



Zebrafish Cancer Predisposition Models

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Cancer predisposition syndromes are rare, typically monogenic disorders that result from germline mutations that increase the likelihood of developing cancer. Although these disorders are individually rare, resulting cancers collectively represent 5–10% of all malignancies. In addition to a greater incidence of cancer, affected individuals have an earlier tumor onset and are frequently subjected to long-term multi-modal cancer screening protocols for earlier detection and initiation of treatment. *In vivo* models are needed to better understand tumor-driving mechanisms, tailor patient screening approaches and develop targeted therapies to improve patient care and disease prognosis. The zebrafish (*Danio rerio*) has emerged as a robust model for cancer research due to its high fecundity, time- and cost-efficient genetic manipulation and real-time high-resolution imaging. Tumors developing in zebrafish cancer models are histologically and molecularly similar to their human counterparts, confirming the validity of these models. The zebrafish platform supports both large-scale random mutagenesis screens to identify potential candidate/modifier genes and recently optimized genome editing strategies. These techniques have greatly increased our ability to investigate the impact of certain mutations and how these lesions impact tumorigenesis and disease phenotype. These unique characteristics position the zebrafish as a powerful *in vivo* tool to model cancer predisposition syndromes and as such, several have already been created, including those recapitulating Li-Fraumeni syndrome, familial adenomatous polyposis, RASopathies, inherited bone marrow failure syndromes, and several other pathogenic mutations in cancer predisposition genes. In addition, the zebrafish platform supports medium- to high-throughput preclinical drug screening to identify compounds that may represent novel treatment paradigms or even prevent cancer evolution. This review will highlight and synthesize the findings from zebrafish cancer predisposition models created to date. We will discuss emerging trends in how these zebrafish cancer models can improve our understanding of the genetic mechanisms driving cancer predisposition and their potential to discover therapeutic and/or preventative compounds that change the natural history of disease for these vulnerable children, youth and adults.

Keywords: zebrafish, cancer predisposition, genetic models, cancer, model organism, p53

INTRODUCTION

Cancer Predisposition Genes

Tumor-specific mutations that cause cancer are usually somatic and are cumulatively acquired over a lifetime, which explains the greater cancer prevalence in the older demographic. In contrast, germline mutations in certain genes, termed cancer predisposition genes (CPGs), are associated with an increased cancer risk. Germline mutations in CPGs often lead to hereditary cancer syndromes, which represent 5–10% of all malignancies and typically have a much earlier tumor onset than their sporadic counterparts (Garber and Offit, 2005). Despite the lack of a generally accepted CPG definition, one study identified 114 CPGs as the genes whose rare mutations increase the relative risk of cancer > 2 fold in at least 5% of individuals (Rahman, 2014). Another extensive study analyzed tumors from 10,389 individuals across 33 different cancer types and identified 152 CPGs using CharGer (Characterization of Germline Variants), an automatic variant classification pipeline they developed that ranks the pathogenicity of a certain mutation (Huang et al., 2018).

Unsurprisingly, many frequently mutated cancer driver genes in sporadic cancers have also been classified as CPGs, such as *TP53*, *APC*, *BRCA1/2*, *ATM*, and *NF1*, and as such, the knowledge gained by examining the role of germline CPG mutations will help shed light on the mechanisms of tumor formation for both sporadic and inherited cancers (Lu et al., 2015; Zhang et al., 2015). Additionally, 115 novel ultra-rare cancer-exclusive variants have been associated with known and candidate CPGs (Rasnic et al., 2020). Somatic mutations in 21 of these candidate CPGs were identified in 35% of cancers from 10,953 patients and were associated with a significantly decreased median survival time. This study shows the relevance of CPGs to the broader cancer research field and demonstrates the need to expand the list of known CPGs to assist with personalized diagnostic approaches and prognosis of cancers as well as to better understand the biological mechanisms of tumorigenesis to inform novel targeted therapeutic strategies.

Zebrafish as a Cancer Disease Model System

There are several traditional model systems commonly used to study cancer, including cell culture based, zebrafish and mouse models. These models all have their own unique advantages and limitations (Figure 1) which can be leveraged in different ways to contribute to cancer and developmental biology as well as to human disease research.

The many advantages of the zebrafish position it as a powerful *in vivo* model, especially for preclinical genetic studies to investigate cancer predisposition (Figure 1). Zebrafish share a high degree of genetic conservation with humans, and tumors that develop in zebrafish are histologically similar to their human counterparts. Zebrafish larvae (and some adult strains such as *casper* White et al., 2008) are transparent, which enables live high-resolution imaging to track single cells and visualize tissues, as well as for large-scale and non-invasive

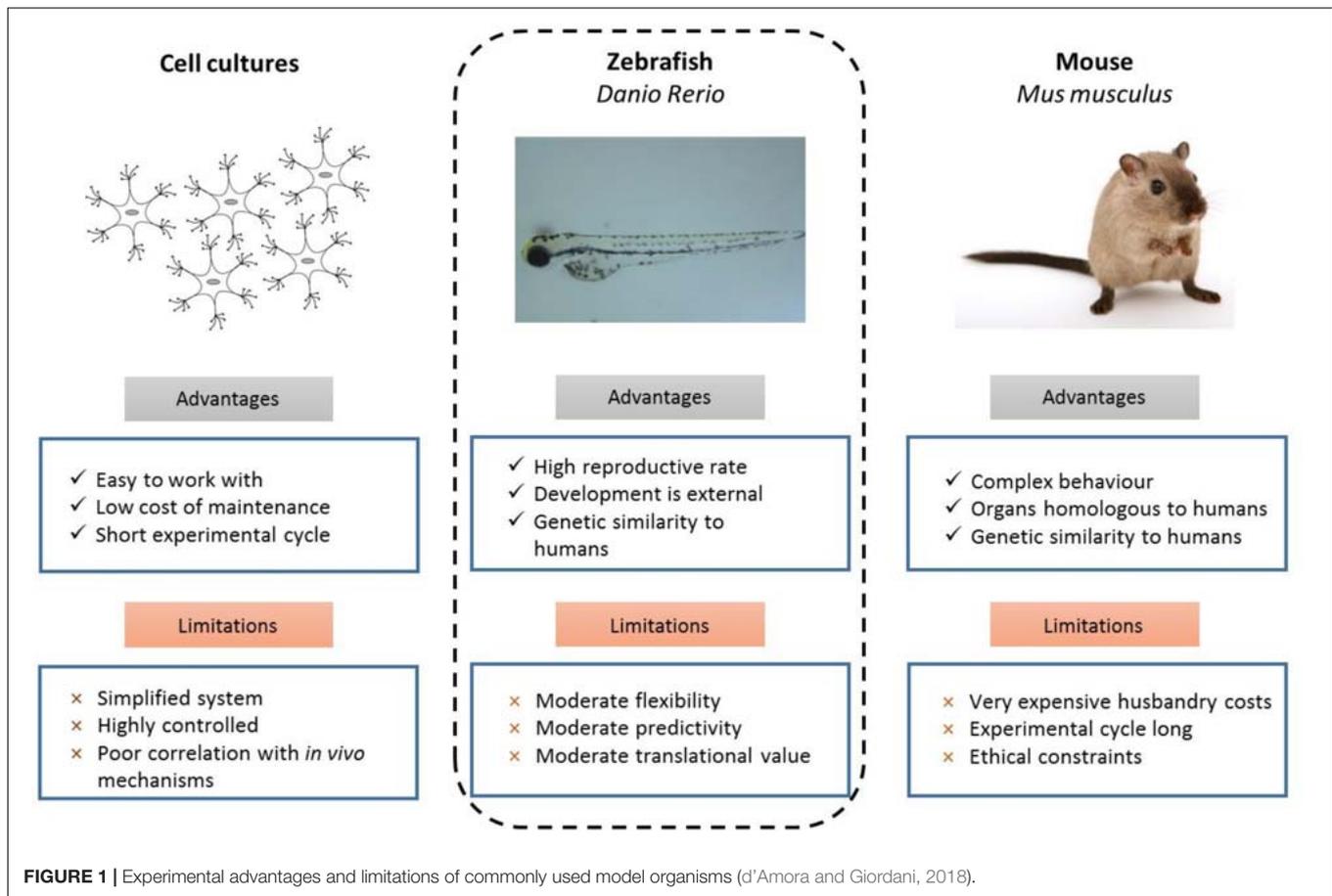
tumor screening studies which is much more feasible than in a mouse model. The high fecundity of zebrafish combined with external fertilization and rapid development to sexual maturity makes the creation of new disease models by genome editing rapid and inexpensive in comparison to the much lower number of offspring, longer time to reach sexual maturity, and higher housing costs of rearing mice. The specificity and adaptability of genome editing technologies available to zebrafish researchers has grown tremendously in the recent years and facilitates both the generation of representative models for specific disease-causing mutations and forward genetic screens to identify genes and pathways involved in cancer predisposition.

Genome Engineering Strategies for Cancer Predisposition Genetic Models in Zebrafish

Genome engineering technologies have been heavily utilized in zebrafish disease modelling. A recent review catalogued and thoroughly discussed available technologies, and we encourage readers to consult this review for information on forward genetic screens using chemical mutagens, TILLING (Targeting Induced Local Lesions IN Genomes), transgenic techniques, gene editing using Zinc Finger Nucleases (ZFN), TAL-effector Endonucleases (TALEN) and Clustered Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 strategies (Raby et al., 2020).

Recent advances have expanded the range of available CRISPR nucleases, guide RNA design tools and other emerging technologies available to edit the zebrafish genome (Prykhozhij et al., 2018a; Liu et al., 2019). Off-target gene editing is a frequent problem when using CRISPR nucleases and this issue has been recently addressed both by the development of more sophisticated guide RNA design tools (Prykhozhij et al., 2018a; Liu et al., 2019) as well as by generating zebrafish-optimized vectors encoding HiFi Cas9 (Vakulskas et al., 2018) and highly specific but less efficient hypaCas9 (Chen et al., 2017) and testing them as mRNAs as well as commercial wild-type and HiFi Cas9 proteins using the same strategies (Prykhozhij et al., 2020).

Generating point mutants efficiently and precisely is one of the most important areas for optimizing genome editing technologies. The use of CRISPR/Cas9 and chemically synthesized oligonucleotides has been the earliest and primary approach to introduce point mutations into the zebrafish genome. We have optimized this technology in zebrafish to generate cancer-relevant point mutants in the *tp53* gene (Prykhozhij et al., 2018b) and have reviewed other studies which applied similar techniques to introduce specific knock-in mutations (Prykhozhij and Berman, 2018). The limitations of this technology are that the double strand breaks and DNA repair machinery are still required for mutant generation, thus limiting efficiency to a maximum of < 10%. CRISPR/Cas9 point mutation knock-ins with enzymatically-synthesized long single-stranded DNA of 300-500 nucleotides as templates have recently been shown to have higher efficiencies in zebrafish compared to shorter oligonucleotides or double-stranded RNA molecules (Bai et al., 2020), but



the efficiencies were measured based on single embryo phenotypes, which is prone to over-estimates. The zLOST (zebrafish long single-stranded DNA template) method is directly analogous to a similar Easi-CRISPR method in mice (Miura et al., 2018).

Among newer technologies for introducing precise mutations are base editors and prime editing, which both remove the need for double-strand breaks (DSB) and template oligonucleotides. Base editors use enzymatically inactive dCas9 fused to deaminase enzymes to mediate mutagenesis in defined parts of guide RNA recognition sites (reviewed in Liu et al., 2019). However, base editors are limited by both the inability of one molecule to introduce different types of mutations and a narrow but not completely defined region of activity. The most anticipated technology to be adopted for use in zebrafish is prime editing as developed by the lab of David Liu (Anzalone et al., 2019), who engineered a fusion of a Cas9 nickase with a reverse transcriptase enzyme to perform edits based on the information encoded in a prime editor guide RNA (pegRNA). The basic mechanism of prime editing consists of the following steps: Cas9 nickase cuts the strand opposite from its pegRNA recognition site, the primer-binding site of pegRNA binds to the released single-strand region of target DNA, and the reverse transcriptase enzyme copies the edited sequence from the pegRNA into the single strand of DNA which is then repaired with the edit being

stably introduced into the DNA. In another strategy, denoted PE3b, the nickase with the regular guide RNA cuts the unedited strand thus promoting repair using the edited DNA strand (Anzalone et al., 2019).

Additionally, the generation of true null mutants in zebrafish is important for robust gene function analysis. While analyzing the transcriptional adaptation to indel mutants, El-Brolosy identified a strategy of deleting the proximal promoter and the first exon to generate “RNA-less” mutants failing to transcribe the gene (El-Brolosy et al., 2019). This strategy is likely highly robust, based on the published data and our own observations, but its utility has been questioned for some long non-coding RNAs (lncRNA), whose transcription was not fully blocked or altered by RNA-less deletions (Lavalou et al., 2019). Instead, insertion of a poly-adenylation signal was more effective for the *malat* lncRNA gene. GeneWeld is a CRISPR/Cas9 and microhomology-based gene insertion technology typically used for protein fusion gene generation (Wierson et al., 2020) that can be easily used to insert a fluorescent protein tag or another insert coupled with a stop-codon and a poly-adenylation signal at the 5' end of a protein-coding gene thus disrupting it coupled with a fluorescent marker. Moreover, GeneWeld allows for a transgenic marker such as fluorescent protein expression in the lens or heart thus marking the mutant allele.

STUDIES OF CPGS IN ZEBRAFISH: DEVELOPMENTAL AND CANCER PREDISPOSITION MODELS

For this review, we focused on zebrafish models of cancer predisposition based on CPGs from published lists (Rahman, 2014; Huang et al., 2018), many of which are implicated in cancer predisposition syndromes such as Li-Fraumeni syndrome (LFS), familial adenomatous polyposis (FAP), Cowden syndrome, Peutz-Jeghers syndrome (PJS), tuberous sclerosis complex (TSC), RASopathies, and certain hematological disorders. We will also discuss CPG mutations that, although not directly linked to a syndrome, are associated with DNA repair and genome stability and thus increase the risk of developing tumors. We have summarized the observations from current zebrafish cancer predisposition models (**Supplementary Table 1**) and classified all CPGs according to their primary functional category, highlighting those where significant zebrafish studies exist (**Table 1**).

