



Zebrafish Models of p53 Functions

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Zebrafish models have significantly contributed to our understanding of vertebrate development and, more recently, human disease. The growing number of genetic tools available in zebrafish research has resulted in the identification of many genes involved in developmental and disease processes. In particular, studies in the zebrafish have clarified roles of the p53 tumor suppressor in the formation of specific tumor types, as well as roles of p53 family members during embryonic development. The zebrafish has also been instrumental in identifying novel mechanisms of p53 regulation and highlighting the importance of these mechanisms *in vivo*. This article will summarize how zebrafish models have been used to reveal numerous, important aspects of p53 function.

The zebrafish, *Danio rerio*, is a small model organism that has long been used to study vertebrate development. Zebrafish embryos are optically clear and develop externally to the mother, facilitating the study of early developmental processes. In addition, zebrafish have increasingly been used in modeling human diseases, including a number of cancers. The availability of forward and reverse genetic tools in the zebrafish has resulted in the identification and characterization of many genes involved in development and disease. One gene that has been extensively studied is the *p53* tumor suppressor gene, which is structurally and functionally conserved in the zebrafish. This article will discuss how studies in the zebrafish have increased our understanding of how p53 contributes to the formation of specific tumor

types, resulted in the identification of novel mechanisms of p53 regulation, and showed how p53 and p53 family members are involved in embryonic development.

CONSERVATION OF p53 STRUCTURE AND FUNCTION IN ZEBRAFISH

Zebrafish p53 is highly similar to mammalian p53 in both structure and function. Full-length zebrafish p53 is 48% identical in amino acid sequence to human p53 (Cheng et al. 1997). As in mice (Rogel et al. 1985; Schmid et al. 1991), p53 is highly expressed early in zebrafish development. *p53* transcript is most highly expressed in embryos within 1 h postfertilization (hpf) (Cheng et al. 1997) and is ubiquitously expressed in the early embryo (Thisse

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et al. 2000). At the pharyngula stage (24–48 hpf), *p53* is predominantly expressed in the head region. By 48 hpf, *p53* transcript and protein levels are barely detectable (Cheng et al. 1997; Thisse et al. 2000; Lee et al. 2008).

To elucidate *p53* function in zebrafish, Langheinrich and colleagues injected zebrafish embryos with *p53* antisense morpholinos that knockdown *p53* expression in the embryo (Langheinrich et al. 2002). The morpholino-injected, or morphant, embryos are developmentally normal. However, as compared with wild-type embryos, the *p53* morphants displayed a decreased induction of apoptosis on DNA-damaging stimuli, including UV irradiation and the topoisomerase inhibitor camptothecin. Studies in zebrafish that harbor a missense mutation, M214K, in the DNA-binding domain of *p53* have yielded similar results (Berghmans et al. 2005). Although developmentally normal, *p53* mutant embryos fail to undergo apoptosis on γ -irradiation. Moreover, a growing body of evidence suggests that in zebrafish, *p53* regulates apoptosis in response to a myriad of stimuli (Langheinrich et al. 2002; Berghmans et al. 2005; Robu et al. 2007; Lee et al. 2008). Thus, as in mammals and other model organisms, *p53* is a key mediator of apoptosis in the zebrafish.

Other aspects of *p53* regulation and function are also conserved in zebrafish. The major functional domains of *p53* negative regulator MDM2, and the physical interaction between *p53* and MDM2, are highly conserved in zebrafish (Thisse et al. 2000). MDM2-deficient zebrafish embryos display growth retardation and high levels of apoptosis. These embryos are completely rescued by *p53* deficiency (Langheinrich et al. 2002), akin to observations in *p53/Mdm2* double knockout mice (Jones et al. 1995; Montes de Oca Luna et al. 1995). However, recent data suggest that the apoptosis seen in MDM2-deficient embryos may be because of off-target effects of the MDM2 morpholino (see following discussion) (Robu et al. 2007), so further experiments will be required to elucidate the interaction of MDM2 and *p53* in zebrafish embryos. In addition, zebrafish *p53* is required for transcriptional regulation of a

number of known *p53* target genes, including *bax*, *mdm2*, and *p21* (Langheinrich et al. 2002; Lee et al. 2008). Lastly, *p53* mutant embryos fail to undergo G1 arrest on γ -irradiation, suggesting a conserved role for zebrafish *p53* in cell cycle regulation (Berghmans et al. 2005). As discussed later, studies on *p53* in the zebrafish have also revealed novel aspects of *p53* function that are important in the function of *p53* in higher vertebrates.

