Title: “Heterozygous inactivation of tsc2 enhances tumorigenesis in p53 mutant zebrafish”

Running title: tumors in tsc2/p53 mutant animals

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Summary

Tuberous Sclerosis Complex (TSC) is a multi-organ disorder caused by mutations of the TSC1 or TSC2 genes. A key function of these genes is to inhibit mTORC1 (mechanistic target of rapamycin complex 1) kinase signaling. Cells deficient for TSC1 or TSC2 have increased mTORC1 signaling and give rise to benign tumors though as a rule, true malignancies are rarely seen. In contrast, other disorders with increased mTOR signaling typically have overt malignancies. A better understanding of genetic mechanisms that govern the transformation to benign cells to malignant ones is critical to understand cancer pathogenesis. We generated a zebrafish model of TSC and cancer progression by placing a heterozygous mutation of the tsc2 gene in a p53 mutant background. Unlike tsc2 heterozygous mutant zebrafish that never exhibited cancers, compound tsc2;p53 mutants had malignant tumors in multiple organs. Tumorigenesis was enhanced compared to p53 mutant zebrafish. p53 mutants also had increased mTORC1 signaling that was further enhanced in tsc2;p53 compound mutants. We found increased expression of Hif1-a, Hif2-a and Vegf-c in tsc2;p53 compound mutant zebrafish. Expression of these proteins likely underlies the increased angiogenesis seen in compound mutant zebrafish compared to p53 mutants and may further drive cancer progression. Treatment of p53 and compound mutant zebrafish with the mTORC1 inhibitor rapamycin caused rapid shrinkage of tumor size and decreased caliber of tumor associated blood vessels.

This is the first report using an animal model to show interactions of tsc2/mTORC1 and p53 during tumorigenesis. These results may explain why patients with TSC rarely have malignant tumors but also suggest that cancer arising in patients without TSC may be influenced by the status of TSC1/TSC2 mutations and be potentially treatable with mTORC1 inhibitors.

Keywords: mTOR, mTORC1, mTORC2, rapamycin, tuberous sclerosis complex
INTRODUCTION

Tuberous sclerosis complex (TSC) is a genetic disorder caused by loss of function of the \textit{TSC1} or \textit{TSC2} genes. Patients with this disorder have multi-organ hamartomas resulting from increased proliferation and abnormal differentiation. While not malignant, these tumors can still cause severe clinical manifestations, particularly in the brain, kidney and lungs (Crino et al., 2006). The protein products of \textit{TSC1} (hamartin) and \textit{TSC2} (tuberin) genes bind to each other and function together to modulate downstream signaling pathways. Rapid advances in our knowledge of TSC were catalyzed by the discovery that hamartin/tuberin normally inhibit the mechanistic target of rapamycin (mTOR, previously known as mammalian target of rapamycin) serine/threonine kinase (Tee et al., 2002). mTOR is found within two functionally and molecularly distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Further intricacies are apparent as loss of \textit{TSC1} or \textit{TSC2} gene function appears to allow broadly dysregulated mTOR activity with constitutively increased mTORC1 activity but also decreased mTORC2 signaling (Inoki et al., 2003; Jacinto et al., 2004; Manning et al., 2005; Sarbassov et al., 2005). Through a very rapid translation of basic science findings, therapies with mTORC1 inhibitors have been developed as effective treatments for some of the clinical manifestations of TSC (Davies et al., 2008; Franz et al., 2006).

It is striking that patients with TSC almost as a rule develop benign tumors instead of malignancies. In contrast, patients with \textit{PTEN} mutations also have constitutive activation of mTORC1 signaling, but develop frank malignancies including aggressive gliomas (Hu et al., 2005). Several models have been proposed to account for the relative lack of malignancy including feedback inhibition of AKT by mTORC1 activation, altered mTORC2 function as well as increased rates of apoptotic cell death in \textit{TSC1/2}-deficient cells. Increased cell death may then balance excessive proliferation and thus prevent the emergence of transformed cells that may ultimately form malignancies. In support of this model, \textit{TSC1/2}-deficient cells \textit{in vitro} are prone to apoptosis, particularly when under metabolic stress (Choo et al., 2010).
**TP53** (*p53*) is one of the most frequently mutated genes in human cancer (Finlay et al., 1989; Levine, 1997; Vogelstein et al., 2000). As a tumor suppressor, **p53** protein regulates growth arrest and apoptosis (Vousden and Prives, 2009) with many identified transcriptional targets (Riley et al., 2008; Veprintsev and Fersht, 2008). Approximately 50% of all human cancers have a mutation in **p53** that alters its transcriptional activity (Edlund et al., 2012) (http://p53.fr/). However, many questions remain regarding downstream molecular pathways involved in tumor cell growth. Recent studies have suggested an emerging role for **p53** in cellular metabolism (Maddocks and Vousden, 2011) during stress conditions including nutrient deprivation and hypoxia. This suggests a functional interaction with the mTOR signaling pathway. In addition, approximately 40% to 90% of human malignancies have increased mTORC1 signaling (Menon and Manning, 2008). **p53** is also known to regulate transcription of the **PTEN** gene, an upstream inhibitor of Akt signaling as well as tuberin, an upstream inhibitor of mTORC1 signaling (Feng et al., 2007; Stambolic et al., 2001). Furthermore, mTORC1 regulates the translation of **p53 in vitro** (Lee et al., 2007). These findings suggest mechanistic links between mTORC1 and **p53** that may be important for TSC pathogenesis, and possibly explain the paucity of cancer in patients with TSC.