Li-Fraumeni Syndrome

Li-Fraumeni syndrome is a hereditary cancer predisposition disorder that albeit rare, is one of the most recognized due to the high penetrance and range of associated tumors. Affected individuals develop a spectrum of malignancies (most commonly breast cancers, bone and soft tissue sarcomas, adrenocortical carcinomas, brain tumors, and leukemia) that typically arise much earlier than their sporadic counterparts (Li et al., 1988; Malkin, 2011; **Figure 2**). More than 30% of LFS patients develop cancer during childhood and adolescence, and those that survive face an 83-fold relative risk of developing additional malignancies (Hisada et al., 1998). Germline mutations in *TP53* have been identified as the causative event in 70–80% of individuals with LFS (Malkin, 1993). *TP53* is the most commonly mutated gene in all cancers and its corresponding protein, p53, is a potent tumor suppressor that orchestrates the response to cellular stress, such as DNA damage, by activating numerous target genes that induce cell cycle arrest, inhibit proliferation, and promote apoptosis among several other cellular processes (Cadwell and Zambetti, 2001). The most frequent *TP53* mutations found in both LFS and sporadic tumors are missense mutations clustered in several hotspot regions in the DNA binding domain at codons 175, 245, 248, 273, and 282, although germline and somatic mutations present at different frequencies (**Figure 2**). However, the wide spectrum of tumors that develop and the varying time to onset cannot be simply attributed to *TP53* mutations but may be partially explained by a number of cooperating factors including copy number variants, shortened telomeres, and somatic mutations in modifier genes (Bond et al., 2004; Tabori et al., 2007; Shlien et al., 2008).

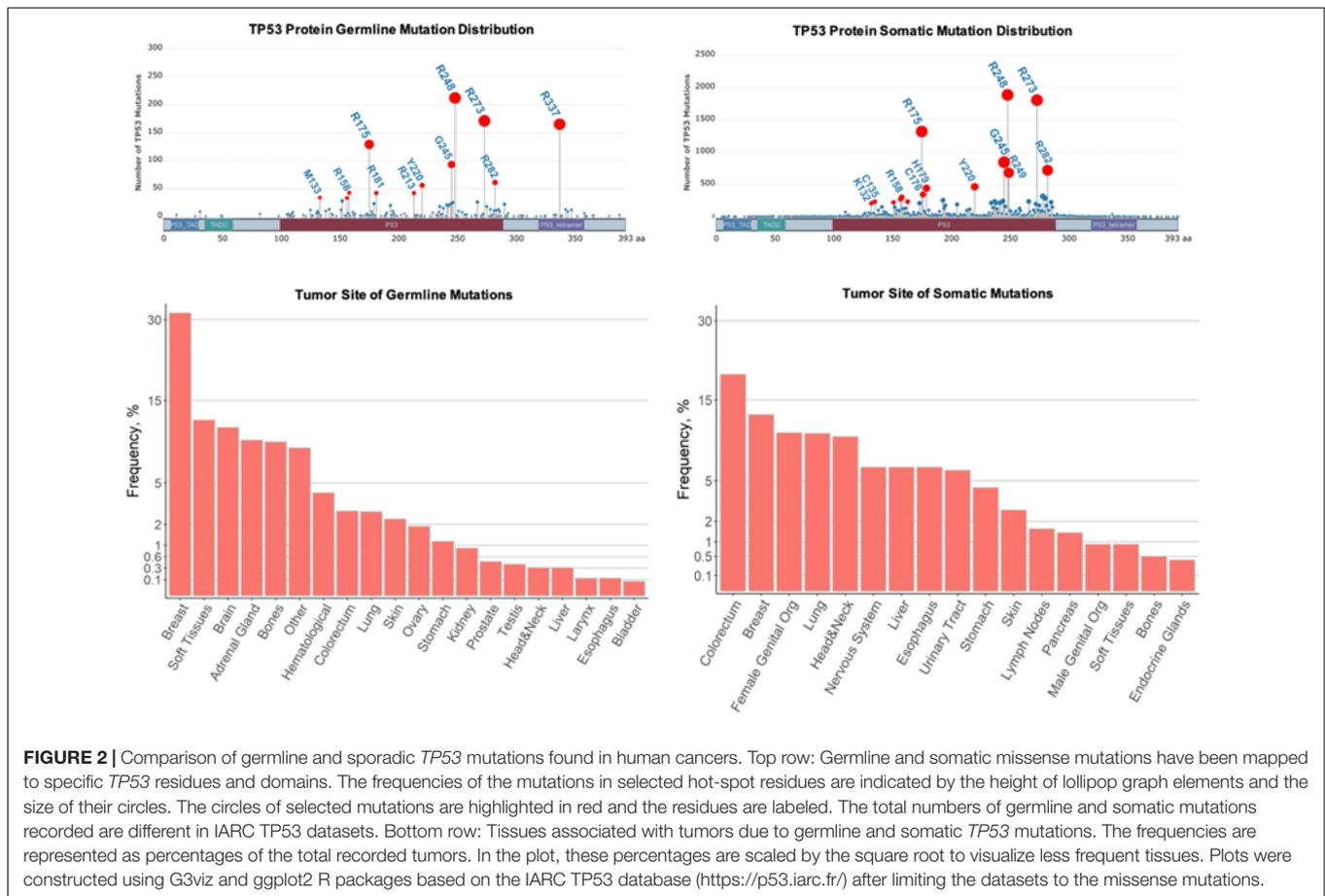
TP53 is highly conserved in zebrafish, especially in the protein DNA-binding domain where ~90% of the mutations identified in human tumors are found (Hainaut and Pfeifer, 2016). Additionally, zebrafish and mammals share induction of the same p53 target genes (e.g., *cdkn1a*, *cytg1*, *gadd45a/b*,

mdm2, *bax1*, *puma*, and *noxa*) following ionizing radiation (IR), which is used to induce DNA damage and trigger p53 pathway activation (Parant et al., 2010). Unsurprisingly, several zebrafish *tp53* mutants have been developed to investigate both sporadic cancers and LFS. One of the initial approaches used to create *tp53* point mutants was through *N*-ethyl-*N*-nitrosourea (ENU) targeted mutagenesis. This strategy was employed to generate two zebrafish *tp53* mutants with missense mutations in the DNA-binding domain, *tp53*^{N168K} and *tp53*^{M214K}, equivalent to human *TP53*^{N200K} and *TP53*^{M246K}, the latter being proximal to several hotspot mutations (Berghmans et al., 2005). These mutants were resistant to IR-induced apoptosis, indicating disrupted p53 signaling; however, the

TABLE 1 | Broad and non-exclusive functional categories of CPG proteins.

Functional category	Proteins
DNA Repair/Genome stability	ATM , ATR , ATRX , BAP1, BLM, BRCA1, BRCA2 , BRIP1 , BUB1B, DDB2, CHEK2 , DICER1 , DROSHA, DIS3L2, ERCC1, ERCC2, ERCC3, ERCC4 , FANCD2 , FANCE , FANCF , FANCA , FANCC , FANCG , FANCM , FANCI , FANCL , POLD1, POLE, POLH, MLH1 , MSH2 , MSH6 , MUTYH, NBN, NTHL1, PALB2 , PMS1, PMS2, RAD51C , RAD51D, RAD51 , RECQL, RECQL4, SPRTN , TP53 , VHL , WRN, XPA, XPC
Cell signaling	ALK, APC , AXIN2, BMPR1A, BRAF, DOCK8, EGFR, FLCN, GPC3, HRAS , ITK, KIT, KRAS , MAP2K1, MAP2K2, MPL, NF1 , NF2, NRAS, PDGFRA , PRKAR1A, PTCH1, PTEN , PTPN11 , RAF1, RET, RHBDF2, SH2B3, SH2D1A, SHOC2, SMAD4, SOS1, STAT3, STK11 , SUFU, TGFBRI1, TMEM127, TNFRSF6, TSC1 , TSC2 , TSHR
Transcriptional regulation and epigenetic modification	CDC73, CEBPA , CTR9, ETV6, GATA2 , HNF1A, JMJD1C, LMO1, MAX, MEN1, MET, MITF, PAX5, PHOX2B, PRDM9, RUNX1 , SETBP1, SMARCA4, SMARCB1, SMARCE1, SRY, WT1
Metabolism	FH, FAH, GBA, HMBS, MTAP, SDHA, SDHAF2, SDHC, SDHD, UROD
Cell adhesion and extracellular matrix	CDH1, COL7A1, EXT1, EXT2, EPCAM, GJB2
Ubiquitination and protein degradation	CBL , CYLD, PRSS1, SERPINA1, TRIM37
Telomere maintenance	DKC1 , NOP10 , NHP2, POT1, TERT
Cell cycle machinery and regulation	CDK4, CDKN1B, CDKN2A, CDKN1C, RB1
Ribosome biogenesis	DKC1 , NHP2, NOP10 , SBDS
Membrane transport	ABCB11, HFE, SLC25A13
Immunity	PRF1, ELANE
Mitochondrial RNA processing	RMRP
Cytoskeleton regulation	WASP

Proteins marked in Bold have been studied in zebrafish in the context of cancer predisposition or developmental phenotypes.



tp53^{N168K} mutants were only capable of this phenomenon when raised at 37°C instead of the normal zebrafish homeostatic temperature of 28.5°C. The *tp53*^{M214K} mutants developed malignant peripheral nerve sheath tumors (MPNSTs) with approximately 28% tumor incidence by 16.5 months. However, a low tumor penetrance combined with a lack of tumor development in heterozygous mutants indicate that this mutant does not completely recapitulate the LFS phenotype (Berghmans et al., 2005).

Another zebrafish mutant, *tp53*^{I166T}, was identified by screening the progeny of ENU-mutagenized zebrafish for the IR-induced apoptosis resistance phenotype (Parant et al., 2010). The location of this mutation is analogous to human codon 195, which is also implicated in several sporadic cancers (Kanda et al., 2013; Mullany et al., 2015). *tp53*^{I166T} mutants have characteristics of the LFS phenotype including highly penetrant tumorigenesis, especially sarcomas, and dominant negative activity of mutant p53 (Parant et al., 2010). Both the *tp53*^{I166T} homozygous and heterozygous mutants developed tumors and the *tp53*^{+/I166T} mutant tumors displayed a high rate of loss of heterozygosity (LOH) of the wild-type *tp53* allele, a characteristic frequently observed in LFS tumors. Mutants experienced aggressive tumorigenesis, with the first tumor appearing by 9 months and overall a dismal 50%

survival rate by 15 months. However, the *tp53*^{I166T} mutation was characterized as a loss of function (LOF) mutation in contrast to gain-of-function (GOF) mutations that are more commonly found in both LFS and sporadic tumors (Parant et al., 2010).

To further investigate the effect of p53 loss on tumorigenesis, a *tp53* knockout zebrafish mutant was engineered using TALEN endonucleases (Ignatius et al., 2018). Although most LFS patients possess deletions at the *TP53* locus. The *tp53*^{-/-} fish are resistant to IR-induced apoptosis and developed a wider spectrum of malignancies than the *tp53*^{M214K} and *tp53*^{I166T} mutants, including MPNSTs, angiosarcomas, germ cell tumors, and a natural killer cell-like leukemia. Homozygous *tp53*^{-/-} mutants developed tumors as early as 5 months with a 37% tumor incidence by 12 months. Heterozygous *tp53*^{+/-} mutants also developed tumors, although infrequently, suggesting LOH also contributes to tumorigenesis in these mutants.

Recently, our group optimized the oligonucleotide-based CRISPR/Cas9 point mutation insertion strategy to generate specific knock-in mutations and employed this strategy to engineer a zebrafish *tp53*^{R217H} mutant, equivalent to the human GOF *TP53*^{R248H} mutation, representing one of the most frequent hotspot mutations found in both LFS and sporadic tumors

(Prykhozij et al., 2018b). Further characterization of these mutants will help shed light on the specific mechanisms and pathways involved in LFS.

CPGs in the Wnt/ β -Catenin and PI3K/Akt/mTOR Pathways

Both the Wnt/ β -catenin pathway and the phosphatidylinositol-3-kinase/Akt/mechanistic target of rapamycin complex 1 (PI3K/Akt/mTOR) pathway are upregulated in many cancers. The Wnt/ β -catenin signaling pathway is essential for development and plays an important regulatory role in cell proliferation, polarity, and fate determination as well as for adult tissue maintenance (Logan and Nusse, 2004; MacDonald et al., 2009; Martin-Orozco et al., 2019). The PI3K/Akt/mTOR signaling pathway regulates many key cellular processes including proliferation, growth, metabolism, and survival (Jiang et al., 2020). Numerous studies have implicated crosstalk between these pathways as contributing to both tumorigenesis and therapeutic resistance in several cancer types including colorectal cancer, T-cell acute lymphoblastic leukemia, gastric cancer, and cervical cancer (Choi et al., 2019; Evangelisti et al., 2020; Guerra et al., 2020; Prossomariti et al., 2020). Oncogenic aberrations in these pathways are implicated in a wide spectrum of malignancies and are present in many syndromes associated with germline mutations in CPGs including FAP(*APC*), PJS (*LKB1*); Cowden syndrome (*PTEN*), and TSC (*TSC1/2*) (Figure 3).