p53 IN ZEBRAFISH MODELS OF CANCER

Tumor Formation in *p53* Mutant Zebrafish

The importance of *p53* in cancer is highlighted by the fact that it is the most frequently mutated tumor suppressor gene in human cancers, with more than half of solid tumors harboring mutations in the *p53* locus. In many other tumors, *p53* is inactivated by more indirect mechanisms, including *MDM2* amplification and *p14ARF* deletion (Vogelstein et al. 2000). Patients with Li-Fraumeni syndrome, characterized by germline inactivating mutations in *p53*, have a significantly increased risk of developing cancer (Kleihues et al. 1997; Nichols et al. 2001). Moreover, mice homozygous for a *p53* null allele also have an increased propensity to develop tumors (Donehower et al. 1992; Jacks et al. 1994).

p53 also acts as a tumor suppressor in zebrafish. Fish homozygous for the *p53*^{M214K} mutation develop tumors at 28% incidence by 16.5 mo of life, whereas wild-type and heterozygous fish seldom develop tumors. Histologically, these tumors most closely resemble human malignant peripheral neural sheath tumors (MPNSTs). The zebrafish MPNSTs displayed morphological features of nerve sheath differentiation by electron microscopy and expressed the neuronal marker *gfap*. The tumors were also clonally aneuploid and had a long latency, suggesting that additional mutations are required for the initiation of zebrafish MPNSTs (Berghmans et al. 2005). Although almost all tumors that developed in *p53*^{M214K} zebrafish were characterized as a MPNST, humans and mice with germline mutations of *p53* do not commonly develop MPNSTs (Donehower et al. 1992; Jacks

et al. 1994; Kleihues et al. 1997; Nichols et al. 2001). The reasons for this difference in tumor spectrum are unclear, but they are likely attributable to species- and tissue-specific differences in the response to DNA damage and unchecked proliferative stimuli (Berghmans et al. 2005).

As in zebrafish, *p53* mutations are likely to be an important contributor to the development of mammalian MPNSTs. *Nf1* knockout mice develop benign nerve sheath tumors, whereas *Nf1;p53* double knockout mice form MPNSTs, indicating that *p53* mutations cooperate with *Nf1* deficiency in the development of murine MPNSTs (Cichowski et al. 1999; Vogel et al. 1999). In addition, deletions and mutations in the *p53* locus (Menon et al. 1990) and deletions in the *CDKN2A/p16* locus (Nielsen et al. 1999) have been identified in human MPNSTs, suggesting a role for *p53* pathway disruption in human MPNST. Thus, *p53* mutant zebrafish can be a useful model in elucidating how *p53* pathway dysregulation contributes to MPNSTs and identifying other pathways that contribute to the pathogenesis of MPNSTs. Moreover, as discussed in the following sections, the *p53* mutant zebrafish has proved useful in clarifying the role of *p53* mutations in the formation of other tumor types.

Cooperativity of *p53* and *BRAF* Mutations in Zebrafish Melanoma

The majority of human melanomas harbor activating mutations in *BRAF*, an effector of RAS/MAPK signaling that mediates cellular proliferation and other processes (Brose et al. 2002; Davies et al. 2002; Tuveson et al. 2003). Activating *BRAF* mutations are also commonly observed in benign nevi (Pollock et al. 2003), or moles, suggesting that mutant *BRAF* cooperates with other mutations to induce melanoma formation. To investigate the effect of activated *BRAF* on melanoma development, Patton and colleagues generated a transgenic construct in which activated human *BRAF*^{V600E} is expressed under the control of the zebrafish melanocyte lineage-specific *mitfa* promoter (Patton et al. 2005). The *mitfa-BRAF*^{V600E} transgene was microinjected into zebrafish embryos, and resulting

mosaic transgenic fish developed focal areas of melanocyte proliferation, termed fish nevi (f-nevi), that resemble their human counterparts. The f-nevi were characterized by an increased number of well-differentiated melanocytes without features of dysplasia or invasion into neighboring tissues. Stable transgenic *mitfa-BRAF*^{V600E} fish that express the transgene in all melanocytes display abnormal stripe patterning. *mitfa-BRAF*^{V600E} fish do not develop melanoma, suggesting that, whereas activated *BRAF* is sufficient to induce melanocyte proliferation, additional mutations are required for the progression to malignant melanoma (Patton et al. 2005).

