Autophagy Induction Is a Tor- and Tp53-Independent Cell Survival Response in a Zebrafish Model of Disrupted Ribosome Biogenesis

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Abstract
Ribosome biogenesis underpins cell growth and division. Disruptions in ribosome biogenesis and translation initiation are deleterious to development and underlie a spectrum of diseases known collectively as ribosomopathies. Here, we describe a novel zebrafish mutant, titania (ttis450), which harbours a recessive lethal mutation in pwp2h, a gene encoding a protein component of the small subunit processome. The biochemical impacts of this lesion are decreased production of mature 18S rRNA molecules, activation of Tp53, and impaired ribosome biogenesis. In tti450 larvae, the growth of the endodermal organs, eyes, brain, and craniofacial structures is severely arrested and autophagy is up-regulated, allowing intestinal epithelial cells to evade cell death. Inhibiting autophagy in tti450 larvae markedly reduces their lifespan. Somewhat surprisingly, autophagy induction in tti450 larvae is independent of the state of the Tor pathway and proceeds unabated in Tp53-mutant larvae. These data demonstrate that autophagy is a survival mechanism invoked in response to ribosomal stress. This response may be of relevance to therapeutic strategies aimed at killing cancer cells by targeting ribosome biogenesis. In certain contexts, these treatments may promote autophagy and contribute to cancer cells evading cell death.


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Introduction
The generation of new ribosomes is the most energy-consuming process in the cell [1]. It requires the coordinated transcription and maturation of 4 different ribosomal RNA (rRNA) molecules and 70 small nuclear RNAs (snRNAs) together with the synthesis of approximately 80 ribosomal proteins (RPs) and an additional 170 associated proteins [2]. The regulation of this complex, multi-step process is the major factor determining the potential of a cell to grow and divide [3]. In times of nutrient availability and/or hormonal and growth factor signalling, the onset of ribosome biogenesis is tightly coupled to the translational requirements of a rapidly proliferating cell. In contrast, ribosome biogenesis is down-regulated to conserve energy and restrict unwarranted cell growth...
and division when the cellular environment is nutrient poor or challenged by harmful stimuli such as hypoxia, reactive oxygen species or genotoxic stress. Inherited impairment mutations in genes that encode components of the ribosome biogenesis machinery or ribosome structure underlie a number of human syndromes, collectively known as ribosomopathies, with a broad range of clinical phenotypes [4]. There is a growing appreciation that sporadically acquired mutations in genes that contribute to ribosome function also increase susceptibility to human cancer, particularly leukemia and lymphoma, although the precise mechanisms involved are only just beginning to emerge [5].

The process of human ribosome biogenesis initiates in the nucleolus with the transcription by RNA polymerase (Pol) I of a 45S pre-rRNA precursor (35S in yeast), which contains the mature 28S, 18S and 5.8S rRNAs interspersed by spacer sequences. A series of processing and chemical modification events mediated by discrete multi-protein/RNA complexes known as the 90S, 66S and 43S pre-ribosomal particles generate the mature 18S, 28S and 5.8S species, respectively and assembles them into the 40S and 60S ribosomal subunits prior to their export from the nucleus to the cytoplasm where they associate to form the functional 80S ribosomes [6]. In yeast, the 90S particle, also identified as the small-subunit processome, has been shown to be strictly required for the production of 40S ribosomal subunits containing 18S rRNA [7].

One of the mechanisms through which ribosome biogenesis is coupled to cell growth and proliferation is the Target of Rapamycin (Tor) pathway, which is activated by cell surface growth factor and insulin receptors and other growth promoting sensors that detect when nutrients such as amino acids are plentiful. Activation of the Tor pathway stimulates the phosphorylation of S6 kinase (S6K) and 4E-Binding Protein 1 (4EBP1), which regulate ribosome biogenesis and mRNA translation [8,9]. Activation of Tor also inhibits macroautophagy (hereafter referred to as autophagy), an evolutionarily conserved process that provides a survival mechanism during periods of cell starvation by promoting intracellular recycling of organelles, such as mitochondria and ribosomes [10,11].

Autophagy describes a complex multi-step process whereby cells sequester a portion of their cytoplasm inside double-membrane vesicles called autophagosomes, which then fuse with lysosomes to form autolysosomes [12]. Inside these vesicles, the captured material, together with the inner membrane, is digested and the released nutrients are recycled. In metazoan, autophagy mediates the catabolic turnover of malfunctions, damaged or superfluous proteins and organelles to maintain cellular homeostasis during development and in adult life [13]. It is activated in response to multiple forms of cellular stress, including nutrient deprivation, endoplasmic reticulum (ER) stress, accumulation of reactive oxygen species, DNA damage, invasion by intracellular pathogens and intense exercise [14,15]. Some of these triggers induce autophagy through activation of Tumour protein 33 (Tp53), which increases the expression of the β1 and β2 subunits of AMP-activated protein kinase (AMPK), an evolutionarily conserved sensor of cellular energy levels [16]. AMPK responds to reductions in the ratio of ATP:AMP nucleotides by phosphorylating multiple targets with functions related to energy metabolism, including the Tuberous sclerosis complex (Tsc) protein, Tsc2 and Raptor. These phosphorylation events indirectly inhibit the Torc1 complex, which in its active state inhibits autophagy by negatively regulating the protein kinase, Ulk1 (mammalian orthologue of yeast Atg1). Ulk1, together with Atg13, Fip200 and Atg101, are the key components of a complex that initiates mammalian autophagosome formation [17,18]. Recent work proposes that AMPK may also induce autophagy independently of Torc1 inhibition by directly phosphorylating Ulk1 [19–21]. However, a clear understanding of the AMPK-Ulk1-Torc1 network is yet to emerge [22].

In this study, we employed a zebrafish intestinal mutant, titania450 (tti450), as an in vivo model to examine the connection between RNA processing and autophagy. tti450 was identified on the basis of its hypoplastic intestinal morphology at 96 hours post-fertilization (hpf) in a focused ENU mutagenesis screen designed to identify mutants with defects in the size and morphology of the endoderm-derived organs [23]. Using positional cloning we identified periodic tryptophan protein 2 homologue (pwp2h) as the mutated gene in tti450. In yeast, Pwp2p has been shown to be an essential scaffold component of the 90S pre-ribosomal particle, facilitating the binding of proteins such as the U3 snoRNP to the 5′ end of the 35S rRNA precursor [24]. Depletion of Pwp2 in yeast cells results in reduced production of mature 18S rRNA and 40S ribosomal subunits [24,25]. In agreement with these results, we show that zebrafish Pwp2h plays a conserved role in rRNA processing and ribosome biogenesis. Moreover, we use this in vivo model system to demonstrate a connection between RNA processing and autophagy which has, to our knowledge, been hitherto unappreciated.

### Results

tti450 larvae exhibit defects in intestinal, liver, pancreas, and craniofacial development

tti450 is one of several intestinal mutants identified in an ENU mutagenesis screen (the Liver450 screen) conducted on a transgenic line of zebrafish (Tg[XlEef1a1:GFP]453, harbouring a GFP transgene (“gutGFP”) expressed specifically in the digestive organs [23,26,27]. Abnormalities in the gross morphology of tti450 larvae are first detectable at 72 hpf and became more severe with time. At 120 hpf, the wildtype (WT) intestinal epithelium exhibits a columnar morphology and starts to elaborate folds; in contrast, the intestinal epithelium in tti450 remains thin and unfolded (Figure 1A and 1B). tti450 larvae also exhibit smaller eyes (microphthalmia), a smaller, misshapen head, an uninflated swim bladder and impaired yolk absorption (Figure 1A). At 120 hpf, the tti450 pancreas and liver are both substantially smaller than in WT (Figure 1C).
By 120 hpf, the rostral intestine (intestinal bulb region) in *tti* larvae is markedly smaller than in WT and the intestinal epithelial cells (IECs) are cuboidal rather than columnar in shape (Figure 1C, 1D). The intestinal lumen appears clear of cellular debris. Cells in the mid and posterior intestine are also smaller and less polarized than in WT (Figure 1D). The mean apicobasal height of the cells in the intestinal bulb region of *tti* larvae is approximately 40% less than that in WT (Figure 1E). However, cellular differentiation...
is not inhibited as similar numbers of mucin-producing goblet cells are found in the mid-intestinal region of 
\( \texttt{itt}^{450} \) larvae as in WT (Figure 1D).

