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Targeted Ablation of Crb1 and Crb2 in Retinal Progenitor Cells Mimics Leber Congenital Amaurosis

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Abstract

Development in the central nervous system is highly dependent on the regulation of the switch from progenitor cell proliferation to differentiation, but the molecular and cellular events controlling this process remain poorly understood. Here, we report that ablation of Crb1 and Crb2 genes results in severe impairment of retinal function, abnormal lamination and thickening of the retina mimicking human Leber congenital amaurosis due to loss of Crb1 function. We show that the levels of Crb1 and Crb2 proteins are crucial for mouse retinal development, as they restrain the proliferation of retinal progenitor cells. The lack of these apical proteins results in altered cell cycle progression and increased number of mitotic cells leading to an increased number of late-born cell types such as rod photoreceptors, bipolar and Müller glia cells in postmitotic retinas. Loss of Crb1 and Crb2 in the retina results in dysregulation of target genes for the Notch1 and Yap/Hippo signaling pathways and increased levels of P120-catenin. Loss of Crb1 and Crb2 result in altered progenitor cell cycle distribution with a decrease in number of late progenitors in G1 and an increase in S and G2/M phase. These findings suggest that Crb1 and Crb2 suppress late progenitor pool expansion by regulating multiple proliferative signaling pathways.

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Introduction

During vertebrate retina development, one type of glial cell and six types of neurons are formed by the orderly generation of postmitotic cells from a common pool of retinal progenitor cells [1,2]. In this temporally fine-tuned process, ganglion cells are generated first, followed by horizontal cells, cone photoreceptors and early born amacrine cells, rod photoreceptors and late born amacrine cells, and finally bipolar cells and Müller glial cells [2]. Retinal progenitor cells are elongated and polarized cells that extend along the apicobasal axis and connect to adjoining cells by adherens junctions via their apical processes. The proliferation of the progenitors is carefully regulated through a combination of intrinsic and extrinsic signals followed by a complete cessation of cell division around 10 days after birth in mice [3]. Many extrinsic soluble or membrane-bound factors directly promote proliferation activity such as Notch, sonic Hedgehog and Wnt signalling pathways [4]. In addition, intrinsic regulatory genes and transcription factors such as Chx10 regulate the cell cycle machinery [3].

Recent work suggests that cell adhesion and cell polarity complex proteins play a critical role in the maintenance of the proliferation of the progenitor cells [6]. The polarity proteins that form the Crumbs complex reside at the subapical region adjacent to the adherens junctions between retinal progenitor cells in the developing retina or between photoreceptors and Müller glial cells in mature retinas. The Crumbs protein was first identified in Drosophila as a key developmental regulator of apical-basal polarity [7]. In mammals, the Crumbs homologue family is composed of three genes, Crb1, Crb2 and Crb3. Crb proteins have a large extracellular domain (which is lacking in CRB3) composed of epidermal growth factor and laminin-globular domains, a single transmembrane domain, and an intracellular domain containing FERM and PDZ protein-binding motifs [8]. Through this PDZ motif CRB proteins interact with PALS1, which binds to MUPP1 or PATJ, thus forming the Crumbs complex [8]. Recently, it has been shown that the CRB-interacting partner PALS1 has a role in regulating the proliferation of neural progenitors. Deletion of PALS1 in the developing cortex caused premature exit of
CRB1 and CRB2 Control the Retina Growth

**Author Summary**

Mutations in the human CRB1 gene lead to one of the most severe forms of retinal dystrophies, called Leber congenital amaurosis. Here, we report that ablation of CRB1 and the second family member CRB2 are crucial for proper retinal development. These mice display severe impairment of retinal function, abnormal lamination and thickening of the retina mimicking human Leber congenital amaurosis due to loss of CRB1 function. The thickening of the retina is due to increased cell proliferation during late retinal development leading to an increased number of late-born retinal cells. We describe in these CRB1 Leber congenital amaurosis mouse models the molecular and cellular events involving CRB proteins during the development of the retina.

progenitors from the cell cycle and massive cell death leading to absence of the cortical structures [9].

Studies suggest a common function of CRB proteins and their partners in regulating growth factor signalling pathways, which orchestrate cell proliferation and cell fate decisions. It has been suggested that Drosophila Crumbs and human CRB2 inhibit Notch1 cleavage and signalling by binding to the presenilin complex, inhibiting γ-secretase activity [10,11]. Zebrafish CRB extracellular domains can directly bind to the extracellular domain of Notch1 and inhibit its activation [12]. The Crumbs complex can negatively modulate the mammalian Target of Rapamycin Complex 1 (mTORC1) pathway via the direct interaction between PATJ and the tumour suppressor gene TSC2 and depletion of PALS1 protein results in loss of mTORC1 activity in the murine developing cortex [9,13]. The Hippo pathway is a key regulator of organ size and tumorigenesis in humans and flies [6,14]. Drosophila Crumbs has been shown to control the Hippo pathway by direct interaction of its FERM domain [15,16]. Furthermore, PALS1 and PATJ can interact with the effectors of the Hippo pathway Yes-associated Protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) proteins and thus promote their inhibition and retention in the cytoplasm [17].