Zebrafish have emerged as a powerful model for the study of genetic diseases as well as cancer (Feitsma and Cuppen, 2008; Goessling et al., 2007; Mione and Trede, 2010). This is due to ease of genetic manipulation in zebrafish, aneuploid nature of cancers arising in zebrafish and their capacity for high-throughput genetic and pharmacologic screens (Zhang et al., 2010; Zon and Peterson, 2005). We previously showed that **tsc2** homozygous mutant (**tsc2**\textsuperscript{nu242/nu242}) zebrafish recapitulate several key aspects of TSC including control of cell size, hamartoma formation in the brain and increased mTORC1 signaling (Kim et al., 2011). **p53** mutant zebrafish models showed a conserved role for **p53** in tumorigenesis (Berghmans et al., 2005; Parant et al., 2010) with the **p53**\textsuperscript{cdfl} allele functionally similar to DNA binding domain mutations identified in cancers from many patients.

To define interactions of the **p53** and mTOR signaling pathways, we first examined
mTORC1 signaling in tumors arising from $p53^{zdf1/zdf1}$ homozygous mutant zebrafish and found increased levels compared to normal tissues from wild type fish. We then explored a potential role for mTORC1 signaling in $p53$ mutation-mediated tumor formation by asking whether further increases of mTORC1 activity enhances tumorigenesis. We used $tsc2^{vu242/+}\, p53^{zdf1/zdf1}$ compound mutant ($tsc2\, p53$ compound mutant) zebrafish for this purpose. These zebrafish had altered mTORC1 and mTORC2 signaling and enhanced rates of tumorigenesis compared to that seen with $p53$ mutations alone. We found increased expression of Hif1-α, Hif2-α and Vgfc that was associated with prominent angiogenesis in tumors from $tsc2\, p53$ compound mutant compared to $p53$ mutant zebrafish. Transient administration of rapamycin, a potent mTORC1 inhibitor, rapidly diminished the tumor-associated blood vessels. These results provide the first in vivo evidence of a critical role of p53 to prevent the formation of malignancies in TSC.

RESULTS

Activation of TORC1 signaling in $p53$ mutant tumors

To examine the contribution of mTOR activity in tumors arising from $p53$ mutant zebrafish, we measured mTORC1 signaling activity in abdominal tumors spontaneously arising from homozygous $p53$ mutant zebrafish (Fig. 1a). Histological analyses of these $p53$ mutant abdominal tumors revealed spindle-like cells reminiscent of malignant sarcomas. These cells were highly invasive, destroying skeletal muscle fibers (Fig. 1b and 2a). Similar pathologic features were previously described in this zebrafish model (Berghmans, Murphey et al. 2005). Using immunofluorescence, we next determined expression of phosphorylated ribosomal protein S6 (phospho-S6) and phosphorylated eukaryotic initiation factor 4E-binding protein 1 (phospho-4E-BP1), well established downstream effectors of the mTOR kinase within mTORC1 (Inoki et al., 2002). These abdominal tumors had high expression of both phospho-S6 and phospho-4E-BP1 (Fig. 1c,d; Supplementary Fig. 1, upper panel) in a subset of tumor cells. Immunoblot analyses also revealed a moderate increase of mTORC1 signaling in normal appearing eyes from $p53^{zdf1/zdf1}$
zebrafish compared to wild type control eyes (data not shown). These results suggest that mTORC1 activation in p53 mutant tissues may facilitate tumor formation and/or growth.

**Heterozygous tsc2<sup>va242</sup> mutation enhanced mTORC1 signaling activity and tumor incidence in p53<sup>df1/df1</sup> zebrafish**

Given the increased mTORC1 signaling in p53 mutant zebrafish tumors, we hypothesized that further augmentation of mTORC1 signaling might enhance tumor formation, cell growth or tumor severity. To test this hypothesis, we introduced a heterozygous tsc2<sup>va242</sup> mutation into p53<sup>df1/df1</sup> mutant zebrafish. We previously reported that heterozygous tsc2<sup>va242</sup> mutant zebrafish are viable and have moderately elevated mTORC1 signaling activity (Kim et al., 2011). Of note, tumors were never seen in heterozygous tsc2<sup>va242/+</sup> zebrafish including zebrafish 2 years of age or older (n>200). In contrast, adult compound mutant tsc2;<i>p53</i> compound mutant zebrafish developed large sarcomatous tumors in the abdomen (Fig. 2a) as well as other regions (Fig. 2c). Immunostaining for phospho-S6 levels confirmed prominent mTORC1 activation within tumors from compound mutant zebrafish, and these levels were further augmented when compared to tumors from p53<sup>df1/df1</sup> zebrafish (Fig. 2a and Supplementary Fig. 1). As previously reported, these malignancies have a long latency with 50% of p53<sup>df1/df1</sup> zebrafish exhibiting tumors by 550 days of life (n=115 total p53<sup>df1/df1</sup> zebrafish, Fig. 2b). In contrast, 50% of tsc2;<i>p53</i> compound mutant zebrafish had tumors by 450 days of life (n=130 total tsc2;<i>p53</i> compound mutant zebrafish, Fig. 2b, compared to p53<sup>df1/df1</sup> zebrafish, Log-rank test, p<0.0001). Types of tumors were broadly similar in p53<sup>df1/df1</sup> and tsc2;<i>p53</i> compound mutant zebrafish with the exception of melanoma-like tumors. These were seen in approximately 7% of tsc2;<i>p53</i> compound mutant zebrafish (Fig. 2c), but never seen in p53<sup>df1/df1</sup> homozygous mutant zebrafish. Melanoma-like tumors were not reported in the original report of the p53<sup>df1/df1</sup> phenotype (Berghmans, Murphey et al. 2005). However, melanoma-like skin tumors were frequently observed in a different zebrafish model featuring a more deleterious <i>p53</i> mutation (Parant et al., 2010).
mTORC1 and mTORC2 signaling in tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish

