Wnt Signaling

Apc1-Mediated Antagonism of Wnt/β-Catenin Signaling Is Required for Retino-Tectal Pathfinding in the Zebrafish

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Abstract

The tumor suppressor Apc1 is an intracellular antagonist of the Wnt/β-catenin pathway. We examined the effects of an Apc1 loss-of-function mutation on retino-tectal axon pathfinding in zebrafish. In apc mutants, the retina is disorganized and optic nerves portray pathfinding defects at the optic chiasm and do not project properly to the tectum. Wild-type cells, transplanted into mutant retinas, acquire retinal ganglion cell fate and project axons that cross at the mispositioned optic chiasm and extend to the contralateral tectum, suggesting a function of apc1 in axon pathfinding. These defects are caused mainly by stabilization of β-catenin. These data demonstrate that Apc1 function is required for correct patterning of the retina and proper retinal ganglion axon projections.

Introduction

In the developing vertebrate brain, the Wnt/β-catenin cascade acts in establishing brain polarity, neuronal differentiation, stem cell induction and maintenance, and axonogenesis.1 Different transduction cascades, including the canonical, planar cell polarity, and Ca2+ pathways have been shown to transmit Wnt-mediated signals to the nucleus. Although it has been proposed that distinct Wnt ligands mediate these separate cascades, it is probable that the same Wnt ligand can elicit different responses depending on the cellular context.2 In the canonical Wnt/β-catenin pathway, in the absence of Wnt ligands, glycogen synthase kinase-3 β, Axin/conductin, and adenomatous polyposis coli (Apc) form a destruction complex that phosphorylates the key effector of the pathway, β-catenin, and target it for degradation by the proteasome. Upon binding of extracellular Wnt ligands to the receptor complex consisting of Frizzled and LRP5/6, the destruction complex of GSK-3, Axin, and Apc is inactivated through the action of Dishevelled. This results in an accumulation of β-catenin in the cytoplasm and subsequent translocation to the nucleus, where it regulates target gene transcription by interacting with TCF/LEF transcription factors.

Wnt signaling also plays a role in axonogenesis.3–4 An important question is how Wnts signal to the cytoskeleton to direct axon growth. Regarding canonical Wnt signaling, it is conceivable that stabilization of β-catenin not only leads to transcriptional target gene regulation, but could also modulate cadherin-mediated adhesion potentially contributing to axon pathfinding or changes in adhesion-mediated axon bundling.5 Apc is a key component of the Wnt/β-catenin cascade, acting as a scaffold to which β-catenin and axin/conductin bind. Mutations in Apc are responsible for familial adenomatous polyposis, a hereditary colorectal cancer syndrome. Apc has been shown to participate in cell fate determination, cell cycle regulation, apoptosis, cell adhesion and migration, microtubule assembly, and chromosome segregation.6–7

This variety of functions is reflected by the presence of a number of specific domains in the Apc protein, such as those that mediate interaction with axin and β-catenin, microtubules, or actin cytoskeleton. Apc’s role in cell adhesion is mediated through interactions with Rho GTPases, such as Asef. Apc has been shown to interact with E-cadherin and plays a role in targeting cytoplasmic β-catenin to the cell membrane, where it promotes interaction of β-catenin with E-cadherin. However, genetic and biochemical data have demonstrated that the tumor suppressor function of Apc is dependent on its capacity to bind and downregulate β-catenin.

Here, we show retinal disorganization upon loss of Apc1 in the zebrafish. Loss of Apc1 results in defective retinal
ganglion cell (RGC) axon extension and pathfinding ensuing from complex interaction between nonautonomous signals from the mispatterned brain in concert with cell-autonomous malfunction of retinal axons. The majority of the observed abnormalities can be explained by stabilization of β-catenin.

Materials and Methods

Zebrafish embryos

Zebrafish embryos were raised and staged as described. ApcCA50a/CAS0a is a lethal recessive zygotic mutant.