The reduction in cell size is accompanied by changes in the proportion of cells in different phases of the cell cycle. At 72 hpf, the intestinal epithelium is the most rapidly proliferating tissue in the zebrafish embryo [28,29]. Using BrdU incorporation analysis, we detected fewer \( \texttt{itt}^{450} \) IECs in S phase than WT IECs (Figure S1A, S1B). Fluorescent activated cell sorting (FACS) of cells significantly accumulated of specifically from the liver, pancreas and intestine. We observed a transgene allowed us to analyze the proliferation of cells derived from the Pwp2h protein in the seventh WD domain (Figure S3).

nonsense mutation in codon 421 (Figure S2A) and truncating upstream of exon 10, thereby generating a frame-shift and (Figure 2C) resulting in utilization of a cryptic splice site 11 bp targeting to 
\( \texttt{pwp2h} \) splice acceptor site in intron 9 of multi-protein complexes and are conserved from yeast to mammals. We identified an A to T base change in the conserved splice acceptor site in intron 9 of \( \texttt{pwp2h} \) in \( \texttt{itt}^{450} \) mutants (Figure 2C) resulting in utilization of a cryptic splice site 11 bp upstream of exon 10, thereby generating a frame-shift and nonsense mutation in codon 421 (Figure S2A) and truncating the Pwp2h protein in the seventh WD domain (Figure S3).

\( \texttt{itt}^{450} \) harbours a mutation in \( \texttt{pwp2h} \)

We identified the mutated gene responsible for the abnormal digestive organ development in \( \texttt{itt}^{450} \) by mapping the \( \texttt{ Witt}^{450} \) locus to a 260-kilobase interval on chromosome 1 encompassing 3 genes (Figure 2A). One of these genes, \( \texttt{pwp2h} \), comprises 21 exons spanning 2928 base pairs (Figure 2B) and encodes a protein of 937 amino acids containing 13 WD-40 repeats. WD-40 repeats generally serve as platforms for the assembly of proteins in multi-protein complexes and are conserved from yeast to mammals. We identified an A to T base change in the conserved splice acceptor site in intron 9 of \( \texttt{pwp2h} \) in \( \texttt{itt}^{450} \) mutants (Figure 2C) resulting in utilization of a cryptic splice site 11 bp upstream of exon 10, thereby generating a frame-shift and nonsense mutation in codon 421 (Figure S2A) and truncating the Pwp2h protein in the seventh WD domain (Figure S3).

The \( \texttt{itt} \) phenotype is completely penetrant, and the animals die at 8–9 days post-fertilization (dpf). Heterozygous \( \texttt{itt}^{450} \) carriers are phenotypically indistinguishable from WT siblings.

\( \texttt{itt}^{450} \) larvae (Figure 2E, 2F). At 72 hpf, the pancreas is the only tissue in which \( \texttt{pwp2h} \) mRNA is detected (Figure 2K, 2L). Expression of \( \texttt{pwp2h} \) is absent in \( \texttt{itt}^{450} \) embryos from 24 hpf onwards (Figure 2M, 2N) indicating that upon exhaustion of maternally deposited supplies of WT \( \texttt{pwp2h} \) mRNA, the zygotically expressed mutant mRNA probably undergoes nonsense-mediated decay (NMD). These expression data are consistent with the eye, brain, pharyngeal cartilages and digestive organs being the most severely affected organs in \( \texttt{itt}^{450} \) larvae.

\( \texttt{pwp2h} \) deficiency leads to impaired ribosome biogenesis in \( \texttt{itt}^{450} \) larvae

In all species, rRNA is transcribed as a large pre-rRNA transcript which undergoes a series of enzymatic cleavage steps within the nucleolus by large ribonucleoprotein complexes to produce mature 18S, 28S and 5.8S rRNAs (Figure 3B). To investigate rRNA processing in \( \texttt{itt}^{450} \) larvae, we conducted Northern blot analysis (Figure 3A) using probes designed to hybridize to the external (ETS) and internal-transcribed (ITS1 and ITS2) spacer regions of zebrafish 45S pre-rRNA (Figure 3B). These probes detect the full-length rRNA precursor and all intermediate species but not the fully mature forms of rRNA. This analysis revealed a 2.5 fold accumulation of the full-length precursor ‘a’ in \( \texttt{itt}^{450} \) and an accumulation of the intermediates ‘b’ and ‘c’ (4.6 fold and 1.3 fold, respectively). These observations are consistent with a block in the processing of the full-length rRNA precursor. We also noted a 2.6 fold decrease in \( \texttt{itt}^{450} \) larvae in the level of ‘d’, the immediate precursor of 18S rRNA (Figure 3A). Furthermore, E-bioanalyzer analysis revealed a marked reduction in the production of mature 18S rRNA in \( \texttt{itt}^{450} \) larvae (Figure 3C); however, the production of mature 28S rRNA was unaffected (Figure 3C). These changes altered the ratio of 28S/18S rRNA in \( \texttt{itt}^{450} \) larvae, which is 2.8 at 120 hpf, compared to 1.8 in WT (Figure 3D).

To investigate the impact of \( \texttt{pwp2h} \) deficiency on ribosome formation, we prepared extracts of WT and \( \texttt{itt} \) zebrafish larvae at 96 hpf and fractionated the ribosomal subunits on sucrose density gradients (Figure 3E). The areas under the peaks corresponding to the 40S subunits and 60S monosomes in \( \texttt{itt}^{450} \) lysates are markedly smaller compared to those in WT (reduced approximately 4 fold and 2-fold, respectively). Meanwhile, the area under the peak corresponding to the 60S subunits is increased by approximately 4.5 fold (Figure 3F). Collectively, these data are consistent with \( \texttt{pwp2h} \) deficiency primarily impacting on 40S subunit formation.

Intestinal epithelial cells in \( \texttt{itt}^{450} \) larvae undergo autophagy

To determine the impact of impaired ribosome biogenesis at the ultrastructural level, we used transmission electron microscopy (TEM) (Figure 4A–4H). While WT intestinal epithelium is folded and the cells exhibit apicobasal polarity and a highly elaborated apical brush border (Figure 4A, 4C, 4E, 4G), IECs in \( \texttt{itt}^{450} \) are smaller and the microvilli are shorter and relatively sparse (Figure 4B, 4D, 4F, 4H). The \( \texttt{itt}^{450} \) nuclei contain prominent condensed nucleoli, suggesting ribosomal stress [32]. Also conspicuous at 96 hpf in the IECs of \( \texttt{itt}^{450} \) larvae, but essentially absent in WT, are cytoplasmic vesicles containing debris (Figure 4B, 4F). At 120 hpf, these structures are bigger in size and electron dense (Figure 4D, 4F). At 144 hpf, vesicles more akin to those observed at 96 hpf are present (Figure 4H, 4I, 4H). Similar transient structures have been previously identified in cells undergoing autophagy. We therefore pursued the

Intestinal epithelial cells in \( \texttt{itt}^{450} \) larvae undergo autophagy
hypothesis that the cytoplasmic vesicles in tti\textsuperscript{450} larvae correspond to autophagosomes and autolysosomes: vesicles that sequester and digest organelles. Autophagy is a dynamic process comprising autophagosome synthesis, delivery of autophagic substrates to lysosomes and substrate degradation in autolysosomes [10,12]. In order to investigate whether the electron dense vesicles observed at 120 hpf (Figure 4D) correspond to autolysosomes, we exposed WT and tti\textsuperscript{450} larvae at 106 hpf for 14 h to chloroquine, an autophagy inhibitor that blocks the fusion of autophagosomes with lysosomes.

