demonstrating the impact of Smurf proteins on tumor cell sensitivity to anticancer therapies. Further in-depth analyses of Smurfs' biological functions and influences on molecular pathways could provide novel therapeutic targets and paradigms for cancer diagnosis and treatment.

Keywords: Smurf1, Smurf2, ubiquitination, protein degradation, cell signaling, cancer

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Abbreviations: aa, amino acids; AR, Androgen receptor; BMP, Bone morphogenetic protein; BMPRI, Bone morphogenetic protein receptor, type 1; β-TrCP, β-transducing repeat containing protein; ccRCC, Clear cell renal cell carcinoma; CDC25A, Cell division Cycle 25A; COSMIC, Catalog of Somatic Mutations in Cancer; Cys, Cystein; DAB2IP, Disabled homolog 2-Interacting Protein; DDR, DNA damage response; E3s, E3 ubiquitin ligases; EGFR, Epidermal growth factor receptor; EMT, Epithelial-mesenchymal transition; ERα, Estrogen receptor alpha; EZH2, Enhancer of zeste homolog 2; GAP, GTPase-activating protein; GC, Gastric cancer; Gly, Glycine; GSK3β, Glycogen synthase kinase 3 beta; GTPase, Guanosine triphosphatases; H2B, Histone H2B; HECT, Homologous to E6-AP carboxyl terminus; HGPS, Hutchinson-Gilford progeria syndrome; HSP27, Heat shock protein 27; ID1, DNA-binding protein inhibitor ID1; ID3, DNA-binding protein inhibitor ID3; I-Smad, Inhibitory Smads; JNK, c-Jun N-terminal protein kinase; KLF5, Krüppel-like factor 5; KRAS, Kirsten rat sarcoma protein; LOH, Loss of heterozygosity; MAD2, Mitotic spindle assembly checkpoint protein; MAPK, Mitogen-activated protein kinase; MAPK, Mitogen activated protein kinase; MLC2, Myosin light chain 2; MEK, Mitogen-activated protein kinase kinase; MEKK2, Mitogen-activated protein kinase kinase kinase 2; MET, Mesenchymal-epithelial transition; NEDD4, Neural precursor cell expressed developmentally down-regulated protein 4; PIAS3, Protein inhibitor of activated STAT3; PKCζ, Protein kinase C zeta; PRC2, Polycomb repressive complex 2; R-Smad, Receptor regulated Smad; RANKL, Receptor activator of nuclear factor kappa-B ligand; RBR, Ring-in-Between-RING fingers; REST, RE1 silencing transcription factor; RhoA, Ras homolog gene family, member A; RING, Really interesting new gene; RNF20, Ring Finger Protein 20; SCF, Skp1–Cullin–F-box proteins; Smurf, Smad ubiquitin regulatory factor; TGF-β, Transforming growth factor beta; TGF-βRI, Transforming growth factor beta receptor I; TNM, Tumor node metastasis; Topo Iiα, DNA Topoisomerase 2 alpha; ubH2B, monoubiquitinated histone H2B; WWP1, WW domain containing E3 ubiquitin protein ligase 1; WWP2, WW domain containing E3 ubiquitin protein ligase 2; YY1, Yin Yang 1.

INTRODUCTION

Protein ubiquitination is a major posttranslational modification that controls a wide spectrum of biological functions, and is critical in maintaining cellular homeostasis under physiological conditions and in diseases.

Ubiquitination is a multi-step enzymatic process which is mediated by the concerted action of three main types of proteins: (i) ubiquitin-activating enzymes (E1s), which bind, adenylate and activate cognate ubiquitin molecules using the energy of ATP hydrolysis; (ii) ubiquitin-conjugating enzymes (E2s), which accept ubiquitin from E1 in the form of a thioester bond to their active-site cysteine; and (iii) ubiquitin protein ligases (E3s) that recruit ubiquitin-charged E2 enzymes and mediate specific transfers of ubiquitin to protein substrates.

It is estimated that the human genome encodes for more than 630 E3s, ∼40 E2s, and only two E1s. E3 ubiquitin ligases are of particular interest since they define the spatio-temporal nature of ubiquitination and, together with other accessory proteins, provide specificity to the cascade.

E3s tightly control protein stability, localization, and function, and thereby regulate a plethora of biological processes. This has instigated intensive investigations of these enzymes as disease biomarkers and drug targets in a variety of human disorders, particularly in cancer (1–3).

Depending on the ubiquitin transfer mechanism and domain characteristics, E3s are classified into three main groups/families: really interesting new gene (RING) family, which is the most abundant in the human genome (∼600 family members), homologous to the E6AP carboxyl terminus (HECT) domain E3s (∼30 members), and RING-in-between-RING (RBR) E3s (∼12 in humans) (4).

Smurf1 and Smurf2 (Smurfs) are two closely related C2-WW-HECT domain E3 ubiquitin ligases, belonging to the NEDD4 subfamily of HECT type E3s. Similar to other NEDD4 family members (nine in total), Smurfs contain: (i) the N-terminal C2 domain, which mediates binding of these E3s to intracellular membranes; (ii) several tryptophan-containing WW domains, which are thought to mediate the protein-protein interactions between the E3s and their interactors and substrates (primarily through association with proline-containing PPxY or LPxY motifs in the binding partners); and (iii) the evolutionary-conserved catalytic HECT domain. Of note, several studies indicate that the HECT domain of NEDD4 E3s are also involved in substrate recognition (5–7).

In mammals, Smurf1 and Smurf2 are encoded by two distinct genes located at chromosomes 7 and 17, respectively (**Figure 1**). Three isoforms of human Smurf1, resulting from alternative splicing, have been reported, and a single protein product has been confirmed for Smurf2 (9).

