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Loss of function *tp53* mutations do not accelerate the onset of *myc*-induced T-ALL in the zebrafish

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Summary

The TP53 tumour suppressor is activated in response to distinct stimuli, including an ARF-dependent response to oncogene stress and an ATM/ATR-dependent response to DNA damage. In human T-cell acute lymphoblastic leukaemia (T-ALL), *TP53*-dependent tumour suppression is typically disabled via biallelic ARF deletions. In murine models, loss of *Arf* (*Cdkn2a*) or *Tp53* markedly accelerates the onset of *Myc*-induced lymphoblastic malignancies. In zebrafish, no ARF ortholog has been identified, but the sequence of ARF is very poorly conserved evolutionarily, making it difficult to exclude the presence of a zebrafish ARF ortholog without functional studies. Here we show that *tp53* mutations have no significant influence on the onset of *myc*-induced T-ALL in zebrafish, consistent with the lack of additional effects of *Tp53* loss on lymphomagenesis in *Arf*-deficient mice. By contrast, irradiation leads to complete T-ALL regression in *tp53* wild-type but not homozygous mutant zebrafish, indicating that the *tp53*-dependent DNA damage response is intact. We conclude that *tp53* inactivation has no impact on the onset of *myc*-induced

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The authors have no conflicts of interest to disclose.

Author Contributions AG designed and performed research and wrote the manuscript. HF, DML and YS designed and performed research and analysed data. OC performed research and analysed data. DSN analysed data. ATL supervised research and co-wrote the manuscript.

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T-ALL in the zebrafish, consistent with the lack of a functional ARF ortholog linking *myc*-induced oncogene stress to *tp53*-dependent tumour suppression. Thus, the zebrafish model is well suited to the study of ARF-independent pathways in T-ALL pathobiology.

Keywords

Myc; Tp53; ARF; tumour suppression; T-cell acute lymphoblastic leukaemia

Introduction

The TP53 transcription factor (also known as P53, or TRP53 in mice) is a central tumour suppressor whose activation induces apoptosis, cell cycle arrest or senescence (Junttila and Evan 2009, Levine 1997, Lu, *et al* 2009). Indeed, *TP53*-dependent tumour suppressor pathways are inactivated in most, if not all, human tumours. The TP53 protein is activated in response to distinct stimuli, including an ARF-dependent response to aberrant oncogene activation and an ATM/ATR-dependent response to DNA damage. The ARF-dependent activation of *TP53*, which is known to be induced by *MYC* overexpression, plays a central role in tumour suppression (Eischen, *et al* 1999, Kamijo, *et al* 1997, Schmitt, *et al* 1999, Zindy, *et al* 1998). Indeed, deletions of *Arf* (*Cdkn2a*) or inactivation of *Tp53* each markedly accelerate the onset of T-cell acute lymphoblastic leukaemia in murine models (Blyth, *et al* 1995, Treanor, *et al* 2011, Volanakis, *et al* 2009). Moreover, in the setting of murine spontaneous or radiation-induced lymphomagenesis, the tumour suppressor function of TP53 is entirely dependent on ARF (Christophorou, *et al* 2006, Efeyan, *et al* 2006). In human T-cell acute lymphoblastic leukaemia (T-ALL), *TP53*-dependent tumour suppression is typically disabled via ARF inactivation resulting from homozygous deletions of the *CDKN2A* locus (encoding both ARF and p16INK4a) (Fizzotti, *et al* 1995, Haidar, *et al* 1995, Hebert, *et al* 1994, Okuda, *et al* 1995). By contrast, *TP53* mutations are very rare in T-ALL clinical samples at initial diagnosis, although they do occur more frequently in heavily treated patients at the time of relapse (Diccianni, *et al* 1994, Hsiao, *et al* 1994, Wada, *et al* 1993).

