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Introduction

The vertebrate eye develops through a series of co-ordinated interactions between tissues of different embryonic origin. The eye field is specified in the anterior neural plate immediately following gastrulation [1]. The lateral walls of the diencephalon then evaginate resulting in the optic vesicle [2]. The distal portion of the optic vesicle makes contact with the surface ectoderm which initiates the formation of the lens placode. Reciprocal interactions between the lens placode and the optic vesicle promote the formation of the optic cup [3]. However, such inductive interactions might not be strictly necessary since it has been shown recently that the optic vesicle can form the optic cup by a self-organising mechanism that is independent of external cues from the lens placode [4]. Lens morphogenesis, establishment of dorsoventral polarity and specification of the neural retina, retinal pigment epithelium (RPE) and optic stalk occurs concurrently with the transformation of the optic vesicle to optic cup [3].

Specification of different cell types in the eye is mediated by a number of key paracrine signalling molecules. Early neural retina specification is mediated by fibroblast growth factor (FGF) emanating from the surface ectoderm in the prospective lens placode leading to expression of the transcription factor Vax2 (also Chx10) in the lateral part of the optic vesicle [5]. There is evidence to suggest that RPE cells are specified by the transforming growth factor β (TGFβ) family member Activin A which is secreted by the extraocular mesenchyme [6]. The RPE cell is a versatile cell type and is involved in many important aspects of eye physiology [7], although whether RPE cells, once specified, influence the development of other cell types within the eye is unclear. The RPE cells are specified in the optic vesicle before pigmentation. The Microphthalmia-associated transcription factor (Mitf) is essential for RPE cell development but is expressed throughout the early optic vesicle where it marks undifferentiated bipotential neuroepithelial precursor cells and following cellular commitment becomes restricted to RPE cells [8]. The Orthodenticle homolog 2 (Otx2) transcription factor is expressed in the eye field before RPE specification but later becomes restricted to the RPE cells [9,10]. Otx2 is required for Mitf expression and activates genes important for pigmentation in co-operation with Mitf [8,10]. In contrast to Mitf, Otx2 is also important for formation of specific cell populations in the neural retina such as photoreceptor cells [11].

During the transformation of the optic vesicle into the optic cup, the opposing actions of bone morphogenetic protein (Bmp) and hedgehog signalling are thought to generate dorsoventral patterning. Hedgehog signalling has been implicated in the specification of ventral structures such as the optic stalk [12], whereas Bmp signalling has also been shown to be involved in optic vesicle development and lens placode induction [13,14,15,16]. The Bmp4 gene is expressed in the dorsal part of the optic vesicle and is involved in dorsal patterning [15,17]. The establishment of dorsoventral identity in the neural retina is manifested by the transcription factors Tbx5 and Vax2, expressed dorsally and ventrally, respectively [18]. The dorsoventral patterning of the neural retina is critical for correct topographic projection of retinal ganglion cell axons to the optic tectum in the brain, and for establishing a cone opsin gradient in the neural retina [19,20].
In addition to dorsoventral patterning the neural retina is composed of functionally distinct cell types organised into a laminar structure. The different cellular layers are referred to as follows (from outside-in), the outer nuclear layer, the inner nuclear layer and the ganglion cell layer. Photoreceptor proteins (opsins) are activated by light (photons) in the rods and cones that are in the outer nuclear layer. The inner nuclear layer consists of amacrine cells, bipolar cells and horizontal cells that are already inactivated in the already specified RPE layer in the optic cup. This phenotype is most likely due to that the RPE layer inactivation of early eye progenitor cells prior to cellular commitment in the optic cup. This phenotype is most likely due to that the RPE layer adhesion is essential for the lamination of the neural retina [36].

Moreover, the function of β-catenin has been shown to be involved in different aspects of eye development. The β-catenin molecule has two different cellular functions; to regulate cellular adhesion by interacting with cadherins, and to mediate the canonical Wnt signalling pathway [27]. In the absence of a Wnt ligand and if not bound to cadherins, β-catenin is phosphorylated and actively degraded by a multi-protein destruction complex [28]. Binding of Wnt ligands to Frizzled (Fz) transmembrane receptors and Lrp5/6 co-receptors starts a series of events leading to inhibition of phosphorylation of β-catenin [29]. The unphosphorylated β-catenin accumulates in the cytoplasm and can become translocated to the nucleus where it acts as a transcriptional activator together with DNA-binding TCF/LEF transcription factors [30]. Canonical Wnt signalling conveyed by β-catenin in the RPE cells is important for maintenance of dorsal identity in zebrafish retina [31], and inactivation of Lrp6, a co-receptor of canonical Wnt signalling, causes a defect in dorsal retinal patterning leading to a coloboma [32,33]. In studies where β-catenin function was investigated in mice it was inactivated in the female germ-line [40]. The β-catenin function was investigated in a specific developmental order that is evolutionary conserved. For example, the retinal ganglion cells are generated first, and rods and bipolar cells are generated last [22,23,24,25,26].

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Materials and Methods

Ethics Statement

The mice were maintained at the animal facility at Umeå University and all experiments involving animals were approved by the Animal Review Board at the Court of Appeal of Northern Norrland in Umeå.

