Tumor Protein Tctp Regulates Axon Development in the Embryonic Visual System

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SUMMARY

The transcript encoding translationally controlled tumor protein (Tctp), a molecule correlated with aggressive breast cancers, was identified among the most abundant in genome-wide screens of axons, suggesting that Tctp is important in neurons. Here, we tested the role of Tctp in retinal axon development in *Xenopus laevis*. We report that Tctp deficiency results in stunted and splayed retinotectal projections that fail to innervate the optic tectum at the normal developmental time due to impaired axon extension. Tctp-deficient axons exhibit defects associated with mitochondrial dysfunction and we show that Tctp interacts in the axonal compartment with myeloid cell leukemia 1 (Mcl1), a pro-survival member of the Bcl-2 family. Mcl1 knockdown gives rise to similar axon misprojection phenotypes, and we provide evidence that Tctp’s anti-apoptotic activity is necessary for the normal development of the retinotectal projection. The findings suggest that Tctp supports the development of the retinotectal projection via its regulation of pro-survival signalling and axonal mitochondrial homeostasis, and establish a novel and fundamental role for Tctp in vertebrate neural circuitry assembly.
INTRODUCTION

Motility and invasiveness are traits central to malignancy and growth cone migration alike. In fact, from the associated changes in adhesion to the build-up of protrusive actin dynamics, or the continuous interaction with the surrounding environment, the initial challenges experienced by a metastatic cancer cell resemble in many ways the obstacles overcome by a navigating growth cone as it progresses through the embryonic brain. Curiously, the four families of guidance cues classically associated with axon guidance – ephrins, semaphorins, netrins and slits, and respective receptors (Tessier-Lavigne and Goodman, 1996) – have emerged as important regulators of cancer progression, in particular during the phases of primary tumor growth and dissemination (Mehlen et al., 2011; Pasquale, 2010; Tamagnone, 2012), suggesting that common signalling may operate in both contexts. Indeed, frequent mutations and copy-number variations were recently discovered in axon guidance genes in tumours derived from patients diagnosed with pancreatic ductal adenocarcinoma (Biankin et al., 2012) and liver fluke-associated cholangiocarcinoma (Ong et al., 2012), and several independent genome-wide screens have found cancer-linked transcripts to be well-represented in axonal mRNA populations (Andreassi et al., 2010; Gumy et al., 2011; Zivraj et al., 2010).

tpt1 transcripts, which encode for Tctp, are ranked among the most enriched in the axonal compartment across diverse embryonic and adult neuronal populations, including retinal ganglion cells (Andreassi et al., 2010; Gumy et al., 2011; Taylor et al., 2009; Zivraj et al., 2010). Tctp is an evolutionarily conserved protein implicated in cell growth (Hsu et al., 2007; Kamath et al., 2003), and particularly well studied in cancer pathogenesis (Amson et al., 2012; Kaarbo et al., 2013; Tuynder et al., 2002).
Initially discovered as an abundant mRNA in untranslated, partially suppressed messenger ribonucleoprotein particles in mouse sarcoma ascites cells (Yenofsky et al., 1982), Tctp was subsequently characterized as a protein whose rate of synthesis is greatly enhanced in growing versus non-growing Ehrlich ascites tumor cells (Benndorf et al., 1988; Bohm et al., 1989). Tctp has since been shown to be involved in cellular functions as diverse as DNA damage (Zhang et al., 2012), cell proliferation (Chen et al., 2007) and allergy responses (MacDonald et al., 1995). In addition, Tctp plays an essential, but still not fully understood, role in development – indeed, loss of tpt1 expression in mice results in increased apoptosis and embryonic lethality (Chen et al., 2007; Susini et al., 2008). Tctp has been shown to interact with the anti-apoptotic oncoproteins myeloid cell leukemia 1 (Mcl1) and Bcl-2-like protein 1 (Bcl-X<sub>L</sub>) (Liu et al., 2005; Yang et al., 2005; Zhang et al., 2002), and to prevent Bcl-2-associated protein X protein (Bax) homodimerization in the mitochondrial outer membrane (Susini et al., 2008). Notably, tpt1 mRNA expression is detected in many areas of the adult human brain (Thiele et al., 2000), and Tctp protein levels are downregulated in the temporal cortex of Alzheimer’s disease patients (Kim et al., 2001), suggesting that its expression in the central nervous system remains important after development.

Here, motivated by the parallels between axon growth and cancer cell invasion, we have investigated the role of cancer-associated Tctp in the context of neural connectivity using Xenopus laevis larvae as an in vivo model system. We report that Tctp functions as a checkpoint for the normal development of the retinotectal projection. Our results also reveal that mitochondrial function and distribution are affected in axons deficient for Tctp. Finally, we link Tctp to the survival machinery of
the axon via its interaction with Mc11, an anti-apoptotic member of the Bcl-2 protein family.
RESULTS

Expression of \textit{tpt1} in the neural retina

Immunohistochemistry using an antibody raised against the \textit{X. laevis} protein revealed that Tctp is broadly present in the retina, including the ganglion cell layer (GCL) and the optic fibre layer (OFL) (Fig. 1A; Bazile et al., 2009). A strong positive signal is also evident in the optic nerve head (Fig 1A), where retinal ganglion cell (RGC) axons collect to exit the eye, and in RGC axons and growth cones \textit{in vitro} (Fig. 1B). \textit{In situ} hybridization (ISH) showed a similarly broad expression in the retina that was surprisingly robust in the OFL and optic nerve head (ONH) indicating the presence of \textit{tpt1} mRNA in retinal axons \textit{in vivo} (Fig. 1C; supplementary material Fig. S1A,B). This was confirmed by fluorescent ISH on retinal axons and growth cones \textit{in vitro} (Fig. 1D,E; supplementary material Fig. S1C). The inner and outer plexiform layers (IPL and OPL, respectively) were also positive for Tctp protein and mRNA, suggestive of widespread localization in the neurites of retinal neurons (Fig. 1A, 1C). Apart from the retinal neuropil, \textit{tpt1} expression was observed in the photoreceptor layer and the ciliary marginal zone (CMZ), a well-characterized retinal neurogenic niche (Fig. 1A, 1C).