We next quantitated mTOR signaling levels using protein extracts from a consecutive series of abdominal tumors arising in p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish. In support of our immunostaining data presented in Figures 1 and 2, we again found increased phospho-S6 levels in a series of tumors arising from tsc2;p53 compound mutant zebrafish compared to those from p53<sup>zdf1/zdf1</sup> zebrafish (Fig. 3a, b). When normalized for total S6, the ratio of phospho-S6 to total S6 was significantly higher in abdominal tumors arising from tsc2;p53 compound mutant zebrafish compared to p53<sup>zdf1/zdf1</sup> zebrafish (Fig. 3d). We also looked at other tumor types and again found increased mTORC1 signaling in tsc2;p53 compound mutant zebrafish compared to tumors from p53<sup>zdf1/zdf1</sup> zebrafish (data not shown). Finally, we quantified mTORC2 signaling in abdominal tumors extracts by immunoblotting for Akt (Serine 473) levels and found no significant differences between tsc2;p53 compound mutant and p53<sup>zdf1/zdf1</sup> zebrafish (Supplementary Fig. 2).

Elevated AKT kinase activity in tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish

p53 activates transcription of the PTEN gene and increased levels of PTEN protein in vitro can suppress AKT activation. This can relieve the inhibitory phosphorylation of tuberin at Ser939 and Thr1462 by AKT kinases (Manning et al., 2002) (Feng et al., 2007). Though these signaling cascades have been described, genetic interactions of AKT and mTOR signaling have not been well characterized in vivo. To further address AKT activity in tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish, we examined phospho-tuberin (Ser939) using immunofluorescence. We found strongly positive cells within both tumor types though there was a clear elevation in abdominal tumors from tsc2;p53 compound mutant zebrafish (Fig. 4 a-f and Supplementary Fig. 3). We attempted to use this phospho-antibody for immunoblotting but did not obtain interpretable results. To further address AKT activity in these tumors, we also performed immunofluorescence...
using antibodies against total AKT, phospho-AKT (Thr308) and phospho-AKT (Ser473) (Fig. 4 g-l and Supplementary Fig. 4 and 5). Interestingly, we found the total Akt level was diffusely increased in tumor cells compared to adjacent muscle tissues from both p53<sup>zdf1/zdf1</sup> and tsc2;<i>p53</i> compound mutant zebrafish (Fig. 4g and j, n=8/8 and Supplementary Fig. 4). Phospho-Akt (Thr308) levels were comparable though the signal from <i>tsc2;<i>p53</i></i> compound mutant tumors were increased compared to <i>p53<sup>zdf1/zdf1</sup></i> tumors (Fig. 4h and 4k, Supplementary Fig. 5). In contrast, phospho-Akt (Ser473) levels appeared to be somewhat decreased in tumor cells (Fig. 4i and l) compared to adjacent muscle. As Serine 473 of Akt is phosphorylated by mTORC2, this reduction may be due to negative feedback by activated mTORC1 (Harrington et al., 2004; Shah et al., 2004).

**Vegf up-regulation in tumors from p53<sup>zdf1/zdf1</sup> and tsc2;<i>p53</i> compound mutant zebrafish**

As we assessed tumors arising in <i>p53<sup>zdf1/zdf1</sup></i> and <i>tsc2;<i>p53</i></i> compound mutant zebrafish, we frequently noted prominent and abnormal appearing blood vessels in the abdomen and eyes from both mutant genotypes (n=25/41 with tumors, Fig. 5a-d). This was consistently enhanced in tumors from <i>tsc2;<i>p53</i></i> compound mutant as compared to <i>p53<sup>zdf1/zdf1</sup></i> zebrafish. Approximately 68% of <i>tsc2;<i>p53</i></i> compound mutant zebrafish with abdomen and eye tumors developed either thin blood vessels (n=10/25) but also massively dilated blood vessels (n=7/25, Fig. 5c and d). By comparison, about 50% of <i>p53<sup>zdf1/zdf1</sup></i> mutant with abdomen and eye tumors exhibited thin (n=5/16, Fig. 5a and b) or dilated blood vessels but not much dilated compared to blood vessels observed in <i>tsc2;<i>p53</i></i> mutants (n=3/16) (comparison not statistically significant with p=0.33 by two-tailed Fisher’s exact test). These findings suggest that angiogenesis is enhanced in tumors arising from <i>tsc2;<i>p53</i></i> compound mutant zebrafish as compared to those arising in <i>p53<sup>zdf1/zdf1</sup></i> zebrafish. To see if there was altered expression of angiogenic factors, we performed immunostaining for Hif1-α, Hif2-α and Vegf-c. All of these angiogenic factors were expressed in <i>p53<sup>zdf1/zdf1</sup></i> tumors with more intensive staining again seen in <i>tsc2;<i>p53</i></i> compound mutant zebrafish (Fig. 5e-k, Supplementary Fig. 6 and 7). These results suggest that further increases in mTORC1 signaling from <i>tsc2</i> heterozygote zebrafish...
in a p53 mutant background possibly increases angiogenesis and vasodilation via up-regulation of Hif1-α, Hif2-α and Vegf-c expression.