Generation and Maintenance of Mice

The Lhx2-Cre transgenic mouse, β-cateninβ-cateninCreFlox/Flox mice (exons 2–6 were flanked by loxP sites) and ROSA26R mice with β-catenin has been described previously [37] [38] [39]. The β-catenin germ-line null allele was generated by crossing β-cateninβ-cateninCreFlox/Flox with β-cateninβ-cateninCreFlox/Flox transgenic mice which inactivate genes in the female germ-line [40]. The Lhx2-Cre;β-cateninβ-cateninCreFlox/Flox and the Lhx2-Cre;β-cateninβ-cateninCreFlox/Flox mice are born but die prior to postnatal day 2 (P2) of unknown causes. The genotypes of the control mice in these crosses were; Lhx2-Creβ-cateninβ-cateninCreFlox/Flox, β-cateninβ-cateninCreFlox/Flox, β-cateninβ-cateninCreFlox/Flox and β-cateninβ-cateninCreFlox/Flox. Genotyping was performed by PCR analysis of genomic DNA extracted from tail biopsies using the following primer pairs: Lhx2-Cre transgene: 5′-TTCGACAGTCTCTGCGGGC-3′ and Lhx2CreR: 5′-CTTGGGATCTCGAAGCATGTG-3′ and β-cateninβ-cateninCreFlox/Flox and the β-cateninβ-cateninCreFlox/Flox alleles, IMR1512: 5′-AACGGAAGATGATGGAAGATTTGTT-3′, IMR1513: 5′-CACCAGTGTCTCTGTCA-3′ and IMR1513: 5′-TACAGTATTTGAATTCAGG-GACCT-3′. ROSA26R mice, IMR8052: 5′-GGGCAAGATTTGCTCCTCAAGC-3′, IMR8545: 5′-AAATGCGCTGCTAGTGTGTTAT-3′ and IMR8546: 5′-GAGGGCGGAGGAAGATTGAT-3′. The morning of the vaginal plug was considered as embryonic day 0.5 (E0.5).

Histology, In situ Hybridisation and Immunohistochemistry

Embryos were isolated and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4 °C. Embryos used for in situ hybridisation were fixed between 30 minutes to 2 hours. After fixation the embryos were transferred to 30% (w/v) sucrose in PBS for 24 hours at 4 °C, mounted in Tissue-tek (Sakura) and stored at −80 °C. Sectioning (8–10 μm) was performed on a cryostat (Microm HM505E), and collected on superfrost plus slides (Menzel-Gläser). In situ hybridisation using DIG labelled probes were performed essentially as previously described [41]. The following probes were used: β-catenin (BC048153, nucleotides 170–758 that covers exons 2–5 which are deleted when β-catenin is inactivated), Lhx2 (NM_010710, nucleotides 460–1750), BMP4 (NM_007554, nucleotides 117–578), Pax6 (NM_013627, nucleotides 799–1605), Sox2 (IMAGE clone: 6143283), Otx2 (NM_144841, nucleotides 338–1158), Six6 (NM_011384, nucleotides 126–932), Six3 (NM_090896, nucleotides 771–1222), Vax2 (IMAGE clone: 6492679), Mif (IMAGE clone: 40047440), Axin2 (BC057338, nucleotides: 1–1520), Tbx5 (BC090639, nucleotides: 1–2924), Raldh1 (BC054386, nucleotides: 755–2078), Vsx2 (IMAGE clone: 40101825), Nr2f1 (IMAGE clone: 6511382), Nr2f2 (IMAGE clone: 629692), Nr1 (BC031440, nucleotides: 1–1123), Crx (BC016502, nucleotides: 1–1728), Trβ2 (IMAGE clone: 40057540) and Wnt2b (IMAGE clone: 8734027).

For hematoyxin-eosin staining, tissue sections were incubated sequentially in the following solutions; Mayer’s hematoxylin solution, in 10% (w/v) aqueous solution of acetic acid for 7–10 minutes, in 95% ethanol for 2–3 minutes, 95% ethanol for 2–3 minutes, 100% ethanol for 2–3 minutes in xylen for 2–3 minutes and xylen for 5 minutes. The slides were mounted with DPX mounting medium (WWR). To get an approximate value of the relative eye size for the different genotypes, the largest diameter of the control eyes and the mutant optic rudiment at E18.5 was measured. The diameter of the control eyes and the mutant optic rudiment at E18.5 was measured. The diameter of the control eyes and the mutant optic rudiment at E18.5 was measured. The diameter of the control eyes and the mutant optic rudiment at E18.5 was measured.
$\gamma$-Catenin Is Essential for Eye Development

The phenotype observed in embryos of other possible genotypes generated in these crosses was indistinguishable from wild type and will be referred to as “control mice” (see Material and Methods).

Eye development was severely affected in all individuals from both the Lhx2-Cre-$\beta$-catenin$^{\text{lox/lox}}$ (n = 30) and Lhx2-Cre-$\beta$-catenin$^{-/-}$ (n = 9) embryos (Figure 1B, 1C, left panels) whereas none of the control mice (n = 156) revealed any defect in eye development (Figure 1A). Histological analysis revealed that both the Lhx2-Cre-$\beta$-catenin$^{\text{lox/lox}}$ and Lhx2-Cre-$\beta$-catenin$^{-/-}$ embryos were anophthalmic and lacked a lens but instead developed a small optic rudiment with no organised eye structures (Figure 1B, 1C, left panels). The most severely affected embryos lacked most optic vesicle-derived eye structures, except for a few pigmented cells (Figure 1C, right panel), and were unable to detect any cells derived from the optic vesicle in some Lhx2-Cre-$\beta$-catenin$^{\text{lox/lox}}$ embryos (n = 2) (data not shown). The optic rudiment was smaller in the Lhx2-Cre-$\beta$-catenin$^{\text{lox/lox}}$—compared with the Lhx2-Cre-$\beta$-catenin$^{-/-}$—mutant embryos at all developmental stages analysed (Figure 1D). Hence, eye development in the Lhx2-Cre-$\beta$-catenin$^{\text{lox/lox}}$—embryos was consistently more severely affected compared with the Lhx2-Cre-$\beta$-catenin$^{-/-}$ embryos. Thus, inactivation of the $\beta$-catenin gene in the early optic vesicle causes anophthalmia showing that $\beta$-catenin is essential for eye development.