Smurfs share a high sequence homology (>70% amino acid sequence identity) and have similar structural characteristics. Despite these high similarities and some redundancy in their substrate repertoire, these proteins exhibit distinct and in some respects opposite biological functions.

In this review, we will discuss the diverse roles of Smurf proteins in pleotropic cellular functions, including cell proliferation and DNA damage response, chromatin organization, dynamics and genomic integrity maintenance, gene expression, carcinogenesis, and metastases. We also highlight studies implicating Smurfs in cellular responses to anticancer therapies.

REGULATORY ROLES OF SMURFS IN THE DECISIVE CELLULAR PROCESSES

Smurfs in TGF-β/BMP Signaling

Smurf1 and Smurf2 were originally identified as negative regulators of BMP/TGF-β signaling pathways. These pathways play crucial roles in embryogenesis and adult tissue homeostasis, as well as in the pathogenesis of various human diseases (10, 11).

In cancer, these pathways appear to have a dual role: operating in both cancer development and suppression (12, 13). Indeed, the activation and/or inhibition of these pathways are highly related to various aspects of carcinogenesis including epithelial-mesenchymal transition (EMT), angiogenesis, behavior of cancer stem cells, metastases, and tumor cell chemo-refractoriness.

For example, TGF-β signaling exerts in normal cells and at the early-stages of carcinogenesis tumor-suppressor functions, including cell-cycle arrest and triggering of apoptosis. However, at late-stages of carcinogenesis, the role is reversed and TGF-β signaling promotes tumorigenesis, metastases and chemoresistance (13).

Smurf1 has been shown to ubiquitinate and degrade the BMP receptor-regulated Smad proteins (R-Smads; i.e., Smad1 and Smad5), which form heteromeric complexes with a common-partner Smad (Co-Smad) Smad4. Following formation, this complex translocates into the nucleus to regulate transcription of a variety of target genes, also related to tumorigenesis, cancer progression, and chemoresistance. The ability of Smurf1 to degrade the BMP-specific Smads as well as BMP receptors, provides negative feedback to the BMP signaling pathway. Noteworthy, Smurf1 can cooperate with inhibitory Smad (I-Smad), Smad6, and Smad7, which repress the TGF-β superfamily signaling by several different mechanisms (14–17).

The E3 ubiquitin ligase functions of Smurf2 were primarily associated with its ability to negatively regulate the TGF-β signaling pathway. Following receptor stimulation with the TGF-β ligand, Smurf2 translocates from the nucleus to the cytosol. For this to occur, nuclear Smurf2 needs to bind to I-Smad, in particular of Smad7, which facilitates the nuclear export of Smurf2.

While in the cytosol, Smurf2 interacts with and promotes the proteasomal degradation of the TGF-β receptor (i.e., TGF-βRI) as well as TGF-β-specific R-Smads such as Smad2 and Smad3 (18–21). Of note, although the Smurf1 and Smurf2 activities are primarily attributed to BMP and TGF-β signaling regulation, respectively, experimental data suggest a model where Smurfs can truncate both of these pathways.

Despite these proceedings, studies conducted on Smurf1- and Smurf2-genetically ablated mice question the role of Smurfs in

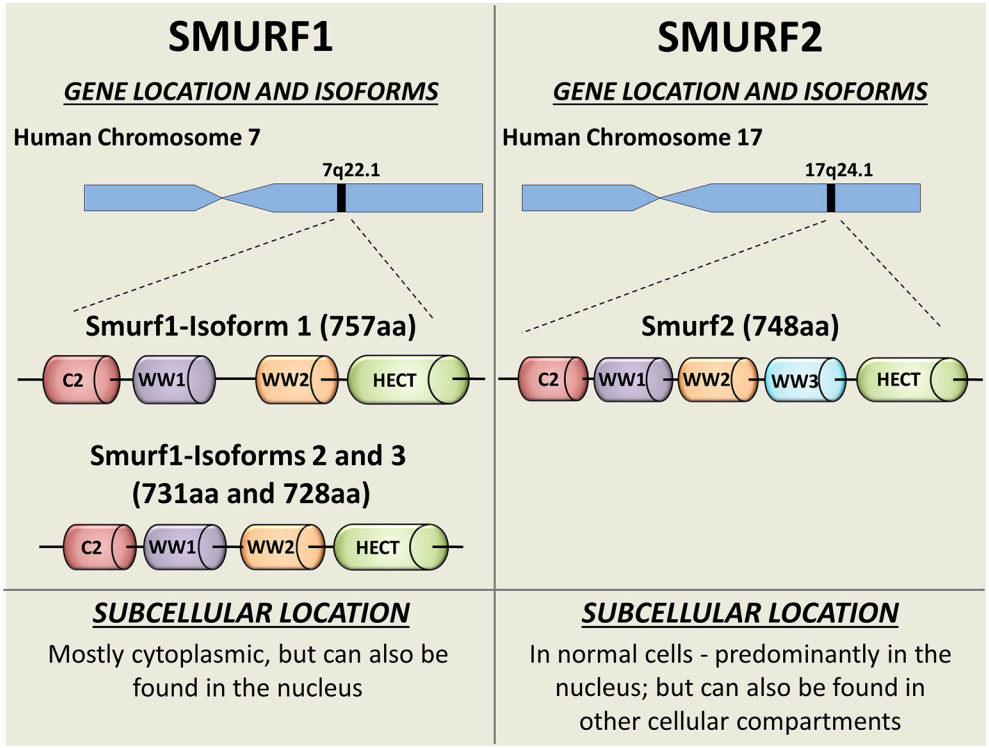

FIGURE 1 | Schematic diagram showing the SMURFs' gene locations, isoforms, and intracellular distribution. Both Smurf1 and Smurf2 possess an N-terminal protein kinase C (PKC)-related C2 domain (Red), 2-3 WW protein interacting domains (purple, orange, and blue) and the catalytical C-terminal HECT domain (green). Smurf1 transcript variant 1 (NP_065162) is the longest Smurf1 isoform (757 aa), and has a 26-residue linker insert between WW domains (8). Catalytical active site—Cys699. Smurf1 transcript variant 2 (NP_851994; 731 aa) lacks an in-frame exon in the coding region, compared to variant 1. Smurf1 isoform 3 (NP_001186776) lacks an in-frame exon in the coding region and uses an alternate in-frame splice site in the 3′ coding region, compared to variant 1. This variant is 728 aa long. Smurf2 (NP_073576) is a 748 aa protein. Catalytical active site—Cys716. Subcellular bio-distributions of Smurf1 and Smurf2 are indicated on the bottom of the figure.