The human \textit{tpt1} gene is transcribed into two distinct mRNA variants that differ only in the length of their 3’ untranslated regions (UTRs) (Thiele et al., 2000). Since most mRNA regulatory elements are situated within the 3’ UTRs (Martin and Ephrussi, 2009), we investigated whether \textit{tpt1} is regulated in an analogous manner in the \textit{X. laevis} retina. Using rapid amplification of 5’ and 3’ cDNA ends (5’ and 3’ RACE), two 3’UTR variants of \textit{tpt1} were obtained from eye RNA extracts, comprising a short (\textit{tpt1}-S, 210 bases) isoform and a longer (\textit{tpt1}-L, 607 bases) version, overlapping in its entirety the short form and possessing a unique stretch at its 3’ end (Fig. 1F). Similar to
Homo sapiens, the exon specifying the 3’UTR in X. laevis contains two alternative polyadenylation signals, resulting in transcripts with 3’UTRs of different length but encoding the same protein (Fig. 1G). A single 5’ end was identified, and, as described in H. sapiens, sequencing it in its entirety revealed the existence of a 5’-terminal oligopyrimidine (TOP) motif previously not annotated in X. laevis (supplementary material Fig. S1E).

Differential processing at alternative polyadenylation sites is known to be physiologically regulated in development or by pathological events such as cancer, and can affect the localization and translational properties of the mRNA (Di Giammartino et al., 2011). For example, the longer importinb1 transcript, equally arising from alternative polyadenylation, harbours a signal that enables axonal localization (Perry et al., 2012). We thus explored whether tpt1 localization in RGC axons is governed in a similar manner. To this end, we used laser-capture microdissection (LCM) to harvest axonal extracts (Zivraj et al., 2010) (Fig. 1H; supplementary material Fig. S1D). To determine the purity of our pool of axonal mRNAs, we tested for the presence of mRNAs encoding nuclear proteins, such as histone H4 (hist1h4a), and for transcripts described in dendrites but not in axons, such as microtubule-associated protein-2 (map2). No such amplification products were detected by reverse transcription-PCR (RT-PCR; Fig. 1J). By contrast, actb, which encodes for β-actin, a transcript previously identified in axons and growth cones (Bassell et al., 1998; Leung et al., 2006), was readily amplified (Fig. 1J). Significantly, tpt1 sequence reads from 5’ and 3’ RACE reactions using RGC axonal extracts were identical to those obtained from whole eye preparations, implying that both isoforms localize in these axons (Fig. 1I).
We next employed quantitative RT-PCR (RT-qPCR) to complement our analysis. We designed two sets of primers: one directed to a segment of the \textit{tptl} protein coding region, thus allowing for an expression readout on both mRNA variants, and a second pair targeting part of the unique region of \textit{tptl}-L (Fig. 1K). In whole eye extracts, an approximately constant 9:1 \textit{tptl}-S to \textit{tptl}-L ratio was obtained in all developmental stages examined. Interestingly, there was a ~16:1 \textit{tptl}-S to \textit{tptl}-L ratio in axonal extracts, indicating that the \textit{tptl}-S variant is locally enriched in the axonal compartment (Fig. 1L). Moreover, we detected a near 10-fold ($\Delta$Cq$_{\text{tptl:actb}} = 3.1$) enrichment over \textit{actb} mRNA, a known axonally enriched mRNA, confirming \textit{tptl} as a highly abundant axonal transcript (Fig. 1M).

**\textbf{Tctp is required to establish correct axonal projections in vivo}**

We next assessed whether Tctp plays a role in retinal axon guidance. To inhibit \textit{tptl} mRNA translation \textit{in vivo}, we used an antisense morpholino oligonucleotide directed against the start site of \textit{tptl} mRNA (\textit{tptl}-MO), which was delivered at the four-cell stage by injection into both dorsal blastomeres (Fig. 2A). In doing so, we targeted both \textit{tptl}-S and \textit{tptl}-L transcripts throughout the central nervous system. Western blot analysis validated the efficient knockdown in Tctp levels (ca. 50% in brain and eye lysates; $P = 0.041$, unpaired \textit{t}-test) (Fig. 2B). Similarly, we observed a 40-60% decrease in Tctp protein expression in \textit{tptl}-MO-positive RGC growth cones ($P < 0.0001$, Mann-Whitney test), demonstrating that the axonal pool of Tctp is targeted by this approach (Fig. 2C). At the morpholino dosage used, Tctp morphants appeared morphologically normal, with no overt delays in development, albeit showing in most individuals small decreases in eye size (an average of 10%, ** $P = 0.0063$, unpaired \textit{t}-test; supplementary material Fig. S2A-D). Of note, we titrated a morpholino dosage
capable of achieving a level of expression knockdown comparable in magnitude to that of $tpt1^{+/−}$ mice, which are reported to be viable and fertile, unlike homozygous $tpt1^{+/-}$ pups (Chen et al., 2007; Susini et al., 2008).

We analysed RGC axon trajectories by anterograde lipophilic dye (DiI) labelling at stage 40 (~3 day-old larvae), when the pioneer population of axons have completed their growth through the optic tract and arrived in the optic tectum (Holt and Harris, 1983). Whereas control projections consistently coursed a normal trajectory and reached the target region by this stage (Fig. 2D), most age-matched Tctp morphants exhibited significantly shorter projections that failed to enter the optic tectum (Fig. 2E,G,H). Additionally, instead of forming the compact axonal bundle typical of normal projections, RGC axons in Tctp morphants grew in a dispersed fashion straying inappropriately into territories in the diencephalon and telencephalon. Indeed, the optic tract in Tctp morphants was on average ~21 μm wider than in controls (Fig. 2E,I). Restoring the levels of Tctp with a morpholino-resistant $tpt1$ mRNA in $tpt1$-MO-injected embryos completely rescued the development of the retinotectal projection both in terms of tract length and tract width, demonstrating that the phenotypes are specific to the loss of Tctp function (Fig. 2F-I; supplementary material Fig. S2E,F). Collectively, these data demonstrate that Tctp is necessary for the accurate and timely development of the retinotectal projection.

**Tctp promotes axon extension in vivo**

The shortened axon projection phenotype in Tctp morphants could arise from a general delay in eye development or a decrease in the rate of RGC axon extension. To distinguish between these possibilities, we first examined the histology of the retina.
Overall, although some disorder in the neuropil and an increase in cell death in the ganglion cell layer were noted, the gross morphology and stratification of the retina appeared unaffected in Tctp morphants, suggesting no major delay in development (Fig. 3A; supplementary material Fig. S3A-D).