The Optic Vesicle is Unable to Transform into an Optic Cup in $\beta$-catenin Mutant Embryos

In order to identify the underlying cause of the anophthalmia we examined at which time point eye development was first perturbed. Although $\beta$-catenin was inactivated in the optic vesicle at E9.5 (Figure 2A), the optic vesicle in mutant and control embryos were indistinguishable at this stage (Figure 2A). However, the subsequent transformation into the optic cup did not occur in either the Lhx2-Cre-$\beta$-catenin$^{\text{lox/lox}}$—or the Lhx2-Cre-$\beta$-catenin$^{-/-}$—mutant embryos (n = 6 and n = 14, respectively, Figure 2B–E). In the mutants the invagination of the epidermal ectoderm is initiated leading to the formation of the lens pit, but this process is delayed compared to the control (Figure 2B–E). This suggests that the lens placode is induced in the mutants and consistent with that we observe expression of lens-specific transcription factors such as Pax6, Sox3 and Sox2 in this presumptive lens tissue (Figure 2C–E). Although $\beta$-catenin is not inactivated in the developing lens using this approach (Figure 2B), lens development did not proceed any further and the primordial lens remained attached to the epidermis (Figure 2B, C). Since no lens-associated structures could be detected in mutant embryos at later stages suggests that the lens cells that are initially formed subsequently deteriorates (Figure 1B and C, right panels). These data show that in the mutant embryos the optic vesicle is unable to transform into the optic cup and the lens does not develop.

Canonical Wnt/$\beta$-catenin Signalling is Required for RPE Cell Commitment

Canonical Wnt/$\beta$-catenin signalling has been shown to be involved in the maintenance of the RPE layer and to maintain dorsal identity of the optic vesicle [31,34,35]. We therefore examined if canonical Wnt signalling was affected in the $\beta$-catenin mutant optic vesicle by analysing expression of the Axin2 gene, which is a downstream target of activated canonical Wnt/$\beta$-catenin signalling [44,45]. Our analyses of control embryos showed that Axin2 expression is not detected at an early stage
Figure 1. β-catenin mutant embryos are anophthalmic. (A–C) The left panels show lateral views of E16.5 embryos of control (A), Lhx2-Cre:β-catenuin^{floxflo} (β-cat^{f/f}) (B) and Lhx2-Cre:β-catenuin^{floxflo} (β-cat^{f/f}) (C) embryos. Arrowheads indicate where the eye should be located in the mutant embryos. The right panels show hematoxylin/eosin staining of coronal tissue sections of the same embryos. Black arrows indicate the optic rudiment surrounded by RPE cells (red arrowheads) that can develop in the mutant embryos. NR: neural retina. L: lens. RPE: retinal pigment epithelium. (D) Relative size of the optic vesicle-derived structure in the Lhx2-Cre:β-catenuin^{floxflo} (f/f) embryos and Lhx2-Cre:β-catenuin^{floxflo} (f–/) embryos compared with control embryos (c) at the indicated embryonic age and postnatal day 1 (P1). To get an approximate value of the relative eye size for the different genotypes, the largest diameter of the control eyes and the mutant optic rudiment at different ages were measured and compared. The
(E9.25) in the optic vesicle (Figure 3B, left panel). At a slightly later stage (E9.75), Axin2 is expressed in the dorsal domain of the optic vesicle, both in prospective RPE cells and prospective dorsal neural retina (Figure 3E, left panel). In agreement with previous findings analysing Axin2 expression or using Wnt/β-catenin signalling reporter mice [34,35,46,47], we find that Axin2 expression becomes restricted to the RPE layer at E10.5 (Figure 3K, left panel). Importantly we find that Axin2 expression was not detected in the mutant optic vesicle at any stage (Figure 3E, K, right panels), which is consistent with the notion that β-catenin is specifically inactivated in this area (Figure 3D, 3J). To evaluate whether the lack of dorsal Wnt/β-catenin signalling influences the commitment to the various eye compartments, the neural retina, the RPE layer, and the optic stalk, we analysed the expression of genes that delineates these domains. During early development expression of the transcription factor Mitf is evenly distributed within the entire optic vesicle, and these cells are thought to represent uncommitted bipotential precursor cells [8]. At this stage inactivation of β-catenin had no effect on Mitf expression (Figure 3A, 3C), showing that Mitf expression in uncommitted precursor cells is independent of Wnt/β-catenin signalling. However, at the time point when Axin2 expression was detected (Wnt/β-catenin signalling was activated) in the control optic vesicle, we found that expression of Mitf was rapidly down-regulated in the dorsal/distal part of the mutant optic vesicle in the area where β-catenin was inactivated (Figure 3F, 3D). At E10.5 Mitf protein becomes undetectable in the area where β-catenin is inactivated (Figure 3L, 3J). Otx2 is also expressed in the prospective RPE cells [10]. Similar to Mitf, Otx2 expression was also rapidly down-regulated in the mutant optic vesicle in the region where β-catenin is inactivated at E9.75 (Figure 3G, 3D) and at E10.5 Otx2 expression becomes undetectable in the area where β-catenin is inactivated (Figure 3M, 3J). The down-regulated expression of Mitf and Otx2 in the mutant optic vesicle was accompanied by a dorsal expansion of the neural retina-specific gene Vsx2 (also Otx10), whose expression domain appeared to replace that of Mitf and Otx2 expression (Figure 3H, 3N). The optic stalk which is located within the ventral part of the optic vesicle/cup express Pax2 [48,49]. In control embryos Pax2 is expressed in the entire optic vesicle at E9.75 (Figure 3I) and becomes down-regulated in the RPE layer at E10.5 (Figure 3O, left panel). Since the RPE layer did not form in the mutant the entire optic vesicle structure remained Pax2+ at E10.5 (Figure 3O, right panel). These results suggest that a critical step in RPE commitment is that Mitf expression transforms from being Wnt/β-catenin-independent to become Wnt/β-catenin-dependent, which extends previously published results showing that β-catenin is important for maintaining RPE cell identity [34,35].