Multiple lines of evidence have suggested that disruption of *p53* pathway activity contributes to melanoma formation. *p53* mutations are found in 10%–25% of human melanomas (Daniotti et al. 2004; Ceol et al. 2008). In addition, a larger proportion of melanomas harbor mutations that inactivate the *CDKN2A* locus, which encodes two distinct tumor suppressor genes, *INK4A* and *ARF*. The *ARF* tumor suppressor inhibits MDM2-mediated ubiquitylation and the ensuing degradation of *p53*. Thus, mutations in the *CDKN2A* locus commonly found in melanoma often result in the disruption of *p53* activity. Furthermore, *p53* pathway activity, as assessed by gene expression changes in response to DNA damage, is attenuated in many melanomas (Kaufmann et al. 2008). However, the requirement of *p53* pathway disruption in the formation of melanoma has been unclear.

To investigate the contribution of *p53* disruption to melanoma formation, the *mitfa-BRAF*^{V600E} construct was microinjected into *p53*^{M214K} mutant zebrafish. Interestingly, half of fish that developed nevi also formed melanoma by 4 mo of age. These tumors resembled human melanoma by histology. In addition, the tumors were highly invasive, transplantable into recipient fish, and often showed aneuploidy. In contrast, melanoma formation is much more rare in wild-type and *p53*^{M214K} zebrafish. Thus, *p53* mutation cooperates with *BRAF* activation in the formation of zebrafish melanoma (Patton et al. 2005). This study importantly showed

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that activating *BRAF* mutations and p53 pathway mutations participate in the formation of melanomas and are not merely bystander mutations formed during tumor development (Ceol et al. 2008). In addition, this zebrafish model can be useful in understanding how p53 loss-of-function contributes to melanoma pathogenesis.

Cooperativity of p53 and RAS Mutations in Zebrafish Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a malignancy with characteristics of muscle differentiation and is the most common soft-tissue sarcoma of childhood (Arndt and Crist 1999). Embryonal rhabdomyosarcoma (ERMS) is the most prevalent subtype of pediatric RMS and is characterized in a minority of cases by mutational activation of RAS family members (Stratton et al. 1989; Chen et al. 2006). Zebrafish embryos were injected with a transgenic construct in which human oncogenic *KRAS*^{G12D} is expressed under the control of a fragment of the zebrafish *rag2* promoter that drives gene expression in satellite cells and myoblasts within skeletal muscle (Langenau et al. 2007). The resulting *rag2-KRAS*^{G12D} mosaic transgenic zebrafish began to develop tumors by 10 dpf that resembled ERMS by histology and were highly invasive. Gene set enrichment analysis of the most up-regulated genes in the zebrafish tumors revealed that the tumors were highly similar to human ERMS by gene expression. Hence, expression of activated *KRAS* in muscle progenitor cells is sufficient to induce ERMS formation in zebrafish (Langenau et al. 2007).

p53 mutations are observed in a subset of ERMS cases (Xia et al. 2002), suggesting that inactivation of the p53 pathway can contribute to the pathogenesis of ERMS. Sequencing of the *p53* locus in zebrafish ERMS tumors failed to identify inactivating mutations in *p53*. However, *mdm2* and *survivin*, negative regulators of p53 pathway activity, are highly expressed in the zebrafish tumors, indicating that the p53 pathway may be disrupted in zebrafish ERMS (Langenau et al. 2007). Overexpression of *MDM2* and *SURVIVIN* is also observed in human RMS

(Xia et al. 2002; Caldas et al. 2006). Interestingly, homozygous and heterozygous *p53*^{M214K} mutants injected with *rag2-KRAS*^{G12D} have a significantly increased tumor incidence in comparison to wild-type siblings, demonstrating that p53 pathway disruption can cooperate with RAS mutations in the formation of ERMS (Langenau et al. 2007). These data suggest that inactivation of the p53 pathway is important in ERMS tumor initiation in both zebrafish and humans.

REGULATION OF p53 IN ZEBRAFISH

p53 plays important roles in controlling cell cycle progression, cellular senescence and apoptosis in response to various cellular stressors. Thus, tight regulation of p53 activity is required for normal cell proliferation and the prevention of tumor formation, and disruption in this regulation is a commonly observed mechanism of p53 pathway inactivation in human cancer. In mammals, p53 stability and function is regulated by a number of posttranslational modifications (Brooks and Gu 2003), whereas in zebrafish, regulation at both the mRNA and protein levels in response to different types of stress has been described (Langheinrich et al. 2002; Lee et al. 2008). Recent studies in zebrafish have highlighted novel and varied ways in which p53 can be regulated during normal development and tumorigenesis.