Familial Adenomatous Polyposis

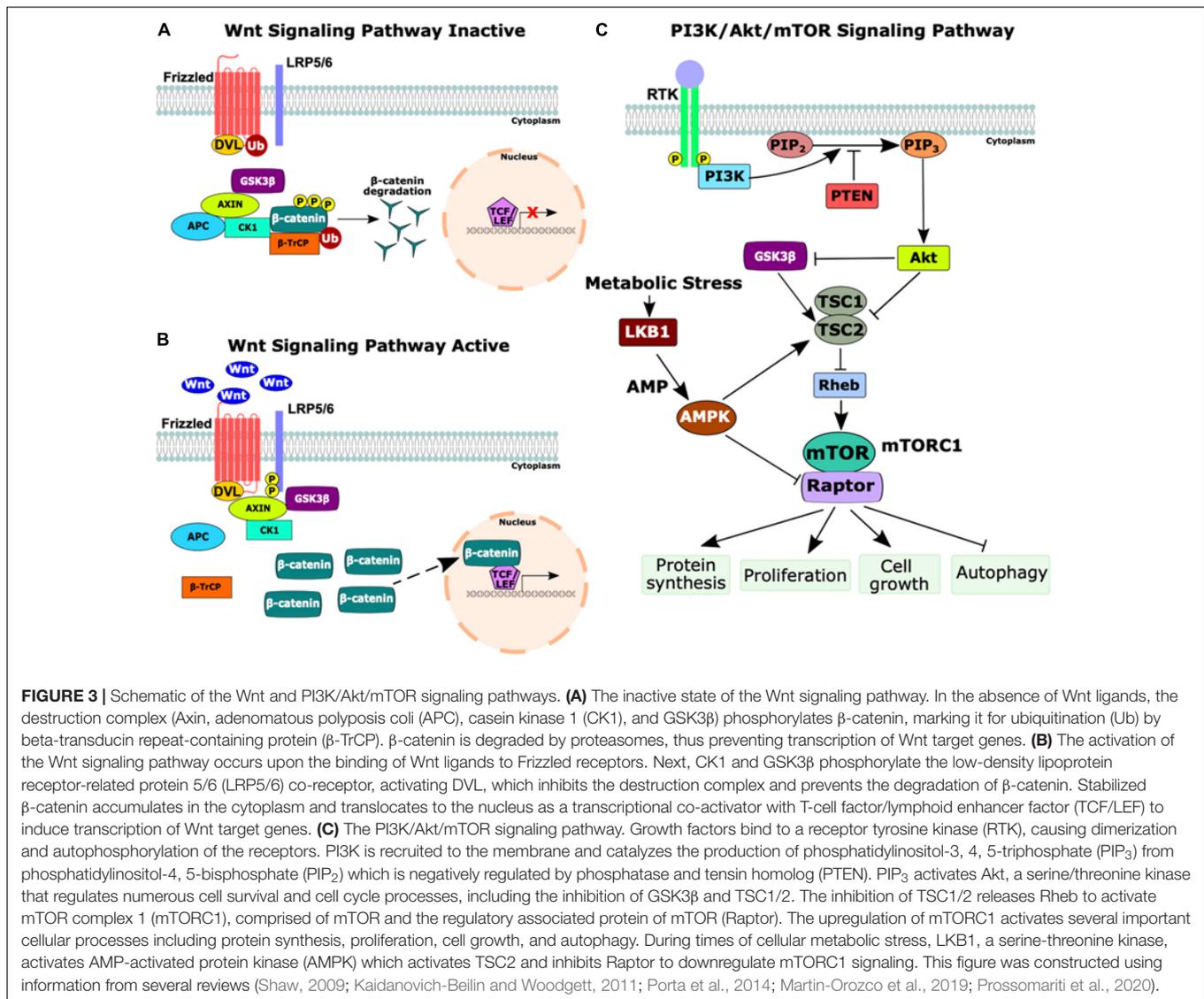
Germline truncating mutations in the *APC* tumor suppressor gene are implicated in FAP, a cancer predisposition syndrome, as loss of *APC* transforms the Wnt/ β -catenin pathway into a constitutively active state. *APC* normally regulates the stability of β -catenin and the loss of *APC* causes the accumulation of β -catenin, greatly increasing transcription of Wnt target genes and ultimately leading to cancer in many cases (Fodde et al., 2001; Figure 3). Mouse models with different truncating *Apc* mutations develop varying numbers of polyps in the small intestine, from 3 to 300 (Moser et al., 1990; Fodde et al., 1994; Oshima et al., 1995), while an *Apc* null mutant engineered with concurrent *Cdx2* mutations, a modifier gene that encodes a transcription factor important for intestinal differentiation, instead developed polyps in the colon which is more representative of human FAP (Aoki et al., 2003). These models show that mice with different truncating *APC* mutations develop tumors, although at different frequencies, and that modifier mutations influence polyp formation.

The first zebrafish *apc* mutant was created using ENU mutagenesis and contains a truncating mutation in the mutation cluster region (MCR; defined by mutations identified in human FAP tumors) (Hurlstone et al., 2003). Homozygous mutant embryos (*apc*^{MCR}) exhibit a curved body type, impaired cardiac valve development, and do not survive past 96 hours post-fertilization (hpf). Cardiac valve defects were rescued by overexpression of *APC* or Dickkopf-1, a Wnt inhibitor. The heterozygous *apc*^{MCR} mutants developed intestinal adenomas

that histologically resembled mammalian polyps with high levels of β -catenin in proliferating cells, as well as liver and intestinal tumors by 15 months following treatment with the carcinogen 7,12-dimethylbenz[a]anthracene (Haramis et al., 2006). LOH of *apc* was not detected in these lesions, which is normally found in human FAP tumors, but the accumulation of β -catenin was suggestive that *APC* functions were disrupted. A later study demonstrated that homozygous *apc*^{MCR} zebrafish embryos had a 34% reduction in intestinal epithelial cells and revealed that β -catenin was upregulated in these cells but was restricted to the cytoplasm (Phelps et al., 2009). Further, they showed that through a β -catenin-independent process, the *apc*^{MCR} embryos had suppressed intestinal cell differentiation associated with increased expression of *ctbp1*, a transcriptional co-repressor. Injection of oncogenic *KRAS*^{G12D} mRNA into *apc*^{MCR} embryos caused the nuclear accumulation of β -catenin, which greatly increased proliferation and resulted in a four-fold increase of the total intestinal cell numbers compared to non-injected *apc*^{MCR} embryos. This was not observed in *KRAS*^{G12D} injected wild-type embryos, suggesting that additional mutations may be required following the loss of *APC* to trigger the nuclear accumulation of β -catenin and robust proliferation. These characteristics are often observed in the malignant transformation of FAP adenomas as well as sporadic carcinomas (Phelps et al., 2009).

For years, the consensus was that *APC* mutations alone were responsible for the initiation of adenomas, but whole-genome sequencing of adenomas from FAP patients has implicated other non-Wnt genes (Delacruz et al., 2019). *CCR4-NOT Transcription Complex Subunit 3 (CNOT3)*, a gene important for regulating mRNA transcription, was identified as the most commonly mutated gene (excluding *APC*) in 20% of 37 FAP adenomas compared to only 1% in the sporadic/inherited colon cancer subset from the TCGA dataset (Gao J. et al., 2013; Delacruz et al., 2019). Two of the most frequent mutations identified were *CNOT3*^{E20K} and *CNOT3*^{E70K}. Knockdown of *cnot3a* in *apc*^{MCR} zebrafish embryos decreased intestinal development and differentiation (Delacruz et al., 2019). Additionally, introducing mutated human *CNOT3*^{E20K} mRNA into *apc*^{MCR} embryos rescued intestinal differentiation while *CNOT3*^{E70K} mRNA did not, suggesting that E70K is an inactivating mutation. *CNOT3*^{E70K} is commonly found in adenoma tissues and a variety of cancers, but the discovery of its inhibitory effect on intestinal differentiation is novel and suggests that *CNOT3*^{E70K} may contribute to the progression of adenomas to carcinomas (Delacruz et al., 2019).

One main tumor-suppressive function of *APC* is to promote intestinal differentiation through regulation of the retinoic acid (RA) biosynthesis pathway (Jette et al., 2004; Nadauld et al., 2004). Zebrafish *apc*^{MCR} embryos demonstrate a lack of colonocyte differentiation which is rescued by treatment with RA (Nadauld et al., 2005). Further, *CYP26A1*, a gene encoding one of the major RA catabolic enzymes, is upregulated in human FAP adenomas and sporadic colon carcinomas that have truncating *APC* mutations as well as in the intestine of



zebrafish *apc*^{MCR} embryos (Shelton et al., 2006). Inhibition of *cyp26a1* in *apc*^{MCR} zebrafish restored intestinal differentiation, further demonstrating the role of APC in RA biosynthesis regulation and implicates CYP26A1 as a potential therapeutic target. A follow-up study demonstrated that deficiencies in the RA biosynthesis pathway in *apc*^{MCR} embryos led to the upregulation of *cebpb*, a transcription factor gene, which was associated with increased cyclooxygenase-2 (*cox-2*) expression, also known as *ptgs2a* (prostaglandin-endoperoxide synthase 2a) (Eisinger et al., 2006). Elevated COX-2 expression is observed in colorectal carcinomas, is associated with the loss of APC, and ultimately drives the activation of Wnt target genes. Additionally, numerous genes important for intestinal cell fate determination are hypomethylated in homozygous *apc*^{MCR} embryos and this methylation loss was reversed upon treatment with RA (Rai et al., 2010). The hypomethylation of these key genes was associated with impaired differentiation, as intestinal cells were maintained in a progenitor-like state.

Given the role of the Wnt/ β -catenin pathway in tumorigenesis, it has been a target for therapy development. Zebrafish embryos were exposed to 6-bromindirubin-3'-oxime, a GSK3 inhibitor that activates Wnt signaling, and underwent a drug screen where axitinib, a clinically approved VEGF inhibitor, was identified as the strongest inhibitor of the Wnt pathway (Qu et al., 2016). Importantly, treatment of adult zebrafish with axitinib for 6 days revealed no observable defects or morphological changes at the concentration required to inhibit Wnt signaling. This provides preclinical evidence that axitinib could be repurposed as a cancer therapy. Further, cross regulation between the Wnt/ β -catenin and the mTOR (previously known as zTOR in zebrafish) pathways has been demonstrated in the *apc*^{MCR} zebrafish mutants. *In vitro* studies have revealed that APC activates GSK3 β which inhibits mTORC1 activity (Inoki et al., 2006; Valvezan et al., 2012). Consistent with this, mTORC1 activity is strongly upregulated in *apc*^{MCR} zebrafish mutants (Valvezan et al., 2014) and inhibition of mTORC1 partially

rescues embryonic *apc*^{MCR} defects, with further rescue of curved body types by inhibiting both mTORC1 and Wnt signaling (Valvezan et al., 2014). This suggests that simultaneous targeting of these pathways is a potentially robust therapeutic strategy.

Peutz-Jeghers Syndrome

Peutz-Jeghers syndrome is a cancer predisposition disorder caused by germline inactivating mutations in *LKB1* (Hemminki et al., 1998). Affected individuals with PJS develop both benign hamartomas and early-onset cancers, especially in the gastrointestinal tract, and females with PJS have an increased risk of developing breast cancer (Giardiello et al., 1987; Hearle et al., 2006). Somatic *LKB1* mutations have been implicated in a spectrum of malignancies, including lung cancer, cervical cancer, breast cancer, malignant melanoma, non-small cell lung carcinomas, and acute myeloid leukemia (AML) (Guldberg et al., 1999; Fenton et al., 2006; Ji et al., 2007; Matsumoto et al., 2007; Wingo et al., 2009; Marinaccio et al., 2020). During periods of cellular metabolic stress, such as low levels of ATP, glucose, or oxygen, *LKB1* plays a major role in energy metabolism control to maintain homeostasis (Kullmann and Krahn, 2018). Normally following a metabolic stress event, *LKB1* activates AMP-activated protein kinase (AMPK), leading to a signaling cascade that inhibits the mTOR pathway (Figure 3C), thus preventing cell replication and growth in unfavorable conditions (Hardie, 2007; Kullmann and Krahn, 2018).

Zebrafish *lkb1*^{-/-} mutants exhibit a starvation phenotype that is lethal by 7–8 dpf (Van Der Velden et al., 2011). Following depletion of the yolk which occurs around 5–7 dpf, homozygous mutants develop an accelerated metabolism that results in rapid depletion of their energy reserves. Treatment with rapamycin, an mTOR inhibitor, slowed the metabolic rate and prolonged survival, but did not fully rescue the mutant phenotype. These mutants demonstrate that *lkb1* is crucial for metabolic control during energetic stress, like yolk depletion, and represent a potential screening tool for compounds that decrease the accelerated metabolic rate, a common characteristic of cancer cells. The *lkb1* null mutants have also demonstrated that loss of *lkb1* prevents the activation of autophagy during the metabolic stress that ensues from the switch of nutrient absorption from the yolk to external nutrients (Mans et al., 2017). Autophagy is a cellular mechanism of self-digestion important for maintaining cellular homeostasis both under basal conditions and during metabolic stress, and defects in this process have been implicated in cancer (Rabinowitz and White, 2010). Impaired autophagy in the *lkb1* mutants was associated with an accumulation of p62, a multifunctional signaling protein important for mTORC1 activation and autophagy regulation (Duran et al., 2011), as well as decreased survival (Mans et al., 2017).

Additionally, zebrafish *lkb1*^{-/-} mutant embryos have glucose homeostasis defects, enhanced glycolysis, and increased lactate production (Kuang et al., 2016). This is similar to the metabolic state observed in cancer cells which often rely on aerobic glycolysis and fermentation of pyruvate to lactate to produce ATP instead of oxidative phosphorylation. This is termed the Warburg effect which, although inefficient for generating ATP, preserves carbon to support anabolic processes for increased proliferation

(Vander Heiden et al., 2009). As a proof-of-principle, treatment with dichloroacetate, an aerobic glycolysis inhibitor, decreased lactate production in *lkb1*^{-/-} embryos, showing the potential of these mutants to be used as a drug screening platform to identify compounds that inhibit aerobic glycolysis (Kuang et al., 2016).