Marked alterations were evident, however, in the photoreceptor layer of Tctp morphants (Fig. 3A). Prompted by the possibility that this defect might provide insight into Tctp’s action, we further evaluated the photoreceptor phenotype. Briefly, the photoreceptor outer segment is an apical structure densely packed with discs of folded membranes containing light sensitive photopigments (opsins), whereas the inner segment, which lies between the outer segment and the nuclear layer, is dedicated to sustaining the energy and protein synthesis needs of the photoreceptor (Wright et al., 2010). First, using opsin markers and a nuclear stain, we pinpointed the localization of Tctp to the mitochondria-rich inner segments of both cone and rod photoreceptors (Fig. 3B,C). Our subsequent analysis revealed that photoreceptors in Tctp morphant retinas have shorter inner segments (cones: 13.7 μm versus 16.1 μm in controls; rods: 11.8 μm versus 15.8 μm in controls), and showed a complete loss of the outer segment in significant proportion of cones (35% versus 13% in controls) and rods (27% versus 5% in controls) (Fig. 3D-G). Collectively, these data indicate that while Tctp is not essential for the timely development of the retina, it lends an unexpected contribution to photoreceptor maintenance.

To measure directly the rate of axon growth in vivo, we made time-lapse movies of control and morphant axons using eye-targeted electroporation to deliver gap-RFP (a membrane-targeted version of RFP) (Fig. 4A). Overall, Tctp-depleted axons were
significantly slower than control axons, advancing through the optic tract at about half the speed (ventral optic tract: 16.5 μm/hour versus 34.4 μm/hour; dorsal optic tract: 16.1 μm/hour versus 27.8 μm/hour) (Fig. 4B-D). In addition, 40% of the morphant axons analysed (33 of 82 axons) stalled along the optic tract, a significantly higher proportion than in control samples (our analysis parameters classified ca. 6% of control axons as ‘stalled’; Fig. 4E). As the fixed DiI-samples had suggested, time-lapse imaging confirmed that axonal growth in Tctp morphants was dispersed and erratic, which translated into significantly wider projections relative to controls (Fig. 4F). Lastly, we tested whether the tortuous trajectories associated with defective pathfinding could account for the shortened axon tract phenotype detected in Tctp morphants by including only normally projecting axons in our analysis. We found that normally projecting Tctp-depleted axons still extended through the optic tract at significantly slower average rates than controls (supplementary material Fig. S3E). Collectively, these findings strongly indicate that Tctp regulates retinal axon growth.

The retinotectal projection develops unerringly in Tctp-deficient brains

Tctp exhibits immunoglobulin E-dependent histamine-releasing activity and other cytokine-like extracellular functions (Kim et al., 2013; MacDonald et al., 1995). It could therefore work in the embryonic environment to promote axon development. To address this possibility, we injected morpholinos into only one of the first two dorsal blastomeres, leading to embryos in which one half of the central nervous system is depleted in Tctp, while the other is wild-type (Fig. 5A-C). Since RGC axons cross the midline at the optic chiasm and project contralaterally, this strategy enables us to test the contribution of the optic tract pathway substrate. Embryos injected with con-MO consistently developed normal projections in both backgrounds, verifying the
suitability of the strategy (Fig. 5D,F). Significantly, Tctp-depleted (*tpt1*-MO-positive) retinal axons navigating into the contralateral normal (*tpt1*-MO-free) hemisphere (Eye-MO:Brain-wild type) replicated the same range of phenotypes observed in global Tctp morphants (Fig. 5E). By contrast, normal (*tpt1*-MO-negative) RGC axons projecting into the contralateral Tctp-depleted (*tpt1*-MO-positive) side of the brain (Eye-wild type:Brain-MO) showed no defects (Fig. 5G-I, supplementary material Fig. S2G). Collectively, these findings show that the retinotectal projection can develop unerringly through a Tctp-depleted optic tract neuroepithelium, and indicate that the axonal phenotype of morphant retinal axons in the optic pathway is independent of Tctp acting extracellularly.

**Tctp knockdown compromises axonal mitochondrial function**

To begin to investigate the mechanism of how Tctp regulates axon growth, we focused on mitochondria. Tctp is documented as part of the mitochondrial proteome (Fountoulakis et al., 2002; Rezaul et al., 2005), and its expression in the brain is downregulated in pathologies associated with mitochondrial abnormalities such as Alzheimer’s disease and Down syndrome (Kim et al., 2001; Nunnari and Suomalainen, 2012; Pagano and Castello, 2012). Furthermore, photoreceptor degeneration is frequently characterized by bioenergetic decline (Wright et al., 2010), and mitochondrial dysfunction is reported in a number of retinal diseases, including photoreceptor-specific age-related macular degeneration (Alexander et al., 2000; Delettre et al., 2000; Wallace et al., 1988; Wright et al., 2010). First we asked whether the overall metabolic status was changed in Tctp morphant retinas using a bioluminescence ATP assay (Agathocleous et al., 2012). Remarkably, the energy content in Tctp-depleted retinas was found to be on average 30% lower relative to
controls (Fig. 6A). We next measured the mitochondrial membrane potential ($\Delta \Psi_m$), a cardinal indicator of mitochondrial function, in retinal explant cultures. Notably, in Tctp morphants, we found a significantly lower accumulation of the cationic fluorescent probe TMRM in the mitochondria-rich growth cone central domain (~20% with respect to control), denoting $\Delta \Psi_m$ depolarization. Analysis of individual mitochondria distributed throughout the axonal compartment showed a comparable $\Delta \Psi_m$ reduction in Tctp morphants (Fig. 6B-D). A significant decrease in the number of axonal mitochondria was also noted in these experiments, which was confirmed in vitro with a mitochondrial stain (ca. 30% fewer mitochondria) and in vivo by co-labelling RGC axons with mitochondrion-targeted GFP (mt-GFP) and gap-RFP (ca. 23% fewer mitochondria) (Fig. 6E,F; supplementary material Fig. S4A). The average length of axonal mitochondria was not different from controls (supplementary material Fig. S4B). Collectively, these results show a significant reduction in mitochondrial density in Tctp-depleted axons, as well as a decrease in axonal mitochondrial function and global energy levels.