Delta113p53 Regulation of p53-Mediated Apoptosis

A number of p53 isoforms generated by alternative splicing and promoter usage have been identified in human cells (Bourdon et al. 2005). One such isoform, $\Delta 133p53$, and its zebrafish ortholog, $\Delta 113p53$, are transcribed from an alternative promoter in intron 4 of *p53* and lack the amino-terminal transactivation domain and part of the DNA binding domain of p53 (Bourdon et al. 2005; Chen et al. 2005). Because the $\Delta 133p53$ and $\Delta 113p53$ isoforms lack the transactivation domain but have a largely intact DNA binding domain, these isoforms are postulated to have a dominant-negative effect

in the regulation of p53 transcriptional activity. In concordance with this hypothesis, cotransfection of $\Delta 133p53$ with *p53* in human cells inhibits p53-induced apoptosis (Bourdon et al. 2005).

The role of this p53 isoform in regulating p53 has been further clarified in the zebrafish (Chen et al. 2005; Chen et al. 2009). The zebrafish $\Delta 113p53$ promoter has a number of p53 consensus binding sites that are required for transcriptional activity and is directly regulated by p53. $\Delta 113p53$ is up-regulated in zebrafish embryos on treatment with DNA-damaging agents in a p53-dependent manner. As in human cells, overexpression of $\Delta 113p53$ rescues p53-induced apoptosis in zebrafish embryos. Moreover, morpholino knockdown of $\Delta 113p53$ strongly enhances p53-mediated apoptosis in response to ionizing radiation treatment. These data suggest that $\Delta 113p53$ expression has a protective effect against p53-mediated apoptosis in zebrafish (Chen et al. 2009).

Interestingly, $\Delta 113p53$ has varying effects on the transcription of p53-regulated genes in zebrafish. $\Delta 113p53$ enhances p53-dependent up-regulation of *p21* and *mdm2*, but inhibits p53-dependent up-regulation of the proapoptotic gene *bax*. In contrast, whereas overexpression of *p53* results in down-regulation of the antiapoptotic gene *bcl2L*, co-overexpression of $\Delta 113p53$ restores *bcl2L* expression. In fact, $\Delta 113p53$ requires *bcl2L* expression to inhibit p53-mediated apoptosis. Thus, $\Delta 113p53$ does not act simply in a dominant negative fashion to inhibit p53 function, but rather modulates p53-dependent transcription in a target-specific manner (Chen et al. 2009).

Studies in zebrafish also suggest that $\Delta 113p53$ plays a role in vertebrate development (Chen et al. 2005; Chen et al. 2009). A loss-of-function mutation in *def* (digestive-organ expansion factor) results in the formation of hypoplastic digestive organs because of cell cycle arrest, rather than apoptosis, in these organs. Mutant embryos also display specific up-regulation of $\Delta 113p53$, but not full-length *p53*, in the digestive tract in zebrafish embryos. Although knockdown of both $\Delta 113p53$ and *p53* partially rescued the mutant phenotype (Chen

et al. 2005), knockdown of $\Delta 113p53$ alone enhances apoptosis in the digestive organs of *def* morphants (Chen et al. 2009). Interestingly, cell cycle-related, but not proapoptotic, p53 target genes are up-regulated in *def* mutant embryos (Chen et al. 2005). Thus, studies in zebrafish have shown that $\Delta 113p53$ functions to regulate p53 activity during development, as well as in response to DNA-damaging agents.

Regulation by Ribosomal Proteins

A large-scale insertional mutagenesis screen in the zebrafish identified more than 300 genes, including 28 ribosomal protein (*rp*) genes, required for early development (Amsterdam et al. 1999; Golling et al. 2002; Amsterdam et al. 2004a). Although homozygous *rp* mutants are embryonic lethals, heterozygous *rp* mutant fish are viable through adulthood. Fish with heterozygous mutations in 17 of the 28 *rp* genes develop malignant peripheral nerve sheath tumors (MPNSTs) with 10%–100% incidence (Amsterdam et al. 2004b; Lai et al. 2009). Interestingly, whereas MPNSTs are exceedingly rare (0.05%) in wild-type zebrafish (Lai et al. 2009), 28% of *p53* homozygous mutant fish develop MPNSTs by 16.5 months of life (Berghmans et al. 2005). In addition, MPNSTs from *rp*^{+/-} and *p53*^{-/-} mutants have very similar gene expression profiles (MacInnes et al. 2008). Collectively, these data hinted at a role for *rp* heterozygosity in p53 pathway regulation.