Cowden Syndrome

Cowden syndrome is a rare hamartoma tumor syndrome that is characterized by mutations in *PTEN* and is associated with an increased susceptibility to benign and malignant tumors including breast, skin, and thyroid cancers (Eng, 2003). *PTEN* is a tumor suppressor gene that is frequently inactivated in many cancers and normally functions as a negative regulator of the PI3K/Akt/mTOR signaling pathway (Sansal and Sellers, 2004; Figure 3C). *PTEN* has two homologs in zebrafish, *ptena* and *ptenb*, and while single homozygous mutants develop normally, double homozygous mutants do not survive past 5 dpf (Faucherre et al., 2008). The *ptenb*^{-/-} single mutants developed spontaneous eye tumors, identified as neuroepitheliomas, around seven months of age. The presence of just one *pten* wild-type allele, as either *ptena*^{-/-}; *ptenb*^{+/-} or *ptena*^{+/-}; *ptenb*^{-/-}, is sufficient for survival to adulthood and the three mutant alleles are associated with spontaneous tumor growth (Faucherre et al., 2008; Choorapoikayil et al., 2012). Double homozygous *pten* embryos have increased proliferation and decreased apoptosis, and treatment with LY294002, a PI3K inhibitor, rescued all developmental phenotypes (Faucherre et al., 2008). Compared to other single and double mutants, the *ptena*^{+/-}; *ptenb*^{-/-} mutants were more susceptible to tumor development as they had a 10% incidence rate with 26/30 tumors found near the eye, while only 1/42 *ptena*^{-/-}; *ptenb*^{+/-} mutants developed a tumor (Choorapoikayil et al., 2012). The tumors were diagnosed as hemangiosarcomas, malignant hamartomas made up of vascular tissue, consistent with the increased frequency of hamartomas found in individuals with Cowden syndrome, and were characterized by increased proliferation, upregulated Akt signaling, and haploinsufficiency of either single *pten* allele (Choorapoikayil et al., 2012; Stumpf et al., 2015). A follow-up study from this group revealed that *ptena*^{-/-}; *ptenb*^{-/-} embryos have enhanced angiogenesis which was rescued by treatment with either LY294002 or sunitinib, a receptor tyrosine kinase (RTK) inhibitor used to reduce angiogenesis in cancer patients (Choorapoikayil et al., 2013). However, treatment with sunitinib greatly increased *vegfaa* expression in both the *ptena*^{-/-}; *ptenb*^{-/-} embryos and the hemangiosarcomas that developed in the adult haploinsufficient mutants (Choorapoikayil et al., 2013). *VEGFA*, the human ortholog of zebrafish *vegfaa*, encodes a ligand crucial for angiogenesis and thus its elevated expression may explain the relapse that is clinically observed following sunitinib treatment in some cases (Tonini et al., 2010; Kikuchi et al., 2012). Importantly, combination treatment with both LY294002 and sunitinib was successful in reducing neovascularization while maintaining normal levels of *vegfaa* in the *ptena*^{-/-}; *ptenb*^{-/-} embryos and thus has potential as a novel treatment strategy for the prevention of tumor angiogenesis (Choorapoikayil et al., 2013).

Tuberous Sclerosis Complex

Tuberous sclerosis complex is an autosomal dominant disorder associated with an increased susceptibility to renal cell carcinomas, as well as benign hamartomas that appear in multiple organs (Crino et al., 2006). This disorder is characterized by mutations in either the *TSC1* or *TSC2* CPGs which normally function to inhibit Rheb, a GTPase that activates the mTOR pathway (Figure 3C). As such, upregulation of the mTOR pathway is commonly observed in individuals with TSC (Zhang et al., 2003). Interestingly, *TSC* mutations are also associated with decreased cell proliferation and increased apoptosis which may counteract the effects of increased mTOR signaling enough to prevent the malignant transformation (Kim et al., 2013). Indeed, individuals with TSC mostly develop benign tumors compared to individuals with Cowden's syndrome who also experience mTOR upregulation but develop multiple malignancies.

Morpholino knockdown of *tsc1a*, one zebrafish paralog of *TSC1*, led to kidney cyst formation, left-right asymmetry defects, increased ciliary length, and upregulation of the mTOR pathway (DiBella et al., 2009). Homozygous *tsc2* mutant embryos (*tsc2^{vu242}*) had greatly increased mTORC1 signaling, consistent with TSC patients, but do not survive past 11 dpf (Kim et al., 2011). Heterozygous mutants had only a moderate increase of mTORC1 signaling and showed no development defects, suggesting there may be a minimum threshold of pathogenic mTORC1 signaling. As both *tsc1a* and *tsc2* homozygous mutations were embryonically lethal and heterozygous mutants did not develop tumors, Kim et al. (2013) introduced the heterozygous *tsc2^{vu242}* mutation into a mutant *tp53* background to investigate the tumorigenic potential of *tsc2* mutations. Compared to the *tp53^{M214K/M214K}* mutants, the *tsc2^{vu242/+}; tp53^{M214K/M214K}* compound mutants had an increased rate of multiple malignancies, mTORC1 signaling, and angiogenesis while treatment with rapamycin caused regression of the tumor and tumor-associated blood vessels.

RASopathies Associated With CPG Mutations

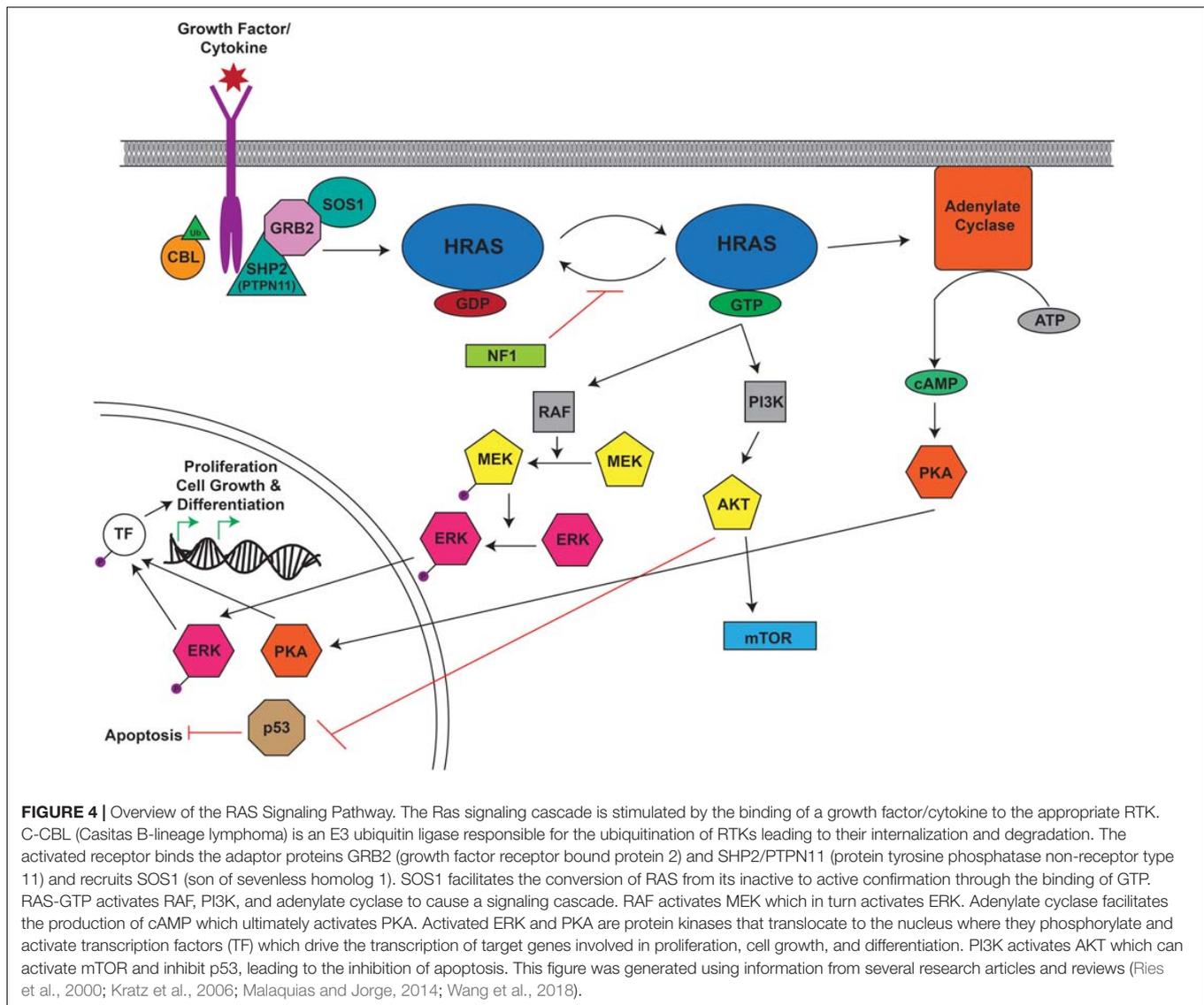
RASopathies are a group of diseases caused by germline mutations in genes that regulate the Ras/MAPK pathway. Several RASopathies are associated with CPG mutations, including Noonan, LEOPARD, *CBL*-mutated, and Costello syndromes as well as neurofibromatosis type 1 (NF1). The Ras/Raf/Mek/Erk (MAPK) pathway is a key signaling cascade that promotes cell growth, division and differentiation (Molina and Adjei, 2006; Figure 4). Ras mutations and aberrant Ras signaling have been extensively studied in the context of malignancy as mutated Ras is found in about 20% of cancers (Prior et al., 2012). Patients with inherited or *de novo* congenital RASopathies are prone to variety of malignancies during childhood as well as later in life through second hit events that eliminate their remaining wild-type allele. Affected individuals also suffer from a variety of developmental phenotypes affecting multiple organs and tissue types which often leads to the initial RASopathy diagnosis.

Noonan/LEOPARD Syndrome

Noonan syndrome (NS) and LEOPARD syndrome (LS) are RASopathies that are typically grouped together as they are genotypically and phenotypically similar (Tartaglia et al., 2011; Martínez-Quintana and Rodríguez-González, 2013) and both syndromes predispose to malignancies such as AML (Jongmans et al., 2011). NS is characterized by growth retardation, skeletal deformities, facial dysmorphism and congenital heart defects, among other abnormalities (Tartaglia et al., 2001) and shares many overlapping features with LS which is defined by multiple lentigines, electrocardiographic abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness (Tartaglia et al., 2011).

Noonan syndrome and LS are heterogenous autosomal dominant diseases caused by mutations in several genes, including the CPG, *PTPN11*. *PTPN11* encodes the ubiquitously expressed Src homology region 2 domain containing phosphatase-2 (SHP2), a protein tyrosine phosphatase involved in relaying growth signals and activating RAS and the MAPK/ERK pathway (Neel et al., 2003; Figure 4). *PTPN11* is essential for many developmental pathways and is frequently deregulated in several types of cancer (Jongmans et al., 2011). Although phenotypically similar, *PTPN11* mutations in LS and NS have biologically distinct functions. GOF *PTPN11* mutations are found in roughly half of NS patients typically as missense mutations that activate PTPN11, while *PTPN11* mutations found in LS mainly affect catalytic residues in the PTP domain that suppress phosphatase activity (Tartaglia et al., 2011). Studies of NS and LS in zebrafish have focused on examining the underlying disease mechanisms of *ptpn11* deregulation and exploring the ways in which overlapping syndromic features can be caused by opposing effects on *ptpn11* catalytic activity. One such explanation implicates the shared activation of RAS/MAPK signaling in overlapping symptoms whereas distinct NS and LS features are the result of differences in PI3K/Akt pathway expression which is uniquely upregulated in LS patients and mouse models (Kontaridis et al., 2006; Serra-Nedelec et al., 2012).

The first of these studies overexpressed either NS or LS mutant mRNA, which most notably resulted in shorter zebrafish larvae that were affected by heart abnormalities and a 'hammer-head' like phenotype reminiscent of both patients and mouse models (Jopling et al., 2007). Furthermore, the simultaneous overexpression of NS and LS mutant mRNA did not increase the severity of the phenotypes; a finding consistent with the established opposing effects of the two mutations. Similar phenotypes have been observed in subsequent studies using morpholinos to knockdown endogenous expression of zebrafish *PTPN11* orthologues, *ptpn11a* and *ptpn11b*, injecting NS or LS mutant mRNA, and in *ptpn11a^{-/-}* and *ptpn11a^{-/+}*; *ptpn11b^{-/-}* mutant fish (Stewart et al., 2010; Bonetti et al., 2014a,b). In contrast to homozygous knockout mice that die pre-implantation, zebrafish *ptpn11a^{-/-}* and *ptpn11a^{-/+}*; *ptpn11b^{-/-}* mutants live to 5dpf, while *ptpn11b^{-/+}* mutants survive to adulthood and are fertile (Yang et al., 2006;



Bonetti et al., 2014b). Together, mortality and expression data, which demonstrated lack of significant *ptpn11b* expression during early embryogenesis, implicate *ptpn11a* as the more influential ortholog (Bonetti et al., 2014b). Decreased expression of *erk* was observed in morphant and double homozygous knockout models while PI3K signaling remained unaffected (Stewart et al., 2010; Bonetti et al., 2014b). Congruent with previous observations, phospho-Akt levels increased only in embryos injected with LS mutant mRNA (Bonetti et al., 2014a). Additionally, NS/LS zebrafish models have been used to investigate the role of *PTPN11* mutations in both congenital sensorineural hearing loss and heart defects which are found in individuals with NS and LS (Sarkozy et al., 2008; Bonetti et al., 2014a; Gao et al., 2020).

c-CBL Mutation Associated Syndrome

Clinical features of patients with germline *c-CBL* (*Casitas B-lineage Lymphoma*) mutations partially overlap those

of NS, including developmental delays, growth defects, facial dysmorphia, and a predisposition to hematological malignancies, specifically juvenile myelomonocytic leukemia (JMML) (Niemeyer et al., 2010; Perez et al., 2010). CBL is an E3 ubiquitin ligase that targets both active β -catenin and FLT3 (fms related receptor tyrosine kinase 3): two factors important for tumor-induced angiogenesis which have also been implicated in AML (Lyle et al., 2019).

The *c-cbl*^{H382Y} zebrafish ENU-induced line (Peng et al., 2015) harbors a missense mutation within a highly conserved domain critical for transfer of ubiquitin to target molecules (Thien and Langdon, 2001; Deshaies and Joazeiro, 2009). *c-cbl*^{H382Y} zebrafish displayed a myeloproliferative phenotype with increased numbers of hematopoietic stem cells (HSCs), erythrocytes and neutrophils at 5 dpf and a median survival time of only 15 dpf (Peng et al., 2015). phospho-S10 histone H3 (pH3) antibody/staining revealed that these HSCs were highly proliferative and remained more abundant in older fish, despite

no obvious impairments in HSC differentiation. In contrast to the *c-cbl^{H382Y}* line, zebrafish injected with human phospho-tyrosine-inactive mutation *c-CBL^{Y731F}* mRNA, an amino acid residue that when phosphorylated suppresses Wnt signalling, had enhanced angiogenesis and increased expression *vegfa* while those injected with the phosphomimetic mutation *c-CBL^{Y731E}* mRNA displayed impaired angiogenesis and decreased expression *vegfa* (Shivanna et al., 2015). Thus, the different phenotypes were likely observed as the different missense mutations affect the binding of c-cbl to different proteins.