Figure 2. Positional cloning reveals that pwp2h is the mutated gene in tti\textsuperscript{450}. (A) Physical map of chromosome 1 in the region encompassing the tti\textsuperscript{450} locus. Analysis of recombinants from 7376 meioses narrowed the genetic interval containing the mutation to a region flanked by 2 BACs (green boxes) and encompassed by 2 scaffolds zv945445 and zv945446 (blue bars) containing 5 genes (arrows). (B) Schematic representation of the pwp2h gene and the location of the sequence variation in intron 9. (C) The nucleotide sequence of pwp2h cDNA from tti\textsuperscript{450} larvae contains an A\textsuperscript{R}T transversion. Wholemount in situ hybridization (WISH) reveals the pwp2h mRNA expression pattern from 4–144 hpf in WT larvae (D–L). pwp2h expression is ubiquitous from 4–12 hpf (D–F), restricted to the retina at 24 hpf (G, black arrow) and encompasses the pharyngeal cartilages (black arrowhead), liver (white arrow), intestine (bracket) and pancreas (white arrowhead) at 48 hpf (H), 72 hpf (I) and 96 hpf (J). From 120–144 hpf pwp2h expression is restricted to the pancreas (K–L, white arrowhead). pwp2h expression is barely detectable at 24 hpf (M) and 72 hpf (N) in tti\textsuperscript{450} larvae. Staining is absent in the sense control at 72 hpf (O) and at all other time points (data not shown).

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lysosomes and thereby prevents digestion of the vesicle contents [33]. After chloroquine treatment, few, if any, electron dense cytoplasmic vesicles (autolysosomes) are found in the intestinal epithelium of $tti^{450}$ larvae (Figure 4F). Instead, the IECs in $tti^{450}$ larvae contain vesicles more reminiscent of autophagosomes (Figure 4F, 4F'). We counted >3 autophagosomes/cell (3.25 ± 0.144, n = 60) in the IECs of $tti^{450}$ larvae, compared to <1 (0.6 ± 0.058, n = 60) in WT IECs. Thus chloroquine inhibition of autophagic flux results in a significantly higher number of autophagosome-like structures in $tti^{450}$ larvae compared to WT.

To investigate this further, we examined LC3 localisation in WT and $tti^{450}$ larvae using wholemount immunocytochemistry (Figure 5A–5G). LC3, the mammalian orthologue of yeast Atg8, is a robust marker of autophagosomes. Upon induction of autophagy, the cytoplasmic form of LC3 (LC3I) is converted by cleavage and lipidation to a transient, autophagosomal membrane-bound Figure 3.

**Figure 3.** $tti^{450}$ larvae display defects in ribosome biogenesis. (A) Northern analysis of RNA isolated from WT and $tti^{450}$ larvae at 120 hpf using 5’ETS, ITS1, and ITS2 probes to detect precursor forms of rRNA. Elf1α is a loading control. a–d correspond to the rRNA intermediates depicted in Figure 3B. (B) Schematic diagram showing the rRNA processing pathway in zebrafish [60]. The sites of hybridization of the 5’ETS, ITS1 and ITS2 probes are indicated. (C) Representative E-Bioanalyser analysis of total RNA isolated from WT and $tti^{450}$ larvae at 120 hpf demonstrates a reduction in the 18S peak in $tti^{450}$ larvae resulting in an elevated 28S/18S rRNA ratio in $tti^{450}$ (D). Graphical representation of the experiment shown in C. Data are represented as mean ±/− SD (n = 5). (E) Representative polysome fractionation analysis performed on WT and $tti^{450}$ larvae at 96 hpf demonstrates reduced levels of 40S ribosomal subunits and 80S monosomes and an increase in free 60S subunits in $tti^{450}$ larvae compared to WT. (F) Graphical representation of the experiment shown in E. Data are represented as mean ±/− SD (n = 5) *p<0.05.

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Figure 4. The intestinal epithelial cells (IECs) in tti^{+4S} larvae contain autophagosome- and autolysosome-like structures. (A–H) Transmission electron micrographs of WT and tti^{+4S} larvae at 96 hpf (A, B), 120 hpf (C–F) and 144 hpf (G, H). Sections are transverse through the yolk in the region of the intestinal bulb. WT IECs demonstrate well-developed apicobasal polarity as evidenced by basally positioned nuclei (n) and the elaboration of microvilli (mv) projecting from the apical surface into the intestinal lumen. Mitochondria (m) are abundant and plasma membranes...
form of LC3 (LC3II). Disrupting the fusion of autophagosomes with lysosomes with chloroquine prolongs the half-life of LC3II and facilitates the accumulation of LC3II-containing autophagosomes, which appear as punctate structures using LC3 immunocytochemistry. We observed more puncta in the IECs of chloroquine-treated WT larvae (Figure 5C) compared to untreated WT larvae (Figure 5A). Consistent with impaired ribosome biogenesis stimulating autophagy, we counted approximately 5 times more puncta in the IECs of chloroquine-treated ttt5450 larvae (Figure 5D) compared to the IECs of chloroquine-treated WT siblings (Figure 5C; compare 2nd and 4th bars in Figure 5G). We next exposed WT and ttt5450 larvae to rapamycin, which through its specific inhibition of Torc1 [34,35] provides a powerful stimulus to autophagy in yeast, zebrafish and mice. We found that the number of puncta in WT larvae treated with rapamycin and chloroquine together (Figure 5E, 5G) was similar to the number of puncta in ttt5450 larvae treated with chloroquine alone (Figure 5D, 5G). Finally, treating ttt5450 larvae with rapamycin and chloroquine together (Figure 5F) resulted in more abundant puncta than in both chloroquine-treated ttt5450 larvae (Figure 5D) compared to the IECs of chloroquine-treated WT larvae (Figure 5G). Upon Western blot analysis of whole larval lysates (Figure 5H, 5I), we found that LC3II levels in chloroquine-treated ttt5450 larvae were significantly higher than in both WT larvae but not significantly different from those in WT larvae treated with rapamycin and chloroquine together (Figure 5I). Together these experiments demonstrate that the vesicles identified in the IECs of ttt5450 larvae are autophagosomes, and, to the best of our knowledge, provide the first evidence for a link between impaired ribosome biogenesis and autophagy.

To determine the extent of autophagy in ttt5450 larvae, we injected RNA encoding a mCherry-LC3 fusion protein into the yolk of 1–4 cell stage zebrafish embryos and evaluated the formation of puncta after prior treatment with chloroquine for 14 h at three time-points (Figure S4). At 72 hpf, abundant puncta are present in the eye (Figure S4B) and brain (Figure S4B′) of ttt5450 larvae compared to WT larvae (Figure S4A, S4A′). At this time-point, there are very few puncta in the digestive organs (Figure S4C, S4D). A similar picture was observed at 96 hpf (data not shown). At 120 hpf, the number of puncta in the brain (Figure S4F) in ttt5450 larvae is now comparable to that observed in WT (Figure S4E′), while higher numbers of puncta are still found in the eye (Figure S4F). At 120 hpf there are more abundant puncta in the intestine and pancreas of ttt5450 larvae (Figure S4H) compared to these organs in WT (Figure S4E and S4G, respectively). This pattern of autophagy induction mirrors the tempo-spatial expression of pwp2h during zebrafish development, and is consistent with these tissues being the most affected by impaired ribosome biogenesis in ttt5450 larvae.