Since new mitochondria are generated in the neuronal soma, being transported from there to the cell periphery (Sheng and Cai, 2012), we reasoned that the decrease in axonal mitochondrial density observed in Tctp morphants could arise through impaired global mitochondrial biogenesis. We documented comparable mitochondrial DNA copy numbers (i.e. the ratio of mitochondrial to nuclear DNA) in Tctp-depleted retinas, as evaluated by quantitative PCR (Fig. 7A). Western blot analysis of Tctp-depleted tissues showed, in addition, unaltered expression levels of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (Pgc1α), a master inducer of mitochondrial biogenesis and regulator of mitochondrial density in neurons (Wareski
et al., 2009) (Fig. 7B; supplementary material Fig. S4C-E). In agreement with these findings, the expression levels of the nuclear-encoded mitochondrial genes examined (\textit{idh3a}, isocitrate dehydrogenase 3 (NAD$^+$) alpha; \textit{cox5a}, cytochrome c oxidase subunit Va; \textit{cysc}, cytochrome c, somatic; \textit{miro1}, mitochondrial Rho GTPase 1) were unchanged relative to control retinas (Fig. 7C,D). Additionally, we detected similar \textit{cox5a} mRNA expression levels in the ganglion cell layer and in the inner plexiform layer (made up of RGC dendrites and processes of other retinal neurons) in both backgrounds (Fig. 7E). Taken together, these data strongly indicate that mitochondrial biogenesis and mass are unaffected in Tctp morphants.

Having excluded impaired mitochondrial biogenesis, and because mitochondrial transport depends on mitochondrial function (Miller and Sheetz, 2004; Rintoul et al., 2003; Zala et al., 2013), we next investigated whether Tctp deficiency affects mitochondrial dynamics in axons. Analysis of 5-minute time-lapse movies of labelled mitochondria showed a higher proportion of mitochondria moving in the retrograde direction (8.7% versus 1.9% in controls, \( P = 0.0358 \)) and fewer mitochondria moving anterogradely (21.7% versus 35.2% in controls, \( P = 0.0353 \)). In addition, the mean net displacement of mitochondria, including stationary, anterogradely, and retrogradely trafficked organelles, was smaller in Tctp-depleted axons, although the bias was still in the anterograde direction (on average, each mitochondrion moved distally +4.4 \( \mu m \), compared to +8.9 \( \mu m \) in controls; Fig. 7F-H). However, we found that the velocity of mitochondrial transport in the anterograde and retrograde directions, as well as the frequency and duration of mitochondrial pauses, were not significantly different between both groups (Fig. 7I-K; supplementary material Fig. S4F), suggesting that the mitochondrial transport machinery is not compromised in Tctp morphants.
Tctp acts via the survival machinery to promote axon development

Several studies indicate that Tctp interacts with members of the B cell lymphoma-2 (Bcl-2) family of proteins, which function as key mediators of mitochondrial integrity and apoptosis (Czabotar et al., 2014). Interestingly, the Bcl-2 family is implicated in many instances of photoreceptor disease (Chen et al., 1996; Nir et al., 2000; Yang et al., 2004), and embryonic sensory neurons depleted of Bcl-2, the prototypic member of this family, have reduced axon growth rates (Hilton et al., 1997), a phenotype we observe in Tctp morphants. Particularly well corroborated is Tctp’s association with Mcl1 (Liu et al., 2005; Yang et al., 2005; Zhang et al., 2002), a pro-survival Bcl-2-related factor linked to neuroprotection responses in the central nervous system (Mori et al., 2004), prompting us to explore a potential interaction between these proteins in neurons.

First, we investigated if Mcl1 is expressed in vivo by RGCs using an antibody raised against the *X. laevis* protein (Tsuchiya and Yamashita, 2011). Similar to Tctp, Mcl1 is expressed in the inner plexiform layer, the outer plexiform layer and the inner segment of photoreceptors. Mcl1 is also present in the ganglion cell layer, the optic fibre layer and the optic nerve head, indicating that Mcl1 localizes to RGCs and their axons in vivo (Fig. 8A; supplementary material Fig. S5A,B). In line with these data, Mcl1 was detected in the axonal and dendritic compartments of rat cortical neurons, confirming that, like Tctp, Mcl1 is present in neurites (supplementary material Fig. S5C,D). We next tested whether Tctp physically interacts in axons with Mcl1 using a proximity ligation assay (PLA) (Soderberg et al., 2006; Yoon et al., 2012). We used rat cortical neurons in these studies due to the availability of specific primary antibodies raised in
different hosts, a central requirement of this methodology. Positive Tctp-Mcl1 PLA spots were abundantly detected in the cell body, but also along the neurites of cortical neurons (E18.5 + 3 DIV) (Fig. 8B,C; supplementary material Fig. S6A), indicative of a close association between Tctp and Mcl1 (maximum working distance of the assay is in the range of 30-40 nm). We obtained an even more profuse signal in cultures aged in vitro for 14 days, suggesting that Tctp-Mcl1 interactions are not transient phenomena (supplementary material Fig. S6B). Of particular note, ca. 5-10% Tctp-Mcl1 positive puncta co-localised with mitochondria in neurites (supplementary material Fig. S6C). Collectively, these data validate previous biochemical reports and add a hitherto unexplored subcellular dimension to them, revealing that Tctp interacts with Mcl1 in the cell body and processes of neuronal cells.