the *canonical* TGF-β superfamily signaling. *Smurf1* knockout mice revealed no significant disruption in the Smad-mediated TGF-β/BMP signaling pathways. Instead, these animals exhibited an age-dependent increase of bone mass due to enhanced osteoblast activity. This activity was related to activation of the MEKK2-JNK signaling cascade (22).

Targeted disruption of *Smurf2* in mice has also revealed that the protein levels and stability of the TGF-β receptor and Smad proteins (i.e., Smad2/3) were unaffected by *Smurf2* depletion, despite the enhanced cellular response to TGF-β stimulation (23). This phenomenon was explained by the uncovered ability of Smurf2 to monoubiquitinate Smad3 and inhibit the formation of Smad3 complexes (23). The activity of these complexes is required for the TGF-β-mediated transcriptional response.

Collectively, these findings stipulate that at least in mouse experimental models, Smurf proteins do not directly regulate the stability and turnover of the BMP/TGF-β receptors and Smad transducers. Of note, both murine and human Smurf proteins share a very high homology and amino acid identity: 95% for Smurf1 and 99% for Smurf2, suggesting that mouse models can appropriately investigate the biological roles of Smurfs in humans.

In addition, experimental evidence shows that Smurfs are not the only E3 ubiquitin ligases regulating TGF-β signaling. Other NEDD4 E3 ubiquitin ligase family members, including ITCH, NEDD2L, WWP1 and WWP2, can also mitigate this cascade (7). These findings suggest that NEDD4 E3s have overlapping functions in the TGF-β signaling regulation. Moreover, Smurf2 has been reported to ubiquitinate and promote the degradation of Smurf1, introducing further complexities in TGF-β/BMP signaling regulation by Smurfs (24).

Interestingly, a recent study shows that mice deficient for *Smurf2* exhibit decreased bone mass due to severe osteoporosis. This phenotype is opposite to the phenotype observed in *Smurf1*-ablated animals (25). Moreover, the authors demonstrate that elimination of Smurf2, but not Smurf1, significantly increases the expression of RANKL, a key regulator in osteoclastogenesis and bone physiology. Through mechanistic studies, they showed that this phenomenon is associated with the ability of Smurf2 to monoubiquitinate and inactivate transcriptional activity of Smad3, as previously reported (23). In the absence of Smurf2, Smad3 activity remains unrestrained and results in enhanced transactivation activity of the vitamin D receptor (VDR) signaling, ultimately leading to elevated expression of RANKL.

Smurfs in Carcinogenesis

Smurf2 Acts as a Tumor Suppressor

The most surprising and exciting finding on the involvement of Smurfs, in particular of Smurf2, in cancer we, and subsequently another group, obtained using Smurf2-depleted mice (*Smurf2*$^{-/-}$ mice). We found that while relatively normal in their early lives, these mice developed a wide spectrum of tumors in different organs and tissues as they aged. The majority of tumors appeared in mice older than 90 weeks (7, 26), equivalent to ∼70 years of age in humans (27). About 70% of the uncovered tumors were of epithelial origin including hepatocellular carcinoma, lung alveolar carcinoma, mammary gland carcinoma, and others. Hematological malignancies were also detected, in ∼30% of the cases.

To best of our knowledge, there are only a few, if any, mouse cancer models which so closely mimic two key characteristics of human cancer: (i) the late cancer onset (∼77% of all cancers are diagnosed in persons 55 years of age and older); and (ii) the epithelial origin of tumors. For example, the vast majority of tumors in *p53*-null mice are hematological malignancies (mostly lymphomas), which develop within a few months after the animal's birth (28, 29).

These findings suggest that the *Smurf2*-ablated animals are highly relevant to human carcinogenesis model, and could be advantageous when studying cancer-related processes at the whole organism level.

Moreover, Zhang's group further demonstrated that mice heterozygote for Smurf2 (*Smurf2*$^{+/-}$) are also susceptible to spontaneous tumorigenesis (30). Further analysis of tumors from these animals revealed the loss of heterozygosity (LOH) at *Smurf2*. LOH is a common genetic event inactivating residual wild type allele of genes, in particular of tumor suppressors.

Altogether, these findings establish Smurf2 as a potent tumor suppressor, preventing the transformation of normal cells into cancerous ones.

Smurf2 Regulates Chromatin Organization, Dynamics, and Integrity

Our subsequent studies revealed that inactivation of Smurf2 triggers a series of cascading events in cells, and creates the "mutator phenotype," which under the stress of aging leads to carcinogenesis (26). Mechanistically, we found that Smurf2 regulates chromatin structure landscape and, thereby, affects gene expression, DNA damage response (DDR), and genomic integrity maintenance. We further demonstrated that these Smurf2 activities were associated with and at least in part relied on its ability to ubiquitinate and degrade RNF20 (**Figure 2**), a RING type E3 ubiquitin ligase responsible for monoubiquitination of histone H2B (ubH2B). The RNF20-ubH2B module regulates chromatin compaction, DNA damage response, and gene expression, and acts both as a tumor suppressor and an oncogene depending on the cellular context (26, 31–39).