Several Changes in Cellular Characteristics Occur in the Mutant Optic Vesicle

The correct timing of formation of the RPE layer appears to be essential for the expansion of the neural retina. To obtain further molecular insights into how the lack of RPE cell formation affects the development of the neural retina, we initially wanted to elucidate if there is a difference in the number of proliferating cells and/or dying cells in the prospective neural retina in the mutant compared to the control embryos. To address this issue we measured cell proliferation by assessing the relative number of cells incorporating BrdU (i.e. cells in the S-phase of the cell cycle) in the prospective neural retina in the control and mutant embryos at a stage when optic cup formation was initiated. At this stage 57% (±6) of the cells in the control embryos and 34% (±4) of the cells in the mutant embryos were BrdU+ (p<0.00001) (Figure 4A), revealing that already at E10.5 the relative number of BrdU+ cells was decreased by 40% in the prospective neural retina in the mutant embryos compared to the control embryos. To quantify the number of dying cells we compared the presence of activated Caspase-3 (Casp-3), a marker for apoptotic cells, in the control and mutant embryos at E10.5. There was a slight increase in the number of cells containing activated Casp-3 cells in the mutant neural retina (4.5/section of neural retina ±2.7) compared to control embryos (1.6/section of neural retina ±1.2) (p<0.001) (Figure 4B). Thus, the lack of commitment to RPE cells in the mutant embryos also causes a significant decrease in cell proliferation and some increase in apoptotic cells in the developing neural retina.

It has been suggested that the transition of the optic vesicle to the optic cup is due to a self-organizing mechanism where the RPE layer imposes a structural constraint on the neural retina leading to an apically convex invagination of the neural retina into the RPE layer [50]. This is caused by a difference in mechanical rigidity between the neural retina and the adjacent RPE layer. At the cellular level this results from a high level of phosphorylated myosin light chain 2 (pMLC2) in the RPE cells reflecting local actomyosin activation [51], which is absent in the retinal cells causing a higher mechanical rigidity of the RPE layer compared to the neural retina [4,50]. Consistent with this we found in the control embryos that pMLC2 level was high in the RPE cells and diminished in the neural retina (Figure 4C). However, in the mutant embryos the pMLC2 level was reduced throughout the entire structure (Figure 4D), consistent with the notion that the mutant optic vesicle adopts a neural retinal fate. This suggests that a critical component of the self-organizing mechanism for optic cup formation is lost in the mutant eye.

**β-catenin is Important for Maintenance of Dorsoventral Patterning of the Retina**

To evaluate if lack of Wnt/β-catenin signalling in the dorsal optic vesicle in the β-catenin mutant affects dorsal patterning in general, we analysed the expression of a number of genes known to be involved in dorsal patterning of the optic vesicle. Bmp4, Tbx5 and Raldh1 are normally expressed in the dorsal domain of the optic vesicle at E9.5 and in the optic cup at E10.5 (Figure S1A–C, and S1D–F, left panels). The expression of these genes was attenuated in the mutant optic vesicle at E9.5 (Figure S1A–C, right panels) and by E10.5 expression was either not detected or significantly down-regulated (Figure S1D–F, right panels). To address whether β-catenin is important for initiating dorsal patterning we analysed gene expression of Bmp4 at an earlier time point. At E9.25 Bmp4 was clearly expressed in the dorsal part of the optic vesicle although β-catenin was inactivated in the mutants (Figure S1G, S1H). This suggests that consistent with previous findings in zebrafish, induction of dorsal patterning is not dependent on Wnt/β-catenin signalling whereas maintenance of dorsal patterning is [31]. The down-regulated expression of dorsal markers in the mutant optic vesicle also leads to an apparent expansion of the expression domain of ventral specific genes, such as Vsx2 (Figure 5C, 5G). Later during embryonic development essentially all cells in the mutant optic rudiment adopt a ventral
identity as the remaining cells almost exclusively expressed Vax2 (Figure 5K) and expression of Tbx5 was virtually absent (Figure 5L). Wnt2b is a ligand eliciting canonical Wnt signalling that is expressed in the dorsal part of the optic vesicle and in the prospective RPE cell layer area at the stages analysed herein (Figure 5D, 5H) [52,53]. Expression of Wnt2b was down-regulated in the dorsal part of the mutant optic vesicle at E9.5 (Figure 5D), and was undetectable at E10.5 (Figure 5H). Moreover, the transcription factors Nr2f1 and Nr2f2 (also COUP-TF1 and COUP-TF2, respectively) have recently been shown to be involved in dorsoventral patterning of the optic vesicle [20,54]. Nr2f1 is preferentially expressed in the ventral part and Nr2f2 is preferentially expressed in the prospective RPE cells in the optic cup (Figure 5I, 5J), but in the mutant optic vesicle we observe that the expression domain of Nr2f1 is expanded dorsally whereas expression of Nr2f2 is not detected in this area (Figure 5I, 5J). Therefore, the initial unaffected expression of Bmp4 in the mutant together with the latter ventralisation and loss of dorsal retinal markers in the optic rudiment, support the conclusion that induction of dorsal identity in the mutant is normal whereas β-catenin is essential for maintaining dorsal identity.

Transcription Factors that are Preferentially Expressed in the Neural Retina become Expressed in the Domain Where the RPE Cells Should Develop

Since lack of dorsal Wnt/β-catenin signalling had such severe effects on eye development, we wanted to determine if expression of transcription factors known to be critical for the progression of eye development were affected in mutant optic vesicles. This includes transcription factors Lhx2, Pax6, Six3, Six6 and Rx. Lhx2 and Pax6 are ubiquitously expressed in the control and mutant optic vesicle (Figure 6B and 2C). However, expression of Six3, Six6 and Rx that ultimately becomes restricted to the neural retina in the controls fail to become restricted in the mutants, but instead expand into the region where RPE cells should develop (Figure 6C–E). Thus, these findings are in agreement with our previous data suggesting that the cells that normally adopt an RPE cell fate instead become committed to neural retina fate in the mutant optic vesicle/cup. This study complements previous results when β-catenin is inactivated in the RPE layer [34,35], since further development of any organised eye structure is completely blocked in the β-catenin mutant embryos presented in this study.