A closer examination of *rp*^{+/-} tumors revealed that tumors expressed wild-type *p53* mRNA at detectable levels. However, MPNSTs from all 17 *rp*^{+/-} lines expressed little or no p53 protein, whereas *p53*^{-/-} MPNSTs express high levels of mutant p53. Moreover, *rp*^{+/-} MPNSTs do not display a p53-dependent induction of *p53* mRNA expression on γ -irradiation, suggesting a loss of p53 protein function in these tumors. p53 protein levels remain low in *rp*^{+/-} MPNSTs even after exposure to γ -irradiation or the proteasome inhibitor MG132, indicating that the low p53 protein levels observed are not because of enhanced protein degradation. Although overall translation capacity is normal in *rp*^{+/-} MPNSTs,

p53 protein synthesis is specifically impaired in the tumors (MacInnes et al. 2008). These observations show that *rp* heterozygosity specifically impairs translation of *p53* mRNA and highlight a novel mechanism of p53 regulation. Because this effect was observed for all 17 *rp* genes studied, it is likely that p53 regulation in these lines occurs by a common mechanism, perhaps by deficiency in ribosomal function. Furthermore, these data also suggest a role for *rp* gene dosage in tumorigenesis.

Although this effect on p53 protein levels was seen in 17 different *rp* lines, down-regulation of p53 by *rp* deficiency is not universally observed. First, p53 protein loss was only observed in tumor cells, and not in all cells, in the 17 *rp*^{+/-} lines that were studied (MacInnes et al. 2008). Thus, defects in p53 synthesis may be tumor- or tissue-specific. In addition, homozygous mutant embryos for three of the 17 *rp* lines display p53 pathway activation (Danilova et al. 2008). Similarly, injection of morpholinos for two other *rp* genes results in activation of p53 target genes (Danilova et al. 2008; Chakraborty et al. 2009). Hence, a complete or near complete loss of *rp* expression does not inhibit p53 function, but rather can result in p53-dependent apoptosis (Chakraborty et al. 2009). Zebrafish embryos with deficiencies in rRNA synthesis and processing, and presumably ribosome biogenesis, also activate the p53 pathway (Azuma et al. 2006; Skarie and Link 2008). Thus, regulation of p53 by rps may depend on cellular context, *rp* expression level, and level of ribosomal function. This work has revealed an important role of rps in the specific regulation of p53, and deficiency in rps may be a mechanism of p53 dysregulation in human tumors.

Regulation by MicroRNA-125b

MicroRNAs (miRNAs) are a large group of small, noncoding RNAs that regulate gene expression by mRNA cleavage or translational repression, largely through binding with partial complementarity to the 3' untranslated region (UTR) of target mRNAs (Bartel 2004). miRNAs have been increasingly implicated in a number of biological and disease processes, including

cancer (Esquela-Kerscher and Slack 2006). Recent work has shown that a miRNA, miR-125b, directly regulates p53 in zebrafish and in human cells (Le et al. 2009). miR-125b is able to bind to the 3'UTR of both zebrafish and human *p53* mRNA and negatively regulates zebrafish and human p53 protein levels when ectopically expressed. This regulation requires an intact, predicted miR-125b binding site in the zebrafish and human 3'UTR. In addition, miR-125b negatively regulates endogenous p53 protein levels and levels of apoptosis in human neuroblastoma cells and in primary human lung fibroblasts (Le et al. 2009).

The regulation of p53 by miR-125b is also important during development. Expression of miR-125b in the developing zebrafish embryo begins at 19 hpf. It is expressed throughout the embryo, but is enriched in the brain, eyes, and somites. Interestingly, there is an inverse correlation between miR-125b expression levels and *p53* or *p21* expression levels in the developing embryo. Embryos injected with morpholinos against miR-125b display neural death specific to miR-125b knockdown and morphological defects including loss of the midbrain-hindbrain boundary and deformed somites. Morphant embryos have elevated levels of p53 protein and *p21* mRNA, suggesting p53 pathway activation in embryos on miR-125b loss. In addition, morphant embryos displayed elevated neural apoptosis by TUNEL assay that was rescued by overexpression of a miR-125b duplex. The neural apoptosis and many of the morphological defects could be rescued by p53 loss-of-function, suggesting that the p53 pathway is the major target of miR-125b during development (Le et al. 2009).