Furthermore, an interesting finding in *Smurf2*$^{-/-}$ cell genome was the accumulation of multiple chromosomal abnormalities, with translocations being the most notable hallmark (26). Subsequent investigation of this phenomenon showed that Smurf2 expression is essential in preventing the formation of pathological chromatin bridges, also known as anaphase bridges (40). These bridges are a major cause of chromosomal translocations, and are often an output of the compromised decatenation checkpoint.

The decatenation checkpoint is normally mediated by DNA topoisomerase IIα (Topo IIα), a core enzyme in chromatin organization, dynamics and unaltered chromosome inheritance (41).

We found that Smurf2 operates as a molecular editor of Topo IIα, switching its ubiquitination code from the degradation-promoting K48 polyubiquitination to monoubiquitination, and stabilizing the enzyme (40) (**Figure 2**). Unaltered E3 ubiquitin ligase functions of Smurf2 were indispensable for this regulation. Moreover, we showed that Smurf2 depletion phenocopied Topo IIα depletion and increased the formation of anaphase bridges. Introduction of Topo IIα into Smurf2-depleted cells rescued this phenomenon. Our studies also uncovered that Smurf2 is a determinant of Topo IIα protein levels in cancer cells and tissues, and is a factor affecting tumor cell sensitivity to the Topo II-targeting drug, etoposide (40).

Collectively, these findings establish Smurf2 as a key cellular factor governing chromatin organization, dynamics and genome integrity maintenance. They also indicate Smurf2 as a potent tumor suppressor.

Other Putative Mechanisms for Smurf2-mediated Tumor Suppression

In addition to RNF20 and Topo IIα, Smurf2 has been shown to regulate stability and/or subcellular localization of other decisive cellular proteins implicated in carcinogenesis and drug resistance. These molecules include the molecular chaperone and apoptosis inhibitor HSP27, transcription factors KLF5, YY1, ID1/ID3, histone methyltransferase EZH2, and others (**Figure 2**).

HSP27 (heat shock protein 27) is one of the central molecules shown to upregulate EMT and affect activities of the matrix metalloproteinases (MMPs), stimulate tumor cell proliferation, migration, invasion, as well as to mediate chemo- and radio-resistance (42). Smurf2 overexpression was reported to alter HSP27 subcellular distribution and induce its ubiquitin-dependent degradation in the human lung adenocarcinoma A549 cell model (43). However, it currently remains unknown whether these Smurf2 activities are pertinent to its tumor suppressor functions in lung cancer, and/or in other types of tumors.

Smurf2 was also shown to promote the degradation of a few principal transcription factors whose activities are associated with carcinogenesis, drug resistance, patient prognosis and survival. Krüppel-like factor 5 (KLF5) is one of these factors with cell growth-promoting and pro-survival activities. KLF5 is also implicated in cell differentiation, migration and stemness and its expression levels are frequently abnormal in different types of cancer (44). Smurf2 was shown to polyubiquitinate and promote the proteasomal degradation of KLF5 in a Smurf2 E3 ligase-dependent manner, thereby inhibiting the transcriptional and pro-proliferative activities of KLF5 (45). Interestingly, KLF5 levels were specifically reduced by Smurf2, but not by Smurf1.

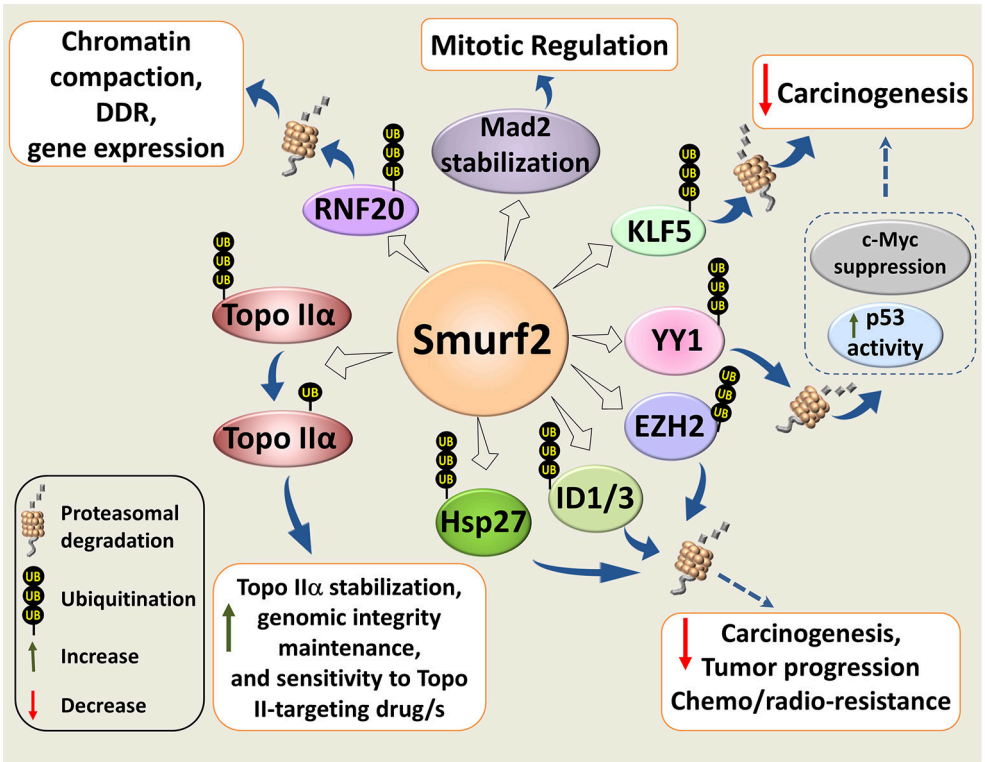

FIGURE 2 | Molecular mechanisms underlying Smurf2 tumor suppressor functions. Smurf2 regulates chromatin compaction, DDR, and gene expression through the ubiquitin-proteasomal degradation of RING-type E3 ubiquitin ligase and histone H2B modifier RNF20. Smurf2 also controls chromatin organization, dynamics and unaltered chromosomal inheritance through stability regulation of Topo IIα. In addition, Smurf2 could affect carcinogenesis, tumor progression and sensitivity to anticancer therapies through the ubiquitin-mediated proteasomal turnover of KLF5, ID1/3, YY1, and others. Degradation of YY1, for example, relieves the suppression of p53 activity by YY1, and decreases the expression of c-Myc. Smurf2-mediated stabilization of the mitotic spindle checkpoint protein Mad2, is also shown in the diagram. Dotted arrows specify potential mechanisms determined from existing evidence.