**Rapamycin treatment suppresses Vegf expression in tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish**

Given the increased expression of angiogenesis factors in these tumors, we next determined whether inhibition of mTORC1 signaling could suppress blood vessel formation and tumor size. Application of rapamycin daily appeared to shrink the caliber of existing blood vessels (example of an eye tumor in Fig. 6a, b). A representative vessel (Fig. 6b, black arrow heads) seemed to completely disappear at the end of both rapamycin treatment cycles (day 3 and day 14) but became visible again during the rapamycin off period (day 6 and day 9) in the same location. This result supports a vasoconstrictive effect of rapamycin, likely through inhibition of Vegf-induced vasodilation (Kim et al., 2010). During a rapamycin 6 day “off period” in tsc2;p53 compound mutant zebrafish, re-dilated vessels appeared (Fig. 6b, black arrow head) as well as possible neo-angiogenesis where vessels were not previously seen (example Fig. 6b, blue arrow head at day 6). These results suggest that activation of mTORC1 signaling is essential for neo-angiogenesis and dilation of blood vessels in tumors arising in a p53<sup>zdf1/zdf1</sup> background. To verify the effect of rapamycin treatment, we performed immunostaining with antibodies against phospho-S6, phospho-4E-BP1, Akt and Vegf-c in abdominal tumors treated with vehicle (DMSO) or rapamycin daily for two days (Fig. 6c). Rapamycin treatment decreased mTORC1 signaling as evidenced by lower levels of phospho-S6, phospho-4E-BP1 in rapamycin treated tumors (Fig. 6c). Furthermore, we found that total Akt expression levels were also downregulated by rapamycin although this suppression was mainly observed in the inner region of tumors.

**mTORC1 inhibitors decrease tumor size**

Given the rapid effect of rapamycin on blood vessel caliber and regression, we asked if continued
treatment could reduce the size of tumors. We treated several types of rapidly growing tumors and found either stabilization of tumor size or a reduction in size during daily rapamycin treatments (Fig. 6 and Supplementary Fig. 7). This effect was seen in tumors from \( p53^{cdo1/cdo1} \) zebrafish as well as those from \( tsc2;p53 \) compound mutants. In all cases, there was rapid resumption of tumor growth after rapamycin was stopped.

**DISCUSSION**

This study was designed to investigate why patients with TSC almost never develop cancer despite high levels of mTORC1 signaling in multiple organs. This is in contrast to the malignancies typically seen in patients with increased mTORC1 signaling due to \( PTEN \) mutations (Hu et al., 2005; Inoki et al., 2005). Various models have been proposed to explain this striking discrepancy including feedback inhibition of AKT through mTORC1 and direct or indirect reductions of mTORC2 activity (Manning et al., 2005). Alternatively, decreased cell proliferation and enhanced cell death in \( TSC1 \) or \( TSC2 \) deficient cells may compensate for any excessive proliferation induced by increased mTORC1 activity. This model is also supported by complications reported during the generation of mouse embryonic fibroblasts (MEFs) from \( Tsc2 \) knock-out mice (Zhang et al., 2003). While initially derived \( Tsc2 \) mutant MEFs grew as expected, they rapidly underwent senescence and died. However, they circumvented this problem by crossing the \( Tsc2 \) knock-out allele into \( p53 \) deficient mice. The resulting \( Tsc2/p53 \) mutant embryos were then used to generate MEFs that were capable of continuous growth. Our *in vivo* results using a zebrafish animal model of TSC further support that senescence in \( Tsc2 \) mutant cells may be mediated by \( p53 \). The loss of both \( tsc2 \) and \( p53 \) gene function may then permit uncontrolled proliferation leading to malignancies.

There are multiple types of benign tumors in TSC including brain subependymal giant cell astrocytomas (SEGAs), angiomyolipomas in the kidney and rhabdomyomas in the heart. One notable exception is renal cell carcinoma (RCC). While RCC is rarely seen in patients with TSC, it has been reported in relatively young patients (Lendvay et al., 2002). This is potentially quite
relevant to our results as RCC usually harbor \( p53 \) mutations (Gad et al., 2007; Habib et al., 2011; Noon et al., 2010). This further suggests that in TSC, an additional mutation of \( p53 \) may allow progression to malignancy. Direct support for this model will require analyses of \( p53 \) status in RCC obtained from patients with TSC. Additional deep sequencing of TSC associated hamartomas for any \( p53 \) mutations would also support or refute this model.

Our finding of enhanced tumor formation in \( tsc2;p53 \) compound mutant zebrafish is supported by previous reports of substantial cross talk between \( p53 \), AKT and mTOR signaling during tumorigenesis. For example, \( p53 \) activates transcription of \( PTEN \), a phosphoinositide phosphatase that normally inhibits AKT signaling (Feng et al., 2007). In addition, AKT/mTORC1 is frequently activated in ovarian (Altomare et al., 2004) and lung cancers (Memmott and Dennis, 2010) that usually have \( p53 \) mutations. Furthermore, \( p53 \) \textit{in vitro} can inhibit mTOR signaling via the hamartin/tuberin complex (Feng et al., 2005). A recent study also indicated that signaling components downstream of mTOR can modulate \( p53 \) through the signaling of ribosomal S6 kinase to MDM2, an ubiquitin ligase that controls \( p53 \) stability (Lai et al., 2010). Thus, the \( p53 \) signaling pathway incorporates AKT and mTORC1 (Levine et al., 2006). However, interactions between \( p53 \) and mTOR pathway components during tumor formation have not been previously well studied \textit{in vivo}.

