TOPORS, Implicated in Retinal Degeneration, is a Cilia-Centrosomal Protein

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Abstract

We recently reported that mutations in the widely expressed nuclear protein TOPORS (topoisomerase I-binding arginine/serine rich) are associated with autosomal dominant retinal degeneration. However, the precise localisation and a functional role of TOPORS in the retina remain unknown. Here, we demonstrate that TOPORS is a novel component of the photoreceptor sensory cilium, which is a modified primary cilium involved with polarized trafficking of proteins. In photoreceptors TOPORS localises primarily to the basal bodies of connecting cilium and in the centrosomes of cultured cells. Morpholino-mediated silencing of topors in zebrafish embryos demonstrates in another species a comparable retinal problem as seen in humans, resulting in defective retinal development and failure to form outer segments. These defects can be rescued by mRNA encoding human TOPORS. Taken together, our data suggest that TOPORS may play a key role in regulating primary cilia-dependent photoreceptor development and function. Additionally, it is well known that mutations in other ciliary proteins cause retinal degeneration, which may explain why mutations in TOPORS result in the same phenotype.
INTRODUCTION

Progressive dysfunction and consequent death of cone and rod photoreceptors in retinal degenerative diseases constitute a major cause of adult blindness. The retina provides extremely sensitive light detection, which is based on the integrity of a highly vulnerable cell - the photoreceptor. Retinal degeneration is genetically the most heterogeneous disease known, with mutations in 146 genes identified to date with an additional 38 loci genetically mapped but the respective genes not yet found. It is arguable that mutations in genes affecting almost every aspect of photoreceptor structure or function can trigger retinal degeneration (1,2).

Amongst the retinal degenerations, retinitis pigmentosa (RP) is clinically and genetically the most heterogeneous with an incidence of almost 1 in 3500 people worldwide. Affected individuals suffer from a progressive degeneration of the photoreceptors, eventually resulting in severe visual impairment. The mode of inheritance of RP can be autosomal dominant (adRP), recessive (arRP), X-linked or digenic (3). To date, twenty causative genes have been identified for adRP (RetNet). The products of these genes are associated with diverse functions, including photoreceptor structure, ciliary transport, phototransduction, and gene expression, e.g. transcription and mRNA-splicing.

Recently, we identified a novel gene mapping to chromosome 9p21.1 causing autosomal dominant retinitis pigmentosa (RP31) (4). The gene encoding topoisomerase I – RS protein (TOPORS, MIM 609923, NM_005802) was found to be mutated in two families of French-Canadian and German origin. Both mutations, a heterozygous 1 bp insertion (c.2474_2475insA) and a 2 bp deletion (c.2552_2553delGA) in exon 3 lead to premature stop codons, p.Tyr825fs and p.Arg851fs respectively (4). Two additional TOPORS mutations were later found to
cause frameshift and termination of the protein prematurely – c.2569delA (p.Arg857GlyfsX9) and c.G2422T (p.Glu808X) (5). A nucleotide substitution c.1205A>C, leading to missense change (p.Q402P) has also been identified in a family with autosomal dominant pericentral retinal dystrophy (6). Prevalence studies indicate that mutations in TOPORS is a rare cause of retinal degeneration and likely to account for 1-2% of autosomal dominant RP.