As mentioned earlier, zebrafish embryos undergo p53-mediated apoptosis on exposure to DNA-damaging agents, such as γ -irradiation and camptothecin. Notably, γ -irradiation or camptothecin treatment results in a significant down-regulation of miR-125b expression and concomitant increase in p53 protein levels in zebrafish embryos. Ectopic expression of miR-125b in these embryos substantially decreases p53 levels and rescues stress-induced apoptosis. Moreover, treatment of human neuroblastoma

cells with etoposide, a topoisomerase inhibitor that induces DNA damage and activates p53, results in a down-regulation of miR-125b levels. Thus, down-regulation of miR-125b may be a conserved mechanism to activate p53 and subsequent p53-mediated apoptosis in response to DNA damage and perhaps other cellular stressors (Le et al. 2009).

In summary, the direct regulation of p53 protein levels by miR-125b plays important roles during development and in the regulation of stress-induced apoptosis. In addition, recent data also suggest that upstream regulators of p53 may also be targets of miR-125b (Sinha et al. 2008). Hence, miR-125b is likely to play multiple, important roles in regulating the p53 pathway. Given its importance as a negative regulator of p53, it will be intriguing to evaluate the oncogenic potential of miR-125b and its role in p53 regulation in tumors.

ROLE OF p53 IN MORPHOLINO TOXICITY

Morpholino antisense oligonucleotides and short interfering RNA (siRNA) are commonly used reagents to elicit gene-specific knockdown in a variety of systems. However, a major limitation of both technologies is the induction of off-target effects (Ekker and Larson 2001; Jackson and Linsley 2004). In particular, injection of morpholinos into zebrafish embryos often results in a characteristic neural death by 22 hpf (Ekker and Larson 2001). This phenotype is not specific to the genes targeted for knockdown, as available loss-of-function mutations in corresponding genes do not elicit this neural death. Also, the off-target effect is morpholino sequence-specific—that is, unique morpholinos targeting different sequences in the same gene may or may not elicit an off-target effect (Robu et al. 2007).

Closer examination of Smo and Wnt5 morphant embryos displaying the off-target neural phenotype revealed *p21* up-regulation and extensive neural apoptosis by TUNEL assay from 14 to 26 hpf, suggesting p53 activation in these embryos. Simultaneous morpholino knockdown of p53 in these embryos rescued the neural apoptosis without affecting gene-specific

phenotypes. Importantly, p53 knockdown does not affect gene-specific apoptosis in the tails of *chordin* morphants, and does not affect gene-specific phenotypes in *nacre*, *no tail* and *UROD* morphants. p53 knockdown is also able to distinguish craniofacial defects that are gene knockdown-specific from those that are the result of off-target effects (Robu et al. 2007).

These results highlight the importance of discerning gene-specific versus off-target effects in morpholino knockdown experiments (Robu et al. 2007). Coknockdown of p53 can distinguish off-target effects, although caution is warranted as some morphant phenotypes may be the result of gene-specific activation of the p53 pathway. Other experiments can also aid in identifying knockdown phenotypes that are gene-specific. Effects are likely gene-specific if they can be rescued by DNA/RNA of the gene of interest, although such rescue experiments can often be difficult to interpret. Moreover, effects that are elicited with multiple, different morpholino sequences targeting the same gene are also likely to be gene-specific. Finally, non-specific activation of p53 has been described in siRNA-treated cells (Scacheri et al. 2004), suggesting that inhibition of p53 may be effective in reducing the off-target effects of other knockdown technologies (Robu et al. 2007).

p53 FAMILY MEMBERS IN DEVELOPMENT

Although p53 plays a critical role in cell cycle control and the regulation of apoptosis, it is largely dispensable for normal embryonic development in fish and mice. *p53^{M214K}* mutant zebrafish embryos and p53 morphant embryos develop normally and are morphologically indistinguishable from wild-type embryos (Langheinrich et al. 2002; Berghmans et al. 2005). In addition, heterozygous and homozygous *p53^{M214K}* mutant fish display normal viability, yet also show a decreased fecundity, in comparison to wild-type siblings (Langheinrich et al. 2002). Similarly, the majority of mice homozygous for a *p53* null allele appear developmentally normal by morphology and histology (Donehower et al. 1992). However, a minority of homozygous mutant mice displays



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exencephaly and defects in neural tube closure, phenotypes predominantly associated with female embryos (Armstrong et al. 1995; Sah et al. 1995). The penetrance of these defects varies with genetic background, suggesting that other modifying genes can interact with *p53* mutations to affect neural tube closure (Sah et al. 1995). Collectively, these data indicate that, in general, *p53* is not essential for normal embryonic development.