Mutations in the human CRB1 gene cause autosomal-recessive progressive retinitis pigmentosa and Leber congenital amaurosis (LCA) [18]. LCA is one of the most severe forms of retinal dystrophy leading to blindness around birth due to defects in the progressive abnormal lamination of newborn rod photoreceptors and disruption of adherens junctions in postnatal developing retina [22]. Here, we study the effects of loss of CRB1 and CRB2 and their potential overlapping functions during early retinal development. Loss of both CRB1 and CRB2 results in absence of a separate photoreceptor layer, misplaced cell types throughout the retina and loss of retinal function mimicking the phenotype observed in human LCA patients. Our data suggests that the pool of late progenitor cells during retinal development is suppressed by CRB1 and CRB2 through the regulation of mitogenic signaling pathways.

**Results**

Lack of CRB1 and CRB2 severely impairs retinal function in adult mice

We crossed Cbh1 KO mice with conditionally floxed Cbh2 mice [22,24]. The mice were bred with Chx10Cre transgenic mice, which express Cre recombinase fused to GFP throughout the developing retina starting at E11.5 [28]. We showed previously that efficient recombination of the floxed Cbh2 alleles occurred around E12.5 [22]. In this study, double homozygote Cbh1<sup>−/−</sup>/Cbh2<sup>F/F</sup>Chx10Cre<sup>+/+</sup> conditional knockout retinas (Cbh1<sup>Cbh2</sup>cKO) were compared to littermate Cbh1<sup>−/−</sup>/Cbh2<sup>P/W</sup> and Cbh1<sup>−/−</sup>/Cbh2<sup>F/W</sup>Chx10Cre<sup>+/+</sup> retinas. Cbh1<sup>−/−</sup>Cbh2<sup>F/F</sup>/Chx10Cre<sup>+/+</sup> (Cbh1<sup>Cbh2</sup>cKO) retinas were compared to littermate double heterozygote Cbh1<sup>−/+</sup>/Cbh2<sup>2+/−</sup>/Chx10Cre<sup>+/−</sup> (Cbh1<sup>Cbh2</sup>cKO) retinas. We verified the loss of CRB1 and CRB2 proteins in the Cbh1Cbh2 cKO at E13.5 and P14 (Figures S1D and S3D).

In vivo functional and structural analysis were performed on 1 to 6 month (M) old Cbh1Cbh2 cKO, Cbh1<sup>−/−</sup>/Cbh2 cKO and control mice, using electoretinography, spectral domain optical coherence tomography and scanning laser ophthalmoscopy. Already at 1M, Cbh1<sup>−/−</sup>/Cbh2 cKO and Cbh1Gbh2 cKO mice showed more pronounced reduction in amplitudes of electroretinogram responses than Cbh2 cKO mice (Figures 1A and S1A). Both scotopic and photopic responses were affected, which indicate alterations of both rod and cone system components. At 3 and 6M (Figures 1B and S1B–C), electoretinogram responses were below detection level, although Cbh1<sup>−/−</sup>/Cbh2 cKO responses were more variable (Figures 1B and S1B).

In vivo imaging analysis revealed changes in Cbh1<sup>−/−</sup>/Cbh2 cKO retinas in fundus appearance as well as in retinal layer morphology in contrast to Cbh1<sup>−/−</sup>/Cbh2<sup>F/F</sup> cKO control retinas (Figure S2). With native scanning laser ophthalmoscopy, many spots and patchy areas were visible throughout the retina, corresponding to pseudo-rosettes in the photoreceptor layer and in histological sections (Figures S2B and 2A–B). Already at 1M, spectral domain optical coherence tomography revealed an aberrant layering in Cbh1Gbh2 cKO retinas (Figure 3E–F). The retina consisted of a single inner plexiform layer, an abnormal thick ganglion cell layer and a second broad nuclear layer (Figure 2A–B). All retinal cell types appeared to be generated, but a separate photoreceptor nuclear layer, inner and outer segment layer and outer plexiform layer were not formed. Two types of rosettes in the broad nuclear layer could be identified and were primarily formed of photoreceptors or ganglion cells and inner nuclear layer cells (Figure 2A–B black arrowheads and asterisks, respectively). Using electron microscopy and immunohistochemistry, we found ectopically localized photoreceptor outer segments, delocalized basal bodies of cilia, adherens junctions and ribbon synapses in the Cbh1Gbh2 cKO at 1M (Figures 2E–F and S3A–C).