TOPORS was originally identified in a screen for proteins that bind to the N-terminus of topoisomerase I (7), and it interacts with p53 (8). TOPORS is a tumor suppressor protein and functions as an E3 ligase for both ubiquitin and small ubiquitin-like modifier 1 (SUMO-1) (9,10). It was recently reported that DNA-damage-inducible SUMOylation of IKK-related kinase - IKK3ε depends on TOPORS (11). Cumulatively, these studies indicate that TOPORS is a ubiquitously expressed gene, encoding essentially a nuclear protein showing multi-functional character with function that varies according to cell type. The multi-functional nature of this protein may be also related to the different protein domains of TOPORS (3). Although extensive studies have helped define the function of TOPORS in cultured cells, and despite being widely expressed, mutations in TOPORS are only known to cause adRP. Mutations in tissue specific genes leading to tissue specific phenotypes define common mechanisms in understanding human genetic diseases. However, TOPORS mutations are beginning to highlight a small group of unique genes that are widely expressed and yet cause a tissue specific phenotype (such as splicing factor mutations and adRP) (12). This could be due to a unique role for TOPORS in the retina and therefore we may see an altered sub-cellular localisation in the retina, away from the photoreceptor nucleus. Conversely, an altered localisation may confer a novel role for TOPORS in the retina.
In the absence of information regarding sub-cellular localisation of TOPORS in the retina of any organism it is critical to perform a comprehensive study, which will formally establish localisation of TOPORS in the retina. For this reason we undertook this study to delineate the role of TOPORS in the vertebrate retina and to understand why mutations cause only retinitis pigmentosa. This may help elucidate its specific role in the retina and define general cellular pathways implicated in retinal degeneration, which may have wider consequences for the development of future therapies for this group of patients. The immunolocalisation studies presented here show TOPORS localisation to the connecting cilium (CC), between inner and outer segment of the photoreceptor cell. Mutations in other proteins such as RP1 (13) and RPGR (14) that also localise to the CC are associated with retinal degeneration thereby providing a basis for a similar explanation for TOPORS mutations causing only RP. All previously published work involving non-retinal cell types demonstrated nuclear localisation supporting a nuclear role for TOPORS.

RESULTS

TOPORS localises to photoreceptor cilia

To determine the cellular localization of TOPORS in mammalian retina, we performed indirect immunofluorescence staining of cryosections of adult mouse, porcine and human retinas using an anti-TOPORS antibody. The specificity of the anti-TOPORS antibody was extensively checked in these mammalian species and as expected yielded a consistent band of 150 kDa (Fig. 1F). In all species, we observed TOPORS immunoreactivity predominantly at the junction between photoreceptor inner and outer segment, called the connecting cilium (Fig. 1A-D). In addition, TOPORS immunostaining was detected in the nuclei of retinal ganglion cells.
We next sought to define more precisely the spatial distribution of TOPORS along the photoreceptor cilium. We employed double immunostaining of retinal cryosections with anti-TOPORS antibody and molecular markers for the ciliary subcompartment: anti-RP1 as a marker for the ciliary axoneme extending from the transition region (13); anti-rhodopsin and anti-MAP2 which stain the outer segments and the microtubules in the inner segments (15); anti-centrin-3 to visualize the transition zone, basal body and adjacent centriole (16), and anti-γ-tubulin as a marker for basal body. Detailed examination of the photoreceptor cilium by high-resolution immunofluorescence microscopy (17,18) revealed that anti-TOPORS antibody stains a pattern of two adjacent dot-like structures (Fig. 2 and 3A). This TOPORS staining did not overlap the rhodopsin, RP1 and MAP2 staining (Fig. 2A-C), thus excluding its presence at the outer segment, axoneme and inner segment of photoreceptors. Proximal TOPORS staining colocalised with centrin-3 (Fig. 2D) and γ-tubulin (Fig. 2E), demonstrating the localisation of TOPORS to the basal body of the connecting cilium of the photoreceptor cells. In comparison with the centriole markers, anti-TOPORS stained a broader dot indicating additional presence of TOPORS in the pericentriolar region (Fig. 3 A-D). In conclusion, our detailed inspection of the subciliary molecular distribution in retinal photoreceptor cells revealed specific localisation of TOPORS in the basal body of the connecting cilium and its daughter centriole.

**TOPORS co-immunoprecipitates with ciliary-centrosomal proteins in retina**

We next examined the interaction of TOPORS with cilia-centrosomal proteins by co-immunoprecipitation in bovine retina extracts. First we looked at proteins implicated in retinal degeneration and known to localise to the connecting cilia. TOPORS antibody
did not immunoprecipitate RPGR – ORF15 (14,19), CEP290/NPHP6 (20), RP1 (13) (Fig. S1 A-C), pericentrin (21) or centrin (16,22) which are part of the ciliary apparatus of the photoreceptor cells. We found co-immunoprecipitation with centrosomal/basal body protein γ-tubulin (Fig. S1 D-F). This prompted co-immunoprecipitation experiments with proteins involved in intraflagellar transport. We consistently detected the association of TOPORS with dynein-dynactin complex proteins (p150<sub>glued</sub>, p50-dynamitin, and cytoplasmic dynein intermediate chain, DIC) that are part of the retrograde transport mechanism (23). Little or no interaction of TOPORS was observed with anterograde motor kinesin-II complex (KIF3A, KAP3 and IFT88) (Fig. S1 G-L). Reverse co-immunoprecipitation experiments did not reveal TOPORS immunoreactive bands, probably due to its lower abundance in these complexes (data not shown).