Evolutionarily, *ARF* is conserved in mammals and chickens, but this gene has not been identified in amphibians or bony fish (including zebrafish and fugu) (Gilley and Fried 2001, Kim, *et al* 2003, Sherr 2006). A previous attempt to identify an *ARF* ortholog expressed in the normal liver of fugu, using 5'RACE from the locus syntenic to mammalian *CDKN2A*, failed to reveal an alternative reading frame transcript expressed from this locus (Gilley and Fried 2001). However, this study suffered from the fact that *ARF* is not expressed in normal liver cells in the absence of oncogene stress (Gromley, *et al* 2009, Zindy, *et al* 2003). In addition, the chicken *ARF* locus differs from its mammalian counterpart in that it encodes only ARF, with no associated CDK inhibitor expressed from the same locus, thus making chicken ARF impossible to identify via 5'RACE from a CDK locus (Kim, *et al* 2003). Because sequence of ARF is extremely poorly conserved evolutionarily, even among closely related mammals (Sherr 2006), it is very difficult to exclude the presence of a zebrafish ARF ortholog based on bioinformatics approaches alone, and it remains unclear whether or not zebrafish harbour a functional ortholog of ARF.

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Here, we show that *tp53* mutations fail to accelerate the onset of *myc*-induced T-ALL in the zebrafish, consistent with the lack of a functional ARF ortholog linking *myc*-induced oncogene stress to *tp53*-dependent tumour suppression in zebrafish thymocytes. We additionally find that the *tp53*-dependent apoptotic response to DNA damage is retained during thymocyte transformation, indicating that the *tp53* axis is intact, but fails to be activated by the *myc*-induced stress response that is triggered by ARF in mammalian cells.

Materials and methods

Genomic sequence analysis

Human (Homo sapiens), mouse (Mus musculus), chicken (Gallus gallus), and frog (Xenopus tropicalis) genomes were accessed using the UCSC genome browser (http://genome.ucsc.edu). The zebrafish genome was accessed using the Vega Sanger genome browser (http://vega.sanger.ac.uk). Genome builds utilized were as follows: Human, GRCh37/hg19; Mouse, NCBI37/mm9; Chicken, WUGSC 2.1/galGal3; Frog, JGI 4.2/xenTro3; Zebrafish, VEGA47. Databases were accessed on November 6, 2013.

Transgenic and mutant zebrafish lines

The *tp53*^{M214K} mutant, *rag2:LoxP-dsRed2-stop-LoxP-EGFP-Myc* transgenic, *hsp70:Cre* transgenic, and *rag2:MYC-ER* transgenic zebrafish lines have previously been described (Berghmans, *et al* 2005, Feng, *et al* 2007a, Gutierrez, *et al* 2011a, Langenau, *et al* 2005). Genotyping for *tp53, hsp70:Cre*, *rag2:LoxP-dsRed2-stop-LoxP-EGFP-Myc*, and *rag2:MYC-ER* transgenes was performed via polymerase chain reaction (PCR) on genomic DNA, as previously described (Berghmans, *et al* 2005, Feng, *et al* 2007a, Feng, *et al* 2007b, Gutierrez, *et al* 2011a).

Activation of conditional myc transgenes

Activation of *myc* expression in *rag2:LoxP-dsRed2-stop-LoxP-EGFP-Myc; hsp70:Cre* double-transgenic zebrafish larvae was performed via heat shock treatment at 37°C × 45 min at 6 days post-fertilization, before returning zebrafish to their baseline 28°C ambient temperature, as previously described (Feng, *et al* 2007a). Post-translational activation of the MYC-ER oncoprotein in *rag2:MYC-ER* transgenic zebrafish was performed by raising animals off-line in 750 ml of water containing 50 μg/L (129 nM) 4-hydroxytamoxifen beginning at 5 days post-fertilization, with weekly water changes, as previously described (Gutierrez, *et al* 2011a).

T-ALL monitoring

Zebrafish were monitored for T-ALL development via weekly fluorescence microscopy beginning at 4 weeks of age. Tumour onset was defined as development of a fluorescent mass arising from the thymus that was greater than twice the diameter of normal thymus, together with invasion of adjacent tissues and structures. Fluorescence microscopy was performed using a Nikon SMZ1500 microscope and an EXFO X-Cite 120 Fluorescence Illumination System. Images were captured using a Nikon DS2MBWc camera and Nikon NIS-Elements F Package version 3.00 software. All zebrafish images shown represent merged fluorescence (shown in green for green fluorescence protein [GFP] fluorescence)

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and brightfield (shown in grayscale) images. Fluorescence and brightfield images were merged using Adobe Photoshop 7.0.