Costello Syndrome

Costello syndrome (CS), another NS-like syndrome, is caused by inherited activating mutations in *HRAS*, typically in the form of missense mutations (Aoki et al., 2005). CS has phenotypic features similar to NS, as well as the distinctive and common formation of benign cutaneous papillomata within the perinasal and perianal regions (Aoki et al., 2005). Santoriello and colleagues developed a zebrafish model of CS that ubiquitously expresses the constitutively active *HRAS^{G12V}* gene (Santoriello et al., 2009). *HRAS^{G12V}* transgenic fish were shorter, had a smaller heart with thicker walls, a flattened head and suffered from scoliosis; features indicative of Costello syndrome. Tumors frequently formed in fish between 5–12 months and included melanoma, gastrointestinal carcinoma, hepatocarcinoma and rhabdomyosarcoma. Congruent with mouse models (Schuhmacher et al., 2008), ERK1/2 and Akt phosphorylation were not upregulated in *HRAS^{G12V}* transgenic zebrafish and remain unlikely drivers of CS pathology; rather, the ability of HRAS to induce hyperproliferation, DNA damage, and cellular senescence through the DNA damage response (DDR) is a well-documented cause (Di Micco et al., 2006). Indeed, *HRAS^{G12V}* transgenic zebrafish demonstrated oncogene-induced senescence in regions of the heart and brain as well as increased expression of DDR markers within various tissues (Santoriello et al., 2009).

Neurofibromatosis Type 1

Neurofibromatosis type 1 is an autosomal dominant disorder caused by mutations to the RAS GTPase activating domain of the *NF1* gene. This mutation renders NF1 unable to hydrolyze RAS-bound GTP to GDP, ultimately activating the RAS signaling pathway (Cichowski and Jacks, 2001; **Figure 4**). Affected individuals develop cardiovascular abnormalities, nervous system defects including cognitive defects, and an increased incidence of malignancies, including JMML, gliomas, and MPNSTs. Homozygous deletion of murine *Nf1* results in embryonic lethality mid-gestation while heterozygous mice remain viable and fertile, but this mutant does not fully recapitulate the human phenotype (Brannan et al., 1994; Silva et al., 1997). More complex mouse models that use Cre/lox technology to express mutant *NF1* within specific cell populations better reproduce specific features of NF1 but lack the ability to study the disease as a whole (Zhu, 2002; Gitler et al., 2003).

Zebrafish have been successfully used to widen the scope of *in vivo* NF1 research using ZFN-mediated knockout of

NF1 paralogues, *nf1a* and *nf1b*, and allogenic transplantation techniques. Double homozygous *nf1* mutant zebrafish display macrocephaly as well as an increase in oligodendrocyte precursor cell (OPC) numbers and migration within the spinal cord (Shin et al., 2012). Further, defects in myelin structure formation and axon wrapping lead to decreased myelination in double homozygous *nf1* mutant fish, despite no change in *myb* (*myelin basic protein*) expression within the central nervous system. Several studies have postulated *NF1* as a positive regulator of adenylate cyclase (Guo et al., 2000; Tong et al., 2002; Dasgupta et al., 2003) and linked cAMP signaling to the expression of myelin components. *NF1* mutations associated with cognitive dysfunction have been linked to regions outside of the GTPase-activating protein-related domain (GRD) (Fahsold et al., 2000), suggesting that some features of NF1 may be caused by aberrant cAMP signaling rather than activated Ras. The memory formation of *nf1* mutant larvae was tested by evaluating the O-bend response to light/dark stimulus (Wolman et al., 2014). Trained *nf1* mutant larvae had a delayed O-bend response which was improved upon treatment with chemical stimulants for the cAMP pathway, but not MAPK or PI3K inhibitors. Similarly, when testing memory recall, *nf1* mutants again showed deficits, however these fish showed improvements over the course of the experiment, suggesting that cognitive defects are reversible and that patients may benefit from specialized learning environments and cAMP pathway effectors. Additionally, when crossed into a p53 null background, *nf1* mutants develop high-grade gliomas and MPNSTs, similar to what is commonly observed in NF1 patients (Shin et al., 2012).

Neurofibromatosis type 1 modulates neuronal differentiation (Hegedus et al., 2007), and NF1 patients frequently develop benign neurofibromas that can undergo malignant transformation to MPNSTs, commonly through the loss of CDKN2A and p53 as well as amplification of *PDGFRA* (platelet-derived growth factor receptor A) (Mantripragada et al., 2008; Zietsch et al., 2010; Lee et al., 2014). The over-expression of *PDGFRA* has been linked to the malignant transformation of MPNSTs (Holtkamp et al., 2004). When modeled in transgenic zebrafish, overexpressing *PDGFRA* in a *nf1a^{+/-}*; *nf1b^{-/-}*; *tp53^{M214K/M214K}* background accelerated the onset of MPNSTs (Ki et al., 2017). Interestingly, over-expression of wild-type *PDGFRA*, was better at accelerating tumor development than the constitutively active *PDGFRA* which activated Ras signaling to levels higher than optimal for tumor growth. MPNST tumors from wild-type *PDGFRA* transgenic fish were harvested and transplanted into *nf1a^{+/-}*; *nf1b^{-/-}*; *tp53^{M214K/M214K}* larvae, establishing an allogenic transplant model that can rapidly test effectiveness of therapies. Indeed, sunitinib treatment effectively arrested progression of the transplanted MPNSTs in these mutants, and treatment with both sunitinib and trametinib, a MEK inhibitor, enhanced this therapeutic effect. Additionally, both DNA topoisomerase I (Topo I) targeting drugs and mTOR kinase inhibitors were identified as the most effective single agent therapies that avoided excessive toxicity (Ki et al., 2019). Treating with Topo I inhibitor Irinotecan and mTOR inhibitor AZD2014, acted synergistically to induce apoptosis and block protein synthesis resulting in tumor cell death.

Hematopoietic Disorders Associated With CPG Mutations

Several familial hematologic syndromes are associated with mutations in CPGs. Inherited bone marrow failure syndromes (IBMFS), including dyskeratosis congenita (DC), Schwachman-Diamond syndrome (SDS) and Fanconi anemia (FA) (discussed in a later section) are rare heterogeneous disorders caused by genetic defects that impair normal hematopoiesis, leading to anemia, cytopenia, and a variety of solid tissues/organ abnormalities. Additionally, these disorders predispose patients to develop malignancies. Zebrafish have long been used to study blood development and model hematologic diseases due to their high level of genetic conservation, especially within gene pathways critical to hematopoiesis (Jing and Zon, 2011). Similar to humans, zebrafish blood development occurs in two waves and within analogous structures recapitulating all blood cell types (Figure 5) (Davidson and Zon, 2004).

Dyskeratosis Congenita

Dyskeratosis congenita is a heterogenous disease caused by mutations in multiple genes involved with telomere maintenance including telomerase reverse transcriptase (*TERT*) and members of the H/ACA ribonucleoprotein (RNP) complex. H/ACA RNPs, NOP10 and DKC1 (dyskerin pseudouridine synthase 1) are involved in ribosome biogenesis, splicing and processing of various RNAs, and are also part of the telomerase complex together with TERT and the telomerase RNA component (Kiss et al., 2010). Telomerase is important for counteracting the progressive shortening of replicated DNA, especially in highly proliferative cell types and stem cells, and prevents activation of DDR pathways that could result in cellular senescence or apoptosis. Affected individuals develop nail dystrophy, mucosal leukoplakia, skin hyperpigmentation, increased risk of both hematological and solid cancers as well as bone marrow failure (BMF) (Cole et al., 1930; Alter et al., 2009). DC mortality is predominantly due to BMF, although the cumulative incidence of cancer is high with roughly 40% of patients developing a malignancy by the age of 50 (Alter et al., 2009). Mouse models of DC are limited because mouse telomeres are roughly 5–10 times longer than their human counterparts (10–15 kb) (Kipling and Cooke, 1990) while zebrafish telomeres are more similar to humans at 15–20 kb (Carneiro et al., 2016).

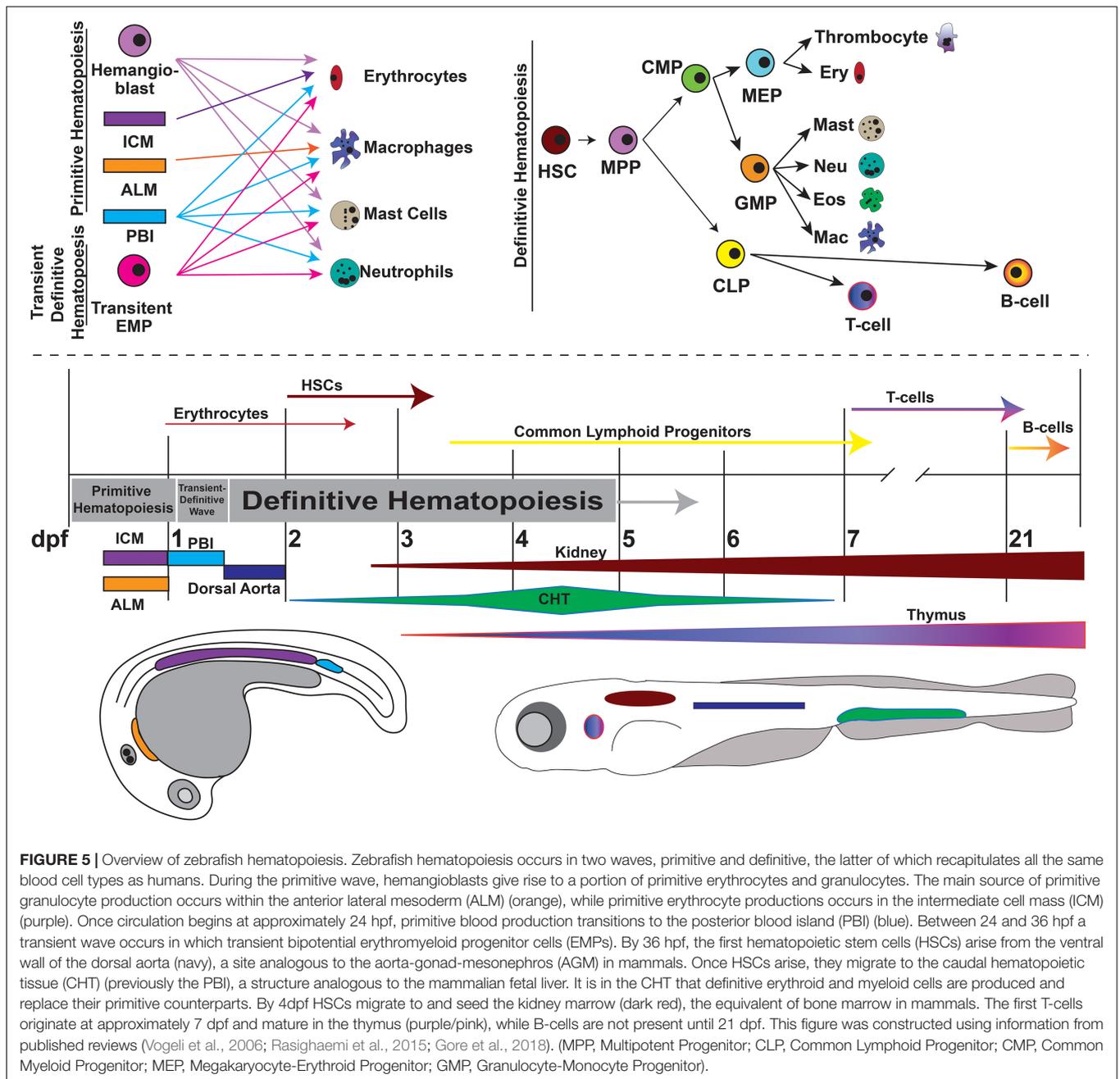
Zebrafish represent an advantageous platform for modeling DC and telomere deficiencies, and *tert* mutant zebrafish have reduced telomere length, premature aging, decreased fertility, and shorter lifespans (Anchelin et al., 2013). Dramatic telomere shortening triggered p53 activation, whereas p53 knockdown rescued apoptosis in larvae although telomere length remained short. Adult *tert* mutants had increased levels of apoptosis within the kidney marrow (the equivalent of human bone marrow), which also showed signs of depletion upon histopathological analysis (Henriques et al., 2013). Notably, reintroducing *tert* into second-generation mutants which typically died by 7dpf prevented both mortality and further telomere shortening (Anchelin et al., 2013). This suggests

that drugs that activate wild-type *TERT* expression could be a potential therapy for DC.

The most severe form of DC, Hoyeraal-Hreidarsson syndrome, is caused by mutations to the catalytic domain of *dyskerin* (*DKC1*), suggesting that telomere-independent mechanisms contribute to DC (Knight et al., 1999; Zhang et al., 2012). Morpholino-mediated knockdown of zebrafish *dkc1* significantly reduced the development of all blood cell lineages during definitive hematopoiesis (Zhang et al., 2012). Similar to the knockdown of *tert*, *dkc1* morphants had a significant increase in the expression of p53 and pro-apoptotic genes such as *bax1*. Hematopoietic defects could be reversed by inhibiting p53 expression in *dkc1* morphants, again suggesting that DC-associated cytopenia is partially p53 dependent. Interestingly, investigators observed significant defects in 18S rRNA production prior to the onset of abnormal blood development in *dkc1* morphants and *nop10* mutant fish (Pereboom et al., 2011; Zhang et al., 2012). Extensive p53-mediated apoptosis in *nop10* mutants was linked to increased binding of Mdm2 to Rps7 resulting in degradation of Mdm2 protein which is normally responsible for p53 degradation. Furthermore, *nop10* mutants failed to form HSCs despite no evidence of telomere shortening, and cytopenia was rescued through the inactivation of p53, providing additional evidence that p53-mediated apoptosis of HSCs is driven by ribosomal biogenesis defects and not solely by telomere shortening (Pereboom et al., 2011).