Yin Yang 1 (YY1) is another example of the Krüppel-like zinc finger transcriptional factors negatively regulated by Smurf2. YY1 is overexpressed in multiple cancer types, and its overexpression correlates with poor clinical outcomes, although several studies suggested that in some types of cancer YY1 acts as a tumor suppressor (46). Two research groups reported that Smurf2 ubiquitinates and promotes the degradation of YY1 (47, 48). The outcomes of these Smurf2-mediated effects were a decrease in the YY1-mediated suppression of p53 activity (47), and a reduction of B-cell proliferation and lymphomagenesis (48). The latter was supposedly mediated via the suppression of YY1-c-Myc regulatory axis (**Figure 2**).

The ability of Smurf2 to ubiquitinate and degrade two dominant inhibitors of helix-loop-helix transcription factors ID1 and ID3 (49) might also be relevant to Smurf2's tumor suppressor activities. Overexpression of these IDs was shown to facilitate tumor growth, angiogenesis, stem cell maintenance, invasiveness, metastasis, as well as correlating with unfavorable clinical prognoses (50, 51). Moreover, ID1 has been shown to confer chemoresistance to different types of cancer (52–55).

In addition, Smurf2 was shown to polyubiquitinate and induce a proteasome-mediated degradation of EZH2, the catalytic subunit of the polycomb repressive complex 2 (PRC2) and histone H3-K27 methyltransferase (56). This was reported in human mesenchymal stem cells during their neuronal differentiation. If this finding is corroborated in tumor cell models it might be highly pertinent to the ability of Smurf2 to interfere with carcinogenesis, as EZH2 was documented as a pro-oncogenic factor involved in neoplastic transformation, cancer cell stemness, metastases and immune evasion. However, it should be mentioned that several studies show that under some circumstances EZH2 also exhibits tumor suppressive activities (57).

Smurf2 has also been implicated in the formation of the functional mitotic spindle checkpoint by regulating the localization and stability of the MAD2 protein (58). Knockdown of Smurf2 or overexpression of its E3 ligase-deficient mutant generated misaligned and lagging chromosomes, premature anaphase onset, and defective cytokinesis in human cervix carcinoma HeLa cells (58). Interestingly, in our study, Smurf2 depletion did not affect the formation of lagging chromosomes, but instead increased the formation of anaphase bridges in osteosarcoma U2OS cells (40). These discrepancies could be explained by different types of cancer cell models used in these studies: HeLa vs. U2OS cells, implying that biological effects of

Smurf2 should be very carefully interpreted taking into account cellular context, genetic make-up, and experimental settings.

Altogether, these findings designate Smurf2 as a pleotropic cellular factor that regulates a wide spectrum of molecular pathways and networks to control transcription, DNA damage response and genomic integrity maintenance. When these pathways are compromised, carcinogenic processes can be set in motion, leading to cell transformation and the development of a wide spectrum of tumors, as observed in *Smurf2*-null mice (7, 26).

Furthermore, the Smurf2-Smad3-RANKL axis described in the previous section could also potentially be involved in tumor formation in Smurf2-deficient animals. This appears to be most relevant to mammary gland carcinomas developed in *Smurf2*$^{-/-}$ mice, as the upregulated RANKL/RANK signaling pathway could promote mammary stem cell expansion, proliferation and the formation of hormone-induced breast cancer (59).

Interestingly, genomic studies showed that the SMURF2 gene is not frequently mutated in human malignancies (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=SMURF2). However, changes in Smurf2 expression are common in many cancers (7, 26, 40), similar to some other cancer-related genes such as the two TP53 paralogs, TP63 and TP73 (60), and the members of the FOXO transcription factors family (61).

The Duality of Smurf2 in Cancer

As described above, evidence points to Smurf2 as a potent tumor suppressor operating in *normal* cells to prevent cell transformation and carcinogenesis. However, results obtained in established cancer cell models argue that Smurf2 has a dual role and under some circumstances acts as an oncogene rather than a tumor suppressor (7, 9, 62). Additionally, the expression levels of Smurf2 were reported to be significantly elevated in several types of cancers including esophageal squamous cell carcinoma tumors (63) and chemo-refractory tumors such as recurrent hepatocellular carcinomas (64). The data available in the COSMIC dataset portal also indicate that Smurf2 is overexpressed in ∼49% of ovarian cancer, about 18% of breast cancer, and in ∼17% of soft tissue neoplasms.

Furthermore, ours and other studies show that subcellular biodistribution of Smurf2 is prominently altered in cancer *vs.* normal cells, with a notable accumulation/sequestration of Smurf2 in the cytoplasm of tumor cells (7, 26, 65). It is possible that overexpressed and mislocalized Smurf2 is employed by the carcinogenic machinery to promote oncogenesis, at least in some types of cancer.

The possible pro-oncogenic functions of Smurf2 in genetically-compromised tumor cells could be related to the reported ability of Smurf2 to interfere with the RAS, Wnt/β-catenin, and EGFR-mediated signaling pathways, three central modules in cancer progression and chemoresistance (**Figure 3**).