To determine whether autophagy is a specific response to impaired ribosome biogenesis, we conducted LC3 analysis of two additional zebrafish intestinal mutants, setebos (set5453) and caliban (cbln5456), which exhibit phenotypes that are essentially indistinguishable from that of ttt5450 when viewed under the light microscope or upon histological analysis. Whereas set5453 harbours a mutation in a gene which impairs 28S rRNA production and ribosome biogenesis (APB et al., in preparation), the mutation in cbln5456 lies in a gene encoding an essential mRNA splicing factor (SJMG et al., in preparation). We observed that set5453 larvae, like ttt5450 larvae, contain higher LC3II levels compared to WT siblings in the presence of chloroquine (Figure S5A, S5B) and their IECs contain abundant autophagosome-like structures when analysed by TEM (data not shown). In contrast, the LC3II levels in cbln5456 larvae are indistinguishable from those in WT siblings (Figure S5A, S5B) and the intestinal epithelium of cbln5456 mutants do not contain autophagosomes or autolysosomes when inspected at the ultrastructural level (Figure S5C–S5H). These data suggest that the induction of autophagy in IECs is a specific response to impaired ribosome biogenesis, rather than a non-specific response to impaired cell growth.

**Autophagy induction in ttt5450 larvae prolongs their survival**

We followed the morphological changes in the intestinal epithelium and liver of ttt5450 larvae until 7 dpf, just before the larvae die at 8–9 dpf. At 7 dpf, the IECs are substantially smaller in ttt5450 larvae than in their WT counterparts and neither ttt5450 nor WT larvae contain detached cells in the intestinal lumen (Figure S6A–S6D). The ttt5450 IECs no longer contain conspicuous autophagosomes, though electron dense vesicles are present in abundance in adjacent liver cells (Figure S6E–S6F). To investigate the impact of inhibiting autophagy in ttt5450 larvae, we blocked autophagosome formation by injecting 1 ng of an antisense morpholino oligonucleotide (MO), which targets the translation start-site of atg5 mRNA [36], into 1–4 cell stage embryos derived from pair-wise matings of heterozygous ttt5450 adults. At 72 hpf, uninjected, vehicle-injected and atg5 MO-injected ttt5450 larvae were identified and subjected to LC3 analysis. We found significantly lower LC3II levels in the atg5 MO-injected ttt5450 larvae compared to uninjected and vehicle-injected controls (Figure 6A). Moreover, from 72–120 hpf, we noticed that atg5 MO-injected ttt5450 larvae start to develop oedema around the head, eye, heart and intestine (Figure S7D). As a consequence, 50% of atg5 MO-injected ttt5450 larvae die by 5 dpf and all atg5 MO-injected ttt5450 larvae are dead by 7 dpf (Figure 6B). This contrasts markedly with untreated or vehicle-injected ttt5450 larvae, which survive until 8–9 dpf (Figure 6B). The longevity of WT larvae injected with the atg5 MO is not affected. Ultrastructural analysis at 120 hpf revealed detached, shrunken cells in the intestinal lumen of atg5 MO-treated ttt5450 larvae (Figure 6D–6F) that were never seen in the intestinal lumen of ttt5450 larvae injected with vehicle or WT siblings injected with atg5 MO (Figure 6C). Together these data demonstrate that autophagy extends the lifespan of ttt5450 larvae and prolongs the survival of IECs.

**Autophagy induction in ttt5450 larvae is independent of Tor pathway activity and p-RPS6**

To explore the relationship between the Tor pathway and autophagy in ttt5450 larvae, we analysed the levels of phosphorylated RPS6 (p-RPS6), a downstream target of Torc1 activity. Using Western blot analysis, we found that p-RPS6 levels decrease.
markedly in WT larvae between 72–120 hpf as previously reported [37] (Figure 7A, 7B). Somewhat surprisingly, p-RPS6 levels persist in ttis450 larvae until 120 hpf, when they are 4-fold higher than in WT siblings (Figure 7A, 7B). We also noticed that the overall level of RPS6 protein is less in ttis450 larvae compared to WT, perhaps reflecting the fact that RPS6 is a structural...
component of the 40S subunits, which are fewer in **tti** larvae. Using immunocytochemistry we examined p-RPS6 expression in histological sections of WT and **tti** larvae. At 96 hpf, we observed robust p-RPS6 expression in the intestinal epithelium and liver of WT and **tti** larvae (Figure 7C). The high p-RPS6 levels in the **tti** intestinal epithelium raise the possibility that elevated p-RPS6 stimulates autophagy directly in **tti** larvae, as this occurrence has been recognised previously, including in the

**Figure 6.** Disrupting autophagy in **tti** larvae results in the death of IECs and a reduced lifespan. (A) Western blot analysis of lysates of **tti** larvae (72 hpf) that had been injected at the 1–4 cell stage with an antisense morpholino oligonucleotide (MO) targeted to the start codon of *atg5* mRNA reveals decreased levels of LC3II compared to untreated and vehicle controls, both in the presence and absence of chloroquine. Data are represented as mean ±/− SD, *p<0.05. (B) Survival curve of untreated WT and **tti** larvae compared to WT and **tti** larvae that had been injected at the 1–4 cell stage with vehicle or *atg5* MO (n=85 larvae per group). The lifespan of WT embryos/larvae is completely unaffected by injection with the *atg5* MO since all three groups of WT larvae (untreated, vehicle-treated and *atg5* MO-treated) progress normally through the first 10 days of development, when the experiment was terminated. The horizontal line represents untreated WT embryos (maroon squares), vehicle-injected WT embryos (green triangles) and *atg5* MO-injected WT embryos (blue triangles). In contrast, **tti** embryos respond to microinjection of the *atg5* MO by impaired survival. Whereas all untreated (yellow diamonds) or vehicle-injected (purple circles) **tti** larvae are still alive at 7 dpf, all the *atg5* MO-injected **tti** larvae are dead at this time-point (red squares). Indeed, 20% of the *atg5* MO-injected **tti** larvae have already succumbed by 3 dpf. (C–F) TEMs of WT (C) and **tti** larvae at 120 hpf (D–F), injected at the 1–4 cell stage with the *atg5* MO-targeted MO. Inhibiting autophagy in **tti** larvae results in the appearance of detached and shrunken IECs in the intestinal lumen (black arrow in D, E and F [boxed area in D]) but has no impact on WT IECs (C). Scale bars = 10 μm.

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Figure 7. *ttis450* larvae exhibit elevated levels of Torc1 activity. (A) Western blot analysis of RPS6, p-RPS6 and Actin (loading control) in whole cell lysates of WT and *ttis450* larvae between 72–120 hpf. (B) Graphical representation of the data shown in A combined with two additional experiments (each bar represents the mean +/- SD, *p<0.05).*ttis450* larvae exhibit increased levels of p-RPS6 at 96–120 hpf and decreased levels of total RPS6 between 72–120 hpf compared to WT siblings. (C) Immunohistochemical analysis of transverse sections of *ttis450* and WT larvae at 96 hpf reveals robust p-RPS6 expression in the digestive organs. Scale bars = 50 μM. (D) The persistent expression of p-RPS6 expression in *ttis450* larvae at 96 hpf compared to WT is due entirely to up-regulated Torc1 activity as shown by the disappearance of the p-RPS6 signal when larvae are pre-treated with rapamycin. (E) Inhibiting the Tor pathway in *ttis450* larvae with rapamycin in the presence of chloroquine reduces p-RPS6 expression and at the same time increases autophagic flux as shown by the increase in LC3II level. In the graphical representation of the data, each bar represents the mean +/- SD (n = 3), *p<0.05.
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in the 90S pre-ribosomal particle or small subunit processome is conserved from yeast to vertebrates.

In our \(pwp2h\)-deficient model, \(titania\) (\(tt\)), the growth of the endodermal organs, eyes, brain and craniofacial structures is severely arrested and autophagy is markedly up-regulated. To the best of our knowledge, this is the first time that a link between impaired ribosome biogenesis and autophagy has been demonstrated. We further show that elevated rates of autophagy support the survival of intestinal epithelial cells and increase the lifespan of \(tt\) larvae, thereby demonstrating that autophagy is a survival mechanism invoked in response to ribosomal stress. In our zebrafish model, autophagy induction does not depend on inhibition of the Tor pathway or activation of \(Tp53\).