Shwachman-Diamond Syndrome

Schwachman-Diamond syndrome is an autosomal recessive disorder caused by mutations in *SBDS* that either interrupt transcript splice sites or result in a truncated protein (Boocock et al., 2003). Affected individuals develop exocrine pancreatic insufficiency, impaired hematopoiesis, skeletal abnormalities, gastrointestinal dysfunction and an increased risk of leukemia with ~36% of patients developing myelodysplastic syndrome (MDS) (a syndrome which represents a pre-leukemia state) or AML by age 30 (Burroughs et al., 2009; McReynolds and Savage, 2017). *SBDS* is involved in ribosome biogenesis and function with a role in 40S and 60S ribosomal subunit maturation and assembly (Stepensky et al., 2017; Warren, 2018; Tan et al., 2019). Zebrafish *slds* shares 86% amino acid identity with humans and retains the same intron/exon boundaries, making it an advantageous model for SDS modelling (Oyarbide et al., 2019). Morpholino-mediated knockdown of *slds* significantly decreases neutrophil proliferation and migration as well as results in pancreatic hypoplasia and abnormal morphology (Venkatasubramani and Mayer, 2008; Provost et al., 2012). Additionally, *slds* morphant embryos displayed broad changes in ribosomal gene expression. Simultaneous knockdown of p53 did not restore normal neutrophil numbers or normal pancreatic morphology, suggesting SDS pathology is not p53 dependent. Two CRISPR/Cas9 generated *slds* mutant zebrafish, *slds^{nu167}* producing a truncated protein and *slds^{nu132}* with a 7 amino acid in-frame deletion, had reduced length and weight and died by 21 dpf, which was not rescued by crossing *slds* mutants into a *tp53* mutant (*p53^{M214K/M214K}*)



background (Oyarbide et al., 2020). Similar to the morphant studies, *slds^{nu132}* displayed neutropenia, while the number of macrophages and hemoglobin production remained normal. In addition, kidney marrow morphology remained normal at 21 dpf. In contrast to morphant studies, neither pancreatic hypoplasia nor changes to *ptfla* expression were observed in *slds* mutant larvae up to 15 dpf but rather pancreatic atrophy began in 15–21 dpf mutant fish after seemingly normal development. Therefore, zebrafish *slds* models recapitulate several patient phenotypes including neutropenia and pancreatic defects, although the mechanisms that lead to pancreatic atrophy have not yet been explained.

Wiskott-Aldrich Syndrome and X-Linked Congenital Neutropenia

Wiskott-Aldrich syndrome (WAS) and X-linked congenital neutropenia (XLN) are severe immunodeficiency diseases caused by both LOF and GOF mutations in the Wiskott-Aldrich syndrome protein (WASp), respectively. WASp is an important regulator of the actin cytoskeleton in hematopoietic cells and is therefore a key player in leukocyte motility, phagocytosis, and a productive immune response (Tsuboi and Meerloo, 2007; Thrasher and Burns, 2010). Individuals with WAS or XLN develop frequent and recurrent infections while WAS patients also suffer from autoimmunity, bleeding disorders, and various

types of leukemia and lymphoma at an incidence of roughly 13–22%, typically before 18 years of age (Sullivan et al., 1994; Imai et al., 2004; Keszei et al., 2018). One explanation for such predisposition is a failure to identify and eradicate malignant cells prior to tumor formation due to compromised immune function. Zebrafish offer a unique advantage over mouse models as the optical clarity of zebrafish larvae allows for the live imaging and analysis of immune cell response and migration.

Both zebrafish homologs of WASp, *wasp1* (*waspb*) and *wasp2* (*waspa*), are well conserved with 52% and 41% amino acid similarity to humans, respectively, and are hematopoietically restricted (Cvejic et al., 2008). Live imaging of *lyz:eGFP* transgenic zebrafish (fluorescently labeled neutrophils and macrophages) with knockdown of *wasp1* revealed a reduction in neutrophils and macrophage migration to wounds in morphant larvae, while the number of immune cells remained normal. Furthermore, *wasp* morphants had decreased survival when inoculated with pneumococci (Rounioja et al., 2012), resembling life-threatening pneumococcal infections seen in individuals with WAS (Sullivan et al., 1994). Homozygous *wasp1* mutant zebrafish recapitulated the ineffective immune responses to both inflammatory wounds and bacterial challenges seen in morphant studies and further revealed that the ineffective formation, maintenance and retraction of neutrophil pseudopods and uropods contributed to the defective immune response (Jones et al., 2013). Expression of *wasp* was replaced in the *wasp1* null background using a variety of patient-specific WASp transgenes and the expression of wild-type human WASp rescued neutrophil migration and wound recruitment and restored phagocytosis when challenged with *S. aureus*. In comparison, phospho-dead WASp^{Y291F} provided minimal improvement to neutrophil migration and did not improve microbe uptake by macrophages. Moreover, expression of constitutively active WASp^{I294T}, observed in patients with XLN, resulted in an overall reduction of neutrophils but upon wound-response, WASp^{I294T} expressing neutrophils had significantly increased cell velocity (Jones et al., 2013).

GATA2 Deficiency Syndromes

GATA2 is a transcription factor critical to both primitive and definitive blood development and lymphatic vessel formation. GATA2 haploinsufficiency, from inherited or spontaneous heterozygous mutations, produces a range of hematopoietic disorders in humans including monocytopenia, mycobacterium avium complex (MonoMAC) syndrome; dendritic cell, monocyte, B and NK lymphoid (DCML) deficiency; congenital neutropenia; and familial MDS/AML (Bigley and Collin, 2011; Dickinson et al., 2011; Hahn et al., 2011; Hsu et al., 2011). Affected individuals often present with infection, cytopenias, aplastic anemia and hypocellular bone marrow, of whom 75% will develop MDS/AML with a median age of onset of 20 years (Wlodarski et al., 2016, 2017). Control of Gata2 expression has been linked to a conserved +9.5 kb enhancer region (Hsu et al., 2013), where deletions are found in ~10% of patients with MonoMAC syndrome (Wlodarski et al., 2016). Zebrafish have two highly conserved homologs of GATA2, *gata2a* and *gata2b*, with 57% and 67% conserved amino acid identity (Jin et al.,

2004; Lutskiy et al., 2005; Gillis et al., 2009; Butko et al., 2015). Comprehensive expression and lineage tracing analysis indicates *gata2a* and *gata2b* zebrafish have distinct patterns of expression; *gata2a* is expressed throughout the endothelium and is more broadly required for vascular morphogenesis while *gata2b* is only present within the hemogenic endothelium of the dorsal aorta and has a more specialized role to activate *runx1* expression required for HSC formation (Butko et al., 2015). Thus, this study suggests segregated endothelial and hematopoietic functions of the two zebrafish *gata2* paralogs.

Using CRISPR/Cas9, Dobrzycki and colleagues introduced a deletion to the conserved +9.5 enhancer within intron 4 of zebrafish *gata2a* (*gata2a*^{Δi4/Δi4}) (Dobrzycki et al., 2020). Unlike mouse Gata2^{Δ+9.5} models (Gao X. et al., 2013), *gata2a*^{Δi4/Δi4} fish survive past embryogenesis, and had decreased expression of HSC markers, *runx1* and *cmyb*, during primitive hematopoiesis. Despite the recovery of *runx1* and *cmyb* expression to wild-type levels by 48hpf, adult *gata2a*^{Δi4/Δi4} fish had half the number of blood cells within their kidney marrow and a surplus of immature blasts was found in 10% mutants. Additionally, these fish had a high incidence of cardiac edema and infection within less than 6 months of age, suggestive of the immune deficiency and lymphatic defects seen in MonoMAC syndrome (Dobrzycki et al., 2020).

Preliminary results from two *gata2b* mutant zebrafish models highlight how zebrafish can elucidate the link between GATA2 deficiencies and MDS/AML predisposition as *gata2b*^{+/-} mutants have reduced myeloid differentiation and dysplastic myeloid cells in older animals (Avagyan et al., 2017; Gioacchino et al., 2019). Cre/Lox fluorescent lineage tracing technology is being used to assess the expansion of single-color clones within the kidney marrow of 3dpf *gata2b*^{+/-} larvae with/without concurrent mutations (Avagyan et al., 2017). Juvenile *gata2b*^{+/-} zebrafish had a decreased myeloid compartment in the kidney marrow of which 30% also had an expansion of single-color clones by 3 months post-fertilization, an event not observed in wild-type fish. This myelocytopenic phenotype is more profound in *gata2b*^{+/-} fish when challenged with similar concurrent mutations, as compared to wild-type, and is indicative of a predisposition to MDS/AML.

Familial AML Associated With CEBPA Mutations

CEBPA, a transcription factor important for normal myelopoiesis and granulocyte differentiation, is mutated in ~10% of individuals with AML (Pabst et al., 2001; Gombart et al., 2002). Of these, about half have biallelic mutations consisting of mutations in both hotspot N- and C- terminal regions that are typically acquired somatically. Mutations in the N-terminal region frequently produce frameshifts that lead to premature stop codons and translation of a dominant negative proteins, while C-terminal mutations are usually in-frame indels (Pabst et al., 2001; Nerlov, 2004). Germline mutations typically occur at the N-terminal hotspot and these individuals acquire a second C-terminal mutation somatically (Fuchs et al., 2008; Tawana et al., 2015). Although a rare occurrence, individuals with germline CEBPA mutations develop AML much earlier

than sporadic AML at a median age of 25 versus 67 years (McReynolds and Savage, 2017).

Zebrafish studies reveal a conserved role for *cebpa* and its involvement in primitive erythropoiesis and HSC formation (Liu et al., 2007; Yuan et al., 2011, 2015). Myeloid defects, including a lack of embryonic neutrophils and macrophages were observed in three different *cebpa* mutant lines (*moli^{h_{kz7}}*, *cebpa^{sum2}*, *cebpa^{sum3}*) (Dai et al., 2016). Myeloid defects in *cebpa* deficient *moli^{h_{kz7}}* mutants could be explained, in part, by the aberrant cell cycle arrest of myeloid progenitors once the definitive wave of hematopoiesis began, suggesting that *cebpa* is more important for myeloid progenitor migration and maintenance than initiation. To more accurately model *CEBPA*-associated MDS/AML, two new *cebpa* mutant zebrafish lines were engineered with either N- or C- terminal mutations (Hockings et al., 2018). Preliminary evidence demonstrates striking defects in mature myelocytes and monocytes in all double mutant larvae, while leukemic transformation was only observed in mutants between 4-6 weeks of age that carried at least one N-terminal mutation.

Familial Platelet Disorder With Predisposition to AML

Patients with familial platelet disorder with predisposition to AML (FPD/AML) suffer from thrombocytopenia, platelet dysfunction, increased risk of bleeding and of developing hematological malignancies, as 35–40% will develop MDS/AML by 33 years of age (Sood et al., 2017). FPD/AML is associated with germline mutations in *RUNX1*, a master transcription factor regulator of hematopoiesis and blood vessel development, most commonly small indels or point mutations that can lead to haploinsufficiency or dominant negative effects (Latger-Cannard et al., 2016). Monoallelic *RUNX1* mutations alone are insufficient to initiation leukemogenesis, rather secondary somatic mutations are required within *RUNX1* or other AML-associated genes (Song et al., 1999; Preudhomme et al., 2009; Ripperger et al., 2009; Schmit et al., 2015; Antony-Debré et al., 2016). Neither heterozygous *RUNX1* murine or zebrafish models develop FPD phenotypes, however a germline homozygous *runx1* deletion is embryonically lethal in mice (Okuda et al., 1996) while zebrafish *runx1^{W84X/W84X}* mutants are viable to adulthood (Jin et al., 2012). Like human patients, homozygous *runx1^{W84X}* larvae have a severe reduction in both immature and mature neutrophils as well as in mature thrombocytes (Sood et al., 2010; Jin et al., 2012), mimicking findings in conditional knockout mice (Ichikawa et al., 2004; Growney et al., 2005). Furthermore, adult *runx1^{W84X/W84X}* fish display reduced number of B-cells (Chi et al., 2018), a phenotype reminiscent of common variable immunodeficiency disorder (Li et al., 2015). Critically, neither myelocyte dysplasia nor an MDS-like phenotype have been reported in *runx1^{W84X/W84X}* fish and it is likely that additional models involving second-hit mutations are needed to more accurately model FPD/AML in zebrafish (Chi et al., 2018).

Congenital Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare IBMFS that often presents in infancy with mutations in the gene

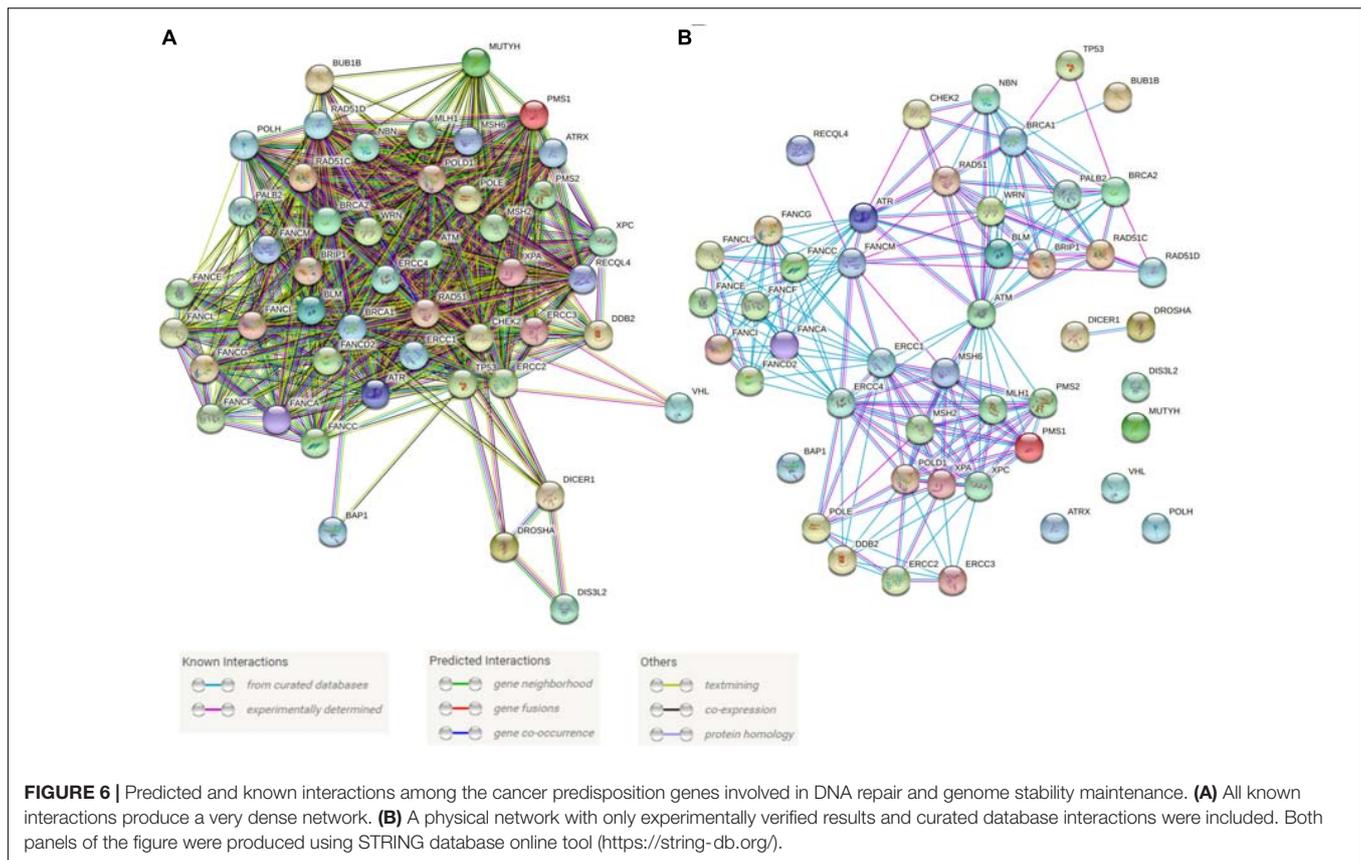
MPL which encodes the receptor for thrombopoietin. Affected individuals develop severe thrombocytopenia, aplastic anemia, hemorrhage and a predisposition to leukemia (Germeshausen et al., 2006; Al-Qahtani, 2010). To date, only one CAMT zebrafish model exists as *mpl^{smu3}*, a LOF mutant that experiences severe thrombocytopenia during embryonic development (Lin et al., 2017). Although the development of HSC and progenitor populations are unaffected, there was a significant reduction in thrombocyte precursor proliferation and thrombocyte markers remained significantly low in adult fish. Similar to the high risk of prolonged bleeding observed in CAMT, *mpl^{smu3}* larvae displayed reduced hemostasis and abnormal bleeding. To further adapt their model as a platform for high-throughput drug screening, Lin et al. generated the thrombocyte-specific fluorescent transgenic reporter line Tg(*mpl:eGFP*)*smu4; mpl^{smu3}*, and as proof-of-principle, showed that treatment with recombinant human IL-11, a cytokine important for megakaryopoiesis (Peeters et al., 2008), modestly increased thrombocyte populations in *mpl^{smu3}* larvae.