The retina thickness in the Cbh1Gbh2 cKO was significantly increased compared to control retinas at P10 (276.1±13.2 µm vs 199.7±5.4 µm, respectively) and P14 (247.8±6.9 µm vs 211±7.7 µm, respectively; Figure 2G). Both Cbh1Gbh2 cKO and Cbh1<sup>−/−</sup>/Cbh2 cKO retinas degenerate rapidly after 1M, which was associated with retinal vasculature defects leading to the thinning of the retinas in 3–6M retinas (Figures 2C–D, S2 and S3). Quantification of cleaved caspase 3 positive cells showed an increase in the number of apoptotic cells in Cbh1Gbh2 cKO retinas at P10, P14 and 3M (Figure 2H). Cleaved caspase 3 positive cells

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Figure 1. Retinal function in Crb1Crb2 mutant retinas is severely impaired. Retinal function in Crb1 KO (black), Crb2 cKO (green), Crb1–/– Crb2 cKO (purple) and Crb1Crb2 cKO affected mice (red) based on single-flash electroretinogram data from 1M (A), and 3M (B) old animals. (left)
Representative single-flash electroretinogram traces recorded from the indicated genotypes under scotopic (top) and photopic (bottom) conditions. (right) Scotopic (top) and photopic (bottom) b-wave amplitude data plotted as a function of the logarithm of the flash intensity. Boxes indicate the 25% and 75% quantile range, whiskers indicate the 5% and 95% quantiles, and the asterisks indicate the median of the data. In Crb1−/− Crb2 cKO and Crb1Crb2 cKO mice, the b-wave amplitude was already considerably reduced at 1M under both scotopic and photopic conditions, and declined even at 3M compared to Crb1 KO and Crb2 cKO.

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Figure 2. Abnormal layering in Crb1Crb2 cKO retinas. Histological sections of P14 (A), 1M (B), 3M (C) and 6M (D) old control (left; Crb1+/−/2 Crb2+/− cKO), Crb1−/− Crb2 cKO (middle) and Crb1Crb2 cKO (right). Crb1Crb2 cKO retinas had a thick ganglion cell layer and a second broad nuclear layer separated by the inner plexiform layer. Crb1−/− Crb2 cKO had perturbed outer and inner nuclear layers. Ectopic localization of dark-pigmented photoreceptors (white arrows), ganglion/inner nuclear layer cells (white asterisks) and rosettes of photoreceptors (black arrowheads) was visible in the two mutant retinas (Figure S5B,D). Both mutant retinas degenerated rapidly with age. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; (E,F) Electron microscopic pictures of 1M old Crb1Crb2 cKO retinas. Some complete segments (E, white asterix), adherens junctions (E, black arrow) and centrioles of cilium (F, bracket) or ribbon synapses with vesicles on the two sides of the cleft (F, black asterix) were identified but in ectopic locations. (G) The thickness of 4–5 control and Crb1Crb2 cKO retinas from P8 to P360. Crb1Crb2 cKO retinas had a thicker retina than littermate controls at P10 and P14, followed by progressive thinning and degeneration. (H) The cleaved caspase 3 positive apoptotic cells were counted at P10, P14 and 3M from 20–30 sections of 3 littermate controls and Crb1Crb2 cKO whole retinas. Mutant retinas showed an increase in the number of apoptotic cells. Data are presented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001. Scale bar, 100 μm (A–D); 1 μm (E,F).

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Figure 3. In vivo retinal imaging in Crb1Crb2 cKO mice. 12M old control Crb1/+ Crb2/+ cKO (A–C) and 1M (D–F), 3M (G–I) and 6M (J–L) old Crb1Crb2 cKO mice were subjected to scanning laser ophthalmoscopy (A,D,G,J) and vertical spectral domain optical coherence tomography (B, E, H, I, L).
at P10 and P14 were identified as rod photoreceptor cells and at 3M mainly as bipolar cells (Figure S3E–F).

CRB1 and CRB2 are essential for proper retinal development

As CRB1 and CRB2 are expressed in the retinal progenitor cells from E12.5 onwards at the subapical region adjacent to adherens junctions [21–22] and due to the severe disorganization of these retinas in adult, we analyzed control, *Crb1<sup>+/−</sup>/Crb2<sup>−/−</sup> cKO and *Crb1/Crb2<sup>−/−</sup> cKO mice from E11.5 to P5. Whereas no visible defects were observed at E11.5 and E12.5, perturbations at the outer limiting membrane and cellular mislocalizations near the retinal pigment epithelium were visible at E13.5 in *Crb1/Crb2<sup>−/−</sup> cKO retinas (Figure 4A, black arrowhead). Between E15.5 and E17.5 in *Crb1/Crb2<sup>−/−</sup> cKO, the adherens junctions were gradually lost and the nuclei of the retinal progenitors showed abnormal orientation, whereas in control retinas, progenitors were arranged radially along the apical-basal axis (Figures 4B–C and S4B). Electron microscopic analyses showed loss of adherens junctions in the neural retina and ectopic nuclei close to the retinal pigment epithelium (Figures 4F–G and S4E–F). During retinogenesis, the photoreceptor layer and the outer plexiform layer formed at P5. However, in the *Crb1/Crb2<sup>−/−</sup> cKO, this process never ensued, as no distinct photoreceptor layer was formed (Figure 4E).

In *Crb1<sup>+/−</sup>/Crb2<sup>−/−</sup> cKO, perturbations at the outer limiting membrane started at the periphery of the retina at E15.5 (Figure 4B, black arrowhead). It progressively extended to the centre of the retinas where rosettes also formed (Figure 4C–E). In late developmental stages, in addition to photoreceptor rosettes, ganglion cell nuclei and inner nuclear layer cells were found in the outer nuclear layer and some photoreceptor nuclei were found in the ganglion cell layer (Figure 2A). These retinas display intermediate phenotypes between the *Crb2<sup>−/−</sup> cKO [22] and *Crb1/Crb2<sup>−/−</sup> cKO.