It has been reported that Smurf2 together with the E2 ubiquitin-conjugating enzyme UBCH5, stabilize the KRAS oncoprotein (66), the most frequently mutated transforming oncogene in human cancers (67). The authors showed that Smurf2 monoubiquitinates UBCH5 to form an active complex for degradation of β-TrCP, the F-box protein and a component of SCF E3 ligase that negatively regulates KRAS (68). Loss of Smurf2 led to the accumulation of β-TrCP, and KRAS degradation. Interestingly, silencing of Smurf2 mostly affected the mutant form/s of KRAS. In addition, the authors demonstrated that knockdown of Smurf2 reduces the clonogenic survival and prolongs tumor latency in the mutant KRAS-driven tumors generated in nude mice with either human colon or lung carcinoma cells (66).

Under the described experimental setting, Smurf2 appears to act as an oncogene, promoting tumor development. However, it should be mentioned that the SCF$^{\beta-TrCP}$ complex mediates the degradation of functionally diverse proteins, and it is capable to downregulate both oncogenes (i.e., RAS, β-catenin, CDC25A, and others) as well as tumor suppressors (e.g., Smad4, IkB, FOXO3 and REST) (69, 70). Thus, the role of the Smurf2/SCF$^{\beta-TrCP}$ module in cancer may vary considerably depending on the cell type and molecular composition, and should be determined in a particular context.

Smurf2 was also reported to promote the Wnt/β-catenin signaling through the degradation of its two negative regulators: GSK3β (71) and Axin (72). Through this route, Smurf2 could potentially facilitate the activities of the proto-oncogene β-catenin.

Interestingly, GSK3β phosphorylates and primes RAS proteins for SCF$^{\beta-TrCP}$-mediated degradation (73). Inhibition of this degradation pathway by aberrant Wnt/β-catenin signaling may contribute to Ras-induced transformation in colorectal tumorigenesis (68). In this regard, it will be even more important to investigate the role of the Smurf2/GSK3β/SCF$^{\beta-TrCP}$/Ras module in animal cancer models and in clinical samples.

Smurf2 has also been shown to ubiquitinate and protect from c-Cbl-mediated degradation the epidermal growth factor receptor (EGFR), which is implicated in a wide range of cell responses ranging from cell division to adhesion, motility, and death (74). The authors also reported that the loss of Smurf2 destabilizes EGFR, and reduces the clonogenic survival of EGFR-expressing cancer cell strains. The effects of Smurf2 depletion on EGFR-negative cancer cells, normal fibroblasts, and on normal epithelial cells were minor. In addition, the authors demonstrated that knockdown of Smurf2 reduces the ability of human head and neck squamous cell carcinoma UMSCC74B cells to form tumors *in vivo*.

Cooperatively, these studies suggest that in immortalized and established cancer cell models Smurf2 operates as an oncogene rather than a tumor suppressor. Of note, Wellbrock's group reported that Smurf2 depletion can significantly increase melanoma cell sensitivity to the cytotoxic effects of the MEK inhibitor selumetinib (AZD6244), both *in vitro* and *in vivo* (75).

This finding implies that at least in this type of tumor, inactivating Smurf2 might overcome tumor cell resistance to MAPK pathway inhibitors experienced in clinics.

Smurfs in Tumor Cell Proliferation, Migration, and Invasion

A few studies have shown that Smurf2 is intrinsically involved in these critical for cancer progression processes. However,

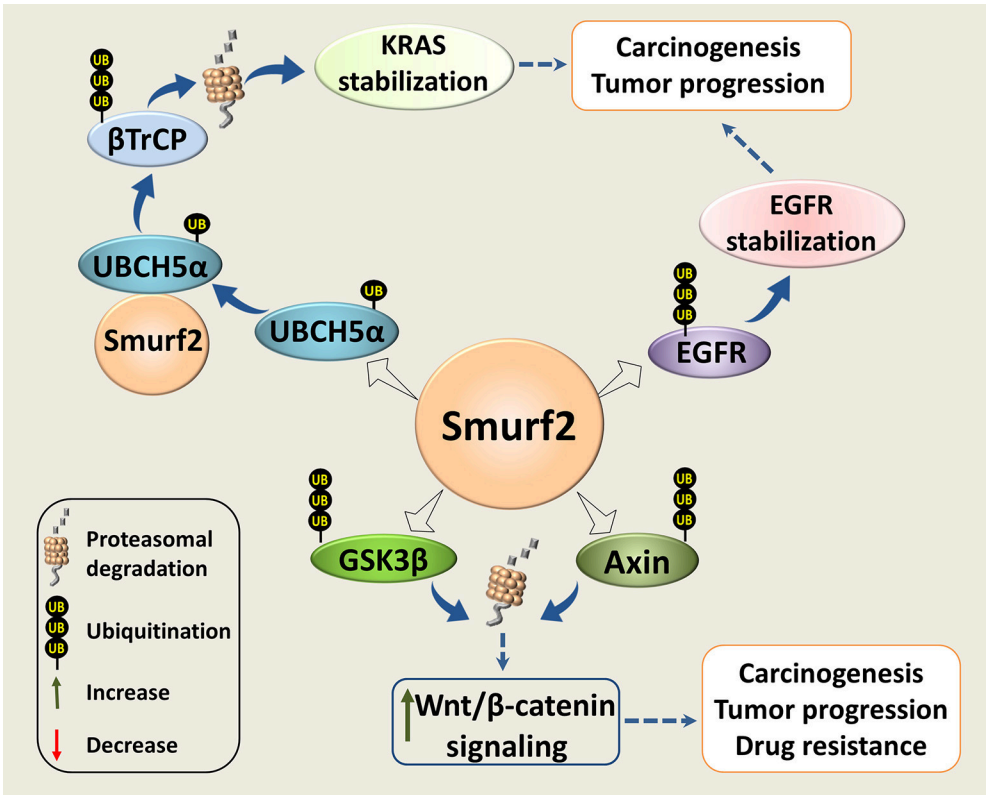

FIGURE 3 | Smurf2 as an oncogene. Smurf2 monoubiquitinates UBCH5α and forms an active complex for the degradation of the KRAS negative regulator, βTrCP. This stabilizes KRAS and could potentiate its pro-oncogenic functions. Smurf2 also ubiquitinates EGFR and protects it from degradation. In addition, Smurf2 through the degradation of GSK3β and Axin could upregulate the Wnt/β-catenin signaling. Through this mechanism, Smurf2 might also increase the pro-oncogenic activities of this pathway. Dotted arrows specify potential mechanisms determined from existing evidence.

