Tumor Suppressors in Zebrafish: From TP53 to PTEN and Beyond

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Abstract Zebrafish are increasingly being used to study cancer. Almost all tumor types have been found in zebrafish. However, tumor incidence is relatively low and tumors develop late in life. Functional inactivation of tumor suppressors is a crucial step in cancer progression and more and more tumor suppressor genes are being studied in zebrafish. Most often tumor suppressors have been inactivated by reverse genetics approaches using targeted disruption. However, some tumor suppressor mutants were identified by forward genetic screens for mutants with a particular phenotype. Some of the latter genes had not been recognized as tumor suppressors yet. Similarly, a screen for genes that suppress tumor formation in zebrafish in vivo led to the identification of a novel tumor suppressor gene. In this review, I will provide an overview of what the zebrafish has taught us about tumor suppressors.

Keywords Tumor suppressor • Zebrafish • tp53 • pten • apc • vhl

Introduction

> A tumor suppressor gene, or antioncogene, is a gene that protects a cell from one step on the path to cancer. When this gene mutates to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes (wikipedia).

Oncogenic activation of proto-oncogenes, for instance by mutation or overexpression of cancer driver genes promotes tumorigenesis. However, in the formation of human cancers, the loss of a tumor suppressor gene may be more important than the gain of an oncogene [1, 2]. Over the years, a large number of tumor suppressor genes have been identified in human cancer and often little is known about the cellular, biological or biochemical function of these genes. The zebrafish is an excellent experimental model to assess gene function in vivo and zebrafish is increasingly

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being used to model human disease [3], including cancer [4]. Particularly the advent of reverse genetics in zebrafish has facilitated the study of tumor suppressors and many tumor suppressors have since been inactivated in zebrafish, often resulting in developmental defects and/or enhanced tumor susceptibility.

TP53

tp53 is the most frequently mutated tumor suppressor in human cancers. More than 50 % of all solid tumors harbor mutations in *tp53* that inactivate TP53 protein function. TP53 has been described as the "guardian of the genome", because it induces cell cycle arrest or apoptosis upon various types of cellular stresses, thus maintaining the integrity of the genome [5–7]. DNA damage and other stressors induce post-translational TP53 protein stabilization, resulting in cell cycle arrest due to TP53-mediated transactivation of *p21* cyclin-dependent kinase inhibitor or to apoptosis in a transcription-dependent or -independent manner [8]. Loss of functional TP53 therefore can result in defective DNA repair and a reduced apoptotic response and hence to accumulation of more mutations in oncogenesis.

The TP53 protein is conserved in zebrafish (48 % at the amino acid level over the full length protein compared to human TP53) [9] and target selected gene inactivation led to the identification of several *tp53* mutants, two of which have missense mutations in orthologous residues as found in human cancer cells (N168K and M214K) [10]. Both mutants fail to transactivate the *p21* response element in SAOS-2 cells, demonstrating that these missense mutations functionally inactivate TP53. Incrosses of heterozygous *tp53*^{N168K} or *tp53*^{M214K} mutant fish result in normal sized clutches of embryos with Mendelian distribution of wild type, heterozygous and homozygous embryos. Development of the heterozygous and homozygous *tp53* mutant embryos is normal and also viability and fertility of these fish is indistinguishable from wild type fish. Yet, homozygous *tp53*^{M214K} mutant zebrafish embryos are resistant to γ-irradiation-induced apoptosis, consistent with TP53 loss of function. The TP53^{N168K} mutant protein turns out to be temperature-sensitive, like the human mutant protein in the orthologous residue. At the permissive temperature (28 °C), the *tp53*^{N168K} allele behaves like wild type, whereas at 37 °C, the non-permissive temperature, *tp53*^{N168K} becomes resistant to γ-irradiation-induced apoptosis. Cell cycle analysis demonstrated that the G1 checkpoint in the *tp53*^{M214K} mutant embryos is defective upon γ-irradiation, which is associated with a lack of upregulation of downstream targets, including *p21* and *mdm2* [10]. All in all, the *tp53*^{M214K} mutant embryos display the hallmarks of cancer cells lacking functional TP53.