Increased number and mislocalization of late born cells in *Crb1/Crb2<sup>−/−</sup> cKO retinas

Due to the severe disorganization of the retinas, we further investigated whether all retinal cell types formed in the absence of CRB1 and CRB2. Using specific markers for the different cell types, we found that all the different cell types formed and there were no indications for hybrid retinal cell types (Figure S5 and data not shown). Several of the retinal cell types appeared to localize ectopically. To further analyze this, we compared the localization of the cell nuclei in the top and bottom parts of the broad nuclear layer in *Crb1/Crb2<sup>−/−</sup> cKO mice to the outer and inner nuclear layer in control retinas (Figures 5A–F and S5A–F). The localization of the earliest born cell types, ganglion cells (marked by Brn3b), cone photoreceptors (Cone arrestin), horizontal cells (Calbindin) and the earliest born amacrine cells (ChAT) was less affected than the late born cell types, rod photoreceptors (Rhodopsin), Müller cells (Sox9) and glutamine synthetase and bipolar cells (PKCα or Cep45-GFP under the Chx10 promoter). In *Crb1<sup>+/−</sup>/Crb2<sup>−/−</sup> cKO retinas, rods, cones and bipolar cells localized ectopically in the ganglion cell layer (Figure S5G–H), and amacrine and ganglion cells surrounded by bipolar cells formed pseudo-rosettes in the photoreceptor layer (Figure S5I–J). These results suggest that all cell types are generated in retinas that lack CRB1 and CRB2 but their normal migration/localization is affected.

To test whether retinal cell types formed in normal numbers, we counted the different cell types at P14 (Figure 5G). The number of early born cells was unchanged whereas the number of late born cells was increased compared to control retinas: GABAergic amacrine cells (19.4±1.6 versus 14.8±0.6 cells/100 μm), late born GlyT1 positive amacrine (38.9±2.8 versus 20.9±1.4 cells/100 μm), Chx10<sup>+</sup> bipolar cells (77.2±5.0 versus 46.7±2.8 cells/100 μm) and Sox9<sup>+</sup> Müller cells (44.3±1.8 versus 18.8±0.6 cells/100 μm). At P14, the number of rod photoreceptors was not significantly increased due to ongoing apoptosis (Figures 3H and S3E). We found at P10 an increase in number of rods (695±44 in *Crb1/Crb2<sup>−/−</sup> cKO and 412±17 cells/100 μm in control; Figure 5H). This finding suggests that CRB1 and CRB2 may play a role in regulating the proliferation of the retinal progenitors.

Increased cell proliferation and apoptosis in developing *Crb1/Crb2<sup>−/−</sup> cKO retinas

In the *Crb1/Crb2<sup>−/−</sup> cKO retinas, the increased number of late born cells might be due to overproliferation of progenitors or reduced apoptosis. Therefore, in control, *Crb1<sup>+/−</sup>/Crb2<sup>−/−</sup> cKO and *Crb1/Crb2<sup>−/−</sup> cKO retinas from E13.5 to P5 animals, we analysed the number of phospho-Histone H3 (phH3) positive cells and cleaved caspase 3 positive cells, which are markers for mitotic and apoptotic cell types respectively (Figures 6A–B and S6C–D). From E15.5 onwards, the number of M-phase cells was significantly increased in *Crb1/Crb2<sup>−/−</sup> cKO retinas, and the number of apoptotic cells was increased at E13.5 and E17.5 onwards. These data showed an increase in both mitosis and apoptosis in retinas lacking CRB1 and CRB2. Furthermore, cells in M-phase are normally located at the apical region in control retinas. However, in E17.5 *Crb1/Crb2<sup>−/−</sup> cKO retinas, where the apical region was almost completely lost, the cells in M-phase localized randomly throughout the entire thickness of the retina (Figure S6C–D). To test whether precursor cells formed in normal numbers, we counted at E17.5 early and late-born precursor cells. The number of Islet1<sup>+</sup> early-born precursor cells (ganglion and amacrine cells) is unchanged in contrast to an increased number of Otx2<sup>+</sup> late-born precursor cells (photoreceptors and bipolar cells; 139.3±5.0 cells/100 μm in *Crb1/Crb2<sup>−/−</sup> cKO versus 110.9±4.1 cells/100 μm in control; Figure 6D).

At E17.5, in *Crb1<sup>+/−</sup>/Crb2<sup>−/−</sup> cKO retinas, the number of mitotic and apoptotic cells was increased like in *Crb1/Crb2<sup>−/−</sup> cKO retinas (Figure S6A–B). However, at P5 an increased number of mitotic cells and a decreased number of apoptotic cells were observed like in *Crb2<sup>−/−</sup> cKO [22], indicating that the *Crb1<sup>+/−</sup>/Crb2<sup>−/−</sup> cKO showed intermediate features between *Crb2<sup>−/−</sup> cKO and *Crb1/Crb2<sup>−/−</sup> cKO.