Differential activation of Hif1-\( \alpha \) and Hif2-\( \alpha \) by mTORC1 and mTORC2 has been reported using \textit{in vitro} assays (Toschi et al., 2008). The expression of Hif1-\( \alpha \) and Hif2-\( \alpha \) was dependent on Akt3 and Akt2, respectively. The effect of rapamycin treatment to blood vessel maintenance is also indicative of a mTORC1 dependent regulation of proangiogenic factors. However, the rapid return of blood vessels when rapamycin treatment was stopped supports a vasoconstrictive mechanism that has been previously suggested (Kim et al., 2010). Vegf-c expression, one of the central regulators of blood vessel formation, was also suppressed by rapamycin treatment, this affect was also most apparent in the inner part of tumor (Fig. 6c). Altogether, these results suggest that mTORC1 activation plays an important role in tumor angiogenesis in \( p53 \) mutant tissues. In support
of this model, we also found increased blood vessel size in the livers of tsc2 homozygous mutants compared to control zebrafish larvae Tg(Fli1:EGFP) zebrafish (Supplementary Fig. 8). An additional feedback pathway resulting from tuberin deficiency is increased PTEN via a mTORC1-dependent mechanism (Bonneau and Longy, 2000; Mahimainathan et al., 2009; Mehenni et al., 2005). We are currently testing this potential mechanism by placing a tsc2 heterozygous allele in a pten mutant background and again analyzing resultant zebrafish for the development of malignant tumors. AMPK may also be involved as this kinase normally interacts with p53 and tuberin to modulate cell proliferation. We found that activated phospho-AMPK (Thr172) was increased in tumor cells from both p53<sup>zdf1/zdf1</sup> and tsc2; p53 compound mutant zebrafish (Supplementary Figure 9). This may be due to a feedback loop uncovered in mutant zebrafish as activated AMPK normally phosphorylates tuberin to activate it and inhibit mTORC1 signaling.

Of note, we have never seen sarcomas arising in tsc2 heterozygous zebrafish. This is broadly consistent to what is observed in human patients with TSC as well as rodent models of this disorder. tsc2; p53 compound mutant zebrafish have an significantly decreased time to tumor detection compared to p53<sup>zdf1/zdf1</sup> zebrafish. However, it is possible that tumors arising in tsc2; p53 compound mutant zebrafish are simply faster growing leading to their earlier detection. This is possible though even within the oldest groups of zebrafish (>2 years old), tsc2; p53 compound mutant zebrafish always exhibited more tumors than p53<sup>zdf1/zdf1</sup> zebrafish.

We conclude that additional genetic changes such as loss of TP53 are likely required for the progression to malignancy in TSC. Our results may also have implications for non-TSC patients if additional tumor-specific heterozygous TSC1 or TSC2 mutations in the setting of existing or subsequent TP53 mutations can potentially lead to more severe forms of cancer. The expected increased mTORC1 signaling in this scenario suggests that mTORC1 inhibitors may be effective therapies for such cancers. Further application of full exome and genome sequencing will likely uncover additional evidence of genetic interactions between TP53 and TSC1/TSC2 in a variety of human cancers.
MATERIALS AND METHODS

Zebrafish strains

Zebrafish strains used in this study included tsc2<sup>v242/+</sup> (Kim et al., 2011) and p53<sup>cdfl/cdfl</sup> (Berghmans et al., 2005). Five pairs of tsc2;p53 compound mutant zebrafish (tsc2<sup>v242/+</sup>p53<sup>cdfl/cdfl</sup>) and p53<sup>cdfl/cdfl</sup> adult fish were mated and embryos raised at 28.5°C. Five month old zebrafish siblings were genotyped and p53<sup>cdfl/cdfl</sup> and tsc2;p53 compound mutant zebrafish identified and raised for up to 20 months in separate tanks. For genotyping of tsc2<sup>v242/+</sup>, we amplified a 151 bp fragment by PCR using forward primer 5’-CCAGCACCACCTGCAGTCTGG-3’ and reverse primer 5’-CTCTTGGGCAGAGCAGAGAAGTTGG-3’ flanking the mutation site. The point mutation was confirmed by absence of a HpyCh4IV restriction site. Tg(Fli1:EGFP) zebrafish (Lawson and Weinstein, 2002) were obtained from ZIRC. All experiments were approved by the Vanderbilt IACUC.

Tumor analyses

For 550 days, adult fish (total of 110 p53<sup>cdfl/cdfl</sup> and 130 tsc2;p53 compound mutant zebrafish) were monitored twice per week for tumor development or death. Fish noted to have a prominent tumor were euthanized in 20% MESAB and dissected into 4 pieces (head, anterior abdomen, posterior abdomen and tail). Pieces containing tumor were fixed in 4% paraformaldehyde for two days at 4°C. Fixed bodies were embedded in 1.2% agarose/5% sucrose and saturated in 30% sucrose. Tissue blocks were frozen in 2-methyl butane. 10 um sections were collected on microscope slides using a Leica cryostat. Sections were then kept at -80°C prior to use.