**TOPORS localises to primary cilia in cell lines**

It is well known that many of the connecting cilia proteins are localised in the cilia of non-dividing cells and in the centrosomes of mitotic cells (24). For these reasons we undertook detailed investigations of TOPORS localisation in cell lines. In dividing MDCK cells, TOPORS exhibits the nuclear localization in small speckles (Fig. 4A) as previously reported (7,25). However, in non-dividing ciliated MDCK cells, the staining of endogenous TOPORS is detected only in the basal body, at the base of the cilium (Fig. 4B). The immunostaining was performed using anti-RPGR antibody. Although both proteins appeared in the ciliary region, TOPORS is proximal to RPGR but shares a small region of overlap at the basal body in this particular cell type. Localisation of TOPORS to the base of the primary cilium was also detected in ARPE-19 (Fig. 4C) and IMCD3 (Fig. 4D) cell lines.
To confirm the localization of TOPORS to the basal body, we double immunostained ciliated IMCD3 cells with anti-TOPORS and anti-γ-tubulin antibodies (Fig. 4E). As expected, γ-tubulin was found at the basal body. TOPORS localised to the basal bodies but did not overlap entirely with γ-tubulin. Triple immunostaining using anti-TOPORS, anti-α-tubulin and anti-γ-tubulin antibodies (Fig. 4F) showed TOPORS at the base of the cilia (with γ-tubulin), and α-tubulin along the length of the cilia axoneme.

**Cell cycle dependent localization of TOPORS to the centrosomes**

As TOPORS was previously reported to be a nuclear protein (7,25) and our results with ciliated cells show a predominant cilia staining of the ciliary apparatus, namely the basal body and the adjacent centriole, we hypothesized that TOPORS might exhibit dynamic association with cilia and the nucleus. When expressed as a GFP-fusion in RPE-1 cells, TOPORS showed localization to the nucleus and also to the centrosome (Fig. 5A). This allowed independent verification of TOPORS localization using the anti-GFP antibody. The control experiment using only the GFP vector showed diffused staining throughout the cell cytoplasm, whereas TOPORS-GFP localization was restricted to nuclei and centrosomes.

We performed a detailed analysis of TOPORS localization during different stages of cell division using synchronized RPE-1 cells. Immunocytochemical studies demonstrated that endogenous TOPORS exhibits cell cycle stage dependent localization in the nucleus, centrosomes and midbody. During G1, G0, S and G2 phase, TOPORS localization is predominantly in the nucleus (associated with PML bodies) and in the centrioles of the centrosomes (co-localised with γ-tubulin) (Fig. 5B). During mitosis, TOPORS is enriched in the centrioles of the spindle poles (Fig.
In addition to centrosome-associated staining, we detected TOPORS at the midbody, the central part of the cytokinetic bridge in telophase, a feature shared by several other centrosomal proteins (24). Altogether, these results show that TOPORS is associated with the centrosome throughout the cell cycle.

**Knockdown of topors results in developmental anomalies in zebrafish**

To delineate the function of TOPORS, we knocked down topors expression in zebrafish embryos. Using BLAST, we identified the human orthologue of topors in zebrafish (Accession no. XM_682803). At the protein level, zebrafish TOPORS displays 38% identity and 67% homology to human TOPORS (Fig. S2). To assess the function of TOPORS, we injected a translation-blocking (AUG) morpholino (MO) against topors at the 1-8 cell stage in zebrafish embryos. Approximately 45% of embryos exhibited developmental anomalies consistent with the effect of depletion of ciliary proteins, such as kinked tail, microphthalmia, hydrocephaly, and pericardial effusion at 4 days post fertilization (dpf) (Fig. 6A and B; Fig. S3). As a control, injection of a mismatch morpholino resulted in only ~15% defective embryos. Further investigation of the specific MO-treated embryos revealed that microphthalmia was present in about 80% of the morphants whereas kinked tail and edema was observed in 70% and 85% of morphants, respectively. Similar results were obtained when a splice-blocking MO was injected in fish embryos (Fig. 6C and data not shown). These phenotypes are specific to the silencing of topors, as co-injection of the topors-MO with mRNA encoding human TOPORS was able to rescue the topors-knockdown phenotypes (Fig. 6D).
TOPORS regulates retinal development in zebrafish