Dysregulation of the cell cycle in *Crb1/Crb2<sup>−/−</sup> cKO retinas

We further investigated, at E17.5, which phases of the cell cycle were affected using a combination of 30 min pulse labelling with BrdU for the S-phase, phospho-Histone H3 (phH3) for the M-phase and Ki67 labelling, a marker for M, G2, S and late G1 phases of the cell cycle (Figures 6C, S6C and S6E). This showed that in *Crb1/Crb2<sup>−/−</sup> cKO retinas the number of phH3<sup>+</sup> (6.1±0.2) in control
Figure 4. Retinal development is impaired in crb1crb2 cKO. (A–E) Histological sections from E13.5 to P5 control (left), Crb1+/+Crb2 cKO (middle) and Crb1Crb2 cKO (right). From E13.5 onwards, disruption of the outer limiting membrane (A right, black arrowhead) accompanied with ectopic localization of cells extended in Crb1Crb2 cKO developing retinas (A,B right). At E17.5 and P1, in contrast to control retinas no proper ganglion cell layer was formed (C,D). The separation of the outer nuclear/photoreceptor layer formed around P5, which never happened in the Crb1Crb2 cKO retinas (E). Crb1Crb2 cKO retinas showed the first disruption in the outer limiting membrane at the periphery at E15.5 (B middle, black arrowhead), which progressively extended to the centre accompanied with rosette formation (B–E middle). Electron microscopic pictures from E17.5 littermate control (F) and Crb1Crb2 cKO (G) retinas. Control retinas showed an organized outer limiting membrane with adherens junctions (white arrowheads), retinal pigment epithelium and retinal nuclei alignments. Crb1Crb2 cKO retinas showed absence of layer organization and adherens junctions. GCL, ganglion cell layer; INL, inner nuclear layer; NBL, neuroblast layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium. Scale bar, 100 μm (A–E); 5 μm (F,G).
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The number of late born cell types is increased in Crb1Crb2 cKO retinas. The distribution of early (A–C) and late born (D–F) cell types in the three layers was quantified as a percentage of each cell type in outer, inner and ganglion cell nuclear layer in the control (white bars), and top and bottom half of nuclear layer (tNL and bNL) and ganglion cell layer in the Crb1Crb2 cKO (black bars) retinas at P14 (3–4 different animals/genotype). The distribution of early-born ganglion cells (Brn3b), cone photoreceptors (cone arrestin) and cholinergic amacrine cells (choline acetyltransferase, ChAT) was slightly affected in contrast to late-born rod photoreceptors (rhodopsin), Müller cells (Sox9) and bipolar cells (Chx10), which were to a larger extent wrongly distributed in the two nuclear layers. (G,H) The number of cells for each cell types was quantified at P14 (G) and the rods at P10 (H) in 3–4 retinas of control and Crb1Crb2 cKO, and represented by the mean ± s.e.m. The number of early born cells was not affected whereas the number of late born cells was increased in Crb1Crb2 cKO compared to control retinas at P14 and the rods at P10. bNL, bottom nuclear layer; Calb, calbindin positive horizontal cells; CAR, cone arrestin; GCL, ganglion cell layer; GlyT1, glycinergic amacrine cells; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; RHO, Rhodopsin; tNL, top nuclear layer. **P<0.01; ***P<0.001.

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In contrast to the control, some Ki67 positive cells were still proliferating even after 24 hours of BrdU labelling. We further investigated this phenomenon using the Ki67 marker to analyse the cell cycle of cells that had already exited the cell cycle in each condition (Figures 6E, G, H and S6G). At E17.5, the proportion of cells that had already exited the cell cycle in G0 was reduced whereas the proportion of cells in S and G2/M was increased and G0 unchanged. At P1 and P5, the proportion of cells in E1Chb2 cKO returned to control proportions. In addition, levels of cyclin D1, cyclin E and c-myc transcripts (Figure 7A) were changed suggesting also an aberrant regulation of the cell cycle in E1Chb2 cKO retinas at E17.5.

We examined how the cell cycle exit was affected in the mutants by injecting BrdU at E16.5 and analysing 24 hours later (Figures 6F and S6F) [29–30]. The proportion of cells which exit the cell cycle (BrdU-Ki67−) in the total population of BrdU labelled cells was significantly decreased in E1Chb2 cKO retinas (12.3±0.7%) compared to control (16.3±1.3%). However, the number of BrdU-Ki67− cells per 100 μm is not significantly different (40.2±2.9) compared to control (35.3±1.8). In summary, our data suggest that the increased population of late progenitor cells and late born cells is due to dysregulation of the cell cycle at E17.5.

**CRB1 and CRB2 restrain the overproliferation of the progenitors via the regulation of mitogenic signaling pathways**

We investigated which proliferative signalling pathway(s) might be involved in the overproliferation of the mouse progenitors in E1Chb2 cKO retinas at E17.5 and in early postnatal days.