Mouse mutants lacking functional TP53 protein are highly susceptible to tumor formation [11, 12]. In zebrafish, 28 % of the homozygous *tp53*^{M214K} mutant fish spontaneously develop tumors between 8 and 16.5 months of age. The majority of these tumors are diagnosed as malignant peripheral nerve sheath tumors (MPNSTs) [10]. This is surprising because MPNSTs are rare in humans and are not observed in mouse models lacking functional TP53 [12]. The difference in spectrum of spontaneous tumor formation between mammals and zebrafish remains to be determined [9].

Whereas the spontaneous tumors in *tp53* mutant zebrafish are predominantly MPNSTs, a broad spectrum of tumors has been identified in *tp53* mutant zebrafish upon expression of a range of oncogenes or cancer drivers. The first driver that was reported is $BRAF^{V600E}$ [13]. This mutation is commonly detected in human nevi and melanoma. Expression of $BRAF^{V600E}$ in wild type zebrafish melanocytes using the *mitfa* promoter induces nevi in ~10 % of the adult fish, but no melanoma. Expression of $BRAF^{V600E}$ in melanocytes of *tp53* mutant fish induces nevi in 13.6 % of the animals, of which almost half progressed to malignant melanoma. The difference in fates of $BRAF^{V600E}$-expressing melanocytes between wild type and *tp53* mutant zebrafish illustrates the tumor suppressive role of TP53 in wild type zebrafish. Histologically, zebrafish melanoma is highly similar to human melanoma, thus establishing the zebrafish as a melanoma model [13].

Similar to expression of $BRAF^{V600E}$, expression of oncogenic $NRAS^{Q61K}$ under the control of the *mitfa* promoter in wild type zebrafish also results in spots of hyperproliferative melanocytes from 3 to 4 weeks of age onwards. Malignant melanoma is not observed in these fish. However, expression of $NRAS^{Q61K}$ in homozygous *tp53* mutant zebrafish results in malignant melanoma in 100 % of the cases. Interestingly, melanoma is also enhanced in *tp53* heterozygous mutants and genotyping is indicative of *tp53* loss-of-heterozygosity in the tumors. Interestingly, comparison of the gene expression patterns from human melanomas to melanomas from *tp53* mutant zebrafish expressing $NRAS^{Q61K}$ indicates a striking similarity, confirming that zebrafish is a good model for human melanoma [14].

NRAS acts upstream of BRAF in the RAS/MAPK signaling pathway and activating mutations in either NRAS or BRAF are identified in 95 % of human melanomas [15, 16]. Therefore, it is perhaps not surprising that expression of mutant $BRAF^{V600E}$ or $NRAS^{Q61K}$ in melanocytes leads to melanoma in zebrafish. However, mutations in *tp53* are rare in human melanoma and the observation that melanoma occurs in a *tp53* mutant, but not wild type zebrafish, suggests that TP53 has a strong protective role. Moreover, the relatively late onset of melanoma—8 months in $BRAF^{V600E}$ expressing fish [13] and 10 weeks in $NRAS^{Q61K}$ expressing fish [14]—suggests that additional mutations are required in the *tp53* mutant background to proceed to malignant melanoma.

Whereas the role of TP53 in protection against malignant melanoma is clear in the $BRAF^{V600E}$ and $NRAS^{Q61K}$ expressing zebrafish described above, expression of oncogenic $HRAS^{G12V}$ in melanocytes under the control of the *kita* promoter induces malignant melanoma in the presence of functional *tp53* [17]. The difference may be due to higher expression of $HRAS^{G12V}$ from the *kita* promoter than $NRAS^{Q61K}$ from the *mitfa* promoter, to differences in the cell specificity of the *mitfa* and *kita* promoters, or to differences in signaling of the oncogenic RAS paralogs, $HRAS^{G12V}$ and $NRAS^{Q61K}$.

In this respect it is noteworthy that expression of oncogenic $KRAS^{G12D}$ in wild type zebrafish also induces tumors in the presence of functional TP53. $KRAS^{G12D}$ expression under the control of the *rag2* promoter, driving expression in satellite cells and myoblasts within skeletal muscle, induces tumors that resemble embryonal rhabdomyosarcoma [18]. *Tp53* mutations are associated with a subset of embryonal rhabdomyosarcomas in humans [19] and expression of $KRAS^{G12D}$ in

homozygous *tp53* mutant embryos induces a significant increase in the incidence of embryonal rhabdomyosarcoma [18], demonstrating that inactivation of *tp53* cooperates with oncogenic activation of KRAS in the formation of embryonal rhabdomyosarcoma.