The death of \(tt\) larvae at 8–9 dpf demonstrates that \(pwp2h\) encodes a protein that is indispensable for life. However, the development of \(tt\) larvae until 72 hpf is supported by the deposition of maternal, wild-type \(pwp2h\) mRNA (and/or protein) into oocytes by their heterozygous mother. At 72 hpf, the tissues in which \(pwp2h\) is most highly expressed are the intestinal epithelium, pharyngeal arches, liver, dorsal midbrain, cerebellum, dorsal hindbrain, retinal epithelium and pancreas. These tissues are also the most rapidly proliferating tissues in WT larvae at 72 hpf [28] and the most severely affected tissues in \(tt\) larvae. Thus the tissue-specific phenotype of \(tt\) larvae may be explained by maternally-derived WT \(pwp2h\) mRNA being exhausted first in developing organs containing highly proliferative cells.

In WT zebrafish larvae there is a transient spike in Torc1 activity [as measured by p-RPS6] at around 72 hpf that is coincident with the activation of anabolic pathways required for cell growth and proliferation during the endoderm to intestine transition [37]. Torc1 is thought to play a role in developing organs as an organ size checkpoint, potentiating growth signals that promote the rapid expansion of organs until they reach a genetically programmed cell size [44]. Therefore the persistent and robust activity of Torc1 we observe in the intestinal epithelium of \(tt\) larvae may be explained by maternally-derived WT \(pwp2h\) mRNA being exhausted first in developing organs containing highly proliferative cells.

The gross phenotype of \(tt\) is highly reminiscent of another zebrafish mutant, \(nil per os\) (\(npo\)), in which the morphogenesis of the intestinal epithelium is also arrested. In \(npo\) the failure of the primitive gut endoderm to transform into a monolayer of polarized and differentiated epithelium is caused by a mutation in \(rhm19\), a gene encoding a protein with six RNA recognition motifs that is also thought to play a role in ribosome biogenesis [45]. The same authors showed that essentially the same hypoplastic intestinal phenotype was recapitulated by exposure of WT zebrafish larvae to the Tor1 inhibitor, rapamycin [46], which presumably stimulated autophagy. It would be interesting to determine whether the growth arrest of the digestive organs in the \(npo\) mutant is also accompanied by autophagy.

The degree of activation of the Tor pathway is thought to be one of the major factors governing autophagy. However, Tor inhibition is not the mechanism responsible for autophagy in \(tt\) larvae and recent work suggests that autophagy regulation is a very complex process involving the integration of signals from many diverse signalling pathways [47]. Indeed, proteomic analysis of binding partners of components of the autophagy machinery suggests that several hundred molecules participate in the regulation of the human autophagy network [48]. While much recent attention has been focused on the direct phosphorylation of Ulk1/Atg1 by AMPK, acting either cooperatively or independently of Tor to exert autophagy control [19–21], there are many reports of other kinases capable of controlling autophagy by a

Discussion

This study shows, in the context of an intact vertebrate organism, that Pvp2h is critical for the production of mature 18S rRNA, an integral component of the 40S ribosomal subunit. In zebrafish, as in yeast, Pvp2h depletion results in reduced levels of the immediate precursor to mature 18S rRNA and a concomitant decrease in the production of mature 18S rRNA and assembly of 40S ribosomal subunits. Thus the role of Pvp2h in the 90S pre-ribosomal particle or small subunit processome is conserved from yeast to vertebrates. 

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\(Drosophila\) fat body during starvation [38,39]. To test this, we blocked p-RPS6 accumulation using rapamycin. We found that prior exposure to rapamycin for 14 h eliminated the p-RPS6 signal in both WT and \(tt\) larvae at 96 hpf (Figure 7D), thereby unequivocally linking the persistent and elevated p-RPS6 signal in \(tt\) larvae to Torc1 activity. Moreover, rapamycin treatment of \(tt\) larvae in the presence and absence of chloroquine results in elevated levels of LC3II (Figure 7E) and LC3II-containing autophagosome formation (Figure 5F, 5G). These augmented levels of autophagy, achieved through rapamycin blockade of RPS6 phosphorylation, exclude the possibility that elevated p-RPS6 is responsible for the induction of autophagy in \(tt\) larvae. Indeed, these data suggest that autophagy induction in \(tt\) larvae is independent of the level of activation of the Tor pathway and the levels of p-RPS6.

We corroborated this finding with a genetic approach by crossing \(tt\) onto the tuc\(^{724/222,242}\) background [40]. Tsc2 is a negative regulator of Tor1 and tuc\(^{724/222,242}\) zebrafish larvae exhibit a variety of defects including an enlarged liver at 7 dpf [40], consistent with Tor playing a positive role in digestive organ growth. The development of the \(tt\) phenotype, including the induction of autophagy, is not perturbed on the tuc\(^{724/222,242}\) background (Figure S8A–S8F). Interestingly, \(tt\) larvae at 96 hpf contain higher levels of p-RPS6 than tuc\(^{724/222,242}\) larvae (Figure S8E, S8F) and the levels of p-RPS6 are higher still in compound \(tt\); tuc\(^{724/222,242}\) mutants (Figure S8E, S8F). In conclusion, these data show that impaired ribosome biogenesis induces autophagy in \(tt\) larvae through a mechanism that does not require inhibition of the Tor pathway and is independent of p-RPS6 levels.

Autophagy induction in \(tt\) larvae is independent of \(Tp53\)

Defects in 18S and 28S rRNA processing have been shown to activate \(Tp53\) [41], which in turn can stimulate autophagy [42]. While WT larvae contain negligible levels of \(Tp53\) protein at 96 hpf, \(tt\) larvae display readily detectable levels of \(Tp53\) protein at this time-point (Figure 8A) and increased transcription of \(Tp53\) target genes, including \(b3p3\), \(p21\), \(cytl\) and \(mdm2\) (Figure 8B–8E). To determine whether \(Tp53\) plays a role in the induction of autophagy in \(tt\), we generated \(tt\) larvae expressing a mutant form of \(Tp53\) (\(Tpm2\)) with negligible DNA-binding activity [43]. While this mutation severely diminished the elevated \(Tm13\); \(p21\), \(cytl\) and \(mdm2\) expression levels in \(tt\) larvae at 96 hpf as expected (Figure 8B–8E), the level of LC3II in compound \(tt\); \(p21\), \(cytl\) and \(mdm2\) mutants in the presence of chloroquine was significantly higher than in \(tt\) larvae at 120 hpf, independent of whether they were on the \(tt\) or \(tt\) background or not (Figure 8H). Therefore the induction of autophagy in response to Pvp2h depletion proceeds unabated in \(tt\) larvae that are devoid of functional \(Tp53\) protein.