We then investigated the effect of knockdown of topors on retinal development. To this end, we first assessed the extent of knockdown of TOPORS. In contrast to the staining observed in the mouse, porcine and human retinas, TOPORS immunoreactivity in the retina of zebrafish embryos treated with mismatch control was detected in all cellular layers; however, in photoreceptors, the staining was predominantly detected in the inner segment. No detectable signal was observed in the defective embryos, which were injected with the specific translation-blocking MO against topors (Fig. 6E). We then investigated retinal development in the defective embryos. Consistent with broad developmental defects in embryonic development, histological analysis of retinas from controls and topors-knockdown embryos revealed that depletion of topors results in lamination defects in the retina (the different layers of the retina could not be detected) as well as failure to form outer segments (Fig. 6F). We then analyzed the expression of photoreceptor proteins in the topors-knockdown embryos. Cone (Zpr1) and rod (Zpr3) photoreceptor staining were absent in the defective embryos as compared to robust expression in control treated group (Fig. 6G).

We then examined whether knockdown of topors results in cell death in the retina, by TUNEL staining of control and topors-MO treated embryos at 3 dpf and 4 dpf. We found that lack of topors resulted in 28-30% TUNEL positive cells indicating increase in apoptotic cell death as compared to control injected embryo retina (6-8% TUNEL-positive cells) (Fig. S3).
DISCUSSION

Primary (or sensory) cilia are near-ubiquitous microtubule-based organelles that regulate diverse intracellular processes. Mutations in ciliary proteins are associated with severe neurodegenerative disorders, including isolated and syndromic forms of Retinitis Pigmentosa (RP), such as Bardet-Biedl Syndrome (BBS), Senior-Loken Syndrome, and Joubert Syndrome (20, 26-30) and Usher syndrome (31-33). Photoreceptors are post-mitotic sensory neurons, which display a unique polarized structure. They are divided into a biosynthetically active inner segment (IS) and a photosensitive outer segment (OS) connected via a bridge-like structure, the connecting (or sensory) cilium, which represents the transition zone of a prototypical cilium (34,35). The OS of the photoreceptor cell is analogous to the primary ciliary structure; here in particular its highly specialised function pertains to phototransduction reliant on a number of proteins that are shuttled in and out of the OS via the CC. Owing to high trafficking demands, defects in cilia-associated proteins are associated with forms of severe retinal degeneration and blindness (13,14).

The RP associated with TOPORS is inherited as an autosomal dominant trait and likely to be due to haploinsufficiency (3). Although TOPORS is a widely expressed protein, patients with mutations in TOPORS do not exhibit a wide range of developmental anomalies, unlike the phenotype detected in topors-knockdown zebrafish embryos. We believe that the residual protein expressed in patients is able to rescue extra-retinal defects. However, as photoreceptors are highly metabolically active neurons, slight perturbations in protein trafficking pathways result in degeneration and eventually blindness. Support of this hypothesis comes from our previous observations of analysis of the function of other ciliary proteins, such as RPGR, CEP290, and RPGRIP1L (36,20,30)
So far TOPORS was known as a multifunctional protein and all previously published work involving non-retinal cell types demonstrated nuclear localisation supporting a nuclear role for TOPORS. It also localises to the centrioles of centrosomes and spindle poles as well as basal bodies in dividing and quiescent cells, respectively. The association of TOPORS with the centrioles and midbody indicates that it may also be involved in the regulation of cell division. Its co-localization and close association with γ-tubulin leads us to suggest a possible role for TOPORS in microtubule nucleation, establishment of a bipolar mitotic spindle or normal cell division. It will be important to understand whether TOPORS has a role in the centrosome cycle as a structural component for the centriole/centrosome assembly or is involved in the maintenance of centrosomal adherence.