Whereas tissue-specific expression of $KRAS^{G12D}$ cooperates with loss of TP53 function in embryonal rhabdomyosarcoma formation, several other factors induce hepatocellular carcinoma in *tp53*-negative zebrafish. Liver-specific expression of the Hepatitis B virus X antigen (HBx) using the *l-fabp* promoter in wild type zebrafish causes steatosis, fibrosis and glycogen accumulation, but not tumor formation. Only in the *tp53*-negative background hepatocellular carcinoma is observed, which is accompanied by upregulation of *src* expression and activation of the Src tyrosine kinase signaling pathway. Interestingly, expression of exogenous Src in *tp53*-negative zebrafish also induces hepatocellular carcinoma, suggesting that upregulation of *src* expression is the mechanism underlying HBx-induced hepatocellular carcinoma in mutant zebrafish lacking functional TP53 [20].

Unlike HBx, which induces hepatocellular carcinoma in *tp53* mutant, but not wild type zebrafish, liver-specific, *fabp10* promoter-driven expression of UHRF1 (Ubiquitin-like with PHD and RING finger domains 1), an essential regulator of DNA methylation that is highly expressed in many cancers, induces hepatocellular carcinoma by itself in wild type zebrafish. However, the tumor incidence is enhanced in heterozygous *tp53* mutant zebrafish from 50 % to 87 % and in one case, the tumor type was distinct, suggesting that *tp53* does suppress tumor formation and may have a role in the spectrum of tumors caused by UHFR1 overexpression [21].

Well-differentiated liposarcoma is yet another tumor type in which TP53 is involved in humans. The majority (91 %) of tumors that are observed in response to expression of myristoylated Akt2, a constitutively active form of Akt2, in mesenchymal cells (*rag2* promoter) display hallmarks of well-differentiated liposarcoma. TP53 suppresses tumor formation, because expression of myristoylated Akt2 induced tumors in 8 % of wild type zebrafish, 6 % of *tp53* heterozygous fish and 29 % of *tp53* homozygous mutants. Akt2 is a kinase that acts downstream of phosphatidylinositol-3kinase in a signaling pathway that promotes cell survival and proliferation. The link of Akt signaling to well-differentiated liposarcoma is novel and may provide new leads to combat these tumors [22].

Not only expression of oncogenic proteins in the *tp53* mutant background induces tumors, but also the additional loss of another tumor suppressor in the *tp53* mutant background leads to tumor formation in zebrafish. *Early mitotic inhibitor 1*, *emi1*, is essential for genomic stability and the human homolog of *emi1* may have a function in leukemia. Zebrafish mutants with a proviral insertion in *emi1* that impairs its function display widespread developmental defects from 20 hpf onwards [23]. Whereas *tp53* mutant zebrafish develop MPNST tumors at a relatively low incidence, compound *tp53* mutants that also harbor an inactivating proviral insertion in *emi1* display a significant increase in MPNST formation. These results provide evidence that *emi1* is a tumor suppressor in *tp53* mutant zebrafish [24].

TSC1 and *TSC2* are two other tumor suppressor genes and loss of function mutations in *TSC1* and *TSC2* in humans are associated with tuberous sclerosis complex,

a genetic disorder that is characterized by multi-organ hamartomas, i.e. benign tumors. mTORC1 signaling is elevated in patients that lack functional TSC1 or TSC2. Mutant zebrafish harboring a nonsense mutation in the *tsc2* gene that truncates the Tsc2 protein and impairs its function display increased cell size in the brain and liver and these mutants are not viable beyond the larval stage [25]. Whereas *tsc2* mutants do not develop cancer, compound heterozygous *tsc2*/homozygous *tp53* mutants display enhanced incidence of malignant tumors, evidenced by large sarcomatous tumors in multiple organs, compared to *tp53* homozygous mutants [26]. Therefore, loss of functional *tp53* is a genetic change that may be required for tuberous sclerosis complex to progress to malignancy on the one hand and in humans, loss of a single allele of *TSC1* or *TSC2* in a *TP53*-negative background may lead to severe forms of cancer too. Interestingly, treatment with the mTORC1 inhibitor rapamycin led to a rapid reduction in tumor size [26], suggesting that *TP53* negative tumors that are heterozygous for mutations in *TSC1* or *TSC2* may be treatable with rapamycin.