18S rRNA, an integral component of the 40S ribosomal subunit. In zebrafish, as in yeast, Pvp2h depletion results in reduced levels of the immediate precursor to mature 18S rRNA and a concomitant decrease in the production of mature 18S rRNA and assembly of 40S ribosomal subunits. Thus the role of Pvp2h
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A

B

C

D

E

F

G

H
Figure 8. Autophagy in ttk+/− larvae is not due to Tp53 activation. (A) Western blot analysis of Tp53 protein in whole cell lysates of WT (lane 1) and ttk+/− (lane 2) larvae at 96 hpf reveals up-regulation of Tp53 expression in ttk+/−. Larvae treated with roscovitine (ROS, lane 3) to induce Tp53 protein expression or untreated larvae (lane 4) are positive and negative controls, respectively. The Actin signal provides a loading control. (B–E) Relative expression of N113p33β, mdm2, cyclinG1 (D) and p21 (E) mRNAs in WT, ttk+/− (pwp2h+/−), tp53+/− (tp53+/−) and ttk+/−, pwp2h+/−, tp53+/− larvae at 96 hpf (n = 3) demonstrates that the expression of Tp53 target genes is increased in ttk+/− compared to WT larvae (compare first 2 bars in all graphs). The Tp53 response is diminished on the tp53+/− background, as expected (compare 2nd and 4th bars). Data were normalised by reference to Elongation factor alpha (Elf-a) expression. (F) Western blot analysis of LC3 in whole cell lysates of ttk+/− (pwp2h+/−) and ttk+/−, pwp2h+/−, tp53+/− larvae at 96 hpf. The elevated autophagic flux in ttk+/− larvae due to ribosomal stress is not diminished on a pwp2h+ background. (G) Graphical representation of the data shown in F and two additional experiments. Bars represent the mean ± SD (n = 3), *p < 0.05. (H) Transmission electron micrographs of IECs of ttk+/−, pwp2h+/−, tp53+/− larvae at 120 hpf (right panel) reveal electron dense vesicles, resembling autolysosomes (white arrowhead), in comparable numbers to those found in ttk+/− larvae with WT Tp53 expression (left panel).

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variety of Tor-independent mechanisms [49–51]. The dissociation of the key BH3 domain-containing autophagy protein, Bcl2 (mammalian orthologue of yeast Apg6) from its inhibitors Bcl2 and Bcl-XL as a result of phosphorylation of one or other components is also a critical determinant in the induction of autophagy [52]. In the case of ttk+/− larvae, it is plausible that autophagy induction may involve a targeted pathway, selective for ribosomes [11], which by analogy with mitophagy [53], is invoked to digest damaged cargo such as non-functional organelles.

Somewhat surprisingly, we also ruled out involvement of Tp53 in the induction of autophagy in ttk+/− larvae, even though Tp53 protein is active in ttk+/− larvae at 96 hpf. However, we believe the increased expression of Tp53 target genes such as p21 and cyclinG1 may be responsible, at least in part, for the reduction in the number of cells in the S phase of the cell cycle we observed at this time-point. To explain this, we surmise that as ribosome biogenesis is progressively impaired, the ttk+/− larvae mount a two-stage response to Pwp2h depletion. Initially, the cells undergo a Tp53-mediated cell cycle arrest. However, as the synthesis of new proteins, including Tp53 and its targets, is progressively impaired, the cells invoke autophagy to prolong their survival.

The notion of the existence of a second type of programmed cell death, distinct from apoptosis, which emanates from catastrophic levels of autophagy, is a hotly debated topic [54]. Using TEM, we did not see any evidence of cell death in the IECs of ttk+/− larvae, even at 7–8 dpf just before the larvae die, affirming that the levels of autophagy induced in the IECs of ttk+/− larvae prolong cell survival rather than trigger cell death. We proved this by disrupting the formation of the early autophagosome by inhibiting the translation of atg5 mRNA. This resulted in the death of IECs in ttk+/− larvae and a markedly reduced lifespan.

As mentioned previously, ttk+/− larvae exhibit impaired development of the craniofacial cartilages, exocrine pancreas and brain, tissues that are often clinically abnormal in patients with certain human ribosomopathies, including Diamond-Blackfan anaemia and Schwachman Diamond syndrome [4]. Recently, two new zebrafish models of dyskeratosis congenita (DC) based on mutations in components of the H/ACA RNP complex were described [55,56]. Like ttk+/−, these mutants display impaired production of 18S rRNA and induction of Tp53 target genes, consistent with previous studies demonstrating that defects in ribosome biogenesis induce Tp53 activation and cell cycle arrest [41]. Moreover, hematopoietic stem cells in these mutants were depleted via a Tp53-dependent mechanism, providing a plausible explanation for why DC patients are susceptible to bone marrow failure [55,56]. In one of these mutants, the gut and craniofacial structures were also reported to be underdeveloped and, as observed in ttk+/−, these defects persisted on a Tp53 mutant background [55]. We speculate that the p53-independent features of this model of DC may be caused by elevated rates of autophagy. If so, and these findings are confirmed in human DC, it will be important to determine whether elevated autophagic activity contributes to prolonged cell survival prior to considering clinical interventions to limit this process.

There is currently a great deal of interest in the development of novel therapeutics that target the cancerous translation apparatus through the combined inhibition of ribosome biogenesis, translation initiation and translation elongation [5]. To avoid inadvertently prolonging cancer cell survival, these approaches could benefit from a detailed understanding of the mechanisms and cellular contexts that induce autophagy in response to ribosomal stress. While such insights may be forthcoming from studies performed on cell lines, it is likely that complementary experiments carried out in the context of an entire vertebrate organism, such as the zebrafish model introduced here, may also be fruitful.

Materials and Methods

Ethics statement

All experimental procedures on zebrafish embryos and larvae were approved by the Ludwig Institute for Cancer Research/Department of Surgery - Royal Melbourne Hospital Animal Ethics Committee.

Zebrafish strains and embryo collection

Zebrafish embryos were obtained from pair-wise matings of heterozygous ttk+/− setebos+/− and caliban+/− zebras, on the Tg(Xlaef1a1:GFP)263 (gutGFP) background and from ttk+/− heterozygotes carrying two mutant alleles of Tp53 (tp53+/−;tp53+/−;Tg(XlEef1a1:GFP)s854, [43] and raised at 28.5°C. ttk+/− was propagated on the Tg(ins:dsRed)f1085, Tg(fabr10:dsRed):zebraGFP+p2h− [2-CLIP] background [51]. The Tp53+/−;Tg(XlEef1a1:GFP)s854 line (gift of Thomas Look and David Lane) and tsc2+/- line were obtained through TILLING [40.43]. The tsc2 and pwp2h loci in zebrafish are both on chromosome 1 so in order to generate sufficient ttk−/−tsc2−/− compound mutants for analysis, we identified and in-crossed recombinants harbouring the two mutations in a cis configuration. To prevent melanization and maintain transparency, embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) in embryo medium. Imaging of live larvae was carried out using a LeicaM2 FLIII microscope after anaesthetizing with 200 mg/L benzocaine (Sigma-Aldrich) in embryo medium. All images were imported into CorelDRAWX4 (Corel Corporation, Ottawa, Ontario, Canada). Image manipulation was limited to levels, hue and saturation adjustments.

Histology and whole-mount in situ hybridisation

Histology was performed as described [27]. Mucins and other carbohydrates secreted by intestinal goblet cells were stained using alcan blue-periodic acid-Schiff reagent [27]. For WISH, larvae were processed as described [57,58] To generate pwp2h riboprobes
a cDNA template was amplified by RT-PCR. For primer sequences see Text S1. These were then transcribed using the digoxigenin DNA Labelling Kit (Roche Diagnostics) according to the manufacturer’s instructions. Hybridized riboprobes were detected using an anti-DIG antibody conjugated to alkaline phosphatase according to the manufacturer’s instructions (Roche Diagnostics). Larvae were imaged on a Nikon SMZ 1500 microscope.

Detection of cells in the S-Phase of the cell cycle and cell height determination
To identify cells in the S-phase of the cell cycle, the incorporation of bromodeoxyuridine (BrdU) by live larvae was analysed as described [27]. To measure cell height, images of sagittal histological sections were captured on a Nikon Eclipse 80i microscope and then analysed using MetaMorph Microscopy Automation & Image Analysis Software.

Genetic mapping and positional cloning of ttt450
For genetic mapping, ttt450 heterozygotes on the gutGFP background were crossed onto the polymorphic WIK strain. Mutant larvae were identified by craniofacial and intestinal defects visible at 96 hpf under brightfield and fluorescence illumination. Subsequent mapping was performed as described [29].

Sequence alignment and domain determination
Protein sequence alignment of Pwp2h from zebrafish, yeast, mouse and human was performed using the Simple Modular Architecture Research Tool (SMART) software.