Statistical analyses

Differences in tumour-free survival were assessed by the log-rank test, and time-to-event distributions were estimated using the Kaplan-Meier method. Statistical significance was defined as $P < 0.05$.

Results

Evolution of the INK4a/ARF/INK4b locus

The mammalian *CDKN2A* locus encodes two proteins, p16INK4a and ARF (p14ARF in humans, p19ARF in mice), whose transcripts originate from distinct promoters and first exons that are then spliced into the same second and third exons (Quelle, *et al* 1995). However, p16INK4a and ARF are transcribed using distinct reading frames through exons 2 and 3, thus they harbour no sequence homology at the protein level. In mammals, *CDKN2A* is located immediately downstream of *CDKN2B*, a locus that encodes p15INK4b (a distinct CDK inhibitor that is related to p16INK4a) but that does not contain an alternative reading frame or encode an ARF paralog (Fig 1a and b). Chickens have an ortholog of *CDKN2B* and ARF is also conserved in chickens, in a genomic location syntenic to mammalian *ARF* immediately downstream of *CDKN2B*, but, in the chicken, ARF is encoded by itself and the chicken does not contain an INK4a ortholog (Fig 1c) (Kim, *et al* 2003). In frogs and teleosts (bony fish, such as zebrafish and fugu), this genomic locus is simpler, in that they each harbour a single CDK inhibitor gene at the location syntenic to mammalian *CDKN2B*, but no ARF or *CDKN2A* orthologs have been identified within this locus or elsewhere in their respective genomes (Fig 1d and e) (Gilley and Fried 2001). Thus, the divergence of teleosts from mammals appears to have occurred prior to the duplication of INK4a/b and the evolutionary appearance of ARF.

tp53 mutations do not accelerate onset of myc-induced T-ALL in the zebrafish

Experimental studies in mammalian systems have revealed that ARF is activated downstream of oncogene stress, which leads to induction of a robust *tp53*-dependent tumour suppressor response (Eischen, *et al* 1999, Schmitt, *et al* 1999, Zindy, *et al* 1998). To functionally test whether zebrafish harbour a functional ARF ortholog linking *myc*-induced oncogene stress to *tp53*-dependent tumour suppression, we crossed zebrafish harbouring a $tp53^{M214K}$ mutation, which encodes a transactivation-defective Tp53 protein (Berghmans, *et al* 2005), into our Cre/Lox-regulated model of *myc*-induced T-ALL (Fig 2a) (Feng, *et al* 2007a). *tp53*-heterozygous mutant zebrafish that also expressed *rag2:LoxP-dsRed2-stop-LoxP-EGFP-Myc* and *hsp70:Cre* transgenes were crossed to *tp53*-heterozygous mutant animals. Cre expression was induced in all offspring from this cross using heat shock treatment at 6 days post-fertilization (dpf), in order to excise the dsRed2-stop cassette and activate expression of *EGFP-Myc*. Following heat shock treatment, T-ALL onset was monitored by weekly fluorescence imaging beginning at 4 weeks of age, and *tp53* genotyping was performed at the end of the experiment. We thus compared T-ALL incidence amongst *tp53* wild-type (n = 42), heterozygous (n = 80), or homozygous mutant (n

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= 26) zebrafish that expressed both the *rag2:LoxP-dsRed2-stop-LoxP-EGFP-Myc* and *hsp70:Cre* transgenes. These numbers provided 82% power to detect a 30% difference between *tp53* wild-type and homozygous mutant zebrafish, but this experiment revealed no significant influence of *tp53* mutations on T-ALL onset in this line ($P = 0.58$; Fig 2b).