CPG Mutations Involved in DNA Repair and Genome Stability Maintenance

Genes linked to DNA repair and genome stability comprise the single most numerous group of CPGs and represent 50 out of 152 genes currently identified, further emphasizing the fundamental importance of genome stability maintenance in cancer development (Table 1). We undertook an analysis of the interactions within this group of 50 genes using the STRING database (Szklarczyk et al., 2015). The inclusion of all known associations results in a highly dense interaction network with only the DICER1-DROSHA-DIS3L2 group, VHL, and BAP1 exhibiting minimal links (Figure 6A) while focusing on well-established physical interactions results in a sparser network highlighting the best-known interactions (Figure 6B). Further, these 50 proteins are part of many dynamic protein complexes with several hundred members, but their discussion is beyond the scope of this review. Instead, we will focus on the contributions that zebrafish model research has made towards understanding the fundamental biology and cancer models associated with these CPGs.

Ataxia-Telangiectasia (A-T) Genes (*ATM*, *ATR* and *CHEK2*)

ATM (A-T mutated) and *ATR* (A-T and Rad3 mutated) are key regulators of the initiation of the DDR (Awasthi et al., 2016). *ATM* mutations are responsible for Ataxia-Telangiectasia (A-T) which is characterized by progressive ataxia, dilatation of small vessels (telangiectasia), immune defects, genome instability and malignancy while *ATR* mutations are implicated in Seckel syndrome which is associated with dwarfism, microcephaly, growth defects and intellectual disabilities (Weber and Ryan, 2015). *ATM* is activated by DNA DSBs while *ATR* is activated primarily by single-stranded DNA filaments resulting from replication stress (Figure 7A). Both *ATM* and *ATR* belong to the PI3K-related kinase (PIKK) family and upon activation they phosphorylate many target proteins including p53, Chk2 and Chk1 kinases, which mediate many of the downstream effects



of ATM and ATR (Awasthi et al., 2016; **Figure 7**). *CHEK2* is also an established CPG confirming the importance of this pathway in cancer.

Zebrafish ATM is highly conserved in specific domains, exhibits a broad pattern of expression at early stages and morpholino knockdown results in increased sensitivity to γ -irradiation (Imamura and Kishi, 2005). Further, in *prim1* (DNA primase subunit 1) zebrafish mutants, knockdown of *atm* or *atr* is too toxic for retinal development, but retinal apoptosis due to DNA damage from faulty DNA replication is mostly suppressed with chemical inhibition of ATM (via KU55933) or ATM/ATR (via CGK733), or knockdown of *chk2* (Yamaguchi et al., 2008). Similar observations were made in the *ugly duckling* (*udu*) zebrafish mutant now mapped to the *gon4l* gene which has been recently implicated in regulating several pleiotropic mechanisms including DNA repair and genome stability (Lim et al., 2009; Tsai et al., 2020). p53-mediated apoptosis in this mutant could be successfully inhibited by either knockdown of *tp53*, *chk2* or chemical inhibition of ATM.

Even though zebrafish ATR does not appear to control the DDR directly, it has been linked to cilia formation in Kupffer's vesicle cells involved in establishment of left-right asymmetry (Stiff et al., 2016). ATR knockdown in zebrafish reduces the size of the Kupffer's vesicle, shortens cilia in this area, reduces *gli1* levels, a Hedgehog pathway target, and induces p53 targets *p21* and *puma*. The authors linked p53 target induction to Hedgehog signaling inhibition, but it is more likely to be a direct ATR

knockdown effect due to limited evidence of Hedgehog pathway inhibition. Morphologically, ATR knockdown produced left-right asymmetry defects, curved bodies, small eyes and narrow heads in zebrafish embryos, phenotypes supportive of significant Hedgehog signaling inactivation. As of this review, there are no characterized zebrafish mutants of either *atm* or *atr*. Based on the above studies, null mutants may have strong phenotypes and generation of both null and precise point mutants would help identify the spectrum of potential developmental and cancer predisposition phenotypes of *ATM* and *ATR* mutations in zebrafish to discern the relative roles of these kinases in genome stability maintenance and tumor suppression.

Fanconi Anemia Genes

Fanconi anemia is a genetic disorder caused by mutations in a group of genes involved in several aspects of DNA repair. Collectively known as the Fanconi anemia pathway, this group (22 known members) of FA proteins forms multiple complexes and mediate DNA interstrand crosslink (ICL) repair resulting from endogenous aldehydes or exogenous crosslinking agents (Liu et al., 2020). Briefly, this involves fork convergence, ICL recognition by the FA core complex, and removal of DNA replication components, involving FA genes such as *BRCA2*, *RAD51* and others (**Figure 7B**). FA is an IBMFS with disease characteristics that include cancer predisposition, fertility issues, growth retardation, and congenital abnormalities affecting many tissue types (Ramanagoudr-Bhojappa et al., 2018). Individuals

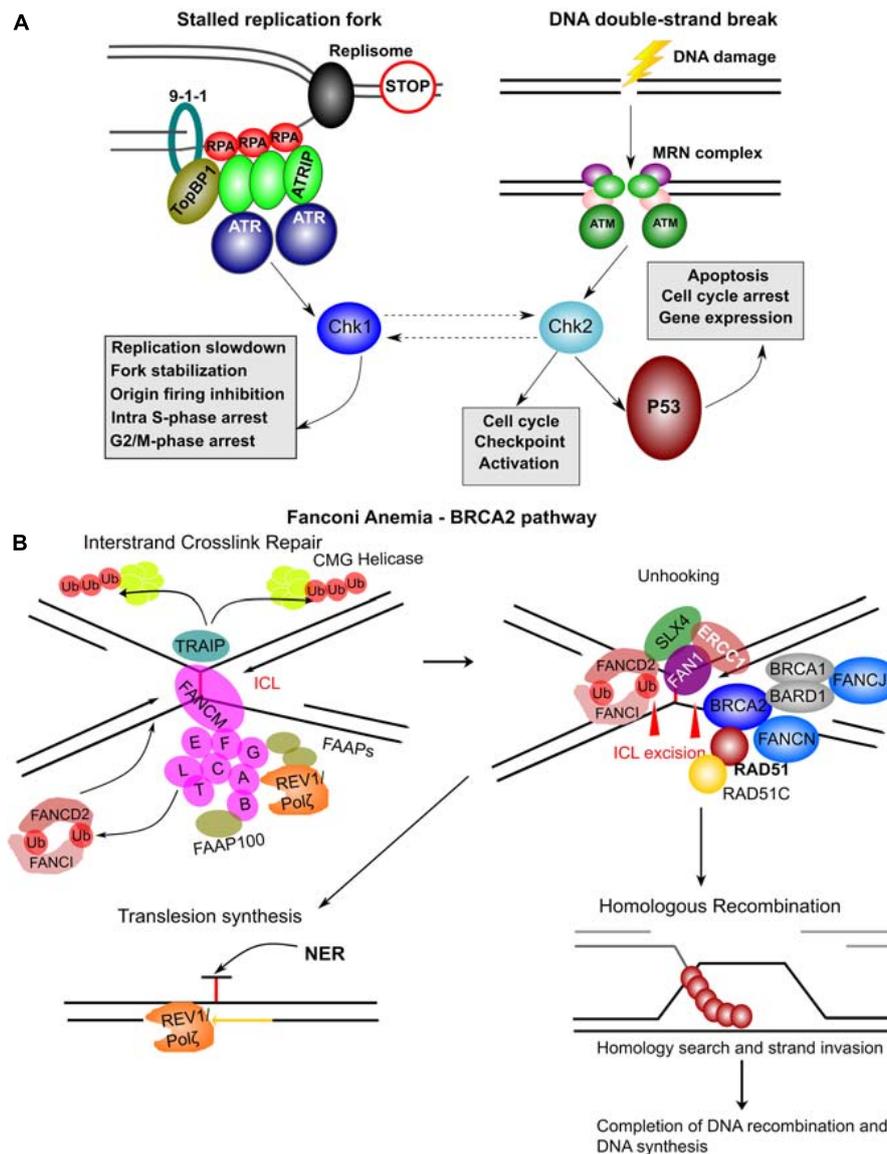


FIGURE 7 | Molecular complexes formed and DNA repair pathways mediated by CPG proteins. **(A)** Mechanisms of ATR and ATM activation by different types of DNA damage. Stalled replication forks resulting from a blockage of replisome progression (indicated by the “STOP” diagram). The single-stranded DNA (ssDNA) region is recognized by Replication Protein A (RPA) forming the filaments which can then recruit ATR-ATRIP (ATR Interacting Protein) complexes. The RAD17-RFC complex (not shown) loads the 9-1-1 complex (RAD9-RAD1-HUS1) onto the ssDNA-dsDNA junction. RAD9 S387 phosphorylation provides a binding site for DNA topoisomerase 2-binding protein 1 (TopBP1), which in turn binds and activates ATR kinase. ATR phosphorylates and activates Chk1 kinase mediating many of its functional effects and directly phosphorylates many other target proteins. DNA double-strand breaks results in a complex signaling cascade resulting in binding of the MRN complex (MRE11-RAD50-NBN) to the DNA ends. MRN provides a DNA damage sensor platform for recruitment and activation of ATM, which then phosphorylates multiple targets including Chk2 responsible for cell cycle checkpoint activation and p53 activation. Chk2 and Chk1 can also functionally interact (hashed lines with arrows). p53 activation leads to apoptosis, cell cycle arrest and gene expression changes. **(B)** FA – BRCA2 pathway. A major role of this pathway is to respond to inter-strand crosslinks (ICL), which can be especially problematic during DNA replication since ICLs prevent replication fork convergence. At such blocked replication forks, TRAIP ubiquitin ligase ubiquitinates the CMG (CDC45-MCM-GINS) Helicase leading to its unloading. The ICL itself is recognized by FANCM / FAAP24 complex. FANCM then recruits the core FA complex components and FAAPs (FA associated proteins). E2 ubiquitin-conjugating enzyme FANCT and E3 ubiquitin ligase FANCL then cooperate to perform mono-ubiquitination of FANCD2 and FANCI forming the ID2 complex. Monoubiquitinated ID2 complex gets recruited the ICL lesion. In the process of unhooking, DNA near the ICL gets processed by nucleases to enable specific DNA repair pathways. The strand with the ICL structure gets repaired by Nucleotide Excision Repair (NER) and the translesion synthesis by REV1/Pol ζ , which gets recruited by the FA core complex. ID2 recruits FANCP (SLX4), FANCP (ERCC1) and the nuclease FAN1 to mediate the ICL incision. The ID2 also interacts with BRCA2 (FANCD1), which gets recruited to the parts of blocked replication forks that will have to go through DNA recombination. BRCA2 in complex with BRCA1, BARD1, FANCD1, FANCD3, FANCD4, FANCD5, FANCD6, FANCD7, FANCD8, FANCD9, FANCD10, FANCD11, FANCD12, FANCD13, FANCD14, FANCD15, FANCD16, FANCD17, FANCD18, FANCD19, FANCD20, FANCD21, FANCD22, FANCD23, FANCD24, FANCD25, FANCD26, FANCD27, FANCD28, FANCD29, FANCD30, FANCD31, FANCD32, FANCD33, FANCD34, FANCD35, FANCD36, FANCD37, FANCD38, FANCD39, FANCD40, FANCD41, FANCD42, FANCD43, FANCD44, FANCD45, FANCD46, FANCD47, FANCD48, FANCD49, FANCD50, FANCD51, FANCD52, FANCD53, FANCD54, FANCD55, FANCD56, FANCD57, FANCD58, FANCD59, FANCD60, FANCD61, FANCD62, FANCD63, FANCD64, FANCD65, FANCD66, FANCD67, FANCD68, FANCD69, FANCD70, FANCD71, FANCD72, FANCD73, FANCD74, FANCD75, FANCD76, FANCD77, FANCD78, FANCD79, FANCD80, FANCD81, FANCD82, FANCD83, FANCD84, FANCD85, FANCD86, FANCD87, FANCD88, FANCD89, FANCD90, FANCD91, FANCD92, FANCD93, FANCD94, FANCD95, FANCD96, FANCD97, FANCD98, FANCD99, FANCD100. The figure was constructed based on the information in several recent reviews (Awasthi et al., 2016; Fradet-Turcotte et al., 2016; Bonilla et al., 2020; Liu et al., 2020).

with FA have defective HSCs that result in aplastic anemia due to BMF, MDS, and AML (Kutler et al., 2003). The frequency of BMF is estimated at 80% in affected individuals, of whom 30% develop hematologic and solid tumors including leukemias, head and neck carcinomas, liver tumors and gynecologic malignancies by 40 years of age with rates several hundred-fold higher than the general population and the age of onset frequently in childhood or early adulthood (Kutler et al., 2003; Liu et al., 2020).