Genotyping
A novel EcoRI restriction enzyme site created by the ttt450 mutation produced a restriction fragment length polymorphism (RFLP) that was exploited for genotyping. Primers were used to amplify a 653-base pair (bp) fragment spanning exons 9 to 11 containing the ttt450 mutation. For primer sequences see Text S1.

RNA preparation and Northern blot analysis
Total cellular RNA was prepared from WT and ttt450 larvae (120 hpf) by homogenizing 20–50 larvae in Solution D (4.2 M guanidinium thiocyanate, 25 mM NaCitrate, 30% Sarkosyl BDH NL30) as described [59]. Northern blot analysis was conducted on 2 μg samples using α-32P-labelled probes designed to hybridize to zebrafish 5’ETS, ITS1 and ITS2 sequences, which were PCR-amplified from genomic DNA using previously described primers [60]. Radioactive signals were detected using a Phosphorimager and Storm 820 scanner (Amersham Biosciences) and analysed using ImageQuant TL software.

Analysis of 18S and 28S rRNA levels
Solutions of total RNA extracted from WT and ttt450 larvae were analysed on an Agilent 2100 E-Bioanalyzer according to the manufacturer’s instructions.

Polysome fractionation
50–100 WT and ttt450 larvae at 96 hpf were resuspended in cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM KCl, 2.5 mM MgCl2, 1% Triton X-100, 0.5% sodium deoxycholate, 3 mM DTT) containing 120 U/mL RNase inhibitor (Invitrogen) and Complete Protease Inhibitor Cocktail (Roche) and sheared through a 23G needle. Cytoplasmic extract (2 mg) was loaded onto a continuous low salt (80 mM NaCl) 3.1–30.1% (w/v) sucrose gradient (14 mL) [61] generated using an ISCO gradient maker. Samples were separated by centrifugation using a SW41 rotor at 40000 rpm for 4 h at 4°C, and fractionated (1 mL) using a Foxy Jr fraction collector. Absorbance at 260 nM was determined with an ISCO UA-6 absorbance detector. In each case, quantititation of 40S, 60S, and 80S was performed by measuring the area under the relevant peak using Metamorph Image Analysis Software.

Transmission electron microscopy (TEM)
For TEM, larvae were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 2 h at R.T, rinsed in 0.08 M Sorensen’s Phosphate buffer pH 7.4 and then stored in 0.08 M Sorensen’s buffer with 5% sucrose. Post-fixation was with 2% osmium tetroxide in PBS followed by dehydration through a graded series of alcohols, 2 ace tone rinses and embedding in Spurrs resin [62]. Sections approximately 80 nm thick were cut with a diamond knife (Diatome, Switzerland) on a Ultracut-S ultramicrotome (Leica, Mannheim, Germany) and contrasted with uranyl acetate and lead citrate. Images were captured with a Megaview II cooled CCD camera (Soft Imaging Solutions, Olympus, Australia) in a JEOL 1011 TEM. Transverse sections were obtained through the anterior intestinal region known as the intestinal bulb.

Immunocytchemistry
For transverse sections, embryos were fixed in 2% paraformaldehyde overnight at 4°C, embedded vertically in 4% low melting temperature agarose (Cambrex BioScience, East Rutherford, NJ) in disposable cryomolds (Sakura Finetek, Torrance, CA), and sectioned at 200 μm intervals using a Leica (Solms, Germany) VT1000S vibrating microtome. Floating sections were transferred to the wells of a 24-well plate containing PBS (PBS containing 0.1% Tween-20 and 0.5% Triton-X) and then replaced with antibody blocking solution (PBS containing 1% (w/v) BSA and 1% (v/v) FCS) for 2 h at RT. The blocking solution was removed and the sections incubated with LC3B primary antibody diluted to 1:500 in PBS containing 0.2% (w/v) BSA at 4°C overnight. The sections were rinsed three times in PBS (PBS containing 0.1% Tween-20) for 20 min at RT, followed by antibody blocking solution for 2 h at RT. The sections were then incubated overnight at 4°C in PBS containing 0.2% (w/v) BSA, Alexa

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Fluor 488 (1:500), rhodamine-phalloidin (1:150; Biotium, Hayward, CA) and 5 μg/mL Hoechst33342 (Sigma Aldrich). Sections were rinsed three times in PBST for 20 min at RT prior to imaging on an Olympus FV1000 scanning confocal microscope. Enumeration of LC3 puncta was performed using Metamorph. Details of antibodies and stains are available in Text S1.

Western blot analysis

Larvae were lysed (2 μL per embryo) in cold RIPA cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) containing Complete Protease Inhibitor Cocktail (Roche) and sheared through a 23G needle. Lysates were incubated on ice for 30 min and then centrifuged for 20 min at 13,000 rpm at 4°C to pellet nuclei and cellular debris. Samples containing 40–80 μg of protein were heated to 95°C for 5 min with 5X Protein Loading Dye (0.03 M Tris-HCl, pH 6.8, 13.8% glycerol, 1% SDS, 0.05% bromophenol blue, 2.7% β-mercaptoethanol) and loaded onto a 12% polyacrylamide gel. The proteins were transferred to PVDF membranes using an iBlot Gel Transfer Device (Invitrogen) according to the manufacturer’s instructions. For RPS6, p-RPS6, LC3 and Actin, subsequent blocking, antibody incubation and membrane exposure were performed using the Odyssey system (LI-COR Biosciences). For Tp53, blocking and antibody incubation were performed in PBST/5% skim milk powder and membranes developed using the SuperSignal West Femto Chemilluminescent Substrate (Thermo Scientific). Signals were quantitated by densitometry and expressed as relative levels by reference to the level in untreated WT larvae, which was set at 1. Details of antibodies are provided in Text S1.

Expression of mCherry-LC3 fusion protein

DNA encoding the fluorophore mCherry fused to the N terminus of LC3 was PCR amplified and transcribed into mRNA using the mMessage mMachine SP6 kit (Ambion Life Technologies, Mulgrave, Australia). For primer sequences see Text S1. mRNA (400 pg) was injected into the yolk of 1–4 cell stage embryos and exposed to 2.5 μM chloroquine (Fluka Sigma-Aldrich, Sydney, Australia) in embryo medium for 14 h at various time-points during development prior to mounting in 1.5% low melting point agarose for imaging with an Olympus FV1000 scanning confocal microscope.

Drug treatment

Live WT, tilapia, tilapia, tilapia larvae were exposed to 2.5 μM chloroquine and/or 10 μM rapamycin in embryo medium at 28°C. Larvae were collected 14 h later for protein extraction and Western blot analysis of LC3II levels as described above.

Knockdown of Pwp2h and Atg5 protein expression

Antisense morpholino oligonucleotides (MOs) targeted to the initiation of translation codons of pwp2h or atg5 mRNA were injected into the yolk of 1–4 cell stage WT or tilapia embryos. 2 nL of MO at a concentration of 120 x 10^−11 mol (total = 1 ng) and 180 x 10^−11 mol (total = 15 ng) were used to knockdown atg5 and pwp2h mRNA translation, respectively. For MO sequences see Text S1.

Quantification of autophagosomes

Using immunocytochemical analysis, LC3II-containing autophagosomes were identified as puncta in thick transverse sections of tilapia larvae. Puncta in 20 cells in 3 independent sections were counted using Metamorph. For TEM sections, the numbers of autophagosome-like structures in 20 cells in 3 independent sections were counted manually.

Quantitative reverse transcription polymerase chain (qRT–PCR)

cDNA was reverse transcribed from total RNA (1–2 μg) extracted from WT and tilapia larvae at 96 hpf using the Superscript III First Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. qRT-PCR was performed using the SensiMix SYBR Kit (Bioline) according to the manufacturer’s instructions. For primer sequences see Text S1.

Statistical methods

Student’s t-test was used to compare the means of two populations in Graphpad Prism 5.0. Error bars represent the mean ±/− standard deviation (n=3). A P value<0.05 was used to define statistical significance.