Human cancers that are associated with mutations in the tumor suppressor *BRCA2* (breast cancer 2) often also have mutations in *TP53*. BRCA2 is a component of the DNA repair machinery and mutations in *BRCA2* perturb double-strand DNA break repair, resulting in chromosomal aberrations. Target selected gene inactivation led to the identification of a nonsense mutation in the zebrafish gene encoding *brca2* that results in a Glutamine 658 to Stop mutation and hence to a severely truncated Brca2 protein that is not functional. Incrosses of heterozygous *brca2*^{Q658X} mutants result in Mendelian ratios of wild type, heterozygous and homozygous *brca2* mutants. Homozygous *brca2*^{Q658X} mutants are viable and at sexual maturity, all homozygous mutants are phenotypically male. At 10–16 months of age, 31 % of the homozygous *brca2*^{Q658X} mutants develop testicular neoplasia, consistent with the notion that *brca2* is a tumor suppressor gene. Compound *brca2*/*tp53* mutants display an accelerated onset of tumor formation, compared to homozygous *tp53* mutants. Interestingly, tumor onset in *tp53*-negative heterozygous and homozygous *brca2* mutants is similar. The majority of tumors are MPNSTs, but also some ovarian tumors are observed [27]. Interestingly, resequencing indicates loss-of-heterozygosity of both tumor suppressor genes in malignant tumors from zebrafish with heterozygous mutations in both *brca2* and *tp53* [28].

In humans, loss of TP53 is also known to cooperate with neurofibromin 1 (NF1). Human neurofibromatosis type 1 patients develop benign peripheral nerve sheath tumors and hyperpigmented skin lesions, caused by dominantly inherited mutations in NF1. These patients are predisposed to develop malignancies, including juvenile myelomonocytic leukemia, glioma and MPNSTs. Zebrafish have two *nf1* genes, *nf1a* and *nf1b*, and genetic models have been generated by target selected gene inactivation as well as using Zn-finger nuclease technology. Zebrafish lacking functional Nf1 display overproliferation of oligodendrocyte progenitor cells and these larvae do not live beyond 10 days post fertilization. Zebrafish that retain a single wild type *nf1* allele are viable and fertile and these fish do not develop tumors until 18 months of age. However, compound zebrafish mutants lacking functional Tp53 and Nf1b that retain a single wild type *nf1a* allele show a marked acceleration in

tumor onset and increase in tumor penetrance, compared to zebrafish mutants lacking TP53 that are wild type for *nf1a* and *nf1b*. The observed brain tumors display hallmarks of diffuse high-grade gliomas, whereas the other tumors are consistent with MPNSTs [29].

Although *TP53* is the most frequently mutated tumor suppressor in human cancer, spontaneous tumor formation in *tp53* mutant zebrafish occurs relatively late with relatively low penetrance and the resulting tumors are predominantly a relatively rare tumor type, MPNST. Several tumor drivers have been expressed in *tp53* mutant zebrafish and compound mutants have been generated lacking functional Tp53 and other tumor suppressors, resulting in accelerated tumor onset, enhanced tumor penetrance and broadening of the tumor spectrum. Future analysis of additional tumor drivers and tumor suppressors that cooperate with loss of functional Tp53 in tumor formation in zebrafish will enhance our understanding of the role of Tp53 in tumorigenesis and may provide new leads how to combat cancer in humans.

PTEN

PTEN is the second most frequently mutated tumor suppressor gene in human cancer [30, 31]. Many different types of cancers have missense and/or nonsense mutations in PTEN [32–34]. Moreover, missense mutations have been identified in human syndromes that render carrier patients more prone to cancer [35–37]. Mouse models lacking functional PTEN are not viable beyond embryonic day 8.5 and heterozygous mice are tumor-prone [38–40]. In fact, a reduction of PTEN protein levels in mouse models by as little as 20 % already enhances tumor susceptibility [41].

Many functions have been ascribed to PTEN at the cellular and biochemical level [42]. However, PTEN is best known for its lipid phosphatase activity. PTEN dephosphorylates phosphatidylinositol(3,4,5)trisphosphates and is selective for the 3-position [43, 44]. Therefore, PTEN antagonizes phosphatidylinositol-3kinase and loss of PTEN results in hyperactivation of Akt (also known as PKB) [45, 46]. Further downstream signaling leads to enhanced cell survival and proliferation and hence, PTEN acts as a tumor suppressor gene.