In situ hybridizations and immunohistochemistry

Whole-mount in situ hybridizations (WISH) were carried out as previously described. Antisense DIG (Boehringer, Ingelheim, Germany) riboprobes were synthesized: (PCR-amplified with primers 5'-ccggttgatctgaggtgag-3'and 5'-aagaaagtctcttaagcc-3'), fgf8, gap43, irx1a, pax2a, and TOPdGFP. Whole-mount in situ hybridization was performed using antiacetylated tubulin (T6793; Sigma, St. Louis, MO; 1:250), anti-zn8 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA; 1:100), and Cy3-labeled secondary antibody (Jackson ImmunoResearch, Suffolk, UK). Histology was performed as described. Images were obtained using a Zeiss Axioplan Stereomicroscope (Oberkochen, Germany) equipped with a Leica (Wetzlar, Germany) digital camera and were adjusted for brightness and contrast using Adobe Photoshop 7.0.

Embryo genotyping

DNA sequencing was performed using the primers forward 5'-cacaactcaaaagccttca-3' and reverse 5'-acaaattgaagattggagattggtc-3' and using competitive allele-specific PCR (KASPar; Kbioscience, Hertfordshire, UK) with the primers WT 5'-gaaggtgacaccgttcccggagc-3', mutant 5'-gaaggtgacaccgttcccggagct-3', and common primer 5'-atctgccacgctgctgctg-3'.

Microinjection of mRNAs

One nanoliter of synthetic mRNA, prepared from ApcGFP construct, using the SP6 mMessage mMachine kit (Ambion, Austin, TX), was injected into one-cell-stage embryos.

Transplantations

For eye transplantations, cells from late blastula donor embryos, injected with dextran-fluorescein (10.000 MW; D1820, Invitrogen, Carlsbad, CA), were transplanted into the presumptive eye field of 1-somite apc1/−/− hosts. Transplanted cells were detected using anti-fluorescein antibody (Roche) and alkaline phosphatase– or Alexa 543–conjugated secondary antibody.

Dil labeling of optic nerve

Dil dissolved in chloroform (D282; Invitrogen) was injected into the eye of fixed embryos embedded in 1% agarose in PBS.

Imaging and quantifications

Fluorescent labelings were imaged using a Leica TCS SPE confocal microscope, and measurements were made using Volocity (Improvision) and ImageJ (NIH, Bethesda, MD). The optic nerve head area and eye size were measured at their largest diameter on three confocal z-sections with the eye mounted facing the lens using 500x magnification. The area size of the ganglion cell layer (GCL) was determined by measuring the number of zn8-labeled pixels on maximum z-projections using ImageJ (using thresholded images) and dividing it by the total area of the eye. For statistics, a two-tailed Student’s t-test was performed. For 3D projections of zn8-labeled retinas, an z-stack containing >120 sections at 0.5 μm each was rendered using Volocity software. A two-tailed Fisher’s exact probability test for a table of frequency data was performed (Fig. 5: http://faculty.vassar.edu/lowry/fisher.html).

Results

Zebrafish apcCA50a/CAS0a mutation results in hyperactivation of LEF/β-catenin signaling in the retina

In a mutagenesis screen for mutations causing axon-pathfinding errors in the developing zebrafish brain (D.Z. and C.H., unpublished data), we recovered a mutant containing a premature stop codon truncation of the encoded gene product at a Leu residue corresponding to position 613 of the human protein. This apcCA50a allele, further referred to as apc, was identified in a noncomplementation assay with mutant apc1hu745. From 36 hpf, the apc−/− mutation affects development of various embryonic structures, among which the eye, leading to lethality from 3 dpf. Similar to apc1hu745 mutants, the apc mutant eye displayed coloboma (failure to close the choroid fissure) and hyperpigmentation (not shown). Heterozygous apc+− embryos have normal eyes.