Mechanistically, pro-survival members of the Bcl-2 family (e.g. Mcl1) operate by sequestering pro-apoptotic proteins (e.g. Bax), thus preventing the release of Cytochrome c from the mitochondrial intermembrane space and subsequent activation of caspases (Pease and Segal, 2014). Tctp is reported to stabilize and enhance Mcl1 biological activity (Liu et al., 2005), and promote the degradation of P53 (Amson et al., 2012; Rho et al., 2011), which itself neutralizes the pro-survival actions of Bcl-2 and Mcl1 at the mitochondria (Leu et al., 2004; Vaseva and Moll, 2009). We found using quantitative immunofluorescence that P53 expression was significantly upregulated in Tctp-depleted growth cones (Fig. 8D). In addition, we measured a 50% increase in active Caspase-3 mean signal relative to controls (Fig. 8E), consistent with a detrimental balance between pro- and anti-apoptotic signalling in Tctp morphants.
Finally, we investigated whether Tctp acts via Mcl1 and the survival machinery to promote axon development. The retinotectal projection in Mcl1 morphants was found to be significantly wider along the ventral optic tract relative to controls, and axons often extended erroneously into the telencephalon (Fig. 9A-D; supplementary material Fig. S6D). Furthermore, we detected outgrowth defects in subsets of axons coursing through the dorsal optic tract, although the absolute length of the projection was comparable to controls (Fig. 9C,E,F). We also noted a high frequency of degenerating axon profiles, distinguished by their beaded morphology, similar to that observed in Tctp morphants (Fig. 9B,F; supplementary material Fig. S7A-D). Overall, these results suggest that Tctp and Mcl1 are functionally related, albeit the qualitatively milder phenotypes detected in Mcl1 morphants. This may be due to compensation by other Bcl-2-related proteins, as our data indicate that Tctp also interacts with Bcl-X\textsubscript{L} in neurons (supplementary material Fig. S8A,B). To test directly whether Tctp’s pro-survival interactions are required for retinal axon development, we designed a mutated \textit{tpt1} rescue transgene encoding an N-terminally truncated Tctp protein lacking anti-apoptotic properties (Tctp\textsubscript{40-172aa}). Tctp\textsubscript{40-172aa} retains Tctp’s signature motifs and the interaction domains of several known Tctp-interacting proteins (Yang et al., 2005), but not those necessary for the association with Mcl1 and Bcl-X\textsubscript{L} (Yang et al., 2005; Zhang et al., 2002). Delivery of \textit{tpt1}\textsubscript{40-172} mRNA together with \textit{tpt1}-MO by blastomere microinjection failed to mitigate the effects of Tctp depletion on the development of the retinotectal projection both in terms of tract length and tract width (Fig. 9G-I; supplementary material Fig. S8C). Collectively, the findings are consistent with Tctp pro-survival actions being necessary for the normal development of the retinotectal projection.
DISCUSSION

Uncontrolled growth and heightened survival are hallmarks of malignancy, allowing cancer cells to out-compete their neighbours and eventually dominate tissues. Tctp has previously been associated with cell growth, including during bone development and cancer pathogenesis (Amson et al., 2012; Brioudes et al., 2010; Kaarbo et al., 2013; Miao et al., 2013; Zhang et al., 2008), and is suggested to function as a pro-survival factor through its interplay with the Bcl-2 protein family (Liu et al., 2005; Susini et al., 2008; Yang et al., 2005; Zhang et al., 2002). Thus, Tctp upregulation, as described in a variety of malignant tumours (Amson et al., 2012; Lo et al., 2012; Miao et al., 2013), likely reflects the growth and survival advantages Tctp confers to the cell. In line with the many parallels that can be drawn between the normal processes of migratory growth cones during axon development and the disease mechanisms of cancer cell invasion, here we document that Tctp regulates the development of the retinotectal projection by impacting on axon growth and guidance, and link Tctp to the survival machinery of the axon.

Tctp deficiency leads to multiple mitochondria-related abnormalities in axons, including a substantially diminished mitochondrial membrane potential and decreased mitochondrial density. This evidence indicates that axonal Tctp contributes to the maintenance of mitochondrial function in this subcellular domain. Unlike vesicular fast axonal transport, which is reliant on the glycolytic pathway for its energetic needs (Zala et al., 2013), the trafficking of mitochondria is dependent on ATP generated by oxidative phosphorylation (Rintoul et al., 2003; Zala et al., 2013). Thus, considering the disruption of the mitochondrial membrane potential observed in Tctp-depleted axons, a parameter that directly influences mitochondrial ATP production, the
defective accumulation of mitochondria at the neuronal periphery is perhaps a predictable outcome of compromised mitochondrial operation. Significantly, the general reduction in axonal mitochondrial density detected in these axons is not accompanied by alterations in mitochondrial biogenesis or mass, arguing that this deficit does not result from an inability of the neuron to generate mitochondria. While the potential involvement of axonal mitophagy was not addressed herein (Ashrafí et al., 2014), our analysis also indicates that more mitochondria are trafficked retrogradely in Tctp-depleted axons. Consistent with these findings, previously reported evidence indicates that dysfunctional mitochondria are selectively returned to the cell body for repair and/or degradation (Miller and Sheetz, 2004; Sheng and Cai, 2012). Hence, the insult to mitochondria in axons depleted of Tctp may, in effect, lead to a secondary perturbation on mitochondrial dynamics and an overall more prominent decline in axonal mitochondrial distribution.

How does Tctp promote mitochondrial function? Pro-survival members of the Bcl-2 protein family, such as Mcl1, work primarily by sequestering and neutralizing Bcl-2-related pro-apoptotic factors (e.g. Bax), which, if left uncontrolled, negatively affect the integrity of mitochondria (Shamas-Din et al., 2013). A fitting analogy would be a molecular tug-of-war between pro- and anti-apoptotic Bcl-2-related factors controlling mitochondrial homeostasis. According to the model put forward by Susini and colleagues, Tctp pro-survival activity results from its blocking Bax dimerization, a key mitochondrial outer membrane permeabilization (MOMP)-inducing event, by binding and reconfiguring Mcl1 and Bcl-XL in such a way that their inhibitory actions on Bax are promoted (Susini et al., 2008). Akin to the pivotal role of mitochondria in neutrophil chemotaxis (Bao et al., 2015), we speculate that compromised pro-survival
signalling in axons deficient in Tctp translates into mitochondrial dysfunction and a secondary decline in axonal mitochondrial density, ultimately resulting in an energy and Ca\(^{2+}\)-buffering state insufficient to sustain the normal processes of a growing axon. These effects would be particularly acute in the growth cone, an ATP-intensive distal outpost where mitochondria accumulate (Lathrop and Steketee, 2013), impairing its ability to adequately respond to guidance and growth-promoting cues in the embryonic environment.