In contrast, studies in zebrafish have shown that *p53* family members, *p63* and *p73*, are required in a number of processes in the developing embryo. *p63* and *p73* are each transcribed from two different promoters and are alternatively spliced to generate gene products with differing carboxy-termini (Yang et al. 1998; Romani et al. 2003). Transcription from an upstream promoter results in TA isoforms that, like full-length *p53*, contain a amino-terminal transactivation domain, a DNA binding domain, and an oligomerization domain, whereas transcription from a more downstream site results in amino-terminally (Δ N) truncated isoforms that lack the transactivation domain. Because the Δ N isoforms lack a transactivation domain, they are thought to act in a dominant-negative fashion to inhibit the normal transcriptional activity of full-length *p53* family members. Studies in the zebrafish have highlighted the importance of TA and Δ N isoforms of *p63* and *p73* during embryonic development and have suggested a role for these isoforms in modulating *p53* activity in vivo.

The Role of Δ Np63 in Ectodermal Development

p63 is required for the normal development of ectoderm-derived tissues. Knockout mice lacking both TAp63 and Δ Np63 isoforms fail to develop stratified squamous epithelia and their derivatives, including skin and hair, and do not form limbs (Mills et al. 1999; Yang et al. 1999). In addition, patients harboring *p63* mutations display a number of skin and limb defects (Celli et al. 1999; McGrath et al. 2001). However, it was unclear from these studies whether loss of TAp63 and/or Δ Np63

isoforms contributed to these defects and how loss of *p63* function affected specific stages of ectodermal development.

Studies in zebrafish have clarified the roles of *p63* isoforms during development. Δ Np63 isoforms are conserved in the zebrafish and are the predominant isoforms expressed during embryonic development (Bakkers et al. 2002; Lee and Kimelman 2002). Δ Np63 is expressed in the ventral ectoderm during gastrulation and is later maintained in ectoderm-derived tissues. Embryos injected with high levels of morpholino specifically targeting Δ Np63, and not TAp63, isoforms show a decrease in nonneural (ventral, epidermal) ectoderm and an expansion of dorsal neural ectoderm at mid-gastrula stages, indicating that Δ Np63 is required for proper dorsoventral (DV) patterning. Interestingly, this effect was phenocopied by overexpression of TA forms of *p53* family members. Conversely, overexpression of Δ Np63 in embryos resulted in an expansion of nonneural ectoderm at the expense of anterior neural structures, which could be rescued by overexpression of murine TAp63. Δ Np63 was shown to be a direct transcriptional target of Bmp signaling and to function downstream of Bmp signaling in inhibiting neural cell fates in the ventral ectoderm (Bakkers et al. 2002).

Δ Np63 also functions later in the development of ectoderm-derived tissues. Notably, whereas Δ Np63 protein is present throughout cells during early development, it becomes restricted to the nuclei of the epidermis and eye epithelium at 20hpf, a stage when fin folds begin to protrude from the embryo because of epidermal expansion (Lee and Kimelman 2002). Embryos injected with Δ Np63-specific morpholinos lack pectoral fins, likely because of a failure to maintain the apical fold (Bakkers et al. 2002; Lee and Kimelman 2002). The apical fold of fin buds in zebrafish is homologous to the apical ectodermal ridge (AER) of higher vertebrates, an epidermal structure at the distal end of limb buds that is required for vertebrate limb development (Ng et al. 1999). Morphant embryos also display skin lesions by 3dpf (Bakkers et al. 2002; Lee and Kimelman 2002). Although Δ Np63 marks BrdU-positive,

proliferating epithelial cells in wild-type zebrafish embryos, $\Delta Np63$ -morphant embryos lose all BrdU-positive cells in the epidermis, indicating that $\Delta Np63$ is absolutely required for the proliferation of epidermal cells. This effect of $\Delta Np63$ on epidermal proliferation in zebrafish may be because of the inhibition of p53 activity by $\Delta Np63$. Moreover, induced p53 overexpression in embryos results in fin and skin defects akin to those observed in $\Delta Np63$ morphants, and these defects are rescued by overexpression of an inducible $\Delta Np63$ (Lee and Kimelman 2002).

