ZEBRAFISH
Volume 6, Number 1, 2009

DOI: 10.1089/zeb.2008.0561

Wnt Signaling

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

Judith T.M.L. Paridaen,[1] Catherine Danesin,[2] Abu Tufayal Elas,[2] Sandra van de Water,[1] Corinne Houart,[2] and Danica Zivkovic[1]

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 retinae, 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 Ca^{2+} 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

[1]Hubrecht Institute, Developmental Biology and Stem Cell Research, KNAW and University Medical Center Utrecht, Utrecht, The Netherlands.
[2]MRC Centre for Developmental Neurobiology, Kings College, London, United Kingdom.

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.[8] $Apc^{CA50a/CA50a}$ is a lethal recessive zygotic mutant.

In situ *hybridizations and immunohistochemistry*

Whole-mount *in situ* hybridizations (WISH) were carried out as previously described.[9] Antisense DIG (Boehringer, Ingelheim, Germany) riboprobes were synthesized: *apc1* (PCR-amplified with primers 5′-ccgtgtgtactgtgttgagg-3′and 5′-acaggagtgtcttcaatgga-3′), *fgf8*,[10] *gap43*,[11] *irx1a*,[12] *pax2a*,[13] and *TOPdGFP*.[14]

Whole-mount immunohistochemistry 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.[15]

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′-cacaatcctaacaagccattc-3′ and reverse 5′-acacattggtgag attgtgc-3′ and using competitive allele-specific PCR (KASPar; Kbioscience, Hertfordshire, UK) with the primers WT 5′-gaaggtgaccaagttcatgctggttaaagtgctgactaaaaacgcca-3′, mutant 5′-gaaggtcggagtcaacggattggttaaagtgctgactaaaaacgcct-3′, and common primer 5′-atctgcaccgttcccggagctt-3′.

Microinjection of mRNAs

One nanoliter of synthetic mRNA, prepared from ApcGFP construct[16] 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 antifluorescein antibody (Roche) and alkaline phosphatase– or Alexa 543–conjugated secondary antibody.

DiI labeling of optic nerve

DiI 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 500× 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 $apc^{CA50a/CA50a}$ *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.[17] This $apc1^{CA50a/CA50a}$ allele, further referred to as *apc*, was identified in a noncomplementation assay with mutant $apc1^{hu745/hu745}$. From 36 hpf, the $apc^{-/-}$ mutation affects development of various embryonic structures, among which the eye,[17] leading to lethality from 3 dpf. Similar to $apc1^{hu745/hu745}$ mutants, the *apc* mutant eye displayed coloboma (failure to close the choroid fissure) and hyperpigmentation (not shown[17,18]). Heterozygous $apc^{+/-}$ embryos have normal eyes.

We investigated LEF/β-catenin–mediated transcriptional activity in the retina of *apc* TOPdGFP (TGFP) transgenic embryos.[14] 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 $apc1^{hu745}$ mutant has demonstrated a requirement for Apc1 in proper retinal organization.[18] 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*