In summary, the findings presented here suggest that Tctp functions as a checkpoint for the normal development of the retinotectal projection via its regulation of pro-survival signalling and axonal mitochondrial homeostasis. While the precise role(s) of mitochondria in axon growth and navigation are still unresolved, it will also be interesting in the future to investigate the possibility of axonal Tctp regulating mechanisms extrinsic to mitochondria. Indeed, considering the involvement of local caspase action in axon guidance and branching behaviours (Campbell and Holt, 2003; Campbell and Okamoto, 2013; Ohsawa et al., 2010), future work should address the contribution of dysfunctional caspase activation towards the axon development defects observed in Tctp morphant embryos.
MATERIALS AND METHODS

Embryos

*Xenopus laevis* embryos were obtained by *in vitro* fertilization, raised in 0.1X Modified Barth’s Saline at 14-18 °C, and staged following Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

Retinal cultures

Unless otherwise noted, eye primordia were dissected from anesthetized stage 32 larvae, and plated on culture dishes coated with poly-L-lysine (10 μg/mL, Sigma) and laminin (10 μg/mL, Sigma). Cultures were incubated at 20°C in 60% L15 minimal medium (Life Technologies) for 24 h before further manipulation.

Laser-capture microdissection

Per experiment, ~140 stage 33/34 eye explants were plated on polyethylene terephthalate slides, pre-coated with poly-L-lysine (10 μg/mL) and laminin (10 μg/mL), and cultured for 32-36 hours. Retinal cultures were fixed (4% paraformaldehyde, 4% sucrose in 1X PBS) for 10 minutes, dehydrated through an ethanol series, and air-dried before the microdissection procedure, as described previously (Zivraj et al., 2010). Extract purity was determined by RT-PCR.

Morpholino oligonucleotides

Antisense *tpi1*-MO (translation-blocking), *mcl1*-MO (splice-blocking), and control-MO were supplied by GeneTools: 5’-ATCATGTTGCGGCTAAGTGTTGT-3’, 5’-AGTAGAGTAAGCCATGCTACCCGT-3’, and 5’-CCTCTTACCTCAGTTACAATTTATA-3’, respectively.
**Dil labelling of retinal axons**

Embryos were fixed overnight at 4 °C in 4% paraformaldehyde in PBS and RGC axons labelled by intraocular injection of the fluorescent carbocyanine Dil. The contralateral (in respect to the dye-injected eye) brain hemisphere was later dissected, mounted in 1X PBS and visualized using confocal microscopy. Tract length was normalized to the distance between the optic chiasm and the posterior boundary of the tectum.

**In vivo imaging of axon pathfinding**

Live imaging of pathfinding retinal axons was performed as described elsewhere (Leung et al., 2013). Specimens were mounted in an imaging chamber constructed on oxygenated Permanox slides (Nunc). Images were acquired every 15 minutes during 2 hours. Axons were scored as ‘stalled’ if their outgrowth was ≤ 10 μm over the 2-hour period of analysis.

**Mitochondrial membrane potential assessment**

Retinal cultures were incubated with 20 nM tetramethylrhodamine, methyl ester (TMRM) at 20°C for 20 minutes and washed with culture medium before imaging. ΔΨp-corrected ΔΨm measurements were derived from the ratio of fluorescence intensities between mitochondria (Fm) and mitochondria-poor regions (Fc) (Marks et al., 2005).

**Visualizing mitochondrial dynamics**

Retinal explants were incubated with 25 nM MitoTracker Red (Life Technologies) at 20°C for 20 minutes and washed with culture medium before imaging. Time-lapse
recordings were run for 5 minutes applying 5-second intervals between time points. A mobile mitochondrion was only considered as such if its dislocation was \( \geq 5 \mu m \) (Sheng and Cai, 2012). The subset of mitochondria undergoing fast transport were defined as those moving at average velocities of \( \geq 0.3 \mu m/\text{second} \).

**Statistical Analysis**

Each experiment was repeated at least three times unless otherwise indicated. Details of statistical analysis are included in figure legends or main text. Data were analysed with Prism (GraphPad), except Real-time PCR data (qbase+). For all tests, a significance threshold of \( \alpha = 0.05 \) was used.
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COMPETING INTERESTS

No competing interests declared.

AUTHORS CONTRIBUTIONS

C.E.H. conceived and supervised the project. C.G.R. developed the approach and designed experiments. C.G.R. performed and analysed experiments, except in Fig. 2F and Fig. 9G-I (C.G.R. and J.Q.L.), Fig. 4 and Fig. 6F (H.W.). C.G.R. and C.E.H. wrote the manuscript.

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REFERENCES


Figures

Figure 1. Expression of tpt1 in the neural retina
(A) Coronal section of stage 43 retina probed with an anti-Tctp antibody and counterstained with DAPI (CMZ: ciliary marginal zone; PR: photoreceptor layer; arrowheads indicate the optic fibre layer). The boxed area is shown in the lower panels. The dashed contour delineates the outer plexiform layer.

(B) Stage 32 eye explants grown in vitro for 24 hours were stained with anti-Tctp antibody (left panel: phase contrast image; right panel: Tctp-derived signal). Tctp is detected in the axon shaft, central domain and filopodia.

(C) In situ hybridization detection of tpt1 mRNA expression on coronal sections of stage 43 retinas (GCL: ganglion cell layer; ONH: optic nerve head; IPL/OPL: inner/outer plexiform layer; arrowheads indicate the optic fibre layer).

(D and E) Quantitative in situ hybridization detection of tpt1 mRNA expression in the RGC axonal and growth cone compartments was performed using stage 32 eye explants grown in vitro for 24 hours (mean ± s.e.m.; *** P < 0.0001, one-way ANOVA and Bonferroni).

(F) 5’ (left) and 3’ (right) RACE amplifications of tpt1 mRNAs using retinal RNA extracts (FP, forward primer: RP; reverse primer; UP, universal primer; NUP, nested universal primer).

(G) Organization of the tpt1 gene in X. laevis (cds, coding region; poly(A) signal, polyadenylation signal).

(H) Schematic representation of the laser-capture microdissection procedure used to collect RGC axonal extracts.

(I) 5’ (left) and 3’ (right) RACE amplifications of tpt1 mRNAs using laser-captured axonal extracts.

(J) Purity assessment of laser-captured material by RT-PCR. –RT, RNA samples not reverse transcribed.
(K) Schematic representation of RT-qPCR experimental design.