The zebrafish genome harbors two *pten* genes, *ptena* and *ptenb* [47, 48]. Both *pten* genes encode functional Pten protein with lipid phosphatase activity, selective for the 3-position of phosphatidylinositol-polyphosphates. The two zebrafish *pten* genes are broadly expressed during embryonic development. Using target-selected gene inactivation, nonsense mutations were identified in each of the two *pten* genes, well upstream of the catalytic site. No functional Ptena or Ptenb protein is expressed in homozygous *ptena* or *ptenb* mutants, respectively. Homozygous *ptena* or *ptenb* single mutants do not display developmental defects and are viable and fertile, indicating functional redundancy between Ptena and Ptenb [48].

Whereas developmental defects are not observed in *ptena* or *ptenb* single mutants, homozygous *ptenb* mutant adults spontaneously developed eye tumors from 7 to 18 months of age at a relatively high incidence (33 %).

Immunohistochemistry demonstrated that the highly proliferative regions in these tumors displayed elevated levels of phospho-Akt, indicative of Akt activation [48]. Ocular tumors are extremely rare in wild type zebrafish and also in homozygous *ptena* mutant adult zebrafish, these eye tumors are never observed. Following several rounds of outcrossing, the ocular tumors in homozygous *ptenb* mutant zebrafish were not observed anymore. This may be due to the loss of an enhancer of the eye tumor phenotype in the *ptenb* mutant background, or to the gain of a suppressor of the phenotype. The identity of the supposed enhancer or suppressor remains elusive currently.

Ocular tumors are not observed in homozygous *ptenb* single mutants anymore. However, tumors are observed in compound *ptena/ptenb* mutants that retain a single wild type allele, i.e. *ptena+/-ptenb-/-* and *ptena-/-ptenb+/-* adult zebrafish. In a cohort of 294 *ptena+/-ptenb-/-* adults 10.2 % develop tumors in the first year of their life. Often the tumors are located close to the eye and pathological analysis indicates that the tumors consist of endothelial cells that form blood vessels that are actually filled with blood. These tumors are diagnosed as hemangiosarcomas [49]. Immunohistochemistry and immunoblotting demonstrates that these tumors display elevated Akt signaling. Why these hemangiosarcomas predominantly develop close to the eye remains to be determined. Under normal conditions, a meshwork of blood vessels, *rete mirabile*, is located close to the eye. This may result in production of high levels of proliferative signals for endothelial cells locally. In addition, due to lack of three of the four *pten* alleles, proliferative signaling in response to external signaling is already enhanced in *ptena+/-ptenb-/-* mutants. Together, high levels of signals and elevated intracellular signaling may lead to unusually enhanced endothelial cell proliferation and thus to tumor formation close to the eye [49]. It is noteworthy that *pten* mutant zebrafish are much less susceptible to tumor formation than mice. Double heterozygous mutant zebrafish (*ptena+/-ptenb+/-*), lacking 50 % pten expression, do not show an increase in spontaneous tumor formation [48, 49], whereas a 20 % reduction in PTEN expression in mouse mutants already enhances tumor susceptibility [41].

Despite the lack in enhanced spontaneous tumor formation in *pten* double heterozygous fish [48, 49], partial loss of pten does affect tumorigenesis in a model for T cell acute lymphoblastic leukemia (T-ALL) [50]. Guttierrez et al. expressed a fusion protein of the MYC oncogenic transcription factor and the estrogen receptor (MYC-ER) in the thymus under the control of the rag2 promoter. Treatment of these fish with ligand, 4-hydroxytamoxifen (4HT), from 5 days post fertilization onwards leads to fully penetrant T-ALL starting at 5 weeks post fertilization. Subsequent removal from 4HT leads to MYC downregulation after 4 days and to tumor regression in wild type zebrafish due to apoptosis. Ligand-induced MYC-ER activation does not accelerate the onset of T-ALL in double heterozygous *ptena+/-ptenb+/-* zebrafish. However, tumor regression following removal from 4HT is reduced from 69 % in wild type zebrafish to 10 % in *ptena+/-ptenb+/-* zebrafish [50]. Therefore, mutation of *pten* promotes loss of MYC oncogene dependence in T-ALL.