β-catenin Remains Associated with N-cadherin and F-actin at the Apical Side of the Cells in the Mutant Optic Vesicle

In addition to mediating canonical Wnt signalling, β-catenin has also been shown to be involved in cell-cell adhesion since it interacts with the cytoplasmic part of cadherins and links it to actin filaments (F-actin) in the cytoskeleton [27]. To examine if this function of β-catenin is affected in the mutant embryos, we analysed the cellular distribution of β-catenin, N-cadherin and F-actin in the late optic vesicle/early optic cup. In the control optic vesicle, β-catenin, N-cadherin and F-actin are associated with each other at the cell membrane on the apical side of the cells in the optic vesicle (Figure 7A–D, left panels). In the mutant embryos, β-
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cattenin remains associated with N-cadherin and F-actin at the apical membrane at this early stage (Figure 7A–D, right panels. See also Figure 5A, 5B, 5E and 5F for the distribution of cells with inactivated β-catenin mRNA and decreased levels of β-catenin protein in the optic vesicle). Also at the later stage of development detectable levels of β-catenin remains associated with N-cadherin and F-actin at the cell membrane (E10.5, Figure S2). Thus, although the non-N-cadherin-associated β-catenin is rapidly depleted in the β-catenin-negative part of the mutant optic vesicle, the apical β-catenin appears to be more stable and remains associated with N-cadherin and F-actin for some time. Although we cannot exclude that reduced levels of N-cadherin-associated β-catenin might contribute to the phenotype, the maintained association of β-catenin and N-cadherin at the apical side of the cells suggest that this cellular function of β-catenin may not be affected to any major extent at the early stages of optic vesicle to optic cup transformation.

A Few RPE Cells can Escape β-catenin Inactivation Leading to the Formation of an Optic Rudiment

Since our results suggest that formation of the RPE layer is dependent on active canonical Wnt/β-catenin signalling it was surprising that a small number of RPE cells developed in most of the mutant embryos (Figure 1B, 1C). However, all mutant

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**Figure 3. Canonical Wnt/β-catenin signalling is required for RPE cell commitment.** (A–C) In situ hybridization analyses of the indicated genes on coronal sections of E9.25 somite stage 21–23 (ss21–23) control embryos (left panels) and Lhx2-Cre:β-cateninflox/flox embryos (right panels). (D–I) In situ hybridization analyses of the indicated genes on coronal sections of E9.75 (ss26–28) control embryos (left panels) and Lhx2-Cre:β-cateninflox/flox embryos (right panels). (J–O) In situ hybridization analyses (K, M–O) or immunohistochemical analyses (J, L) for gene expression of the indicated genes on coronal sections of E10.5 (ss32–34) control embryos (left panels) and Lhx2-Cre:β-cateninflox/flox embryos (right panels). β-catenin protein is indicated by red labelling in J and L while Mitf protein is indicated by green labelling in L. Arrows indicate the area where β-catenin has been inactivated in A, D and J. Arrow heads indicate the area of Axin2 expression in E and K. Dorsal-ventral (D–V) orientation for all panels is indicated in A. Scale bars: (A–C and D–O) 100 μm.

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**Figure 4. Decreased proliferation and change in physical properties of mutant optic vesicle.** (A) Immunohistochemical analysis for the presence of BrdU-labelled cells in the prospective neural retina on a coronal section of an E10.5 control embryo (left panel) and an Lhx2-Cre:β-cateninflox/flox embryo (right panel). The part of the prospective neural retina that was analysed for BrdU+ cells in the control and mutant embryos are outlined. (B) Immunohistochemical analysis of the presence apoptotic cells as revealed by the presence of activated Casp-3+ cells on coronal section of an E10.5 control embryo (left panel) and a Lhx2-Cre:β-cateninflox/flox embryo (right panel). (C) Immunohistochemical analyses for the presence of phosphorylated myosin light chain 2 (pMLC2) on a coronal section of an E10.5 control embryo. (D) Immunohistochemical analyses for the presence of pMLC2 on a coronal section on an E10.5 Lhx2-Cre:β-cateninflox/flox embryo. Insets in C and D are a magnification of the areas indicated by the squares. Scale bars: (A, B and C, D) 100 μm.

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**Figure 5.** β-catenin is important for maintenance of dorsoventral patterning of the retina. (A–D) In situ hybridization analyses (A, C, D) and immunohistochemical analysis (B) on coronal sections of E9.5 (ss25–26) optic vesicles from control (left panels) and on Lhx2-Cre:β-catenin<sup>lox/lox</sup> embryos (right panels). (E–H) In situ hybridization analyses (E, G, H) and immunohistochemical analysis (F) on coronal sections of E10.5 (ss32–34) optic cups from control (left panels) and Lhx2-Cre:β-catenin<sup>lox/lox</sup> embryos (right panels). Black arrow heads in A and E, and white arrow heads in B and F, indicate the boundaries of the area where β-catenin has been inactivated. (I, J) In situ hybridization analyses on coronal sections of E10.5 (ss32–34) optic cups from control (left panel) and Lhx2-Cre:β-catenin<sup>lox/lox</sup> embryos (right panels). (K, L) In situ hybridization analyses on coronal sections of an eye from E12.5 control (left panels) and Lhx2-Cre:β-catenin<sup>lox/lox</sup> embryos. Dorsal-ventral (D–V) orientation of all panels is indicated in A. Scale bars: (A–E, G–J, F and K, L) 100 μm.