Recent work has shown that aberrant proliferation can be driven by low-level activation of Myc, below the threshold required to trigger the ARF-Tp53 tumour suppressor response (Murphy, *et al* 2008). Thus, we postulated that cooperation between *TP53* mutations and *MYC* might be unmasked in a different *MYC*-transgenic line in which T-ALL develops with high penetrance and short latency. To address this issue, we took advantage of our *rag2:MYC-ER* line (Fig 3a) (Gutierrez, *et al* 2011a). We crossed the $tp53^{M214K}$ mutation into the *rag2:MYC-ER* line, and subsequently mated *tp53*-heterozygous zebrafish that also expressed *rag2:MYC-ER* and *rag2:GFP* transgenes to *tp53*-heterozygotes. MYC-ER activation was induced by raising offspring in 50 μg/l (129 nM) 4-hydroxytamoxifen, beginning at 5 dpf, and T-ALL onset was monitored by weekly fluorescence imaging, beginning at 4 weeks of age. We thus compared T-ALL onset amongst *tp53* wild-type (n = 9), *tp53* heterozygous (n = 15), or *tp53* homozygous mutant (n = 16) zebrafish that each expressed both *rag2:MYC-ER* and *rag2:GFP*, numbers that provided 81% power to detect a 45% difference between the *tp53* wild-type and homozygous mutant groups. This experiment revealed no detectable effect of *tp53* mutations on T-ALL onset ($P = 0.41$; Fig 3b).

The tp53-dependent DNA damage response is retained during thymocyte transformation

The lack of acceleration of T-ALL onset in our Myc transgenic models with mutant *tp53* raises the question of whether Tp53 is functionally active at all in these tumour cells. To test whether zebrafish thymocytes are capable of inducing Tp53 function, we assessed the response of established T-ALL tumours of *tp53*-wild type or homozygous mutant genotypes to irradiation. Delivery of a 22.5 Gy dose of total body irradiation to *tp53*-wild type, *rag2:MYC-ER; rag2:GFP* zebrafish with T-ALL led to complete loss of all GFP-positive T-ALL cells 72 h post-irradiation in all zebrafish examined (Fig 4a; n=4). By contrast, irradiation had no discernible effect on GFP-positive T-ALL cells in any of the *tp53* homozygous mutant zebrafish examined (Fig 4b; n=4). Thus, we conclude that the *tp53*-dependent DNA damage response is intact in *myc*-induced T-ALL cells of *tp53* wild-type zebrafish.

Discussion

Tumour suppressor pathways have evolved to protect long-lived organisms from malignancy. The TP53 transcription factor is a central suppressor of mammalian tumorigenesis that is activated by distinct pathways, including an ARF-dependent response to oncogene stress and ARF-independent pathways in response to DNA damage (Junttila and Evan 2009, Levine 1997, Lu, *et al* 2009). ARF is conserved in mammals and chickens, but ARF orthologs have not been identified in the genomes of other vertebrates including bony fish and amphibians, although the very poor sequence conservation of ARF among species makes its presence impossible to exclude via bioinformatic approaches (Gilley and

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Fried 2001, Kim, *et al* 2003, Sherr 2006). We now demonstrate that inactivation of Tp53 fails to accelerate the onset of *myc*-induced T-ALL in the zebrafish, consistent with findings in ARF-deficient mouse models of lymphoma (Christophorou, *et al* 2006, Efeyan, *et al* 2006). These data thus strongly suggest that zebrafish thymocytes lack a functional ARF ortholog linking *myc*-induced oncogenic stress to *tp53*-dependent tumour suppression. We have considered an alternative potential explanation for our findings, which is that the zebrafish *tp53*^{M214K} mutation used in our studies may not fully abrogate *tp53*-dependent tumour suppression. Although we cannot rule out modest residual tumour suppressor activity in *tp53*^{M214K} mutant zebrafish, this mutation has a major impact on *tp53*-dependent tumour suppression, as evidenced by its inability to transactivate Cdkn2a (p21) (Berghmans, *et al* 2005), a key mediator of *tp53*-dependent tumour suppression (el-Deiry, *et al* 1993, Martin-Caballero, *et al* 2001), as well as the spontaneous development of tumours in homozygous *tp53*^{M214K} mutant zebrafish (Berghmans, *et al* 2005). Thus, the lack of any detectable interaction between Myc and Tp53 in our studies is most consistent with absence of a functional ARF ortholog in zebrafish. Moreover, we have previously shown that the zebrafish locus syntenic to mammalian INK4a/ARF/INK4b is not deleted in zebrafish *myc*-induced T-ALL (Langenau, *et al* 2005), suggesting that inactivation of ARF drives selection for biallelic INK4/ARF locus deletions in human T-ALL.