Pten double heterozygous fish and even mutant zebrafish that retain a single wild type *pten* allele are viable and fertile. However, complete loss of functional Pten is

lethal around 6 days post fertilization. Double homozygous embryos display pleiotropic defects that are consistent with enhanced proliferation and survival. These developmental defects are largely rescued by microinjection of synthetic mRNA encoding Ptena or Ptenb at the one-cell stage, or by treatment of the embryos with the phosphatidylinositol-3kinase inhibitor, LY294002 [48]. Double mutant embryos display hyperbranching of blood vessels from 3 days post fertilization onwards, suggesting that endothelial cells are particularly sensitive to loss of functional Pten. This is consistent with the observation that *pten* mutant adult zebrafish develop hemangiosarcomas. Interestingly, double homozygous zebrafish embryos express elevated levels of *vegfaa*, encoding a ligand for VEGFRs that have crucial roles in endothelial cell proliferation [51]. Therefore, hyperbranching in *pten* mutant embryos may be caused by elevated VEGFR signaling because of enhanced levels of signal on the one hand and an increase in signaling due to the lack of Pten on the other.

In mammals, PTEN reportedly has an important role in many biological processes, including hematopoiesis. In homozygous double mutant zebrafish embryos lacking functional Pten, the number of hematopoietic stem and progenitor cells (HSPCs) is enhanced at 4 days post fertilization, consistent with enhanced proliferation in these cells. These HSPCs engage in all blood cell lineages, demonstrating that the commitment to differentiate is not impaired. Yet, fully mature blood cells are not observed, indicating that differentiation is blocked in embryos lacking functional Pten [52]. Similarly, increased numbers of myeloid cells are detected in *pten* mutant embryos, which are however immune-deficient [53]. Strikingly, inhibition of phosphatidylinositol-3kinase signaling at relatively late stages restores terminal differentiation of hematopoietic cells [52]. Taken together, loss of functional Pten results in enhanced proliferation of HSPCs and an arrest in terminal differentiation of progenitor cells.

Pten mutant zebrafish that retain a single wild type allele spontaneously develop tumors, hemangiosarcomas. Whether other tumor suppressors or cancer driver genes cooperate with the loss of Pten in tumor formation has not been addressed yet. Insight into which genes cooperate with *pten* in zebrafish tumor formation is highly interesting and will lead to fundamental insights into the function of the tumor suppressor, *pten*, which may provide the first step to combat cancer that is associated with loss of PTEN in human cancer.

APC

Adenomatous polyposis coli (APC) mutations are common in colorectal cancer. *APC* was found to be mutated in familial adenomatous polyposis syndrome in humans and in these patients, somatic mutation of the second allele leads to colorectal cancer [54]. APC has a central role in inhibition of Wnt/β-Catenin signaling. Truncation of APC by mutation leads to accumulation of nuclear β-Catenin and to constitutive further downstream signaling [55]. Mouse models lacking functional APC fail to complete gastrulation and heterozygous *Apc* mutant mice progressively develop intestinal tumors [56].

In zebrafish, a nonsense mutation was identified in *apc* by target selected gene inactivation. This mutation is located in the equivalent of the mutation cluster region in human APC. Heterozygous *apc* mutant zebrafish develop normally and they are viable and fertile. Unlike mice lacking functional APC, homozygous *apc* mutant zebrafish complete gastrulation. Yet, *apc* mutant zebrafish are embryonic lethal at 3–4 days post fertilization and these embryos display multiple developmental defects, of which the cardiac defects, cardiac edema, smaller eyes, and body curvature are most prominent. That these developmental defects are due to loss of functional Apc was confirmed by another mutant with a nonsense mutation in *apc* upstream of the mutation cluster region that was identified in an independent forward genetic screen. The hearts in homozygous *apc* mutants fail to loop properly and form excessive endocardial cushions [57]. Moreover, detailed analysis of the brain in developing embryos indicated that loss of Apc function impaired maintenance of local brain organizers and established brain subdivisions [58]. Whereas heterozygous *apc* mutant fish are viable and fertile, aged fish (>15 months of age) spontaneously develop neoplasias in the liver (18 %) and intestine (12 %). This is rarely (3 %) observed in siblings (liver neoplasia was observed in a single age-matched sibling) [59]. Whereas homozygous *apc* mutant zebrafish are embryonic lethal, they nevertheless provide an attractive model to test genetic cofactors and inhibitors. Transient expression of active $KRAS^{G12D}$ in homozygous *apc* mutant zebrafish embryos enhanced intestinal cell proliferation [60], and inhibition of mTORC1 partially rescued the early developmental defects in zebrafish embryos that lack functional Apc [61]. Further analysis of the *apc* model will provide leads for therapeutic intervention in cancer that is associated with loss of this prominent tumor suppressor.