Supporting Information

Figure S1 tilapia larva contain fewer replicating IECs than WT larvae. (A) Sagittal sections of the intestine of WT and tilapia zebrafish larvae at 72 hpf showing cells that accumulated BrdU (black arrows) during a 30 min exposure to this thymidine analogue at 72 hpf. BrdU-positive nuclei (brown) indicate cells in the S-phase of the cell cycle. Scale bars = 50 μm. (B) Quantitation of BrdU-positive IECs in independent sagittal sections of WT and tilapia larvae at 72 hpf reveals that tilapia larvae contain approximately 50% fewer S-phase IECs than WT. *p<0.05. Data are represented as mean ±/− SD. (TIF)

Figure S2 pwp2h is the mutated gene in tilapia. (A) Sequence of pwp2h in WT and tilapia cDNA reveals that tilapia larvae utilize a cryptic splice site in exon 10 due to a mutation in the splice acceptor site in intron 9. This results in an 11 bp deletion (bracket) which causes a frame-shift in the pwp2h coding sequence resulting in 13 aberrant amino acids and a premature stop codon in exon 10. (B, C) Upon microinjection into the yolk of 1–4 cell WT zebrafish embryos, a pwp2h-targeted MO (15 ng) produces a robust tilapia phenotype at 120 hpf (C). Vehicle-injected controls appear WT (B). (D–G) Non-complementation of 2 independent pwp2h alleles confirms that pwp2h is the mutated gene in tilapia. Heterozygous tilapia carriers were crossed with heterozygous carriers of s927, an independent pwp2h allele identified in the 2-CLIP screen [30]. One quarter of the offspring are compound tilapia, tilapia mutants (E) and exhibit the tilapia phenotype (F) at 120 hpf including impaired development of the digestive organs, eye and craniofacial structures. Other panels show WT (D) and tilapia mutant (G) larvae at 120 hpf. These data indicate that both alleles correspond to the same genetic locus. e, eye; ib, intestinal bulb; sb, swim bladder; y, yolk. (H) The nucleotide sequence of pwp2h cDNA generated from tilapia larvae contains a T→A transversion (arrow). (I) The base change in tilapia results in a highly conserved branched amino acid codon (valine, shaded blue) being replaced by glutamic acid. Alignment was performed using ClustalW. (TIF)

Figure S3 Alignment of human, mouse, zebrafish and yeast Pwp2h protein sequences. Zebrafish Pwp2h protein comprises 937 amino acids, compared with 919 in human and mouse and 923 in yeast. WD domains are highly conserved (shaded in blue). The position of the amino acid change in tilapia larvae occurs at amino acid 113 in the 2nd WD domain (red box). The position where the
frame-shift occurs in $tt^{450}$ is indicated (red arrow) as is the position of the premature stop codon (red star). Sequences used: human (Homo sapiens) NP_003040.2; mouse (Mus musculus) NP_058322.1; zebrafish (Danio rerio) NP_998212.1; yeast (Saccharomyces cerevisiae) NP_009904.1.

**Figure S4** LC3II-containing autophagosomes are found in multiple tissues in $tt^{450}$ larvae at 72 hpf and 120 hpf. (A-H) RNA encoding a mCherry-LC3 fusion protein was injected into the yolk of 1–4 cell zebrafish embryos derived from a pairwise mating of $tt^{450}$/+ heterozygotes (on the gptGFP background) and allowed to develop until the indicated time-point in the presence of chloroquine for the final 14 h. Maximum intensity projection images of a z series of confocal sections through WT [A, A’ (boxed area in A), C, E, E’ (boxed area in E) and G] and $tt^{450}$ larvae [B, B’ (boxed area in B), D, F, F’ (boxed area in F) and H] showing accumulated autophagosomes (red puncta) in the brain, eye and digestive organs (marked by GFP fluorescence in C, D) at 72 hpf (boxed area in A), C, E, E’ and 120 hpf (E-H). Scale bars = 50 μM. b, brain; e, eye; ib, intestinal bulb; f, fin; y, yolk; p, pancreas.

**Figure S5** Up-regulated autophagy is not a shared feature of all zebrafish intestinal mutants. (A) Western blot analysis of LC3 in protein extracts of WT, setebos (set$^{450}$) and caliban (clb$^{460}$) larvae. Actin was used as a loading control. (B) The levels of LC3II were quantitated by densitometric analysis of three independent Western blots. Chloroquine-treated set$^{450}$ larvae at 96 hpf contain significantly higher LC3II levels compared to their chloroquine-treated WT siblings; meanwhile, LC3II levels are similar in chloroquine-treated clb$^{460}$ larvae and WT larvae treated with rapamycin and chloroquine. There are no significant differences between LC3II levels in clb$^{460}$ larvae and their WT siblings at 120 hpf, in the presence and absence of chloroquine. Data are represented as mean $\pm$ SD (n = 3), $p$<0.05. (C-H) Transmission electron micrographs of transverse sections of WT (C, E, G) and $tt^{450}$ larvae (D, F, H) through the intestinal bulb region at 120 hpf. There are negligible numbers of autophagosomes/autolysosomes in the IECs of WT and clb$^{460}$ larvae. Scale bars $= 50 \mu M$ (C, D); $10 \mu M$ (E, F); $5 \mu M$ (G-H); ib, intestinal bulb; n, nucleus; m, mitochondria; mv, microvilli.

**Figure S6** Absence of dead cells in the intestinal lumen of WT and $tt^{450}$ larvae at 7 dpf. (A-F) Transmission electron micrographs of transverse sections of WT and $tt^{450}$ larvae at 168 hpf (7 dpf). The number of conspicuous autophagosome-like structures in the IECs of $tt^{450}$ larvae has diminished by 7 dpf and there are no dead cells in the lumen (D). Meanwhile, liver cells of $tt^{450}$ larvae contain abundant autolysosome-like structures at this time-point (F, white arrows). Scale bars $= 50 \mu M$ (A, B); $10 \mu M$ (C–F), ib, intestinal bulb; n, nucleus; m, mitochondria; mv, microvilli; l, liver; bd, bile duct; a, arteriole.

**Figure S7** Disruption of autophagy in $tt^{450}$ larvae results in severe oedema. Upon microinjection into the yolk of 1–4 cell WT and $tt^{450}$ zebrafish embryos, an aeg3-targeted MO (1 ng) produces severe oedema around the organs of $tt^{450}$ larvae at 120 hpf (D), while WT larvae are unaffected (C). WT and $tt^{450}$ larvae injected at the 1–4 cell stage with vehicle (A, B) are also unaffected.

**Figure S8** Autophagic flux in $tt^{450}$ larvae is not abrogated by Tor pathway activation. (A–D) Enhancing Tore1 activity by ablating Tsc2 activity in $tt^{450}$ larvae does not change their gross morphology at 120 hpf. Compound mutants ($tt^{450}$/Tsc2$^{924/242}/a^{42}$, D) are essentially indistinguishable from $tt^{450}$ larvae (C). Other panels show WT (A) and Tsc2$^{924/242}/a^{42}$ mutant (B) larvae. (E,F) Western blot analysis of p-RPS6 and LC3 demonstrates that $tt^{450}$/Tsc2$^{924/242}/a^{42}$ compound mutants at 96 hpf contain higher levels of p-RPS6 than $tt^{450}$ mutants due to increased Tor activity, yet LC3II levels are comparable between the two genotypes (refer to right hand half of the Western blot, where the larvae were pre-treated with chloroquine). p-RPS6 and LC3II levels are not significantly different between WT and Tsc2$^{924/242}/a^{42}$ larvae in the presence of chloroquine. Actin was used as a loading control. The levels of LC3II were quantitated by densitometric analysis of three independent Western blots. Data are represented as mean $\pm$ SD, $p$<0.05.

**Text S1** Sequences of primers and morpholinos and additional antibody information.

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