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Figure 6. Dorsal shift in expression of retina-specific eye field transcription factors in the mutant optic vesicle. (A–E) In situ hybridization analyses on coronal sections of E10.5 optic cups from control embryos (left panels) and Lhx2-Cre;β-catenin<sup>flx/flx</sup> mutant embryos (right panels). Arrows indicate the dorsal shift in expression of the retina-specific transcription factors Six3, Six6 and Rx in mutant embryos (C–E). Scale bar: 100 μm.
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embryos analysed (n = 4, both eyes) revealed that the few RPE cells that did develop always expressed β-catenin protein (Figure 8A–F), supporting the conclusion that formation of RPE cells is dependent on functional β-catenin expression. The non-RPE cells in the optic rudiment that developed in the mutant embryos were both β-catenin<sup>−</sup> and β-catenin<sup>+</sup> (Figure 8A, 8B), revealing that these cells could develop in a β-catenin-independent manner but consistently became ventralised (Figure 5K, 5L). Of particular note, all the mutant embryos analysed where an optic rudiment could be observed contained RPE cells (n = 19) (Figure 8A, 8B and Figure 1B, 1C). This suggests that maintenance of the non-RPE cells is dependent on the formation of RPE cells. We have previously shown that all of the cells derived from the optic vesicle (neural retina, optic stalk and RPE cells) are progeny of the cells expressing the Cre recombinase in the Lhx2-Cre mouse line since previously shown that all of the cells derived from the optic vesicle (neural retina, optic stalk and RPE cells) are progeny of the cells expressing the Cre recombinase in the Lhx2-Cre mouse line since [37]. Given that a few cells in the optic rudiment expressed β-catenin, we wanted to confirm that the cells escaping β-catenin inactivation were derived from the progenitor cells defined by Cre expression and not from a separate progenitor cell population in the anterior neural plate. To distinguish between these two possibilities, we crossed the Lhx2-Cre; β-catenin<sup>flx/flx</sup> and ROSA26R mouse lines to generate Lhx2-Cre; β-catenin<sup>flx/flx</sup>ROSAX26R mouse embryos. Since all lineage-traced cells express β-Galactosidase (β-Gal) we analysed the mutant embryos for β-Gal and β-catenin expression. All cells in the mutant optic rudiment, including both the β-catenin<sup>−</sup> and the β-catenin<sup>+</sup> cells, were β-Gal<sup>+</sup> (n = 3, both eyes) (Figure 6F–J). These data strongly suggest that floxed β-catenin alleles can escape inactivation even though they originate from the Cre<sup>+</sup> progenitor cells in the anterior neural plate. Since this observation suggests thatCre-mediated recombination of the β-catenin locus is less efficient compared to the ROSA26R locus it could explain why the phenotype is consistently more severe in the mutant optic vesicle, where only one allele of β-catenin has to be inactivated compared to two alleles (Lhx2-Cre; β-catenin<sup>flx/flx</sup> vs. Lhx2-Cre; β-catenin<sup>flx/flx</sup> embryos) to generate β-catenin<sup>−</sup> cells (Figure 1B–D). Furthermore, these data also suggest that the size of the optic rudiment correlates to the number of RPE cells that escape β-catenin inactivation.

Development of Retinal Cell Classes is Independent of β-catenin

As most transcription factors known to be important for eye development were expressed in the mutant optic vesicle, we next wanted to evaluate whether differentiation into more mature cell types occurred in the mutant embryos. We therefore assayed for the presence of different neuronal cell types that normally are located in the various cell layers of the retina. By using markers for more mature committed cells, it appears as if retinal ganglion cells (Bra3<sup>+</sup>) (Figure S3B) [55] rods (Nrl<sup>+</sup>, Gcx<sup>+</sup>) (Figure S3C, S3D) [56,57], cones (Trβ2<sup>+</sup>, Gcx<sup>+</sup>) (Figure S3D, S3E) [57,58], horizontal cells and amacrine cells (Prox1, Ptf1a) [59,60,61] could develop (Figure S3F, S3G). Thus, most cell types within the mature neural retina were present in the mutants, although in an apparent random distribution and reduced number. Moreover, these cell types appear to develop from precursor cells located in both β-catenin<sup>+</sup> as well as β-catenin<sup>−</sup> areas in the mutant optic rudiment (Figure S3B–G), further supporting the view that the neural cell types in the retina can develop independent of β-catenin [56].

Figure 7. β-catenin remains associated with N-cadherin and F-actin at the apical side of the cells in the mutant optic vesicle. (A–D) Immunohistochemical analyses for cellular localisation of the indicated proteins on coronal sections of E10.5 optic vesicles from control embryos (left panels) and Lhx2-Cre; β-catenin<sup>flx/flx</sup> embryos (right panels). (C) is a merge of (A) and (B) to show co-localisation (yellow) in the apical side of the cells in the optic vesicle on both control and mutant embryos (arrows). (D) A serial section of the same embryo as in (A–C) revealing F-actin protein at the apical side of the cells in both control and mutant optic vesicles (arrows). Scale bars: (A–C and D) 50 μm.
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Thus, while some mature neurons develop, the stereotyped organisation that underlies the topography and function of the normal eye is profoundly disrupted in the mutants.