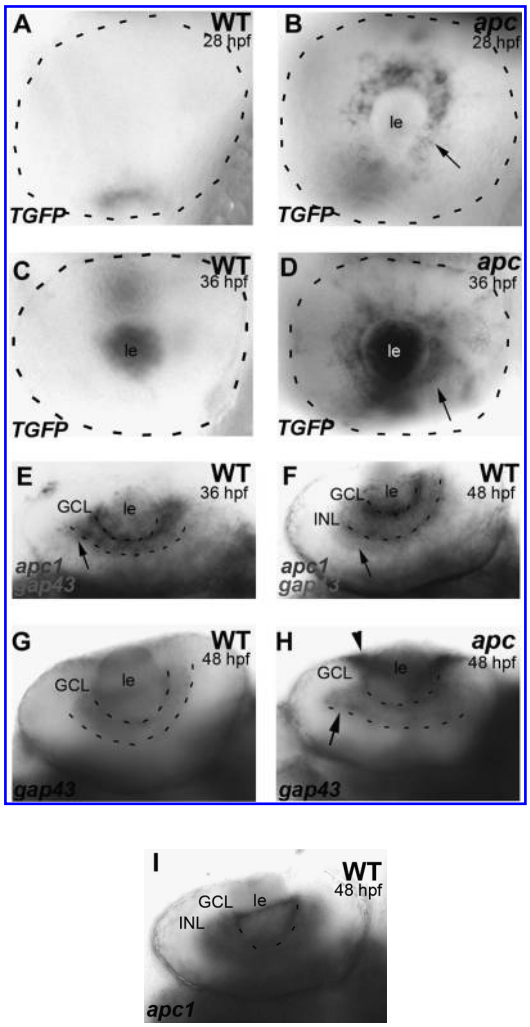

FIG. 1. The $apc^{CA50a/CA50a}$ mutation results in aberrant LEF/β-catenin transcription. (**A, B**) In wild-type embryos at 28 hpf (**A**), *TGFP* is absent from the retina. In *apc* mutants (**B**), *TGFP* is expressed in the retinal epithelium surrounding the lens (arrow). (**C, D**) At 36 hpf, *TGFP* is upregulated in the mutant lens and RGC layer (arrow in **D**). (**E, F**) Double *in situ* hybridization to *apc1* and *gap43*, which marks differentiating neurons developing axons at 36 (**E**) and 48 hpf (**F**), shows that *apc1* is expressed in areas of the retina that undergo differentiation. Dashed line indicates eye circumference (**A–D**) Lateral view, anterior to the left. (**E, F**) Ventral view, anterior to the left. (**G, H**) *gap43* Expression in WT (**G**) and *apc* mutants (**H**) at 48 hpf shows disorganization of the GCL in the mutants. (**I**) *apc1* Expression in the GCL and INL of the WT retina at 48 hpf. GCL, ganglion cell layer; INL, inner nuclear layer; le, lens; RGC, retinal ganglion cell. Scale bar = 50 μm.

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).

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 and cerebellum 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), comparably 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