studies from different groups revealed different and in some respect contradictory results, even when using the same cancer cell model. For example, Zhang's group demonstrated that elevated levels of Smurf2 were required for and promoted migration, invasion and *in vivo* metastatic dissemination of human breast carcinoma MDA-MB-231 cells. Moreover, the authors demonstrated that Smurf2 E3 ligase-defective mutant (Cys716Gly) decreases the metastatic behavior of these cells (76).

In contrast, Imamura's group showed that Smurf2 knockdown in MDA-MB-231 cells enhances cell migration *in vitro* and bone metastasis *in vivo*, implying that under these circumstances Smurf2 is a tumor suppressor (24). The same group also demonstrated that Smurf2 reduces MDA-MB-231 cell migration via Smurf1 degradation. The authors also provided evidence that the motility of Smurf2-knocked down cells is independent of TGF-β-signaling.

In addition, a recent study showed that knock-down of Smurf2 increases the proportion of invasive MDA-MB-231 cell-derived organoids. This group also demonstrated that PIAS3-dependent sumoylation of Smurf2 is important in suppressing the invasive behavior of these cells (77).

The discrepancies in these studies could be explained, at least in part, by different approaches used to manipulate the Smurf2 expression levels (overexpression *vs.* knock-down). However, a more comprehensive investigation is needed to support this notion. One possible approach is to examine these effects in SMURF2 genetically-ablated MDA-MB-231 cells, as well as in other human cancer cell models. These cells were recently generated by our group using the CRISP/Cas9 gene-editing tool (78), and are currently under investigation.

Studies conducted using pancreatic cancer cells also suggest that Smurf2 acts as a tumor suppressor. The authors showed that Smurf2 is downregulated in pancreatic cancer tissues, and its overexpression suppresses migration and invasion of pancreatic cancer cells, while having no effect on cell viability, cell cycle, and senescence (79). Interestingly, the authors also showed that Smurf2 promotes mesenchymal-epithelial transition (MET), and that its expression levels are negatively associated with cancer cell resistance to gemcitabine treatment.

Smurf1 has also been implicated in cancer cell proliferation, migration and invasion (**Figure 4**). Wrana's group found that Smurf1 plays an important role in regulating protrusive activity and the transformed phenotype of HEK293T cells (80). Mechanistically, the authors demonstrated that Smurf1 is recruited by PKCζ to cellular protrusions, where it controls the protein levels of RhoA, a small GTPase implicated in cell shape,

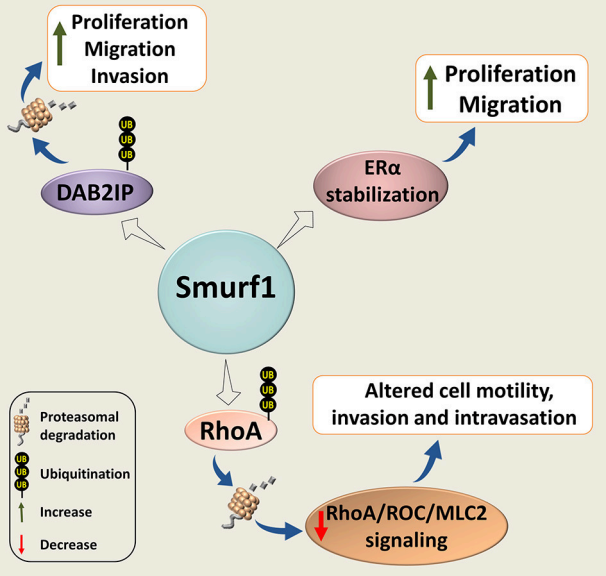

FIGURE 4 | Smurf1 in cancer cell proliferation, motility, and invasion. Smurf1 promotes ubiquitin-dependent degradation of DAB2IP; and stabilizes ERα, resulting in accelerating tumor cell replication, migration and invasion. Smurf1 can also affect cancer cell motility and invasion by proteasomal degradation of RhoA.

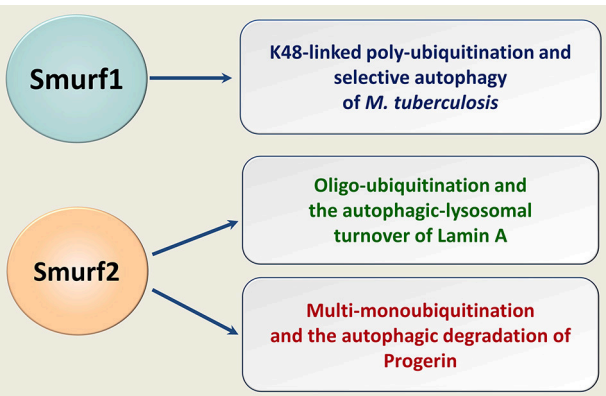

FIGURE 5 | Smurfs in selective autophagy of human pathogens and endogenous cellular proteins. Smurf1 mediates the K48-linked polyubiquitination of human pathogen *M. tuberculosis,* targeting it for the ubiquitin-dependent selective autophagy. Smurf2 oligo-ubiquitinates lamin A and multi-monoubiquitinates progerin. Smurf2 targets both lamin A and progerin for the autophagic-lysosomal turnover.

polarity, adhesion, and motility regulation. Smurf2 however, was not involved in the RhoA stability regulation.