Interestingly, the evolutionary emergence of ARF correlates closely with loss of limb- and organ-regenerative capacity in adult animals, and ARF has been directly implicated in the suppression of mammalian regenerative capacity (Brockes and Kumar 2008, Pajcini, *et al* 2010). These findings thus suggest that emergence of the ARF-Tp53 pathway represents an evolutionary tradeoff between regenerative capacity and tumour suppression, with tumour suppression being evolutionarily favoured in long-lived organisms.

Our findings, that the zebrafish *tp53*^{M214K} mutation fails to accelerate *myc*-induced T-ALL, contrasts with data from our laboratory and others, which demonstrate that this zebrafish *tp53* mutation accelerates the onset of a range of solid tumours induced by other oncogenes, including *AKT*-induced liposarcoma, *RAS*-induced rhabdomyosarcoma and hepatocellular carcinoma, *BRAF*-induced melanoma, and *EWSR1-FLI1*-induced Ewing's sarcoma (Gutierrez, *et al* 2011b, Langenau, *et al* 2007, Leacock, *et al* 2012, Nguyen, *et al* 2011, Patton, *et al* 2005). Moreover, these *tp53*-mutant zebrafish spontaneously develop malignant peripheral nerve sheath tumours (Berghmans, *et al* 2005). These findings highlight the context specificity of *tp53*-dependent tumour suppressor pathways. Indeed, human T-ALL diagnostic specimens almost always inactivate ARF rather than *TP53*, whereas human rhabdomyosarcomas and osteosarcomas preferentially acquire mutations of *TP53* itself. Taken together, these data thus suggest that *TP53*-dependent tumour suppression in *MYC*-induced T-ALL is largely dependent on ARF, whereas ARF-independent functions of TP53 appear to have more prominent tumour suppressor function in solid tumours.

Despite the apparent absence of a functional ARF ortholog in the zebrafish, ARF-independent tumour suppressor and oncogenic pathways are highly conserved between zebrafish and humans (White, *et al* 2013). Indeed, the zebrafish model has already revealed key insights into mechanisms of oncogenic transformation by canonical T-ALL oncogenes and tumour suppressors, including *MYC*, *NOTCH*, and *PTEN* (Blackburn, *et al* 2012,

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Gutierrez, *et al* 2011a, Langenau, *et al* 2003). Moreover, recent studies leveraging findings in the zebrafish model have revealed pathways that regulate the dissemination of localized thymic lymphomas to T-ALL, which are conserved in humans (Feng, *et al* 2010). Furthermore, the zebrafish model is particularly well-suited to small molecule screens to discover novel T-ALL therapeutic agents that act via ARF-independent mechanisms, as recently demonstrated (Gutierrez, *et al* 2014). Thus, we conclude that the zebrafish model system is ideally suited to the study of ARF-independent pathways in the molecular pathogenesis of T-ALL.