The phospho-S6 ribosomal protein (pS6RB), a downstream target of mTOR signalling, localised in the post-mitotic cells of the retina and the number of the pS6RB positive cells or pS6RB protein levels at E17.5 and P1 were unchanged in E1Chb2 cKO retinas, suggesting that mTOR signalling is not affected in the retina upon removal of CRB1 and CRB2 (Figure 7D and data not shown).

No differences were observed in the primary downstream targets Gli1 and Pch1 of sonic hedgehog signalling (Figure 7A). The downregulation of Smoothened and Gli2 might be due to a secondary effect of the loss of CRB proteins. The sonic hedgehog signalling seemed to not be directly involved in the increased number of progenitors.

In E17.5 and P1 retinas, whereas no difference in the amount of cleaved active intracellular form of Notch1 protein was detected, the transcript levels of Notch1 and its primary downstream targets Hey1 and Hey2 were reduced in E1Chb2 cKO compared to control (Figures 7 and S7). The Notch1 signalling might be affected following loss of CRB1 and CRB2.

The role of Wnt-β-catenin canonical signalling in retinal proliferation remains controversial. In E17.5 control retinas, P120-catenin and β-catenin localized mainly in the adherens junctions at the subapical region whereas in the E1Chb2 cKO the adherens junctions were disrupted and the catenins are membrane-associated (Figure S7A–B, white arrowheads). At E17.5, levels of P120-catenin proteins were increased in E1Chb2 cKO retinas, in contrast to β-catenin, whereas transcript levels were unchanged (Figures 7 and S7E–F). Furthermore, we showed that the zinc finger protein Kaiso was expressed in E17.5 and P1 developing retinas, but that its protein levels were not affected in E1Chb2 cKO mice (Figures 7 and S7E). The presence of Kaiso in
the retina and the increased levels of P120-catenin proteins are of interest as the inhibition of Kaiso on Wnt signalling is blocked through its interaction with P120-catenin (Figure 7E) [31,32]. Only recently, YAP, the downstream effector of the Hippo pathway, has been reported to promote the proliferation of the murine progenitors in postnatal retinas, followed by downregulation around P5 during neuronal differentiation [33]. In control mice, YAP protein was detected in progenitor nuclei, overlapping with Chx10Cre-GFP localization (Figure S7C). YAP localized also at the apical region where the adherens junctions and the CRB complex were located. In the Crb1Crb2 cKO retinas, YAP localized at the remaining subapical region and only in the cytoplasm of the progenitors (Figure S7C). Phosphorylation of YAP causes its retention in the cytoplasm and binding to the adherens junctions, thus inactivating the protein [14]. Both YAP and phospho-YAP (pYAP) protein levels and the transcripts of the direct downstream targets genes CTGF and Cyr61 were reduced in Crb1Crb2 cKO retinas at E17.5 and P1 whereas P120-catenin was increased and β-catenin and Kaiso unchanged at E17.5. Data are presented as mean ± s.e.m *P<0.05; **P<0.01. doi:10.1371/journal.pgen.1003976.g007

Discussion

One key element in the construction of the retina during development is the tight control of the proliferation and differentiation of the retinal progenitor cells by a combination of extrinsic and intrinsic influences [2]. In this study, we analyzed the effect of ablation of CRB1 and CRB2 in the murine retina and showed that levels of CRB protein control the lamination and proliferation of the progenitors. Complete loss of CRB1 and CRB2 proteins in the mouse retina mimics human LCA due to mutations in the CRB1 gene.

The adherens junctions play a critical role in the migration of post-mitotic cells from the apical surface to their final destination [34]. Ganglion, bipolar and photoreceptor cells extend basal processes that guide nucleus translocation to their final destination. Bipolar and ganglion cells relinquish their apical attachment when translocation is complete whereas photoreceptors maintain adherens junctions with Müller glial cells. Amacrine and horizontal cells by contrast display active cellular migration without apical attachment by sensing their local environment [34]. Disruption of the apical adherens junctions/subapical region in Crb1Crb2 cKO retinas at E15.5 leads to ectopic localization of some photoreceptor and bipolar cells in the ganglion cell layer and vice versa, ganglion, amacrine and bipolar cells in the outer nuclear layer. In Crb1Crb2 cKO mice, where the disruption occurs two days earlier, the lack of apico-basal axis leads to distribution of all the cell types in two nuclear layers and lack of a separate photoreceptor layer. Photoreceptor, ganglion and bipolar cells may undergo misguided migration due to the lack of apical attachment. The localization of the earliest born cells remains less affected, probably due to completion of migration prior to adherens junction disruption.