VHL

Complete loss of the von Hippel-Lindau (VHL) tumor suppressor in heterozygous *VHL* patients by inactivation of the remaining allele in somatic cells predisposes these patients to the development of highly vascularized tumors and cysts in many organ systems [62]. VHL protein has a role in the adaptive response of cells to hypoxia. VHL is an E3 ubiquitin ligase and regulates hypoxia inducible factor (HIF) protein levels. Mouse *Vhl* knockouts die in utero around embryonic day 11.5–12.5 due to hemorrhagic lesions in the placenta [63]. Two zebrafish lines with nonsense mutations in *vhl* were identified by target selected gene inactivation. Transheterozygotes lack detectable Vhl protein levels and are larval-lethal at 8–11 days post fertilization. *Vhl* mutant embryos display a systemic hypoxic response and a marked increase in the number of red blood cells (polycythemia), reminiscent of the Chuvash form of Polycythemia (CP) that is observed in human patients with a R200W missense mutation in the extreme C-terminus of the VHL protein. It is noteworthy that CP patients are not predisposed to cancer and likewise, heterozygous *vhl* mutant zebrafish do not spontaneously develop tumors [64]. Whereas the *vhl* mutant zebrafish model does not develop tumors, it has been used successfully to model hypoxia, a central modulator of cellular physiology in cancer [65, 66].

Ribosomal Proteins

Heterozygous mutant zebrafish lines with viral insertions were generated in the course of a large-scale insertional mutagenesis screen and were screened for early mortality and tumor development later in live. Twelve lines were identified with enhanced tumor incidence and most tumors that were identified were diagnosed as MPNSTs. One line had an insertion in *nf2*, an established tumor suppressor gene, and surprisingly, all other lines had viral insertions in genes encoding ribosomal proteins (*rp*s), suggesting that these *rp* genes are haploinsufficient tumor suppressor genes [67]. MPNSTs from heterozygous zebrafish with viral insertions in *rp*s encode wild type TP53, but express very low to undetectable levels of TP53 protein. This, together with the notion that *tp53* mutant zebrafish spontaneously develop MPNSTs suggests that lack of TP53 protein may be the underlying mechanism for MPNST formation in the *rp* mutant lines [68].

Rpl36 and *rpl23a* are two of the genes encoding ribosomal proteins that were identified in the viral insertion screen above. Recently, *rpl36*, but not *rpl23a*, was confirmed to act as a tumor suppressor in pancreatic cancer. Expression of $KRAS^{G12V}$ in early pancreatic progenitor cells (*ptf1a* promoter) of heterozygous *rpl36* mutant fish increases pancreatic epithelial cell proliferation, accelerates tumor progression and decreases survival compared to wild type sibling controls, demonstrating that *rpl36* acts as a haploinsufficient tumor suppressor [69].

A mutation in *rps29* was identified in an independent forward genetic screen for hematopoietic mutants. Zebrafish embryos lacking functional Rps29 have less hematopoietic stem cells and display craniofacial defects, which is reminiscent of Diamond-Blackfan anemia in human patients that is associated with mutations in ribosomal proteins. Mutation of *tp53* almost completely rescues the developmental defects observed in *rps29* mutants [70]. Apparently, loss of Tp53 protein mediates MPNST formation in heterozygous rp mutants on the one hand and *tp53* mediates developmental defects caused by loss of Rps29 on the other.

Other Tumor Suppressors

Next to the tumor suppressor genes described above, several more have been knocked out in zebrafish by reverse genetics approaches, some tumor suppressor mutants were identified in forward genetic screens and a candidate tumor suppressor was identified in a screen for genes that suppressed tumor formation.