We investigated Lef1/β-catenin–mediated transcriptional activity in the retina of apc TOPdGFP (TGFP) transgenic embryos. At 28 hpf, TGFP expression was absent from the wild-type retinal epithelium (Fig. 1A). In contrast, TGFP expression was present in the basal retinal epithelium surrounding the lens in apc mutants (Fig. 1B). At later stages, TGFP expression was induced in the mutant lens as well (Fig. 1C, D). In the mutant eye, apc mRNA was strongly diminished from 30 hpf and eventually disappeared, presumably due to nonsense mediated decay (data not shown). TGFP was induced in the mutant eye in the regions corresponding to those that express apc in the wild-type eye, that is, the prospective retinal GCL at 36 hpf (Fig. 1E) from where the transcripts spread to the inner nuclear layer (INL) at 48 hpf (Fig. 1F, I). In wild-type retina, apc partially overlapped with gap43, which marks differentiating neurons that are extending axons (Fig. 1E, F). Interestingly, in apc mutants, gap43 expression in the GCL was disorganized, with aberrant labeling near the ciliary marginal zone (Fig. 1G, H).

Loss of Apc1 results in disorganization of the retina

Previous work in apc1hu745 mutant has demonstrated a requirement for Apc1 in proper retinal organization. Accordingly, histological analysis of apc mutant eyes showed that the neural retina was disorganized (Fig. 2A, B). Because apc1 was expressed in the GCL, we examined RGCs by irx1a
Loss of Apc1 results in retinal axon pathfinding defects

In addition to being thinner, the ON of apc mutants did not cross properly at the optic chiasm (OC) in ~50% of mutant embryos as observed using acetylated tubulin labeling (n = 120; data not shown). Moreover, pleiotropic RGC axon pathfinding defects were observed employing anterograde DiI labeling and zn8 antibody (Fig. 3A–I). In 25% of apc mutants, the ON displayed axons branching off immediately upon exiting the eye (asterisk in Fig. 3E) or when nearing the midline (Fig. 3B). In 50% of mutants, a portion (Fig. 3C, E) of the retinal axons crossed over to the contralateral ON as observed using DiI labeling (n = 35) and zn8 labeling at 48 hpf (n = 20; Fig. 3I). In 25% of mutants, a minority of retinal axons project beyond the OC into the optic tract (arrowhead in Fig. 3C, E).

At 60 hpf, retinal axons were arrested near the midline and/or misprojected into the contralateral ON in 55% of apc mutants as observed with DiI labeling (n = 22; data not shown). In 45% of mutants, a small subset of ON axons was projecting along the optic tract, although only in 13% of apc embryos, some axons were nearing the optic tectum. However, no axons were observed to terminate in the optic tectum (n = 22; Fig. 3F, G).

In spite of RGC mispatterning, several groups of RGCs localized at the boundaries of the RGC field were able to project axons that contributed to the ON (Fig. 3J), suggesting that disturbed retinal patterning does not cause the pathfinding defects. However, in rare cases, mutant RGC axons misprojected within the eye (Fig. 3K).

Defective branching of axons was present in tectal neuropil in >90% of mutants at 48 hpf as well as in the anterior, postoptic, and posterior commissures (not shown), showing that axon pathfinding defects are not limited to RGC axons.

Axon pathfinding defects can be secondary to mispatterning of the diencephalon. Expression of fgf8 and pax2a that are expressed in the optic stalk in early stages is expanded in the optic stalks of apc mutants at 48 hpf. This indicates that mispatterning of the optic stalk/chiasm region may be involved in the pathfinding defects as well.

Apc1 is required within RGCs for correct retinal axon pathfinding

To investigate whether the ON phenotype was caused nonautonomously by mispatterning or by a defect in RGC axon extension, fluorescein-labeled wild-type cells were transplanted into mutant eye anlagen (Fig. 4A, B). Subsequently, RGC axons were examined by antifluorescein and acetylated tubulin immunostaining at 48 hpf. In contrast to the abnormal pathfinding of mutant RGC axons (Fig. 3) the axons of transplanted wild-type RGCs exited the eye properly within the ON bundle and were able to cross the OC, although at a more ventral position than in wild-type embryos (asterisk in Fig. 4A, B), and reach the tectum (n = 5; arrowhead in Fig. 4B), comparable to axons of wild-type cells transplanted into wild-type embryos (Fig. 4A). Unexpectedly, the optic tectum neuropil that was highly disorganized in mutants was partially