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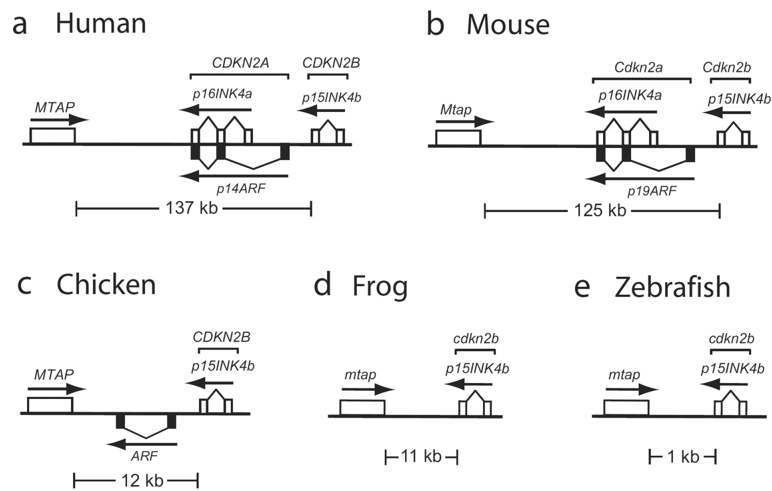
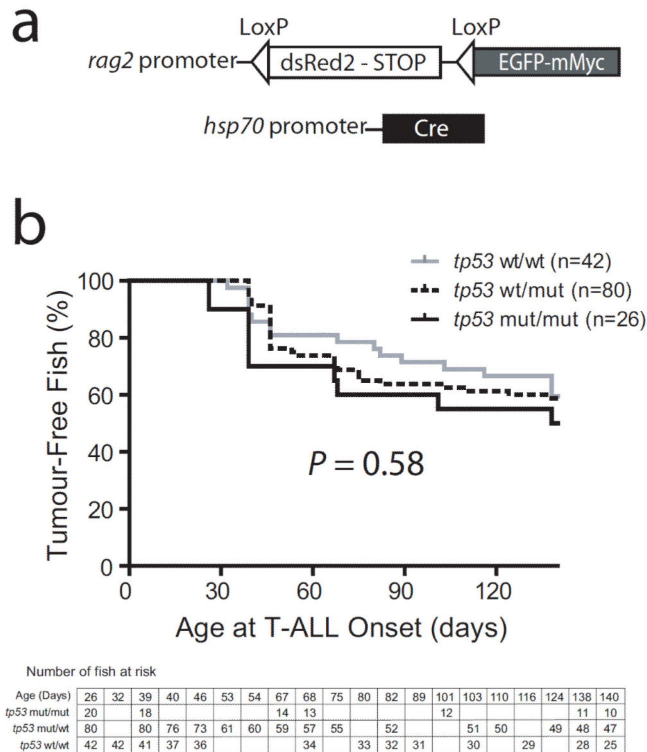


Fig 1.

Evolution of the INK4a/ARF/INK4b locus. (A–B) The human and mouse *CDKN2A* loci, which encode INK4a and ARF, are shown together with the flanking loci *CDKN2B* (encoding INK4b) and *MTAP*. (C) The syntenic locus in chickens (Gallus gallus), where the ARF locus does not encode a corresponding INK4a paralog, but is syntenic to mammalian *CDKN2A*, between *CDKN2B* and *MTAP*. (D–E) Frogs and zebrafish genomes lack recognized ARF orthologs, and have a single cyclin-dependent kinase inhibitor gene at this locus. This CDK inhibitor is annotated as *cdkn2b*, which encodes p15INK4b, but shows similar sequence similarity to mammalian p16INK4a proteins, and probably represents the ancestral gene whose duplication gave rise to mammalian INK4a and INK4b.



Number of fish at risk

Age (Days)	26	32	39	40	46	53	54	67	68	75	80	82	89	101	103	110	116	124	138	140
tp53 mut/mut	20		18					14	13					12					11	10
tp53 mut/wt	80		80	76	73	61	60	59	57	55		52			51	50		49	48	47
tp53 wt/wt	42	42	41	37	36				34		33	32	31		30		29		28	25

Fig 2.

Mutation of *tp53* does not accelerate onset of T-ALL in the Cre-LoxP regulated conditional model. (A) Schematic depiction of the transgenes utilized in the Cre-LoxP regulated conditional model of *myc*-induced T-ALL. (B) Kaplan-Meier analysis of leukaemia-free survival in *tp53* wild-type, heterozygous or homozygous mutant siblings. All zebrafish analysed expressed both *rag2:LoxP-dsRed2-Stop-LoxP-EGFP-mMyc* and *hsp70:Cre* transgenes, and were subjected to heat shock treatment at 6 dpf. The table indicates the number of zebrafish at risk.