LKB1 is a tumor suppressor that encodes a serine/threonine kinase. Inactivating mutations in *LKB1* cause Peutz-Jeghers syndrome, an autosomal disorder characterized by multiple hamartomas and abnormal pigmentation of mucus membranes and predisposition to gastrointestinal cancers. Zebrafish mutants that lack functional Lkb1 develop normally up to 5 days post fertilization, but die at 7–8 days post fertilization. The *lkb1* mutant larvae exhibit a high metabolic rate and display a starvation response, but no increase in tumor susceptibility is reported in heterozygous *lkb1* mutant zebrafish [71].

Somatic loss-of-function mutations of the ten-eleven translocation 2 gene (*TET2*) are frequently observed in human myelodysplastic syndrome. *TET2* encodes a methylcytosine oxidase that has an important role in demethylation of DNA in CpG islands. Zn-finger nuclease technology was used to inactivate *tet2*. Homozygous *tet2* mutant zebrafish embryos do not display hematopoietic defects during embryonic development. Adult fish lacking Tet2 are viable and fertile, but develop progressive myelodysplasia, resulting in myelodysplastic syndrome at 2 years of age, which is highly reminiscent of myelodysplastic syndrome in humans due to loss of functional TET2 [72].

The *lgl* gene was originally identified as a tumor suppressor in *Drosophila*. A mutation in its zebrafish ortholog, *pen/lgl2*, was identified in a forward genetic screen. Homozygous *pen/lgl2* mutant embryos show overgrowth of endothelial cells, which is dependent on erbB signaling, and die at 4–5 days post fertilization and hence tumor formation cannot be assessed in homozygous *pen/lgl2* mutant zebrafish [73].

Space cadet is a mutant that was identified in a forward genetic screen for mutants with defective locomotor behavior and is caused by a nonsense mutation in the retinoblastoma susceptibility gene, *rb1*, a well-known tumor suppressor gene. Biallelic mutations in *RB1* cause intraocular childhood retinoblastoma. Zebrafish *rb1* mutants display defects in retinotectal tract development and visual function, resulting from a delay in cell cycle exit of retinotectal ganglion cells and initial failure of their axons to exit the retina, thus leading to optic nerve hypoplasia [74].

A forward genetic screen for mutants with defects in cilia function led to the identification of *hu255H*, a mutant that lacks motile cilia in the nose and neural tube. Positional cloning led to the identification of Leucine-rich repeat containing protein 50 (*lrrc50*) as the affected gene. Homozygous *lrrc50* mutants are larval-lethal [75]. Heterozygous *lrrc50* mutants are viable and fertile and are highly susceptible to the formation of seminomas. Loss-of-heterozygosity was observed in 44 % of tumor samples, which correlated with tumor progression. It turns out that *LRRC50* mutations are associated with seminomas in humans as well. This finding, together with the zebrafish data justifies the recognition of *LRRC50* as a tumor suppressor [76].

In search of novel tumor suppressors, 19 chromatin factors were selected for further analysis. As described above, expression of $KRAS^{G12D}$ under the control of the *rag2* promoter induces embryonal rhabdomyosarcoma formation [18]. To test for tumor suppressing activity, each of the 19 selected chromatin factors was co-expressed with $KRAS^{G12D}$ and tumor formation was assessed. One of the chromatin factors, SUV39H1, significantly suppressed tumor formation. Interestingly, SUV39H1 has a role in senescence and data in Oncomine is consistent with SUV39H1 being a tumor suppressor in humans [77]. Hence, this approach successfully led to the identification of a novel tumor suppressor gene, SUV39H1.

Conclusion

Zebrafish is maturing as a cancer model. Mutants lacking prominent tumor suppressor genes have been derived and some of these spontaneously develop tumors while others require additional mutations. It is noteworthy that the onset of spontaneous

tumor formation is relatively late in some of the models, suggesting that additional mutations are required. Complete loss of activity of many tumor suppressors is not compatible with life and leads to embryonic or larval lethality. This provides insight into the developmental and cell biological function of these tumor suppressors. In addition, this provides an opportunity to screen for genes or chemical compounds that rescue these developmental defects and/or embryonic lethality. Despite all the work that has been done to date, there is room for discovery of tumor suppressor genes and cooperation between tumor suppressors as well as between tumor suppressors and cancer driver genes and—perhaps more importantly—for development of means of therapeutic intervention, for which the zebrafish is an ideal model system.

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