Defects in the protein transport system responsible for the organization and maintenance of the cilia is implicated as a primary cause for some human diseases also known as ciliopathies. Taken together, we propose that TOPORS dysfunction should be included in the broad category of these diseases. As in other ciliated cells, in photoreceptors, the transport of ciliary components to their destination in the cilium is characterized by different transport modules. In photoreceptor cells newly synthesized outer segment components are transported from the biosynthetically active organelles through the cytoplasm of the inner segment and across the connecting cilium to the outer segment (34,35). At the base of the connecting cilium, the cargo which is destined for the outer segment is transferred from a microtubule-based transport system mediated by minus-end directed cytoplasmic dynein motor complexes (37) to the ciliary transport systems - the intraflagellar transport (IFT) system mediated by Kinesin motors (18). Our present data on TOPORS localization indicate that TOPORS is associated with the specialized periciliary machinery...
essential for the cargo handover from the dynein mediated inner segment transport to the IFT complexes (33,35,38). In this periciliary compartment the ciliary transport complexes are made up of individual IFT molecules (18) and the ciliary cargo delivery into and export from the cilium is mediated by the BBSome (39). Defects of molecules associated with this periciliary reloading complex are common primary causes of retinal degenerations and syndromic ciliopathies (17,26,40,41). Association of TOPORS predominantly with dynactin subunits further supports a possible role for TOPORS in regulating protein trafficking. The dynactin subunits p150\textsuperscript{glued} and p50-dynamitin are responsible for tethering the cargo to the dynein motor and regulate microtubule-associated transport (42). Although direct evidence of a role of retrograde transport in photoreceptor is lacking, the cytoplasmic dynein 2 motor has been identified in the axoneme of photoreceptor outer segments (43). Additionally, retrograde transport via the photoreceptor cilium may be involved in renewal of kinesin motor subunits or for IFT-mediated signaling (44,45,46). Investigations are underway to validate if TOPORS is involved in dynein-dependent trafficking.

Knockdown of *topors* in zebrafish results in defective development, including perturbed retinal lamination and photoreceptor development. This defect is consistent with the phenotype observed in knock out mouse mutants of transcription factors involved in retinal differentiation (47) and in the mutant of *Ift88/Polaris* (40). We hypothesise that microphthalmia could be the result of defective retinal development (dysplasia) and degeneration. The mechanism of such a severe retinal developmental defect associated with TOPORS may be defective Hedgehog or Wnt signalling cascades, as both are at least partly regulated by ciliary function (48,49). Further evidence for potential involvement of TOPORS in cilia-dependent function comes from our observation that TOPORS associates with ciliary and centrosomal proteins.
in the mammalian retina. TOPORS has been shown to function in proteasomal degradation pathway by acting as an E3 ubiquitin ligase for p53. As proteasomes are concentrated around the pericentriolar region and are involved in regulating Wnt signalling (50,51), our studies indicate that TOPORS may regulate proteasomal targeting of signalling proteins or cargo molecules. Analysis of the animal models of TOPORS mutations should further our understanding of this phenomenon and delineate the mechanism of pathogenesis of associated retinal degeneration.

MATERIALS and METHODS

Antibodies

Mouse monoclonal anti-TOPORS antibody (diluted 1:50) and TOPORS Recombinant Protein was obtained from Abnova Corporation (Taiwan). Rabbit anti-γ-tubulin (1:1000) was obtained from Chemicon (Temeculla, CA), anti-KIF3A (1:1000) and anti-KAP3 (1:1000) was from Sigma. Anti-RP1 (1:500) and anti-IFT88 (1:1000) antibodies were a generous gift of Dr. Eric A. Pierce (University of Pennsylvania, PA, USA) and Dr. Bradley K. Yoder (University of Alabama at Birmingham, Birmingham, AL, USA), respectively. Antibody against p150glued (1:500) was purchased from BD Transduction Labs (San Jose, CA). Characterization of the anti-RPGRORF15 (ORF15CP) and anti-CEP290 antibody has been previously described (16, 19, 20, 22). Rat-anti-α-tubulin (1:500) obtained from Abcam, UK; rabbit-anti-centrin 3 (1:400) and goat-anti-MAP2 (1:100) obtained from Santa Cruz Biotechnology, Inc., USA; anti-p50 (1:1000) and mouse-anti-DIC (1:500) from Chemicon; rabbit anti-pericentrin (1:500) from Sigma; and rabbit polyclonal anti-rhodopsin antibody (1:250) (Abcam). Secondary antibodies were FITC-conjugated donkey anti-mouse IgG (1:300), Cy3-conjugated donkey anti-rat IgG (1:300), Cy3-conjugated donkey anti-goat IgG (1:300), Cy3-
conjugated donkey anti-rabbit IgG (1:300); horseradish peroxidase-conjugated goat-anti-rabbit and anti-mouse IgG from Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA.