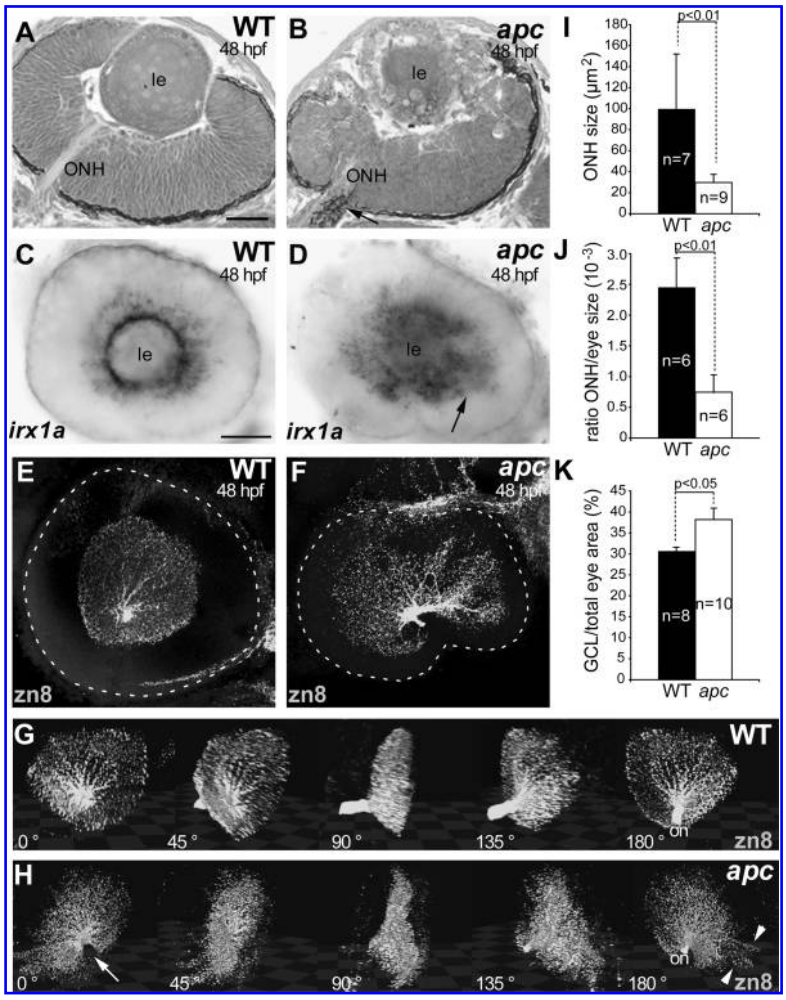

FIG. 2. Loss of Apc function leads to mispatterning of the retina. (**A, B**) Transverse toluidine blue–stained sections of wild-type (**A**) and *apc* mutant (**B**) eyes. *apc* Retina is abnormal, with ectopic retinal pigment around the ONH (arrow). Ventral to the left. (**C, D**) Expression of *irx1a*, 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 α-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 significantly smaller 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 (ApcGFP AA1020-2032[15]).

We examined whether Apc-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 *apcGFP* mRNA resulted in full rescue of 72% of mutant embryos and partial rescue in 16% of mutants. Injection of 450 pg *apcGFP* 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 Apc1.

Discussion

Apc1 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.[19] In addition, a study using $apc1^{hu745/hu745}$ zebrafish showed retinal disorganization and reduced differentiation of photoreceptors. The photoreceptor and retinal pigment epithelium defects were shown to depend on retinoic acid signaling,[18] and TGFP hyperactivation was only observed in the lens. In this study, we used the $apc1^{CA50a}$ allele that truncates the Apc1 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 $apc1^{hu745}$ allele (data not shown).[17] 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