Immunofluorescence

To minimize variation of staining intensity while staining process, 10 tumor sections of each p53<sup>cdfl/cdfl</sup> and tsc2;p53 compound mutant zebrafish were concurrently processed in a Sequenza™
Slide Rack (Thermo Scientific), results are shown in Supplementary figures 1-3. Sections were rehydrated in 1x PBS for 20 min at room temperature and blocked in 5% sheep serum/1xPBS for 2 hours. Antibodies: phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling #2215), phospho-4E-BP1 (Thr37/46) (Cell Signaling #2855), phospho-tuberin (Ser939) (Cell Signaling #3615), Akt (Cell Signaling #9272), phospho-Akt (Thr308) (Cell signaling #2965), phospho-Akt (Ser473) (Cell Signaling #9271), phospho-AMPK (Thr172) (Cell Signaling #2535), Hif1a (abcam ab8366), Hif2a (Thermo Scientific FA1-16510) and Vegf-c (Ana Spec 55877) were diluted 1:300 and added to sections overnight at 4°C. Cy3 labeled goat anti-rabbit secondary antibody (Jackson ImmunoResearch) was incubated overnight at 4°C. Sections were then washed with 1X PBS for 1 hour and mounted in Vectashield with DAPI (Vector Laboratories). Images were acquired using Zeiss Axio Imager Z1 and Zeiss AxioCam MRm digital camera. Digital images were then processed using Adobe Photoshop CS5. Images received only minor modifications of contrast with both control and mutant sections always processed in parallel.

Rapamycin treatment

After anesthesia of zebrafish with MESAB, 10 uL of rapamycin in DMSO (10 mg/ml, LC Laboratories) was added to a gill for ten seconds. Fish were then placed back in water where they rapidly recovered. To monitor angiogenesis in zebrafish with tumors, we treated once a day for one to two weeks. For zebrafish with abdominal tumors (Fig. 6), rapamycin treatment was performed for two days total.

Immunoblots

Tumors and control tissues (mixture of organs derived from brain, kidney, intestine and liver of wild type zebrafish) were lysed in RIPA cell lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0,1% SDS, 1% Triton X-100, 1% Sodium deoxycholate with protease and phosphatase inhibitors added), using a motorized pestle grinder to aid in cell disruption. Protein concentrations
were determined using a BCA protein assay, loading buffer was added to each sample and heated at 80°C for 10 minutes prior to loading on a gel and electrophoresed using the Invitrogen X-Cell II™ system. Gels were transferred to PVDF membranes, blocked in Odyssey buffer (Li-COR #927-40000) for one hour and incubated with primary antibodies overnight. Primary antibodies: phospho-S6 ribosomal protein (Ser240/244; Cell Signaling #2215, 1:1000), S6 ribosomal protein (Cell Signaling #2217; 1:1000) phospho-GSK-3α/β (Ser21/9; Cell Signaling #9331; 1:1000) and β-actin as a loading control (Sigma-Aldrich #A5441; 1:2000). Secondary antibodies include IRDye® 800CW Goat anti-Mouse IgG (Li-COR #926-32210; 1:10,000) and IRDye® 680RD Goat anti-Rabbit IgG (Li-COR #926-68071; 1:10,000). Fluorescent-tagged secondary antibodies were visualized with an Odyssey fluorescence scanner and digitized band densities quantitated using ImageJ (version 1.46).

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COMPETING INTERESTS STATEMENT
The authors declare no financial or competing interests.

AUTHOR CONTRIBUTIONS
S.-H.K. developed the concept, performed experiments, wrote and edited the manuscript, M.K. and L. R. B. performed experiments, R.C. performed experiments and edited the manuscript and K.C.E. developed the concept, wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary Figures 1-9.

TRANSLATIONAL IMPACT

Clinical issue

Tuberous Sclerosis Complex (TSC) is a multi-organ disease that features multi-organ hamartomas. While there are rare exceptions, these lesions are non-cancerous. This is despite enhanced mTORC1 signaling that occurs from loss of function mutations of the TSC1 or TSC2 genes. This seemingly paradox can be explained by several potential mechanisms including negative feedback inhibition by mTORC1 signaling and enhanced apoptosis that may limit excessive proliferation and progression to malignancy.

Results

No cancers were observed in a zebrafish model of TSC that features a heterozygous loss of function mutation of the tsc2 gene. However, after placing this mutation in a p53 mutant background, multi-organ malignancies were seen. These cancers were also seen earlier and in more tissue types as compared to that seen with zebrafish mutant for p53 alone. Alterations of both mTORC1 and mTORC2 signaling was seen as well as increased levels of angiogenic factors and angiogenesis in tumors from tsc2/p53 compound mutant zebrafish. Treatment of tsc2/p53 compound mutants with rapamycin, a mTORC1 inhibitor, decreased tumor size as well as the caliber of tumor associated blood vessels.

Implications and future directions

We provide strong evidence using an animal model that the lack of progression to cancer in patients with TSC is a p53 dependent process that is associated with increased mTORC1 signaling. These findings have clear implications for the progression of cancer and the interactions of tuberin/mTORC1 signaling with p53. Inhibition of mTORC1 signaling may also be a viable therapeutic option for p53 deficient tumors in non-TSC as well as TSC patients.
REFERENCES


Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C. and Blenis, J.