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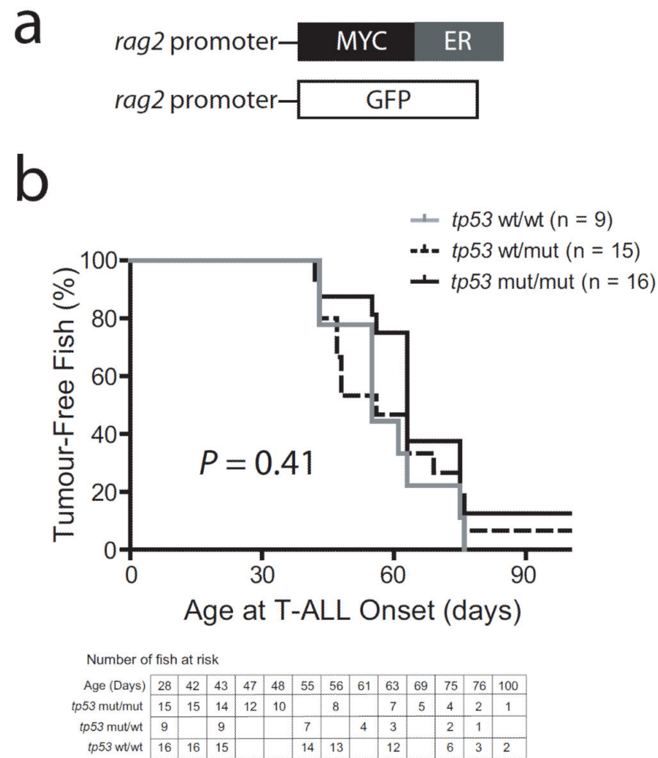

Number of fish at risk

Age (Days)	28	42	43	47	48	55	56	61	63	69	75	76	100
tp53 mut/mut	15	15	14	12	10		8		7	5	4	2	1
tp53 mut/wt	9		9			7		4	3		2	1	
tp53 wt/wt	16	16	15			14	13		12		6	3	2

Fig 3.

Mutation of *tp53* does not accelerate onset of T-ALL in the highly penetrant *rag2:MYC-ER* transgenic line. (A) Schematic depiction of the *rag2:GFP* and *rag2:MYC-ER* transgenes utilized. (B) Kaplan-Meier analysis of leukaemia-free survival in *tp53* wild-type, heterozygous, or homozygous mutant siblings that expressed both *rag2:MYC-ER* and *rag2:GFP* transgenes. All zebrafish were raised in 50 μg/l (129 nM) 4-hydroxytamoxifen, beginning at 5 dpf, to activate the *MYC-ER* transgene. Table indicates the number of zebrafish at risk.

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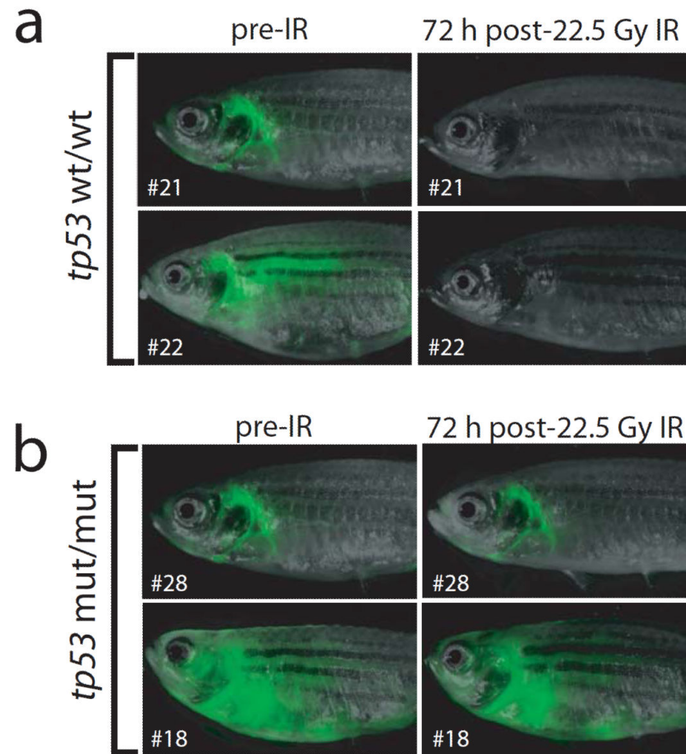

Fig 4.

The *tp53*-dependent DNA damage response is retained during thymocyte transformation. (A) Two representative *tp53*-wild type *rag2:MYC-ER; rag2:GFP* double-transgenic zebrafish, shown at baseline and 72 h post-irradiation with 22.5 gray. (B) Two representative *tp53*-homozygous mutant, *rag2:MYC-ER; rag2:GFP* double-transgenic zebrafish, shown at baseline and 72 h post-irradiation with 22.5 Gray.