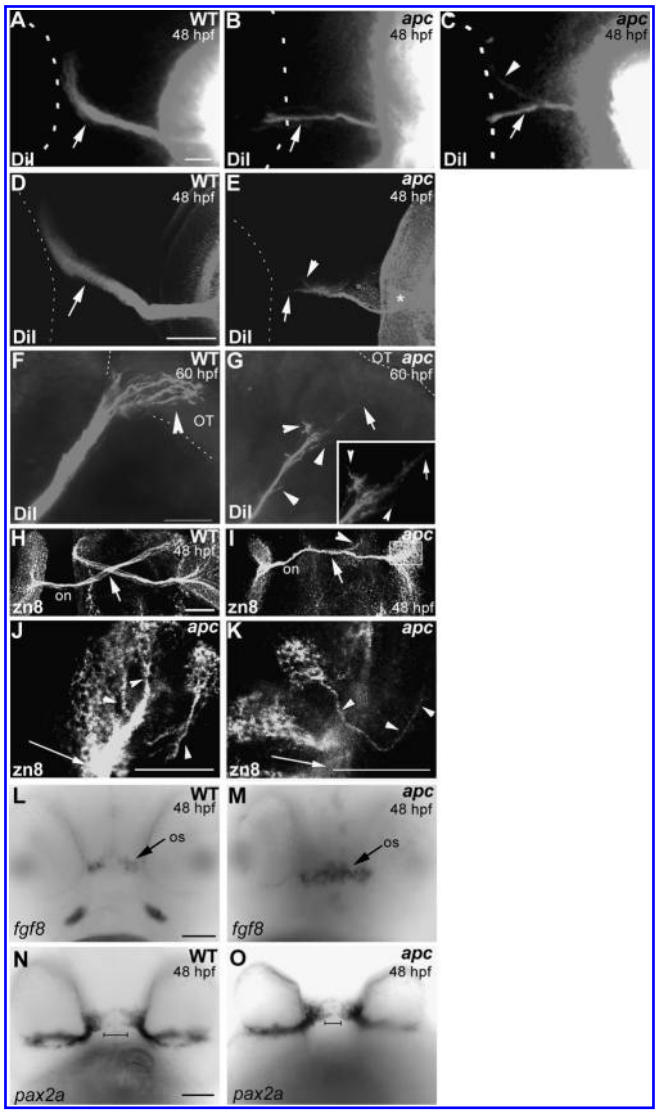

FIG. 3. Axon pathfinding defects in *apc* mutants. (**A–E**) Whole-eye fills with DiI at 48 hpf show that in most *apc* mutants, the ON does not cross the OC properly. In 25% of embryos, the ON does not cross at the OC and displays branching axons (arrow in **B**). In 50% of mutants, a portion of the 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 *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 DiI-labeled ON in wild-type (**F**) and *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 *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 α-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 *apc* retina. (**J**) Most disorganized groups of RGCs are able to extend axons (arrowheads) toward the ONH (arrow). (**K**) In rare cases, RGC misproject within the retina (arrowheads) and do not contribute to the ON (arrow). Anterior is up. (**L, M**) Expanded *fgf8* expression in the os region of *apc* mutants (arrow). (**N, O**) In *apc* mutants, *pax2a* expression is expanded toward the diencephalic midline as indicated by the bars. (**L, M**) Ventral view. ce, Cerebellum; ON, optic nerve; os, optic stalk; OT, optic tectum. Scale bar = 50 μm.

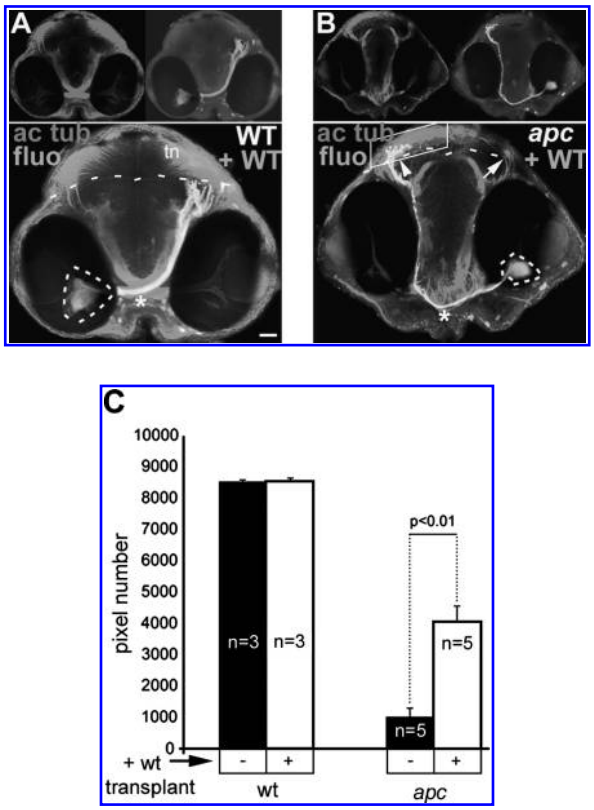

FIG. 4. Axons from transplanted wild-type cells within the $apc^{-/-}$ retina are able to reach the tectum. (**A, B**) Transplantation of wild-type cells in retina of wild-type (**A**) or mutant (**B**) embryos. Frontal view showing location of fluorescein-labeled transplanted wild-type cells (circled with dashed lines) and their axons projecting to tectum (right panel inset) and acetylated tubulin labeling (left panel inset). The boundary between OT and tegmentum is indicated with dashed lines. In mutants (**A**), wild-type axons are able to cross at the OC (asterisk) and project to the mutant tectum (arrow), similarly to the axons of wild-type cells transplanted into wild-type embryos (arrow). Acetylated tubulin labeling in the OT neuropil is increased. Note the more ventral location of the OC (asterisk) in transplanted mutant versus wild-type embryos. (**C**) Quantification of acetylated tubulin labeling in tectum neuropil (boxed area in **B**) shows 4.2-fold increase of the axonal termination zone in *apc* mutants upon transplantation with wild-type cells into the retina (Student's *t*-test $p < 0.01$). Error bars represent standard deviation (SD). tn, Tectal neuropil. Scale bar = 50 μm.

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 $apc1^{hu745/hu745}$ mutant.[18] At the moment, it is unclear whether the RGC defects in *apc* mutants