(L and M) Axonal and whole-eye content of tpt1 mRNAs were analysed by RT-qPCR and normalized to actb expression. In (L), data are plotted as ‘tpt1-S + tpt1-L’ to ‘tpt1-L’ expression ratios \( P = 0.0175 \), one-way ANOVA), whereas in (M) the quantification cycle (Cq) difference relative to actb is shown.

Scale bars: 50 μm in (A) and (C), 5 μm in (B) and (D).
Figure 2. Tctp is required to establish correct retinotectal projections in vivo

(A) Experiment outline.

(B) tpt1-MO leads to a specific knockdown in Tctp protein levels in the CNS, as evaluated by Western blot analysis of stage 37/38 embryos using an anti-Tctp antibody.

(C) Representative con-MO and tpt1-MO-positive growth cones stained for Tctp.
(D-F) Dil-filled retinotectal projections in morpholino-injected stage 40 embryos. Dashed lines approximate the boundary of the optic tectum, where RGC axons terminate.

(G) Relative projection lengths in the various morpholino-injected backgrounds (mean ± s.e.m.; \( n = \) no. of brains analysed; \( P < 0.0001, \) Kruskal-Wallis test).

(H) Number of embryos displaying axon extension defects (‘con-MO’ versus ‘\( tpt1 \)-MO’: \( P < 0.0001 \); ‘\( tpt1 \)-MO’ versus ‘\( tpt1 \)-MO + rescue mRNA’: \( P = 0.0002 \); Fisher’s exact tests, performed on number of observations but plotted as percentages).

(I) Mean optic tract widths (‘con-MO’ versus ‘\( tpt1 \)-MO’: ** \( P < 0.01 \) (C2), * \( P < 0.05 \) (C3), *** \( P < 0.0001 \) (C4), * \( P < 0.05 \) (C5), ** \( P < 0.05 \) (C6), two-way ANOVA and Bonferroni – for detailed statistics see supplementary material Fig. S2F). C2-7 denote imaginary, evenly spaced hemi-circumferences centred on the optic chiasm.

Scale bars: 5 μm in (C), 100 μm in (D-F).
Figure 3. Tctp is not necessary for the timely development of the eye

(A) Representative stage 43 control and Tctp-depleted retinas stained with phalloidin and DAPI. The boxed areas are shown at a higher magnification in the rightmost panels.

(B and C) Immunohistochemistry analysis of the photoreceptor layer in stage 43 wild-type retinas probed with anti-Tctp and anti-Opsin or anti-Rhodopsin antibodies, and counterstained with DAPI (PR: photoreceptor; ONL: outer nuclear layer; IS: photoreceptor inner segment; OS: photoreceptor outer segment).
(D and E) Representative micrographs of the photoreceptor layer in stage 43 control or Tctp morphant retinas probed with anti-Opsin or anti-Rhodopsin antibodies, and counterstained with DAPI.

(F) Average inner segment lengths in control and Tctp morphant retinas (*** $P < 0.0001$, unpaired $t$-tests; boxplot whiskers denote 5-95 percentile).

(G) Proportion of photoreceptors showing a complete loss of the outer segment in control and Tctp morphant retinas ($n =$ no. of photoreceptor layers analysed; *** $P < 0.0001$, unpaired $t$-test).

Scale bars: 50 μm in (A), 25 μm in (B), (D) and (E).
Figure 4. Tctp deficiency impairs axon extension in vivo

(A) Schematic representation of the experiment. con-MO- or tptl-MO-injected stage 28 embryos were electroporated with gap-RFP to label retinal axons and allowed to develop until stage 40 before in vivo brain imaging.

(B and C) Representative time-lapse images of gap-RFP-labelled control (top panels) and Tctp-depleted (bottom panels) RGC axons coursing through the optic tract. Dashed lines approximate the boundary of the optic tectum.
(D) Average extension rates measured from time-lapse recordings of RGC axons coursing through the ventral optic tract (VOT) and dorsal optic tract (DOT) in controls and Tctp morphants (mean ± s.e.m.; \( n \) = no. of axons analysed; VOT: \(* * * P < 0.0001\), unpaired \( t \)-test; DOT: \( * P < 0.0273 \), unpaired \( t \)-test).

(E) Percentage of axons with stalled progression in the control and morphant backgrounds (mean ± s.e.m.; \( n \) = no. of embryos analysed; \(* * P < 0.0035 \), unpaired \( t \)-test).

(F) Retinotectal projection angular spreads in controls and Tctp morphants (mean ± s.e.m.; \( n \) = no. of embryos analysed; pre-turn: \(* * P < 0.0082 \), unpaired \( t \)-test; post-turn: \(* * * P < 0.0001 \), unpaired \( t \)-test).

Scale bars: 25 μm.
Figure 5. The retinotectal projection develops unerringly in Tctp-deficient brains

(A) Experiment outline.

(B) Dorsal view of embryos microinjected unilaterally with fluorescein-tagged
con-MO or tpt1-MO.

(C) Unilateral tpt1-MO injection leads to a targeted knockdown in Tctp expression in half of the central nervous system, as shown by immunoblot analysis of eye or brain lysates. The ‘ipsilateral’ label refers to the MO-positive half of the embryo; likewise, the uninjected half is designated ‘contralateral’.

(D-G) DiI-filled stage 40 retinotectal projections. Dashed lines approximate the boundary of the optic tectum.

(H) Relative projection lengths (mean ± s.e.m.; n = no. of brains analysed; *** $P = 0.0002$, Kruskal-Wallis and Dunn’s Multiple Comparison Test – for detailed statistics see Fig. S2F).

(I) Number of brains displaying axon extension defects (Eye-MO:Brain-wt backgrounds: * $P = 0.0352$, Fisher’s exact test; tpt1-MO backgrounds: * $P = 0.0364$, Fisher’s exact test; analyses performed on frequencies but plotted as percentage).

Scale bars: 100 μm.
Figure 6. Compromised mitochondrial homeostasis in axons deficient for Tctp

(A) Relative ATP levels per retina normalized to total protein (mean ± s.e.m.; \( n = 17 \) per condition, *** \( P < 0.0001 \), unpaired \( t \)-test).

(B) Representative con-MO and tpt1-MO-positive RGC growth cones loaded with TMRM.

(C) Quantification of TMRM fluorescence intensity in the mitochondria-rich growth cone (GC) central domain (mean ± s.e.m.; *** \( P = 0.0002 \), Mann-Whitney test).