expression (n = 15) and zn8 immunolabeling (n = 20). In apc mutants, the RGC layer was highly irregular and disorganized (Fig. 2C–H). Concomitantly to disorganization of the GCL, the size of the optic nerve head was reduced (Fig. 2I). To rule out the possibility that the reduced ON size was due to reduced eye size, we normalized ONH area for eye size (Fig. 2J). The mutant optic nerve head was smaller than the ratio over eye size would predict, suggesting that the number of RGC axons contributing to the optic nerve (ON) was reduced. In addition, the ratio of the GCL area to total eye area was increased, indicating that the reduction in ONH size is not due to reduced RGCs (Fig. 2K).
FIG. 2. Loss of Apc function leads to mis-patterning of the retina. (A, B) Transverse toluidine blue-stained sections of wild-type (A) and apc mutant (B) eyes. apc Retina is abnormal, with ectopic retinal pigment around the ONH (arrow). Ventral to the left. (C, D) Expression of irx1a, a marker for RGCs, is expanded in apc mutants (arrow in D). (E, F) Lateral view of wild-type (E) and mutant (F) eye labeled with z-zn8 antibody. Dashed line indicates eye outline as observed by DAPI. The RGC layer in apc mutants (F) is irregular and disorganized. (G, H) Snapshots of a 3D reconstruction of wild-type (G) and apc (H) GCL as observed using zn8 immunolabeling. The eye is displayed at frontal view in the first frame and is rotated by 45° in each consecutive frame. apc Mutant RGC layer is disorganized, with asymmetric distribution of RGC around the lens and RGCs that are not contained within the RGC layer (H). At the choroid fissure (arrow), RGCs are lacking. Widest diameter of RGC layer is 170 μm. (I–K) The mutant ONH and eyes are smaller in size as determined by measuring ONH area (I) and the ratio of ONH size to eye size (J; Student’s t-test p < 0.01). (K) The percentage of the area covered by RGCs relative to the entire eye area shows that in apc mutants, there is a significant increase in the GCL area size as compared to wild types (p < 0.05). (C–H) Lateral view, anterior to the left. Apc, adenomatous polyposis coli; le, lens; ONH, optic nerve head. Error bars represent standard error of the mean (SEM). Scale bar = 50 μm.

rescued in the tectal lobe contralateral to the transplanted eye (data not shown). Quantification of acetylated tubulin-labeled neuropil in transplanted versus nontransplanted apc mutants shows a fourfold increase in acetylated tubulin-labeled axons (Fig. 4C). Of note is that the neuropil axons do not originate from RGCs and hence somehow nonautonomously respond to proper extension of wild-type RGCs axon that do reach the mutant tectum. As mutant RGC axons do not reach the tectum (Fig. 3E, G) and transplanted wild-type RGC axons from the mutant retinae do, the data suggest a role of Apc in axon growth and extension.

Stabilization of β-catenin underlies the apc phenotype

To verify that the apc phenotypes were due to the inability of mutant Apc to downregulate β-catenin, we injected progeny of apc+/− fish with human Apc fragment containing β-catenin and Axin binding domains (ApclGFP AA1020-2032). We examined whether Apcl-GFP rescued axon phenotypes using acetylated tubulin labeling (Fig. 5A). Full rescue was defined by rescue of brain axon pathways and commissures, including the OC and the projections toward the tectum (Fig. 5B). Injection of 300 pg apclGFP mRNA resulted in full rescue of 72% of mutant embryos and partial rescue in 16% of mutants. Injection of 450 pg apclGFP mRNA rescued the axon phenotype in 86% of mutants (Fig. 5). Nonrescued mutants remained defective in several aspects of axon branching. These data show that axonal defects are mainly the result of the loss of Wnt/β-catenin-dependent function of Apcl.