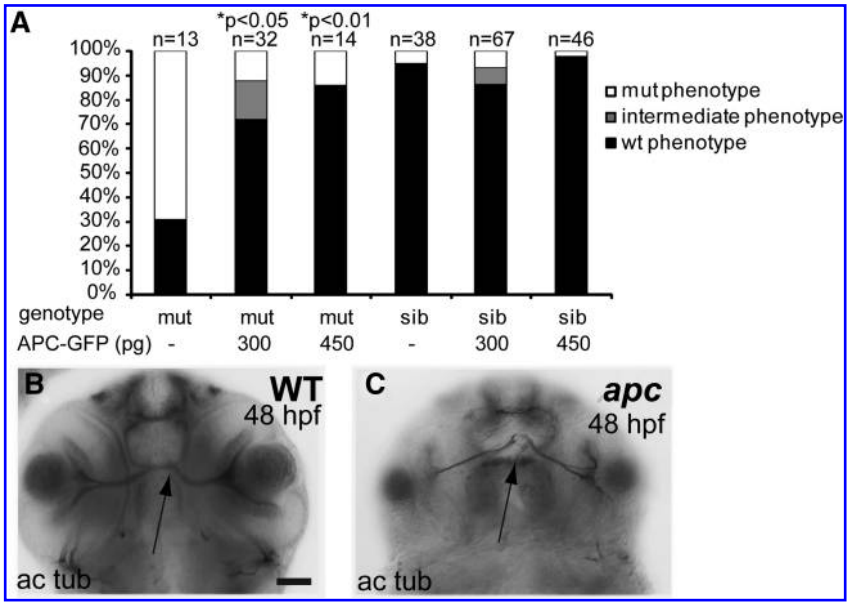

FIG. 5. Apc axon phenotype is mainly the result of β-catenin stabilization. (**A**) Percentage of genotyped mutant, wild-type, and heterozygous sibling embryos, showing mutant (white), intermediate (gray), or wild-type (black) phenotypes upon injection with 300 or 450 pg *apc-GFP* mRNA. The number of 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 mutant controls show variable penetrance of axon pathfinding defects (69%; $n = 13$). Injection with 300 pg *apc-GFP* causes rescue 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 OC 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 Apc1 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 Apc 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] Apc 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 Apc localization at microtubules.[26] Both transcriptionally active and inactive stabilized β-catenin clustered at these Apc-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 Apc1 and Apc2 does not cause axon outgrowth defects in *Drosophila*.[27] Hence, our *in vivo* data show that Apc1 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 *apc* phenotype.

In summary, the zebrafish *apc* 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.*

Acknowledgments

The authors thank S. Diks and M. Wieffer for aid with statistics, J. Korving for histology, and R. Boonen and M. Melis for technical assistance. We are indebted to L. Bally-Cuif, H. Clevers, and B. Hogan for commenting on the manuscript.

We thank R. Dorsky and R. Moon for providing TOPdGFP fish; R. Moon for the Apc-GFP construct; and A.-P. Haramis, R. Dorsky, M. Westerfield, and C. Cheng for providing riboprobes. The Hubrecht Institute Animal Care Facility is thanked for services rendered. J.P. is supported by Netherlands Organization for Scientific Research NWO Genomics Grant #050-10-024 granted to the Zivkovic Laboratory.

Disclosure Statement

No competing financial interests exist.