(D) Quantification of TMRM fluorescence intensity of individual mitochondria along the axonal compartment (mean ± s.e.m.; ** \( P < 0.0012 \), Mann-Whitney test). Up to 10 replicate experiments were performed per condition, totalling 427 growth cones and 4918 single mitochondria analysed.
(E) Schematic representation of the approach used to examine mitochondrial density in RGC axons in vivo.

(F) Micrographs of RGC axons co-labelled with mt-GFP and gap-RFP, plus quantification of axonal mitochondrial density (\(n = \) no. of axons analysed; *** \(P = 0.0002\), unpaired \(t\)-test; boxplot: whiskers cover 5-95 percentile and ‘+’ the mean). Boxed areas are shown in the rightmost panels.

Scale bars: 5 μm.
Figure 7. Altered mitochondrial dynamics in Tctp-depleted axons

(A) Ratio of mitochondrial to nuclear DNA determined by qPCR in control and Tctp-depleted retinas (mean ± 95% confidence interval; \( n = 7 \) paired retinas per condition; n.s., \( P = 0.23 \), Mann-Whitney test).

(B) Tctp morphants show unaltered Pgc1α expression levels in the CNS, as evaluated by Western blot analysis of stage 37/38 embryos using an anti-Pgc1α antibody (\( n = 3 \) independent samples; n.s., \( P = 0.5955 \), unpaired t-test).

(C) Tctp morphants show unaltered expression of mitochondria-related genes, as assessed by RT-qPCR using eye RNA extracts (mean ± 95% confidence interval; \( n = 9 \) retinas per condition, Mann-Whitney test).

(D) Tctp morphants show unaltered Cytochrome c expression levels in the CNS, as evaluated by Western blot analysis of stage 37/38 embryos using an anti-Cytochrome c antibody (\( n = 3 \) independent samples; n.s., \( P = 0.5989 \), unpaired t-test).

(E) Control and Tctp-depleted RGCs have similar levels of cox5α expression (mean ± s.e.m.; \( n \approx 20 \); GCL: n.s., \( P = 0.2026 \), Mann-Whitney test; IPL: mean ± s.e.m.; n.s., \( P = 0.2668 \), Mann-Whitney test).

(F) Representative kymographs of MitoTracker-labelled RGC axonal mitochondria in control and Tctp morphant backgrounds.

(G) Summary of changes in axonal mitochondrial dynamics (statistical significance determined using Fisher’s exact test).

(H) Relative mitochondrial motility and mean net movement in control and Tctp-depleted RGC axons (boxplot whiskers indicate 5-95 percentile; right panel: mean ± s.e.m.; * \( P < 0.0117 \), Mann-Whitney test).
(I) Analysis of fast mitochondrial transport (mean ± s.e.m.; anterograde direction: $P = 0.9468$; retrograde direction: $P = 0.7308$; statistical analyses used Mann-Whitney test).

(J) Average duration of mitochondrial pauses in control and Tctp-depleted RGC axons (boxplot whiskers indicate 5-95 percentile and ‘+’ the mean; n.s. $P = 0.902$, Mann-Whitney test). Permanently stationary mitochondria were excluded from this analysis.

(K) Average number and frequency distributions of mitochondrial pauses (mean ± s.e.m.; n.s. $P = 0.317$, Mann-Whitney test).

Scale bars: 50 μm in (E), 5 μm in (F).
Figure 8. Axonal Tctp interacts with pro-survival Mcl1

(A) Coronal section of stage 43 retina probed with an anti-Mcl1 antibody and counterstained with DAPI.

(B and C) PLA signal for Tctp and Mcl1 in cultured rat cortical neurons (E18.5 + 3 DIV) counterstained with DAPI and phalloidin. The boxed areas are shown in the bottom panels. In (C), anti-Mcl1 serum and blocking peptide were co-incubated before proceeding with the assay.

(D) Representative con-MO and tpt1-MO-positive RGC growth cones stained for P53 (mean ± s.e.m.; n = no. of growth cones analysed; *** P = 0.0002, unpaired t-test).

(E) Representative con-MO and tpt1-MO-positive RGC growth cones stained...
with an antibody that specifically recognizes the cleaved (activated) form of Caspase-3 (mean ± s.e.m.; \( n \) = no. of growth cones analysed; *** \( P = 0.0002 \), unpaired \( t \)-test).

Scale bars: 50 \( \mu \text{m} \) in (A), 10 \( \mu \text{m} \) in (B) and (C), 5 \( \mu \text{m} \) in (D) and (E).
Figure 9. Tctp regulates axon development via its anti-apoptotic effects

(A-C) Lateral view of Dil-filled retinotectal projections in con-MO- or mcl1-MO-injected stage 40 embryos. Dashed lines approximate the boundary of the optic tectum; arrowhead denotes a region of the tract with outgrowth defects; asterisks mark beaded axons, suggestive of degenerating axons; boxed region in (C) shows axon
misprojections into the telencephalon and diencephalon.  

(D) Mean optic tract width in con-MO- and mcll-MO-injected embryos (C2: **
P < 0.01, two-way ANOVA).  

(E) Relative projection lengths in control and Mcll morphant backgrounds (mean ± s.e.m.; n = no. of brains analysed; n.s., Mann-Whitney test).  

(F) Summary of phenotypic changes in Mcll morphant projections.  

(G) Co-delivery of tpt1-MO and tpt1<sub>40-172</sub> mRNA, encoding a truncated Tctp protein devoid of anti-apoptotic activity, fails to rescue the effects of Tctp depletion on the development of the retinotectal projection.  

(H) Relative projection lengths in con-MO-, tpt1-MO- and ‘tpt1-MO + truncated tpt1<sub>40-172</sub> mRNA’-injected embryos (mean ± s.e.m.; n = no. of brains analysed; P = 0.008, Kruskal-Wallis test).  

(I) Mean optic tract widths (‘con-MO’ versus ‘tpt1-MO + truncated tpt1<sub>40-172</sub> mRNA’: * P < 0.05 (C2), * P < 0.05 (C3), * P < 0.05 (C4), ** P < 0.01 (C5), * P < 0.05 (C6), two-way ANOVA and Bonferroni).  

Scale bars: 50 μm.