Discussion

Apcl is required to restrict Wnt/β-catenin signaling during retinal patterning

Previous studies have shown that Wnt/β-catenin signaling is required for promoting proliferation of retinal progenitors. In addition, a study using apclhu745/443 zebrafish showed retinal disorganization and reduced differentiation of photoreceptors. The photoreceptor and retinal pigment epithelium defects were shown to depend on retinoic acid signaling, and TGFP hyperactivation was only observed in the lens. In this study, we used the apclC50a allele that truncates the Apcl protein N-terminally to its mutation cluster region (location of human APC mutations surrounding β-catenin-binding sites) and is therefore expected to cause a more severe phenotype with respect to Wnt/β-catenin signaling than the apclhu745 allele (data not shown). The analysis of the mutant retinal phenotype revealed activation of TGFP in the prospective GCL, suggesting that the ectopic activation of Wnt/β-catenin signaling may underlie aspects of the RGC phenotype. In agreement with the expansion of GCL characterized...
in this study, an expanded expression of ath5 that marks retinal progenitors about to exit the cell cycle and differentiate into RGCs was noted in the \( \text{apc}^{-/-} \) mutant. At the moment, it is unclear whether the RGC defects in \( \text{apc} \) mutants

![FIG. 3](image)

**FIG. 3.** Axon pathfinding defects in \( \text{apc} \) mutants. (A–E) Whole-eye fills with Dil at 48 hpf show that in most \( \text{apc} \) mutants, the ON does not cross the OC properly. In 25% of embryos, the ON does not cross at the OC and displays branching axons (arrow in B). In 50% of mutants, a portion of the ON projects toward the optic tract (arrowhead in C), while the remaining axons project into the contralateral ON (arrow in C). In some of the \( \text{apc} \) mutants with misprojecting ON into the contralateral ON, the ON from the opposite eye appears to initiate correct projection to the OT (arrowhead in E). In addition, axons regularly branch off after exiting the eye (asterisk in E). Ventral view. The outline of the contralateral eye is indicated with dashed lines. (F, G) Lateral view of embryos with Dil-labeled ON in wild-type (F) and \( \text{apc} \) mutant (G) at 60 hpf. Overlay of fluorescence and transmission images. Anterior to the left. In wild-type embryos (F), the ON terminates in the OT (arrowhead). In \( \text{apc} \) mutants (G), some RGC axons branch off the ON (arrowheads) and the ON does not reach the tectum (arrow). Inset—ON magnification. Dashed lines indicate boundary between ventral midbrain and OT. (H–K) Dorsal flatmount view of RGCs and their projections labeled with \( \alpha \)-zn8 antibody at 48 hpf shows pathfinding defects at the OC (arrow), whereas some axons continue along the optic tract (arrowhead). Box in (I) shows magnified area in (J, K). (J, K) Single confocal planes of RGCs and their axons within the \( \text{apc} \) retina. (J) Most dis-
FIG. 5. Apc axon phenotype is mainly the result of β-catenin stabilization. (A) Percentage of genotyped mutant, wild-type, and heterozygous sibling embryos, showing mutant (white), intermediate (gray), or wild-type (black) phenotypes upon injection with 300 or 450 pg apc-GFP mRNA. The number of injected embryos is indicated above the bars. Injection of apc-GFP mRNA partially rescues the axonogenesis defects as demonstrated by acetylated tubulin labeling as scored over all affected brain domains. Uninjected control embryos show variable penetrance of the mutant phenotype (72%; n = 32; p < 0.05). Injecting 450 pg rescues 86% of injected mutant embryos (n = 14; p < 0.01). About 5% of the sibling embryos were erroneously scored as mutants. Statistics: two-tailed Fisher’s exact probability test for a table of frequency data. p-Values are indicated above the bars. (B, C) Example of a wild-type (B) and an apc mutant embryo labeled with α-acetylated tubulin antibody showing a normal and a defective ON crossing (arrow). Acetylated tubulin labeling of fully rescued apc mutants is indistinguishable from wild-type controls. (B, C) Ventral view. Scale bar = 50 μm.

are due to attenuated retinoic acid signaling or activated Wnt/β-catenin signaling.