These studies using the zebrafish show specific roles for the ΔN isoform of p63 in both DV patterning of the ectoderm and epidermal proliferation during development. Both roles of $\Delta Np63$ appear to be at least partially mediated by inhibition of the activity of p53 family members during development. Interestingly, zebrafish $\Delta Np63$ can act as a dominant negative inhibitor of p53 transcriptional activation in both tissue culture and in zebrafish embryos (Lee and Kimelman 2002). Future experiments will likely elucidate how modulation of the transcriptional activity of p53 family members by $\Delta Np63$ is involved in the proper development of ectoderm-derived tissues.

TAp73 is Required for Normal Embryonic Development

Like p63, p73 is also essential for normal embryonic development. In mice, $\Delta Np73\alpha$ is the major isoform expressed in embryos and adults, and can act in a dominant negative fashion to inhibit TAp73-mediated transcriptional activation. Murine p73 is expressed in the nasal epithelium, hippocampus and hypothalamus in developing embryos. p73-deficient mice that lack all p73 isoforms display defects in neuronal development and pheromone sensing, and also show hydrocephalus and chronic inflammation (Yang et al. 2000). $\Delta Np73$ isoforms have an antiapoptotic, prosurvival role in at least some neurons of the central and peripheral nervous systems during neural development and are also important for the long-term maintenance

of adult neurons (Pozniak et al. 2000; Pozniak et al. 2002).

In contrast to the mouse, TAp73 α is the major p73 isoform expressed in zebrafish embryos, and ΔN isoforms have so far been undetectable in zebrafish (Pan et al. 2003; Rentzsch et al. 2003). TAp73 transcript is detected in zebrafish embryos as early as mid-gastrulation, and is expressed in the developing olfactory system and telencephalon, similar to observations in mouse embryos (Yang et al. 2000). In addition, p73 expression can be seen in the pharyngeal endoderm, pronephric ducts and slow muscle cells of developing zebrafish, but not mouse, embryos (Yang et al. 2000; Rentzsch et al. 2003). Zebrafish embryos injected with morpholinos targeting p73 show defects in the formation of the olfactory system and telencephalon, reminiscent of defects in the olfactory system and neurons in p73-knockout mice. In addition, p73 morphant embryos show craniofacial malformations; whereas all cartilage elements are present, they are generally misshapen in the morphants (Rentzsch et al. 2003). This phenotype is specific to zebrafish, as craniofacial development is not affected in p73 null mice (Yang et al. 2000).

Like transcriptionally active forms of other p53 family members, zebrafish TAp73 can antagonize the activity of $\Delta Np63$ overexpression in embryos, suggesting that TAp73 shares some of the transactivating activities of the p53 family. However, whereas overexpression of p53 results in elevated levels of apoptosis in zebrafish embryos, overexpression of TAp73 does not have a proapoptotic effect. In addition, morpholino-induced knockdown of TAp73, unlike knockdown of p53, does not prevent apoptosis induced by UV irradiation (Rentzsch et al. 2003). In contrast, in mammalian cell culture, TAp73 can induce apoptosis through the regulation of p53-responsive genes (Jost et al. 1997). This difference in the regulation of apoptosis may be attributable to differences in the target genes of TAp73 in mice and fish.

ΔN and TA isoforms of p53 family members are generally thought to have antagonizing activities. Although different p73 isoforms are expressed in mice and fish during embryonic



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development (ΔN and TA, respectively), deficiency of p73 in both species results in similar defects in the development of the telencephalon and olfactory system. Thus, it is possible that the ΔN and TA isoforms of p73 have some shared activity, perhaps in the regulation of transcriptional targets. It will be interesting to discern the overlapping and distinct functions of ΔN and TA isoforms of p73 during embryonic development.

CONCLUDING REMARKS

The p53 tumor suppressor is a master regulator of cell cycle progression and apoptosis in response to cellular stressors. The significance of p53 in regulating cellular proliferation is underscored by the fact that it is mutated in more than half of all human solid tumors. Studies in zebrafish have shown the importance of p53 mutations in various tumor types and have clarified how p53 and p53 family members function during embryogenesis. The zebrafish has also been instrumental in identifying novel mechanisms of p53 regulation that may contribute to p53 pathway disruption in human tumors. The ease of performing forward and reverse genetic techniques and chemical screens make the zebrafish ideally suited for the future identification and characterization of genes and drugs that modify p53 pathway activity in vertebrates.

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