Discussion

By inactivating the β-catenin gene prior to the cellular commitment into prospective neural retinal cells and RPE cells in the optic vesicle, we have demonstrated that this gene is essential for optic cup formation. β-catenin has at least two different cellular functions; i) activating canonical Wnt signalling by translocation into the nucleus. ii) regulating cell adhesion by binding to the cadherin family of adhesion molecules [27]. We have shown that β-catenin mutant embryos have significantly down-regulated canonical Wnt signalling in the dorsal optic vesicle. This reduction in canonical Wnt signalling in the dorsal
optic vesicle causes a severely compromised commitment to RPE cells in the mutant embryos as evidenced by a lack of Mitf and Otx2 expression. However, a small number of RPE cells could develop in the mutant embryos leading to the formation of an optic rudiment. While we demonstrate that the RPE cells are derived from the Cre+ eye committed progenitor cells in the anterior neural plate, we clearly demonstrate that these cells have escaped β-catenin inactivation and are hence β-catenin+. Moreover, the significantly reduced expression levels of dorsal markers led to an almost complete ventralisation of the non-RPE cells in the optic rudiment. Moreover, active Wnt/β-catenin signalling in RPE cells at an early stage of optic vesicle development is evolutionary conserved in fish, chicken and frogs [31,52,62,63]. Collectively, our and other observations strongly suggest that the severe eye phenotype observed in the β-catenin mutant embryos is due to the lack of canonical Wnt signalling resulting in blockade of RPE cell commitment in the optic vesicle. RPE cells have been suggested to be specified by the TGFβ family member Activin A which is secreted by the extraocular mesenchyme [6]. Whether TGFβ signalling is upstream of Wnt signalling or if they act in parallel to induce commitment to RPE remains to be elucidated.

In previous studies β-catenin was inactivated during eye development at a stage when the optic cup had been formed [34,35], hence the RPE and the neural retina were already specified in these studies. β-catenin was inactivated in the RPE cell layer which lead to the loss of RPE cell identity and transdifferentiation of the committed RPE cells into neural retinal cells [34,35]. A similar phenotype is also observed when the transcription factors Mitf or Otx2 are inactivated, or when both N2f2l1 and N2f2 are inactivated [3,10,54,64]. β-catenin has been suggested to be important for maintaining RPE cell identity by directly binding to putative Tcf/Lef binding sites in the Mitf and Otx2 enhancers [35]. Moreover, in the previous studies where β-catenin was inactivated during eye development the mutant eye develops relatively mature structures including a lens and neural retina, revealing that the phenotype is considerably milder compared to the phenotype described in this study. It has also been shown that β-catenin and Lef1 can directly interact with Mitf to induce Mitf-specific target genes in melanocytes [65,66], indicating that Wnt/β-catenin signalling is important for maintenance of pigmented cells. Whether this is the case for RPE cells remains to be determined. However, our study extends these findings by suggesting that β-catenin is also essential for the specification of the RPE cells, which is essential for optic cup formation. We show here that β-catenin inactivation prior to the specification of the RPE and the retina extends the Vsx2+ neural retina domain at the expense of the Mitf+/Otx2+ RPE domain. This change in fate is further supported by the expression of the retina-specific genes Six6, Six3 and Rx that appears to replace expression of the RPE-specific genes Otx2, Wnt2b and N2f2. Although delayed, the subsequent transition of the optic vesicle to the optic cup is initiated in the mutant embryos as the optic vesicle invaginates, leading to the formation of a two-layered structure and a simultaneous invagination of the lens placode. At this stage eye morphogenesis is developmentally arrested in the mutant embryos and an optic cup is never formed. This is most likely due to that the mutant optic vesicle is unable to form an RPE layer at the correct time. Thus, the eye phenotype in the Lhx2-Cre-β-catenin+/− and Lhx2-Cre-β-catenin+/−/Lhx2-Cre-β-catenin−/−/Lhx2-Cre-β-catenin−/−/Lhx2-Cre-β-catenin−/− embryos we observe here is much more severe compared with the earlier studies on β-catenin inactivation during eye development. The results presented in this study are reminiscent of the eye phenotype observed when the development of RPE cells are severely compromised using another approach [67]. Together, this suggests that specification and expansion of RPE cells at the correct time are essential steps in the development of the eye during optic vesicle to optic cup transformation. The inability to form the optic cup when the RPE layer formation is severely compromised is in agreement with the “Relaxation-expansion model” for self-driven morphogenesis [50]. This model predicts that the formation of an optic cup is based on the premise that the retina decreases its rigidity during its specification. This is thought to be achieved by down-regulation of the microtubule coordinator pmLC2 levels in the retina compared with the RPE layer which maintains high levels of pmLC2. This results in the retina expanding inside the less flexible RPE layer [4,50]. Thus, disruption of the RPE layer and therefore loss of the more rigid “RPE frame” for the retina to fold against means that the optic cup cannot form. Our results suggest that in the mutants since most cells in the optic cup become committed to retinal fate, the whole optic vesicle down-regulates the level of pmLC2. This general down-regulation of pmLC2 presumably leads to a lack of differential rigidity and hence causes it to collapse and thereby preventing the formation of the optic cup. It has recently been suggested that N-cadherin/β-catenin and F-actin interact with mlc [68]. However, since there is no obvious difference in the levels of β-catenin between the neural retina and the RPE cells during normal eye development, pmLC2 is probably regulated by other cell type specific mechanisms and not directly by β-catenin. Furthermore, the frequency of proliferating cells in the mutant neural retina is significantly reduced and the number of dying cells is slightly increased in the mutant neural retina compared to the control neural retina, suggesting that the RPE cells regulate cell proliferation and to some extent also cell survival in the neural retina in a cell nonautonomous manner. We therefore suggest that the reduced proliferation together with the lack of structural constraint imposed by the RPE cells on the neural retina in the mutant embryos are the major causes of the observed anophthalmia. Most studies conducted thus far studying the supportive role of the RPE layer have addressed its role in regulating photoreceptor survival and differentiation [7]. If these factors overlap with or are distinct from those responsible for regulating the expansion and organisation of the neural retina during early eye development remains to be elucidated.