Most FA genes are highly conserved in zebrafish (Titus et al., 2009) and the most comprehensive study of 17 FA genes (*fanca*, *fancb*, *fancc*, *fancd1/brca2*, *fancd2*, *fance*, *fancf*, *fancg*, *fanci*, *fancj/brip1*, *fancl*, *fanclm*, *fancln/palb2*, *fanco/rad51c*, *fanclp/slx4*, *fanclq/ercc4*, *fanclt/ube2t* and 2 FA-associated genes: *faap100* and *faap24*) revealed that FA mutants do not exhibit overt morphological phenotypes during embryonic development (Ramanagoudr-Bhojappa et al., 2018). However, adult FA zebrafish experience a female-to-male sex reversal as mutants in 12 genes had a complete reversal while 5 others had a partial reversal. This reversal has complicated certain phenotypic analyses due to the need for crosses with heterozygous females, which prevents generation of maternal-zygotic mutants. Additionally, 11 out of 17 mutants have some degree of hypersensitivity to treatment with diepoxybutane (DEB), which serves as a diagnostic test for FA gene mutations. *fanclp* mutants exhibit a decreased body size but no FA mutants demonstrate blood-related phenotypes or increased cancer susceptibility (Ramanagoudr-Bhojappa et al., 2018). Previous work in *fancl* zebrafish mutants similarly demonstrated female-to-male sex reversal, which mechanistically was connected to germ cell apoptosis mediated by p53 activation (Rodríguez-Marí et al., 2010). This reversal was blocked in the *fancl*^{-/-}; *tp53*^{-/-} mutants, confirming the role of p53 in this process and suggested a model where lack of oocyte survival generates signals for testis development and overall male sex determination in *fancl*^{-/-} mutants.

Fanconi anemia genes such as *BRCA2/FANCD1*, *RAD51C* and *RAD51*, homologous to the bacterial RecA, are involved in repair of DNA DSBs by homologous recombination (Bonilla et al., 2020). *BRCA2* is also more broadly important for genome stability due to its involvement in DNA replication, cell cycle progression and telomere homeostasis (Fradet-Turcotte et al., 2016). Zebrafish *brca2/fancl1* has received a significant amount of research attention which has revealed its important functions in genome stability, germ cell development, kidney development and tumor suppression. Female-to-male reversal and male infertility is observed in homozygous *brca2* zebrafish mutants (Q658X and ZM_00057434 referred to as *brca2*^{-/-} and both have a disruption in exon 11 of *brca2* which is commonly observed in hereditary breast and ovarian tumors) due to the disruptions in germ cell meiosis after being correctly specified in development (Shive et al., 2010; Rodríguez-Marí et al., 2011). Loss of *tp53* rescues ovary development but not male fertility. Apoptosis is dramatically increased after DEB treatment in *brca2*^{-/-} zebrafish embryos and *brca2*^{-/-} mutant cell lines have higher rates of chromosomal aberrations, apoptosis and slower growth compared to wild-type

(Rodríguez-Marí et al., 2011). These *brca2* mutant studies represent the first FA zebrafish cancer susceptibility models as both the infertile *brca2*^{-/-} males developed testicular cancer, and *tp53* and *brca2* double heterozygotes (*tp53*^{+/^{M214K}}; *brca2*^{+/⁻}) or homozygotes (*tp53*^{M214K/M214K}; *brca2*^{-/-}) had accelerated cancer development by about 5 months (Rodríguez-Marí et al., 2011). To further analyze tumor development, *brca2*^{Q658X} mutants were crossed to a *tp53* mutant background to drive tumor formation (Shive et al., 2014). After a > 2-year follow-up, all lines, *brca2*^{+/⁺}; *tp53*^{+/^{M214K}}, *brca2*^{+/^{Q658X}}; *tp53*^{+/^{M214K}} and *brca2*^{Q658X/Q658X}; *tp53*^{+/^{M214K}}, had a tumor incidence of 80–100% and *tp53* LOH was observed nearly universally in the *brca2*^{+/⁺}; *tp53*^{+/^{M214K}} tumors while it was only observed in 30% of the *brca2*^{Q658X/Q658X}; *tp53*^{+/^{M214K}} tumors, suggesting that *brca2* homozygous mutants undergo other genetic changes that drive tumorigenesis (Shive et al., 2014). Additionally, the creation of the zebrafish *zeppelin* (*zep*) mutant, mapped to *brca2* by whole-genome sequencing, has revealed that *brca2* also plays a role in kidney development (Kroeger et al., 2017).

RAD51/FANCR has recently been associated with FA and provides genetic support to well-known interactions between *RAD51* and *BRCA2* (Bonilla et al., 2020). This functional interaction is well-conserved in zebrafish as antibody staining in embryos show that endogenous *rad51* forms foci upon induction of DNA DSBs, but these foci do not form in *brca2*^{-/-} mutants and are reduced in *brca2*^{+/⁻} embryos (Vierstraete et al., 2017). A recent zebrafish study showed that *rad51* null embryos exhibit both increased chromosomal abnormalities after DEB treatment and increased sensitivity to gamma irradiation, consistent with the designation of *RAD51* as an FA pathway gene (Botthof et al., 2017). Zebrafish *rad51l1* mutants, a *rad51* paralog, are synthetically lethal when combined with *rad51* mutant, demonstrating the redundancy of their functions. In contrast to all other FA gene mutants, *rad51*^{-/-} mutants exhibit decreased staining for HSC markers during larval stages and lower total cell numbers in kidneys in adults, both of which are rescued by *tp53* loss (Botthof et al., 2017).

DNA Mismatch Repair

The DNA mismatch repair (MMR) pathway maintains chromosome stability and prevents increases in the rate of mutations, therefore it is of great significance for the maintenance of genome stability (Ijsselstein et al., 2020). Indeed, genes involved in the MMR pathway, including *MLH1*, *MSH2*, *MSH6*, *PMS1*, *PMS2*, are known CPGs and their mutations are responsible for Lynch syndrome (hereditary non-polyposis colorectal cancer) (Rahman, 2014). The zebrafish *mlh1* mutant shows the importance of *mlh1* for chromosome segregation in meiosis during gametogenesis as mutant males are infertile and exhibit abnormal testis histology, accumulation of spermatocytes and apoptosis (Feitsma et al., 2007) with a < 1% fertilization rate, as well as higher mutational burden in the resulting embryos (Leal et al., 2008). *mlh1* mutant females are fertile when bred with wild-type males, but still frequently produced aneuploid and triploid embryos. These phenotypes are fully consistent with mouse models of MMR defects and possible links of MMR gene defects to human male infertility (Mukherjee et al., 2010).

Further characterization of *mlh1*, *msh2* and *msh6* mutants shows microsatellite instability in the progeny from mutant males and revealed increased cancer incidence in mutant adults with tumors mainly characterized as neurofibromas and MPNSTs (Feitsma et al., 2008).

CPGs of Diverse Functions Contribute to Genome Stability

The genes discussed so far in this section are directly involved in DNA repair pathways. However, given the complexity of this process and its regulatory mechanisms, it can be expected that additional genes will be implicated in tumor suppression that have indirect or unexpected roles in genome stability. We will discuss several case studies highlighting how the zebrafish model has been employed to examine such genes.

ATRX is a SWI/SNF-like chromatin remodeler specialized in depositing H3.3 histone onto genomic DNA in a complex with the histone chaperone DAXX. Loss of ATRX is associated with both alternative lengthening of telomeres, thus promoting cell immortalization, and a wide range of cancer types, especially those of the nervous system, such as gliomas and neuroendocrine tumors (Nye et al., 2018). However, a recently-identified interaction between ATRX and FANCD2 implicates ATRX and DAXX in repair mechanisms of DNA DSBs, suggesting a direct and active role of ATRX in the DDR (Raghubandan et al., 2020). Although *atrx* null mutant (*atrx*^{-/-}) zebrafish do not survive past larval stages, blood development in mutant embryos is relatively normal with the exception of a single globin gene and more spherical erythrocytes (Oppel et al., 2019). Lethality of *atrx* loss required the authors to investigate its cancer-promoting properties in highly sensitized tumor-prone mutants, *tp53*^{-/-}; *nf1b*^{-/-}; *nf1a*^{+/-}; *atrx*^{+/-} and *tp53*^{-/-}; *nf1b*^{-/-}; *nf1a*^{+/+}; *atrx*^{+/-}. Heterozygous *atrx* loss did not increase the rate of tumor formation but led to a wider spectrum of tumor types, *tert* down-regulation, lengthening of telomeres and up-regulation of polycomb repressive complex 2 genes compared to *atrx*^{+/+} tumors. This model represents an important step for *atrx* investigation in zebrafish and may be further improved by tissue-specific or conditional approaches to *atrx* inactivation to circumvent larval lethality of this mutant.

The designation of *DICER1* and *DROSHA* as CPGs is not surprising given their fundamental roles in microRNA (miRNA) biogenesis as miRNA genes are well-known oncogenes and tumor suppressors (Hammond, 2015) and there is a diverse spectrum of cancers observed in *DICER1* syndrome (Robertson et al., 2018). However, over the last decade, small RNAs and lncRNAs have been implicated in the DDR (Vågbø and Slupphaug, 2020). Genomic DDR foci produce *DICER1*- and *DROSHA*-dependent small RNAs (20–35 nucleotides), which are required for full DDR activation in mammalian cells and zebrafish (Francia et al., 2012). Further, *dicer1* knockdown in zebrafish strongly disrupts the DDR based on reductions in ATM and γ -H2AX phosphorylation in a cell-autonomous manner based on the aberrant DDR in transplanted *dicer1* morphant cells (Francia et al., 2012). This suggests that *DICER1* and *DROSHA* are part of the DNA repair pathway and further evaluation is needed

from the perspectives of both miRNA biogenesis and genome stability maintenance.

The von Hippel-Lindau protein (pVHL) mainly functions in the cellular response to hypoxia where it is responsible for poly-ubiquitination of Hypoxia Inducible Factor (HIF) proteins 1 and 2. Under normoxic conditions, HIF1/2 are hydroxylated and degraded; however, they become less hydroxylated under hypoxic conditions and accumulate, causing transactivation of their target genes. Loss of pVHL leads to a constitutively active hypoxic response and is the cause of von Hippel Lindau disease. Affected individuals develop retinal and central nervous system vascular tumors known as hemangioblastomas, pancreatic and kidneys cysts, pheochromocytoma, and clear cell renal cell carcinomas (ccRCC) (Gossage et al., 2015). Previous work in ccRCC cell lines demonstrates the importance of pVHL for DNA DSB repair (Metcalfe et al., 2014). *VHL* has two paralogs in zebrafish, *vhl* and *vll*, and zebrafish pVHL mutants demonstrate that *vhl* gene loss is mainly responsible for HIF signaling activation, which is significantly enhanced upon *vll* loss, while *vll* is important for genome stability maintenance (Kim et al., 2020). The *vhl*^{-/-}; *vll*^{-/-} double mutants have reduced p53 activation and are resistance to apoptosis following the induction of genotoxic stimuli which was phenocopied by Hif activation. Additionally, genotoxic-induced apoptosis in *brca2*^{-/-} mutants is rescued by *vhl* loss. Thus, these results firmly establish *vhl* and *vll* as VHL homologs with divided but complementary functions that can enable the creation of cancer models involving these genes (Kim et al., 2020).

CONCLUSION

Zebrafish models are amenable to a variety of genetic technologies and represent a powerful *in vivo* tool to investigate the roles and mechanisms of CPGs in development and tumorigenesis. We have reviewed how the zebrafish is well-positioned for preclinical studies focused on identifying novel mutations and genes, as well as elucidating how their molecular mechanisms contribute to biological functioning and cancer development. One major advantage of the zebrafish model is the more rapid and less expensive generation of genetic disease models than is possible in the mouse. Zebrafish also provide excellent opportunities for *in vivo* imaging throughout development and to some extent in adults. Additionally, mutations of certain genes are often more tolerable in zebrafish than in the mouse which is partially explained by the partial genome duplication that occurred in zebrafish and as such, zebrafish models often survive longer and allow for more in-depth studies. This phenomenon also provides the ability to investigate the effect of multiple modifier genes through the creation of compound mutants, especially for CPGs. One common CPG/modifier often investigated is *tp53* due to the prominent role of *tp53* in tumor suppression. Several of the cancer predisposition models we have highlighted investigated the role of p53 in disease progression and have employed *tp53* mutants to facilitate tumor development in their models.

In this way, zebrafish models have tremendous potential to provide preclinical insights to change the natural history of life-threatening cancer predisposition disorders. Mutations in CPGs predispose individuals to an increased risk of cancer via their key roles in both development and homeostatic control/maintenance, especially during times of cellular stress like DNA damage or energy depletion. Thus, the generation and characterization of zebrafish models has tremendous utility for identifying novel therapies for these rare but devastating syndromes. Further, knowledge gained from zebrafish models also holds great significance to the broader cancer research community, as biological insights about CPG mechanisms will also reveal the biological processes underlying sporadic tumorigenesis. However, one current limitation is that several of the aforementioned zebrafish disease models are knockouts while their corresponding syndrome is more typically caused by a missense mutation that does not result in protein truncation; thus, these models may not fully represent the genetic underpinnings of the disease nor the resulting protein interactions. This was likely due to the availability/usability of genome editing technologies at the time of model creation, but the recent optimization of several genome editing techniques, as well as soon anticipated-in-zebrafish technologies including prime editing, will aid future researchers to more easily and efficiently develop more representative disease-specific genetic models. Additionally, once established, genetic zebrafish models can serve as a drug

screening platform to rapidly and efficiently identify novel therapies. However, to date, zebrafish cancer predisposition models have been employed predominantly in proof-of-principle drug studies with known molecular targets to highlight genotype-phenotype correlations. Thus, this represents an exciting and powerful next step for many of these models to provide clinical insights and reveal new avenues of therapeutic intervention.

AUTHOR CONTRIBUTIONS

KK, KC, and SP wrote and edited the manuscript and generated the figures and tables. JB edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.660069/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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