**Preparation of tissue sections and immunostaining**

Eyes of adult mice were dissected after cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and were fixed for overnight at 4°C. The cornea and the lens were removed followed by sucrose gradient infiltration, embedded in optimal cutting temperature (OCT) mounting medium and cryosectioned at 10 µm. For immunostaining, retinal sections were pretreated with blocking solution (5% normal goat serum and 0.1% Triton-100 in PBS) for 1h at room temperature, followed by incubation with primary antibody. The sections were washed and treated with appropriate secondary antibodies. Slides were then visualised using a laser scanning confocal microscope (ZEISS LSM 510, Carl Zeiss, Welwyn Garden City, Herts, UK). Images were processed using LSM5 Image Browser and Adobe Photoshop CS2 (Adobe Systems, USA). Cell nuclei were counterstained in blue with bisbenzimide.

**Immunoblot analysis**

Retinal bovine lysates were subject to SDS-PAGE followed by immunoblotting with antibodies. Equal amounts of protein were run on 7.5% gel and transferred to nitrocellulose membrane (BIORAD). Membranes were blocked in 5% non-fat dried milk in PBS. Primary and secondary antibodies were diluted in concentrations described above. For visualization ECL (Amersham Biosciences) was used.
Immunocytochemistry

MDCK and IMCD3 cells were maintained in Dulbecco’s modified Eagle medium – DMEM (GIBCO) and ARPE-19 and hTERT-RPE-1 cells in DMEM/F-12+GlutaMAX (GIBCO) supplemented with 10% FCS, penicillin-streptomycin (1000 µg/ml) at 37°C in an atmosphere of 5% CO₂. Cells were grown at confluence for at least 10 days (to allow them to form primary cilium), and then subjected to fixation and antibody staining. Slides were treated with ice-cold methanol (5 min at -20°C), dehydrated in PBS (5 min at room temperature) and blocked with PBS-0.5% BSA-20mM glycine-0.1% NaN₃ for 15 min before staining. Cover slips were incubated with the primary antibodies, in dilutions described above, for 1h at room temperature. Cells were washed in blocking solution and incubated with secondary antibodies for 45 min at room temperature in the dark. Cells were again washed in blocking solution and mounted in ProLong Gold antifade reagent (Invitrogen). The hTERT-RPE-1 cells were synchronized by serum starvation for 24h followed by addition of 10% FCS and methanol fixation every hour.

Zebrafish studies

A translation blocking morpholino (Gene Tools) (5’<GCT CCT TAT CTG TGG TGA TGC CAT>3’) and its 5 base mismatch (Mm) were diluted in Danieau’s solution (5mM HEPES pH 7.6, 58mM NaCl, 0.7 mM KCl, 0.4mM MgSO₄, 0.6 mM Ca(NO₃)₂ and injected into wt zebrafish embryos at 1-8 blastomere stage at different concentrations as described. Embryos were assessed for tail extension anomalies, microphthalmia, and edema essentially as described (36). The splice blocking morpholino used in this study is: 5’<GCC ATG ACC TGA CCT GAT AGA AGA AAC ATT>3’. The splice-blocked morphants were screened with zebrafish _topors_ primers (F: 5’<GTC
AGATAATGG CAC CCT CTA AGATGA A>3’ and R: 5’<TCG CTG CAG CTC GTG ATT CTC CG>3’).