FIGURE LEGENDS

Fig. 1. mTORC1 signaling in malignant abdominal tumors arising from p53<sup>zdf1/zdf1</sup> zebrafish. (A) 1 year old p53<sup>zdf1/zdf1</sup> mutant fish with prominent abdominal tumor. (B) Hematoxylin and eosin staining of sections from abdominal tumor showing whorls of spindle shaped cells consistent with an invasive sarcoma. Increased expression of mTORC1 downstream effectors phospho-S6 (C) and phospho-4EBP1 (D) staining in abdominal tumor from p53<sup>zdf1/zdf1</sup> zebrafish. For C and D panels, red fluorescence is immunosignal and blue is DAPI nuclear stain. M, muscle; T, tumor. Scale bar = 100 μm. Similar findings were seen in abdominal tumors from 10/10 p53<sup>zdf1/zdf1</sup> zebrafish.

Fig. 2. Increased mTORC1 signaling, enhanced tumorigenesis and altered tumor spectrum from the addition of a tsc2 heterozygous allele to a p53<sup>zdf1/zdf1</sup> background. (A) Hematoxylin and eosin staining, phospho-S6 and DAPI (nuclei) staining of abdominal tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish. Left panel: invasive region of p53<sup>zdf1/zdf1</sup> tumor, right panel: tsc2;p53 compound mutant tumor. Circular structures are skeletal muscle bundles encircled by invasive tumor cells (pink in the H & E staining, but dark areas with immunofluorescence images). Phospho-S6 signal is red and DAPI is white. Scale bar = 100 μm. (B) Kaplan-Meier tumor-free survival. Blue line is p53<sup>zdf1/zdf1</sup> zebrafish, red line is tsc2;p53 compound mutant zebrafish. 50% of p53<sup>zdf1/zdf1</sup> zebrafish had tumors at approximately 550 days of life compared to 450 days for tsc2;p53 compound mutant zebrafish. n=115 and 130 respectively, this reduction in latency to tumor detection was statistically significant using Log Rank test, p<0.0001. (C) Spectrum of tumor types from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish. Tumor types were broadly similar with the exception of melanoma-like tumors seen only in tsc2;p53 compound mutant zebrafish. (D) Normal zebrafish head and dorsal fin on the top panels and melanoma-like tumors on the head and dorsal fin on the bottom panels. Dorsal fins were sectioned at region marked “1” (normal) and “2” (tumor) and stained with DAPI in D’ and D”.
Fig. 3. Increased mTORC1 signaling in tumors arising from \textit{tsc2};\textit{p53} compound mutant \textit{zebrafish} compared to \textit{p53}^{df1/df1} \textit{zebrafish}. (A) Immunoblotting of a series of abdominal tumors comparing \textit{p53}^{df1/df1} mutant zebrafish (lanes marked Tsc2^{+/+}) and \textit{tsc2};\textit{p53} compound mutants (lanes marked Tsc2^{+/-}). (B) Quantification of phospho-S6 levels were normalized to actin expression, p=0.25. (C) Quantification of total S6 levels were normalized to actin expression, p=0.06. (D) Increased ratio of normalized phospho-S6 to total S6 levels in \textit{tsc2};\textit{p53} compound mutant compared to \textit{p53}^{df1/df1} zebrafish, asterisk denotes significance by two-tailed student’s \textit{t}-test, p=0.03. n=5 for \textit{p53} mutant and n=7 for compound mutant zebrafish though two tumor samples from \textit{tsc2};\textit{p53} compound mutant zebrafish (lanes 4, 5 and 11, 12) were divided in half and processed independently for immunoblotting.

Fig. 4. AKT activity in \textit{p53}^{df1/df1} and \textit{tsc2};\textit{p53} compound mutant tumors. (A-F) Phospho-tuberin (p-Tsc2) staining in \textit{p53}^{df1/df1} and \textit{tsc2};\textit{p53} compound mutant zebrafish. (B, C) and (E, F) higher magnification of a and d, respectively. (C, F) phospho-tuberin (Ser939, red) and (B, E) phospho-tuberin (red) merged with DAPI (blue). (G, J) Total Akt immunofluorescence, (H, K) Phospho-Akt (Thr308) immunofluorescence and (I, L) phospho-Akt (Ser473) immunofluorescence in \textit{p53}^{df1/df1} (g-i) and \textit{tsc2};\textit{p53} compound mutant zebrafish (J-L). Scale bar = 100 μm.