**Novel insights regarding the role of Apc and Wnt/β-catenin signaling in RGC axon pathfinding**

Overactivation of Wnt/β-catenin due to loss of zygotic apc results in variable degrees of retinal axon pathfinding defects along the retino-tectal tract. This phenotype likely results from multiple defects. Retinal axons of wild-type RGCs transplanted into mutant eyes properly exit the eye, suggesting that this phenotype is not due to mispatterning of the mutant retina.

Nonautonomous effects due to mispatterning of the diencephalon are likely to be involved. At the diencephalic midline, the establishment of boundaries between expression domains of certain genes, like pax2a and shh, is important for positioning the optic cup and regulation of axon guidance cues.20,21 The expansion of pax2a and fgf8 at the optic stalks of apc mutants is similar to the *aussicht* mutant that also displays retinal axon defects.20 Possibly, ectopic pax2a at the midline disrupts axon guidance cues in apc mutants. Although transplanted wild-type RGC axons are able to cross the midline in apc mutants, the OC is mispositioned upon transplantation of wild-type cells, suggesting that its mispositioning is secondary to brain mispatterning. Because nonautonomous components of the apc phenotype cannot be excluded by our experiments, further experiments are needed to elucidate the contribution of diencephalic mispatterning to the apc defects. Expression of axon guidance molecules such as Netrins, Semaphorins, Slits, and Eph receptors/Ephrins could be examined, and additional mosaic analysis using mutant whole-eye transplantation into wild-type hosts could be performed.

The mosaic analysis data indicate that in addition to non-cell autonomous effects, cell-autonomous components may also be involved in the apc retinal axon phenotype. Transplanted wild-type RGC axons are able to overcome mispatterning defects at the midline and extend along the optic tract to the tectum. Stabilization of β-catenin caused by Apcl LOF may affect axon outgrowth via altered transcription of potential target axon guidance receptors, such as Ephs, in the navigating axon. Several studies have implicated Apcl and β-catenin in neurite growth in *vitro*. Stabilization of β-catenin in murine retinal explants resulted in inhibition of retinal neurite growth, partly due to overactivation of Tcf-mediated transcription.22 Dendritic growth and arborization is enhanced by stabilization of β-catenin through its function in cadherin/catenin complexes.23 Apcl is specifically enriched at tips of neurites and is required for specifying axon polarity independently of its function in Wnt/β-catenin signaling.24,25 Recently, it was shown that Wnt signals can induce changes in growth cone behavior by affecting Apcl localization at microtubules.26 Both transcriptionally active and inactive stabilized β-catenin clustered at these Apcl-enriched sites and inhibited neurite outgrowth in *vitro*.25 An interesting hypothesis is that subtle differences in β-catenin signaling between neurites determine neuronal polarity; for example, downregulation of β-catenin would promote axons, whereas stabilization would promote dendritic growth. Unexpectedly and in contrast to observations in vertebrates, even a combined loss of Apcl and Apcl2 does not cause axon outgrowth defects in *Drosophila*.27 Hence, *in vivo* data show that Apcl function in axon outgrowth and extension may differ between flies and vertebrates.

The rescue experiments employing ApcGFP containing only β-catenin and Axin-binding sites show that the axon pathfinding phenotype of apc mutants is mainly due to stabilization of β-catenin and is not due to loss of other Apc protein domains. However, in these experiments, we cannot distinguish the contribution of stabilized β-catenin in complexes with N-cadherin from its function in LEF/Tcf-mediated transcription. Rescue experiments using Apc lacking β-catenin and Axin-
binding sites and β-catenin lacking domains necessary for LEF/TCF-dependent transcription could help unravel contributions of Wnt-independent functions of Apc and β-catenin, respectively, to the apec phenotype.

In summary, the zebrafish apec mutation shows that Wnt/β-catenin signaling is involved in in vivo pathfinding of axons and provides an excellent opportunity to further study the contribution of Apc1 structural protein function versus its role as an antagonist of Wnt/β-catenin pathway in vivo.

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References


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1. Wolfgang Hofmeister, Brian Key. 2013. Frizzled-3a and Wnt-8b genetically interact during forebrain commissural formation in embryonic zebrafish. *Brain Research* **1506**, 25-34. [CrossRef]