Although our data strongly suggests that β-catenin is essential for the formation of the RPE layer, a small number of RPE cells were still present in all optic rudiments analysed in the mutants at later developmental stages. However, all the RPE cells that developed in the mutant were β-catenin+ and had thus escaped β-catenin inactivation, emphasising that β-catenin is essential for RPE cell development. It has been suggested that dorsal and ventral RPE cells develop by different mechanisms, and that development of ventral RPE cells might be independent of β-catenin [12,34]. Our data suggest that all the RPE cells require β-catenin for their development, irrespective of their dorsoventral origin. In contrast to the RPE cells, the non-RPE cells that developed in the mutant embryos could be either β-catenin- or β-catenin+. These non-RPE cells did not form an organised retina although various neurons developed suggesting that commitment of neuronal cell types is not solely dependent on β-catenin and tissue organisation. Moreover, our data implies that in the mutant, non-RPE cells are not maintained in the absence of RPE cells, further arguing for a central role of the RPE cells in the development of the eye.

The data presented here suggest that Wnt/β-catenin signalling is important for maintaining dorsal identity of the retina. Previous studies in zebrafish have also suggested that Wnt/β-catenin signalling is important for maintaining dorsal identity of the retina in a cell nonautonomous manner since Wnt/β-catenin signalling is restricted to the RPE cells in zebrafish [31]. However, we provide
evidence that Wnt/β-catenin signalling is active in both prospective RPE cells and the developing dorsal retina, suggesting that the dorsalising effect on the retina mediated by β-catenin is cellular autonomous. Wnt2b, a ligand for the canonical Wnt/β-catenin signalling pathway, is expressed in the dorsal part of the optic vesicle/cup and the RPE cells [69], and this gene is down-regulated in our β-catenin mutant suggesting a positive feedback regulation. It should be noted that additional canonical Wnt ligands are probably required for the RPE cell layer formation and dorsalisation of the neural retina since Wnt2b null mice have no reported eye defects [70].

Another mediator of canonical Wnt signalling is the Fz co-receptor Lrp6. Lrp6 null mice do share some of the phenotypes with the β-catenin mutants presented here, but these are less severe. Although the Lrp6−/− mice have microphthalmia with coloboma, they develop the basic structures of the eye: neural retina, RPE cell layer and the lens [32,71], which is in contrast to the β-catenin mutants presented in this work. However, in agreement with our data the neural retina in the Lrp6−/− mice lose or down-regulate expression of dorsal markers such as Thbx5, Raldh1 and BMP4 and the expression domain of the ventral marker Vax2 extends dorsally [32,71]. Since canonical Wnt/β-catenin signalling is suggested to be mediated via Lrp5 or Lrp6 [72,73,74], it is possible that Lrp5 might compensate for Lrp6 in the Lrp6−/− mice and hence the phenotype will not be as severe as in the β-catenin mutant which presumably block all canonical Wnt signalling. Other mediators of Wnt signalling that have been studied during eye development the Fz receptors Fz5 and Fz8. The Fz5−/− and Fz8−/− compound mutants show a severe retinal coloboma and microphthalmia although the major eye structures, neural retina, RPE cells and lens, developed in this mutant [75]. Since there are a number of Fz receptors expressed in the eye during development [76], it is highly likely that other Fz receptors could compensate for loss of Fz5 expression and decrease in Fz8 expression and hence ameliorate the phenotype compared to the β-catenin mutant mice presented in our study.

In conclusion, β-catenin is important for eye development since it is essential for the transition of the optic vesicle to the optic cup. This defect is due to the fact that commitment of RPE cells depends on β-catenin. Moreover, β-catenin is also important for the dorsoventral patterning of the retina whereas differentiation of the various cells in the retina is independent of β-catenin.

Supporting Information

Figure S1 Maintenance but not induction of dorsal identity of the optic vesicle is dependent on β-catenin. (A–C) In situ hybridization analyses on coronal sections of E9.5 (ss25–26) optic vesicles from control (left panels) and Lhx2-Cre;β-cateninfl/fl mutant embryos (right panels). (D–F) In situ hybridization analyses on coronal sections of E10.5 (ss32–34) optic vesicles from control (left panels) and Lhx2-Cre;β-cateninfl/fl mutant embryos (right panels). Arrow heads indicate the boundaries where β-catenin has been inactivated in the optic vesicle. Arrows indicate Bmp4 expression. Dorsal-ventral (D–V) orientation for all panels is indicated in A. Scale bars: (A–F and G, H) 100 μm.

Figure S2 β-catenin protein remains associated with N-cadherin and F-actin in the mutant optic vesicle at E10.5. (A–D) Immunohistochemical analyses for cellular localisation of the indicated proteins on coronal sections of E10.5 (ss33–35) optic vesicles from control embryos (left panels) and Lhx2-Cre;β-cateninfl/fl mutant embryos (right panels). (A) and (B) are merged in (C) to show co-localisation (yellow) on the apical side of the cells in the optic vesicle. (D) Serial section following those in (A–C). Note that while the RPE and the neural retina have been specified in the control embryo (left panels), the corresponding structure in the mutant embryo contains almost exclusively cells of neural retinal fate at this developmental stage (see Figure 3L, 3M, and 3N). Scale bar: 100 μm.

Figure S3 Retinal cell classes can develop independent of β-catenin. (A) Hematoxylin/eosin staining of coronal sections of an E18.5 control embryo (left panel) and an Lhx2-Cre;β-cateninfl/fl mutant embryo (right panel). Arrow indicates the optic rudiment that can develop in mutant embryos (right panel). (B, F, G) Immunohistochemical analyses for the presence of the indicated proteins on coronal sections of control (left panels) and mutant embryos (right panels). (C–E) In situ hybridization analyses for gene expression analysis of the indicated genes on coronal sections of control (left panels) and mutant embryos (right panels). All the sections from the mutant embryo have been analysed for β-catenin expression on a consecutive section to ensure that the distribution of β-catenin+ and β-catenin− cells shown in panel (B), is maintained in all panels. Scale bars: (A) 500 μm. (B-G, left and right panels) 100 μm.

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Author Contributions

Conceived and designed the experiments: ACH LC. Performed the experiments: ACH AB. Analyzed the data: ACH AB LC. Contributed reagents/materials/analysis tools: ACH AB. Wrote the paper: LC.

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