Apart from the role in orchestration of migration, we suggest a direct role of CRB proteins in regulation of proliferation of retinal
progenitors. Cbh1 KO retinas do not show an obvious developmental phenotype [24], and Cbh2 cKO retinas show an increase in the number of progenitors only at P3 [22]. However, the Chx10CreTg/+ cKO showed increased number of mitotic cells from E15.5 to P10 and Chx10CreTg/+ Cbh2 cKO retinas at E17.5 and P5. Thus, the uncontrolled proliferation of progenitors is proportional to the lack of CRB1 and CRB2 proteins. A study on CRB-interacting protein PALS1 has shown that the CRB complex might be involved in the control of progenitor proliferation in the developing mouse cortex [9]. However, in mouse retinas, conditional knockout or knockdown of Pals1 does not lead to increased proliferation of retinal progenitor cells [35,36]. The role of CRB protein on the proliferation of the progenitors may be independent of PALS1 and involve other partners. Ablation of CRB1 and CRB2 proteins leads to an increased number of proliferating cells and abnormalities in the cell cycle. Hence, CRB proteins restrain the proliferation acting on the cell cycle machinery. Additionally, the lack of the apical CRB1 and CRB2 had an effect on the cell cycle exit potentially directing the decision to re-enter the cell cycle and explaining the increased number of progenitors. The reduced number of cells withdrawing the cell cycle may explain why retinal progenitor cells in Cbh1Cbh2 cKO retinas undergo several more cell cycles compared to control retinas, leading to an increase in number of late-born cell types and significant thinning of the retina.

Here, we report that CRB1 and CRB2 act on the proliferation of the retinal progenitor cells through dysregulation of the proliferative signalling pathways such as Notch1 and YAP/Hippo. In addition, we report the prevalence of Kaiso in the retina and increased level of P120-catenin at E17.5. We hypothesize that the lack of CRB1 and CRB2 leads to disruption of the adherens junction complex and release of available β- and P120-catenins in the cytoplasm and nuclei of progenitors. P120-catenin may retain in the cytoplasm leading to the loss of inhibition of the Wnt target genes. Overexpression of P120-catenin and Kaiso has been linked to aberrant mitosis in cancer cells [37,38]. Lack of CRB proteins affects the YAP/Hippo pathway. Despite its direct role on proliferation, YAP promotes cell survival by inhibiting apoptotic pathways [14]. The decrease in YAP signalling at E17.5 and P1 in Cbh1Cbh2 cKO might explain the increase in apoptosis observed.

Mutations in the CRB1 gene cause progressive autosomal-recessive retinitis pigmentosa and LCA. CRB1-LCA retinas are remarkably thick and lack distinct layers as detected by optical coherence tomography [20]. Mice lacking CRB1 function show limited and mild retinal disorganization in the inferior quadrant [24–27]. Prominent differences were found between the severe loss of retinal function in humans and the mild phenotype in mice [39]. In contrast, Chx10CreTg/+ cKO mice display a severe phenotype with progressive loss of photoreceptors and retinal activity mimicking CRB1-related retinitis pigmentosa [22]. Many genes involved in retinal dystrophies have been reported to show difference in temporal and spatial expression patterns and in their localization inside the retina [40,41]. Furthermore, compensation by other members of the same protein family occurs frequently in mice and humans such as the tumor suppressor genes during retinal development [42]. Further investigations on CRB1 and CRB2 would be needed to completely understand the difference between mice and humans.

From Chx10CreTg/+ cKO, Chx10CreTg/+ Chx10CreTg/+ cKO and Chx10Crb2 cKO retina studies, the severity of the retinal disease is inversely proportional to the amount of CRB1 and CRB2 proteins which seemed to be critical for the development of the retina. As no genotype-phenotype correlation in CRB1 retinal dystrophies has been identified [43], additional down-regulation of CRB2 function in human CRB1-mutant retinas might range from CRB1-retinitis pigmentosa to CRB1-LCA. Several polymorphisms in highly conserved residues have been identified in the CRB2 gene but not directly linked to retinal dystrophies [44]. Further investigations on possible mutations in CRB complex member genes in CRB1-LCA versus CRB1-RP patients might address the question of the genotype-phenotype correlation. Here, we report that Cbh1Cbh2 retinas display a thicker retina due to excessive proliferation of late-born retinal progenitor cells and also immature layering. Moreover, Cbh1Cbh2 and Chx10CreTg/+ Chx10CreTg/+ cKO animals show severe loss of retinal function. Cbh1Cbh2 and Chx10CreTg/+ Chx10CreTg/+ cKO retinas exhibit the characteristics of human CRB1-LCA retinopathies, and are therefore mouse LCA models for the development of therapeutic drugs.

Materials and Methods

Animals

Animal care and use of mice was in accordance with protocols approved by the Animal Care and Use Committee of the Royal Netherlands Academy of Arts and Sciences (KNAW). All mice used were maintained on a 50% C57BL/6J/OlaHsd and 50% 129/Ola genetic background. Animals were maintained on a 12 h dark/dim light cycle and supplied with food and water ad libitum. Cbh1 KO mice [24] and Chx10CreTg/+ Cbh2/+/CreTg/+ clone P1E9 (Cbh2 cKO) generated previously [22] were crossed to generate Chx10CreTg/+ Cbh2/+/Chx10CreTg/+ (Chx10CreTg/+ Chx10CreTg/+ cKO) and Chx10CreTg/+ Cbh2/+/CreTg/+ Chx10CreTg/+ (Chx10CreTg/+ cKO). Chx10CreTg/+ cKO retinas were compared to littermate Cbh2/+/CreTg/+ and Chx10CreTg/+ (Chx10CreTg/+ cKO) and Cbh2/+/CreTg/+ (Chx10CreTg/+ cKO). Chromosomal DNA isolation and genotyping were performed as previously described [22].