For retinal immunohistochemistry, eye cryosections of zebrafish embryos at 4 dpf were stained with different antibodies. Fluorescent images were acquired using Olympus FV500 confocal microscope. Evaluation of cell death in retinal cryosections was performed using Apoptosis Detection Kit (Promega G3250), according to manufacturer’s instructions. Briefly, sections were permeabilised with PBS+ 0.1% Triton X-100 followed by incubation with equilibration buffer (200mM potassium cacodylate pH 6.6, 25mM Tris-HCl pH 6.6, 0.2mM DTT 0.25mg/ml BSA 2.5mM cobalt chloride) for 10 min at room temperature. Slides were then replaced with 50µl rTdT incubation buffer [90µl Equilibration Buffer, 10µl Nucleotide Mix (50µM fluorescein-12-dUTP 100µM dATP 10mM Tris-HCl pH 7.6 1 mM EDTA, 2µl rTdT Enzyme)] at 37°C for 60 min in a humidified chamber. At the end of incubation, the slides were washed with 2X SSC buffer pH 7.6 (87.7g NaCl 44.1g sodium citrate, pH 7.6) and observed for Fluorescein-12-dUTP incorporation (520nm, green fluorescence). Green fluorescent nuclei were counted in a given area of the section.

For eye measurements, the 5 µM thick plastic sections were examined under the microscope and were imaged. The diameter of the eye was measured as number of pixels on the images using ImageJ/Olympus software. They were then converted to mm by multiplying with the calibration factor obtained using a stage micrometer. The data is presented as percent change in diameter as compared to the mismatch controls.
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Conflict of Interest statement. Not declared.
REFERENCES


FIGURE LEGENDS

Figure 1. Localization of TOPORS in retina. (A-D) Indirect immunofluorescence analysis of mouse, porcine and human retina sections using anti-TOPORS antibody (green). (A) TOPORS is localised only in the ciliary region of the photoreceptors and in the nuclei of the ganglion cells in mouse retina. (B) Higher magnification only of the photoreceptor cell layer of mouse retina. (C) Immunostaining of anti-TOPORS antibody in porcine retina. (D) Immunostaining of anti-TOPORS antibody in human retina. (E) As a negative control mouse section was probed with TOPORS antibody blocked with TOPORS recombinant protein (206 a.a.) where no signal could be observed. (F) Immunoblot analysis of the specificity of mouse anti-TOPORS antibody shows a 150 kDa signal detected in mouse, porcine and human retina lysates, where in mouse two bands could be seen with molecular weight of 100 and 150 kDa due to a specific smaller isoform in this species lacking exon 2 of the gene.

OS: outer segment; CC: connecting cilium; IS: inner segment; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Nuclei are stained with DAPI (blue). Scale bars: 10µm.

Figure 2. Subcellular localization of TOPORS at the ciliary region of photoreceptor cell. (A-E) Indirect immunofluorescence analysis of mouse retina sections of stained with anti-TOPORS antibody (green) with ciliary proteins and proteins of the outer and inner photoreceptor segment (red) in retina. (A) Photoreceptor localization of TOPORS with the most abundant protein in these cells in the area of the outer segment – rhodopsin. (B) Subcellular localization of TOPORS and RP1 as a marker for photoreceptor ciliary axoneme (13). TOPORS is localised at the region of the connecting cilium (CC) of the photoreceptor cells. (C) Double labeling with anti-
TOPORS and anti-MAP2. Antibodies against TOPORS stain CC region. MAP2 is present in the inner segment (IS) of the photoreceptor cell and slightly at the CC region. (D) Indirect immunofluorescence double labelling with antibodies against TOPORS and the marker of the CC, basal body (BB) and centriole centrin (Centrin-3) in cryosections through mouse retinas. (D’) Higher magnification of merged signal (TOPORS and Centrin-3) from the CC of a single photoreceptor cell. (E) Double staining with anti-TOPORS and anti-γ-tubulin. TOPORS is localised at the cilium of photoreceptor cell. Anti-γ-tubulin stains the basal body. (E’) Higher magnification of merged signal of TOPORS and γ-tubulin, which partly co-localises in the basal body of the CC of a single photoreceptor cell. Scale bars: 10µm.

OS: outer segment; CC: connecting cilia; IS: inner segment; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.