Subsequently, Vial's group showed that Smurf1 expression is required for lamellipodia formation, tumor cell plasticity, and motility through the regulation of peripheral RhoA/ROCK/MLC2 signaling. Silencing of Smurf1 or expression of its interfering mutants inhibited cell migration (81). Interestingly, their *in vivo* studies showed that Smurf1 reduction induces the mesenchymal-amoeboid transition, facilitates cell motility and increases invasion and intravasation. However, this reduction was insufficient to promote metastasis after cells have entered the vessels.

In another study, induction of Smurf1 expression either by EGF or by overexpression of MEK1, as well as Smurf1 overexpression, significantly increased migration and invasion of breast carcinoma MDA-MB-231 cells, whereas knockdown of Smurf1 suppressed the phenotype (82). These findings are in agreement with results reported by Imamura's group (24).

The pro-oncogenic role of Smurf1 was also noted in other types of cancer. For example, suppression of Smurf1 expression in human ovary carcinoma SK-OV-3 and OVCAR-3 cells significantly decreases cell migration and invasion (83). Similar results were also observed in prostate cancer cell models (84). In addition, the authors demonstrated that expression of Smurf1 in prostate cancer cells is regulated through the androgen receptor (AR) signaling, which is critical for prostate cancer growth and survival.

Another recently published study shows that Smurf1 expression is also triggered through estrogen signaling (85). The authors also demonstrated that Smurf1 stabilizes estrogen receptor alpha (ERα) in breast cancer cells, leading to increased estrogen signaling and enhanced cell proliferation. These findings suggest a forward feedback loop in Smurf1-ERα regulation.

Smurf1 is also overexpressed in human gastric cancer (GC) tissues (86). Moreover, Smurf1 expression levels were shown to be positively associated with more advanced tumor-node-metastasis (TNM) stage of GC, and inversely correlated with patient survival. Knockdown of Smurf1 inhibited proliferation, migration and invasion of GC cells, at least in some GC cell models, while Smurf1 overexpression exacerbated these phenotypes. Furthermore, the authors reported that Smurf1-knockdown in GC cells markedly inhibits tumor growth and liver metastasis *in vivo*. Mechanistically, they linked the Smurf1 pro-oncogenic activities with the ability of Smurf1 to negatively regulate the expression of DAB2IP, a GTPase-activating protein (GAP) and a suggested tumor suppressor (86).

The existence of similar Smurf1-regulated tumor promoting mechanism was also observed in clear cell renal cell carcinoma (ccRCC) cells (87). This mechanism was associated with the ability of Smurf1 to promote proliferation, migration, and invasion of ccRCC cells. In addition, the expression levels of Smurf1 were found to be elevated both in ccRCC cell lines and cancer tissues, and associated with worse patient survival.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this review, we highlighted and discussed the cancer related biological functions of two C2-WW-HECT E3 ligases, Smurf1 and Smurf2. These proteins surfaced as influential, and under some circumstances, as decisive cellular factors regulating a plethora of cellular processes pertinent to cancer onset, progression and therapy.

The currently available data stipulate that Smurf1 acts as an oncogene, whereas Smurf2 operates both as a tumor suppressor and a tumor promoting molecule, depending on the tumor stage, type, molecular binding partners, and other still unidentified factors.

It is now evident that apart from TGF-β/BMP signaling, Smurfs regulate different signaling pathways and networks. Understanding these networks and the Smurfs' impact on their components is important and should be further investigated.

Another line of investigation is elucidating the mechanisms regulating expression, localization and functions of Smurfs. Currently, these mechanisms remain elusive. Understanding of these mechanisms is imperative in explaining the dual role of Smurf2 in cancer and its impact on cancer progression and treatment. In addition, the full spectrum of mechanisms and networks operating under Smurf1/2 auspices is also currently unknown.

Recently, we have shown that Smurf2 in addition to its ability to control protein homeostasis through the proteasomal breakdown, targets some cellular proteins for autophagic-lysosomal turnover (88). Specifically, we found that Smurf2 regulates selective autophagy of nuclear lamins, in particular lamin A, and its mutant form progerin (**Figure 5**). The expression of progerin underlies the pathogenesis of the devastating premature aging syndrome, HGPS (Hutchinson-Gilford progeria syndrome). Remarkable, in addition to HGPS, progerin also accumulates in cells during physiological aging and supposedly in cancer, where it could promote genomic instability and increase tumorigenesis (89–91). This association suggests that targeting progerin through the Smurf2-mediated autophagy might be a promising direction to eradicate tumor cells, though more research is needed in this regard.

Smurf1 has also been implicated in selective autophagy, in particular in eliminating in human cells of Mycobacterium tuberculosis (92) (**Figure 5**). Whether Smurf1 can also mediate the autophagic degradation of endogenous cellular proteins is currently unknown.

Both the ubiquitin-proteasome and autophagic-lysosomal degradation pathways are intrinsically involved in cancer initiation, progression and cure. Thus, understanding mechanisms operating in the intersection of these protein turnover machineries could provide novel therapeutic targets and paradigms in cancer diagnosis and treatment. Further efforts should be directed to characterize the involvement of Smurf proteins in these processes. Another important direction that we believe should be investigated is elucidating whether and how targeting of Smurfs by pharmacological intervention (e.g., by Smurf catalytic inhibitors) affect the ability of tumor cells to escape the destructive impact of anticancer drugs and therapies used in clinics. This is a more long-term goal as such compounds are currently unavailable.

AUTHOR CONTRIBUTIONS

PK and GL-C drafted the initial version of the manuscript, and prepared figures and bibliography. MB conceptualized the study and wrote the manuscript.

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