References

1. Logan CY, Nusse R. The Wnt signalling pathway in development and disease. Annu Rev Cell Dev Biol 2004;20:781–810.
2. van Amerongen R, Mikels A, Nusse R. Alternative Wnt signaling is initiated by distinct receptors. Sci Signal 2008; 1:re9.
3. Charron F, Tessier-Lavigne M. Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development 2005;132:2251–2262.
4. Ciani L, Salinas PC. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat Rev Neurosci 2005;6:351–362.
5. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. Science 2004;303:1483–1487.
6. Senda T, Shimomura A, Iizuka-Kogo A. Adenomatous polyposis coli (Apc) tumor suppressor gene as a multifunctional gene. Anat Sci Int 2005;80:121–131.
7. Willert K, Jones KA. Wnt signalling: is the party in the nucleus? Genes Dev 2006;20:1394–1404.
8. Westerfield M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio*). University of Oregon Press, Eugene, OR, 1995.
9. Joore J, van der Lans GB, Lanser PH, Vervaart JM, Zivkovic D, Speksnijder JE, *et al.* Effects of retinoic acid on the expression of retinoic acid receptors during zebrafish embryogenesis. Mech Dev 1994;46:137–150.
10. Reifers F, Bohli H, Walsh EC, Crossley PH, Stainier D, Brand M. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hindbrain boundary development and somitogenesis. Development 1998;125:2381–2395.
11. Reinhard E, Nedivi E, Wegner J, Skene JH, Westerfield M. Neural selective activation and temporal regulation of a mammalian GAP-43 promoter in zebrafish. Development 1994;120:1767–1775.
12. Cheng CW, Hui C, Strahle U, Chen S. Identification and expression of zebrafish Iroquois homeobox gene irx1. Dev Genes Evol 2001;211:442–444.
13. Kelly GM, Moon RT. Involvement of wnt1 and pax2 in the formation of the midbrainhindbrain boundary in the zebrafish gastrula. Dev Genet 1995;17:129–140.
14. Dorsky RI, Sheldahl LC, Moon RT. A transgenic Lef1/beta-catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development. Dev Biol 2002;241:229–237.
15. Diks SH, Bink RJ, van de Water S, Joore J, van Rooijen C, Verbeek FJ, *et al.* The novel gene asb11: a regulator of the size of the neural progenitor compartment. J Cell Biol 2006;174: 581–592.
16. Miller JR, Moon RT. Analysis of the signalling activities of localization mutants of beta-catenin during axis specification in Xenopus. J Cell Biol 1997;139:229–243.
17. Hurlstone AF, Haramis AP, Wienholds E, Begthel H, Korving J, van Eeden F, *et al.* The Wnt/beta-catenin pathway regulates cardiac valve formation. Nature 2003;425:633–637.
18. Nadauld LD, Chidester S, Shelton DN, Rai K, Broadbent T, Sandoval IT, *et al.* Dual roles for adenomatous polyposis coli in regulating retinoic acid biosynthesis and Wnt during ocular development. Proc Natl Acad Sci USA 2006;103:13409–13414.
19. Yamaguchi M, Tonou-Fujimori N, Komori A, Maeda R, Nojima Y, Li H, *et al.* Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. Development 2005;132:3027–3043.
20. Heisenberg CP, Brennan C, Wilson SW. Zebrafish aussicht mutant embryos exhibit widespread overexpression of ace (fgf8) and coincident defects in CNS development. Development 1999;126:2129–2140.
21. Barresi MJ, Hutson LD, Chien CB, Karlstrom RO. Hedgehog regulated Slit expression determines commissure and glial cell position in the zebrafish forebrain. Development 2005; 132:3643–3656.
22. Ouchi Y, Tabata Y, Arai K, Watanabe S. Negative regulation of retinal-neurite extension by beta-catenin signalling pathway. J Cell Sci 2005;118:4473–4483.
23. Yu X, Malenka RC. Beta-catenin is critical for dendritic morphogenesis. Nat Neurosci 2003;6:1169–1177.
24. Shi SH, Cheng T, Jan LY, Jan YN. APC and GSK-3beta are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. Curr Biol 2004;14:2025–2032.
25. Votin V, Nelson WJ, Barth AI. Neurite outgrowth involves adenomatous polyposis coli protein and beta-catenin. J Cell Sci 2005;118:5699–5708.
26. Purro SA, Ciani L, Hoyos-Flight M, Stamatakou E, Siomou E, Salinas PC. Wnt regulates axon behavior through changes in microtubule growth directionality: a new role for adenomatous polyposis coli. J Neurosci 2008;28:8644–8654.
27. Rusan NM, Akong K, Peifer M. Putting the model to the test: are APC proteins essential for neuronal polarity, axon outgrowth, and axon targeting? J Cell Biol 2008;183:203–212.

Address reprint requests to:
Danica Zivkovic, Ph.D.
Hubrecht Institute
Developmental Biology and Stem Cell Research
KNAW
Uppsalalaan 8
3584 CT Utrecht
The Netherlands

E-mail: d.zivkovic@niob.knaw.nl

This article has been cited by:

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]
2. W. Zac Stephens, Megan Senecal, Minhtu Nguyen, Tatjana Piotrowski. 2010. Loss of adenomatous polyposis coli (apc) results in an expanded ciliary marginal zone in the zebrafish eye. *Developmental Dynamics* **239**, 2066-2077. [CrossRef]
3. Cristina Sánchez-Camacho, Paola Bovolenta. 2009. Emerging mechanisms in morphogen-mediated axon guidance. *BioEssays* **31**, 1013-1025. [CrossRef]