Fig. 5. Angiogenesis and increased expression of angiogenic factors in tumors from \textit{tsc2};\textit{p53} compound mutant \textit{zebrafish}. (A, B) Arrows indicate visible blood vessels on tumors from abdomen (A) and eye tumors (B) from \textit{p53}^{df1/df1} zebrafish (n=8/16). (C, D) Arrows indicate large and tortuous blood vessels observed in abdomen (C) and eye tumor (D) from \textit{tsc2};\textit{p53} compound mutant (n=17/25) zebrafish. Scale bar = 1 mm. (E, H) Hif1a staining, (F, J) Hif2a staining and (G, K) Vegf-c staining in \textit{p53}^{df1/df1} (E-G) and \textit{tsc2};\textit{p53} compound mutant zebrafish (H-K). Scale bar = 100 μm.
Fig. 6. Rapamycin treatment reduced blood vessel caliber in tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish. (A, B) Representative tumors from rapamycin treated fish. (A) Rapamycin treatment of p53<sup>zdf1/zdf1</sup> zebrafish harboring a tumor in its trunk. Arrowheads indicate visible vascular structure on the tumor surface. (B) Rapamycin treatment cycle (on day 0 to 3, 9 to 14, off day 4 to 8) lasting two weeks on compound mutant zebrafish with an expending eye tumor. Pictures were taken at indicated days. Black arrowheads indicate vascular structures that disappeared during rapamycin treatment. Blue arrowhead indicates apparent new vessels formed during the rapamycin-off period in the eye tumor from a tsc2;p53 compound mutant zebrafish. Scale bar = 1 mm. (C) H & E staining, phospho-S6, phospho-4EBP1, Akt and Vegf-c expression (antibody signal shown as white) in p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant abdomen tumors treated with DMSO or rapamycin for two days, (n=3). Scale bar = 100 µm. (D) Rapamycin shrinks tumors in p53<sup>zdf1/zdf1</sup> zebrafish. A representative angioma-like tumor was seen near the pectoral fin and treated daily with rapamycin. After three days of treatment, a clear decrease in size was noted. The tumor continued to shrink over the one week total period of rapamycin treatment. Scale bar = 1 mm.
Figure 1
Figure 3
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. 1. Phospho-S6 staining (red) in a series of abdominal tumors from p53<sup>zdf/zdf</sup> and tsc2;<sup>p53</sup> compound mutant zebrafish tumors. (Left) 8 tumor samples per each line were used for comparison. (Right) Mean values of intensity (arbitrary units) were graphed. M, muscle; T, tumor. Scale bar = 100 µm. Student’s t-test was used to assess statistical significance.
Supple Figure 2
Supplementary Fig. 2. Immunoblotting of whole abdominal tumor extracts do not show any changes in mTORC2 signaling in tumors from tsc2;p53 compound mutant zebrafish compared to p53zdf1/zdf1 zebrafish. (A) Quantification of phospho-Akt (Serine 473) levels normalized to actin expression. (B) Quantification of total Akt levels normalized to actin expression. (C) Increased ratio of phospho-Akt to total Akt in tsc2;p53 compound mutant compared to p53zdf1/zdf1 zebrafish. Student’s t-test was used to assess statistical significance.
Supplementary Fig. 3. Phospho-tuberin Ser939 staining (red) in a series of abdominal tumors from \( p53^{+/+} \) and \( tsc2;p53 \) compound mutant zebrafish. (Left) 8 tumor samples per each line were used for comparison. (Right) Mean values of intensity (arbitrary units) were graphed. M, muscle; T, tumor. Scale bar = 100 \( \mu \)m. Student’s \( t \)-test was used to assess statistical significance.
Supplementary Fig. 4. Total Akt staining (red) in a series of abdominal tumors from $p53^{zdf1/zdf1}$ and $tsc2;p53$ compound mutant zebrafish. (Left) 8 tumor samples per each line were used for comparison. (Right) Mean values of intensity (arbitrary units) were graphed. M, muscle; T, tumor. Scale bar = 100 µm. Student’s $t$-test was used to assess statistical significance.
Supplementary Fig. 5. Phospho-Akt Thr308 (red) staining in a series of abdominal tumors from p53<sup>−/−</sup> and tsc2;p53 compound mutant zebrafish. (Left) 8 tumor samples per each line were used for comparison. (Right) Mean values of intensity (arbitrary units) were graphed. M, muscle; T, tumor. Scale bar = 100 µm. Student’s t-test was used to assess statistical significance.
Supplementary Fig. 6. Hif2a staining (red) in a series of abdominal tumors from p53<sup>zdf1/zdf1</sup> and tsc2;p53 compound mutant zebrafish. (Left) 8 tumor samples per each line were used for comparison. (Right) Mean values of intensity (arbitrary units) were graphed. M, muscle; T, tumor. Scale bar = 100 μm. Student’s t-test was used to assess statistical significance.
Supplementary Fig. 7. Vegf-c staining (red) in a series of abdominal tumors from \(p53^{+/-}\) and \(tsc2;\)\(p53\) compound mutant zebrafish. (Left) 8 tumor samples per each line were used for comparison. (Right) Mean values of intensity (arbitrary units) were graphed. M, muscle; T, tumor. Scale bar = 100 \(\mu\)m. Student’s \(t\)-test was used to assess statistical significance.
Supplementary Fig. 8. Increased size of blood vessels in the liver of \textit{tsc2} homozygous mutant \textit{zebrafish}. The \textit{tsc2} mutant allele was crossed to transgenic fish expressing EGFP under the \textit{Fli1} promoter that directs expression in endothelial cells. Examples shown are liver from 7 day old larvae. Above images are wildtype (wt) control, below are \textit{tsc2} homozygous mutant sections. Left images are \textit{Fli1:EGFP}, and right images hematoxylin and eosin staining. Asterisks (yellow in \textit{Fli1:EGFP}, black in hematoxylin and eosin stain) denote the blood vessel lumen. Scale bar = 100 \mu m.
AMPK (Thr172), DAPI

Supple Figure 9
Supplementary Fig. 9. AMPK Thr172 staining (red) and DAPI (blue) in abdominal tumors from $p53^{zdf1/zdf1}$ and $tsc2;p53$ compound mutant zebrafish. Merged colors above and AMPK Thr172 alone shown below. M, muscle; T, tumor. Scale bar = 100 µm.