Figure 3. TOPORS is localised at the basal body and centriole of photoreceptor cell. (A-D) Indirect immunofluorescence double staining with antibodies against TOPORS and marker proteins for the ciliary region of cryosections in mouse retinas. High magnification images of double immunofluorescences of the ciliary region of photoreceptor cells and schematic presentations (on the left) illustrating subciliary localization of TOPORS (green) and marker molecules (red). (A) Anti-TOPORS and anti-RP1 immunolabeling. RP1 is used as a marker indicating the ciliary axoneme (Ax). TOPORS and RP1 do not co-localise. (B) Anti-TOPORS and anti-MAP2 immunolabeling. MAP2 stains the microtubules in the inner segment (IS) of the photoreceptor cells. (C) Centrin-3 labels connecting cilium (CC), basal body (BB) and centriole (arrowhead). According to this centrin-3 labeling TOPORS is present at the BB and at centriole (arrowhead). (D) Anti-TOPORS and anti-γ-tubulin.
immunostaining. γ-tubulin stains the centriole (arrowhead). TOPORS partly co-localise with γ-tubulin at the BB.

OS – outer segment of photoreceptor cell, IS – inner segment of photoreceptor cell

Figure 4. TOPORS localises to the basal body of primary cilia and associates with microtubule proteins in ciliated cells. (A) Endogenous TOPORS (green) localised in the nucleus stained with DAPI (blue) of mitotic MDCK cells. (B) TOPORS (green) localises to the base of the ciliary axoneme stained with RPGR (red) in ciliated MDCK cells. (C) In ciliated ARPE-19 cells, TOPORS (green) localises to the base of the cilium; the ciliary axoneme is stained with α-tubulin (red). (D) In ciliated IMCD3 cells, TOPORS (green) localises to the base of the cilium; the ciliary axoneme is stained with α-tubulin (red). (D’) Higher magnification of the ciliary structure. (E) Double staining of ciliated IMCD3 cells with TOPORS (green) and γ-tubulin (red) used as a marker for the basal bodies. Both proteins localise in the same area with some overlap but do not co-localise entirely (see also Fig. S3). (E’) Higher magnification of the basal body. (F) Ciliated IMCD3 cells stained with TOPORS (green), α- and γ-tubulin (both in red), highlighting the basal bodies and ciliary axoneme. (F’) Higher magnification of the ciliary apparatus. Nuclei are stained with DAPI. Scale bars: 10µm.

Figure 5. Localization of TOPORS during cell cycle. (A) RPE-1 cells were transfected with plasmid encoding for TOPORS-GFP and GFP alone, fixed and stained for the centrosomal marker γ-tubulin. (B and C) Synchronized RPE-1 cells were fixed and stained for endogenous TOPORS and γ-tubulin. G0 was stained using anti-acetylated α-tubulin antibody. DNA was stained with DAPI. Insets show higher magnifications of
centrosome containing regions. Representative images of each step of the cell cycle are shown. In coloured images, TOPORS staining is in green, centrosomes in red, nuclei in blue and the arrow shows the midbody. Scale bars: 10µm.

Figure 6. Knockdown of Zebrafish topors causes developmental anomalies and effect of inhibition of topors on Zebrafish retina. (A) Injection of anti-sense morpholino (topors-MO) into zebrafish embryos results in developmental disorders, including shortened body axis and hydrocephaly. Embryos injected with the 5 base mismatch (Mm) control are also shown. Asterisk depicts kinked tail; long arrow shows microphthalmia; arrowhead indicates hydrocephaly. (B and C) Histograms showing frequency of occurrence of morphants. (B) Incidence of the different phenotypes observed in the MO-treated embryos compared to Mm-treated controls. (C) Results are representative of at least three independent experiments. (D) The topors-knockdown phenotype can be rescued by injecting indicated doses of human TOPORS (hTOPORS) mRNA. Data are representative of three independent experiments (n>100). Bottom panel shows representative image of an embryo co-injected with the specific MO against topors and mRNA encoding hTOPORS. Majority of the phenotypes seem to be rescued by human TOPORS. (E) Immunohistochemistry of cryosections of 4dpf zebrafish retina was performed using anti-TOPORS antibody (green). DAPI was used to stain the nuclei (blue). IS: inner segment; OS: outer segment; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: Ganglion cell layer. (F) Retinal histology of control (Mm) and topors-MO injected (MO) embryos at 4dpf (40X). Plastic sections from 4dpf zebrafish embryo were stained with toludine blue. (G) Cryosections of 4dpf zebrafish were immunostained with Zpr1 or Zpr3 antibody (red) for marking cone and rod photoreceptors respectively. Nuclear layers are stained with DAPI (blue). Scale bars: 10µm.