In vivo analysis

Scanning laser ophthalmoscopy (SLO), spectral domain optical coherence tomography (SD-OCT) and electroretinography (ERG) measurements were performed at 1, 3, 6 and 12 month in 4 to 6 animals of each genotype. Electroretinograms were recorded binocularly as described previously [45]. Single-flash responses were obtained under scotopic (dark-adapted overnight) and photopic (light-adapted with a background illumination of 30 cd/m2 starting 10 minutes before recording) conditions. Single white-flash stimuli ranged from −4 to 1.5 log cd s/m2 under scotopic and from −2 to 1.5 log cd s/m2 under photopic conditions. Ten responses were averaged with inter-stimulus intervals of 5 s (for −4 to −0.5 log cd s/m2) or 17 s (for 0 to 1.5 log cd s/m2). Retinal morphology of the anesthetized animals was visualized via SLO imaging with a HRA 1 and HRA 2 (Heidelberg Engineering, Heidelberg, Germany) according to previously described procedures (Text S1) [46]. SD-OCT imaging was performed with a commercially available Spectralis HRA+OCT device from Heidelberg Engineering. This equipment features a broadband superluminescent diode at λ = 870 nm as low coherent light source (Text S1) [47].

Morphological analysis

Eyes were collected from embryonic day E11.5 to 12M (n = 3–5/age/group) and were fixed at room temperature with 4% paraformaldehyde in PBS. Eyes were dehydrated in ethanol and embedded in Technovit 7100 (Kulzer, Wehrheim, Germany) and sectioned (3 μm). Slides were dried, counterstained with 0.5% toluidine blue and mounted under coverslips using Entellan (Merck, Darmstadt, Germany). The thickness of the retina in Cbh1Cbh2 cKO mice from P8 to 12M was measured from the outer limiting
membrane to the inner limiting membrane (from top to bottom of Chbh1Cbh2 cKO retinas) at exactly 1 mm apart from the optic nerve and the average of the ventral and dorsal measurement was compared to the dorsal measurement of control mice.

**Standard transmission electron microscopy**

E17.5 and 1M old mice were perfused with 4% paraformaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. After the retinas were dissected free, they were post-fixed in 1% osmium tetroxide. Tissues were thoroughly rinsed and stained with 2% uranyl acetate in 70% ethanol. Samples were then dehydrated in a graded series of ethanol and embedded in epon 812 (Polysciences). Ultrathin sections were examined with a Zeiss 912 electron microscope.

**Quantification of apoptotic, proliferating and retinal cells**

Positive cells (Table S1) from 20–30 representative sections of the whole retina from 3–5 different control or experimental animals were manually counted and corrected by the length of each section (measured using ImageJ® software fiji-win32). Retina sections of E13.5 to P5 were stained with cleaved Caspase 3 (cCasp3; marker for apoptotic cells) and phospho-Histone H3 (pH3); marker for M-phase mitotic cells] antibodies.

To examine the proportion of progenitors in S-phase, pregnant females were injected with BrdU (50 µg/g body weight) at E17.5 and embryos were collected 30 min after BrdU injection. To examine the number of progenitors which exit the cell cycle, pregnant females were injected with BrdU at E16.5 and embryos were collected 24 h later. The number of BrdU+/Ki67 cells represents the number of cells which have exited the cell cycle. The number of retinal cells at P14 was counted on 20–30 representative pictures of retinas stained with specific antibodies for each cell type. Cones, rods, horizontal, Müller and ganglion cells analysis was performed using the flow cytometer BD LSR Fortessa. See more details about the analysis in Text S1.

**Western blotting**

The E17.5 and P1 retinas from at least 3 Chbh1Cbh2 cKO or control littermate mice were isolated, homogenized and incubated on ice in 20 µL of lysis buffer (10% glycerol, 150 mM NaCl, 1 mM EGTA, 0.5% Triton x-100, 1 mM PMSF, 1.5 mM MgCl2, 10 µg/µl aprotin, 50 mM Hepes pH 7.4 and protease inhibitor cocktail). Retina extracts from 3 independent control and Chbh1Cbh2 cKO animals were fractionated by SDS-PAGE electrophoresis, using 4–12% precast gels (NuPage Novex Bis-Tris Mini Gels, Invitrogen). After transfer to nitrocellulose membrane and blocking in 3% BSA in T-TBS buffer (Tris-HCl 50 mM pH7.5, 200 mM NaCl, 0.05% Tween-20), the primary antibodies (table S1) were diluted 1/1000 in T-TBS-5% BSA and incubated overnight at 4°C. After washing, they were incubated with the appropriate secondary antibodies (conjugated to DyLight Dyecolor-conjugated secondary antibodies (conjugated to DyLight Dyecolor-conjugated secondary antibodies (conjugated to DyLight Dye-600, Li-COR Odyssey or to cyanine 5) diluted 1/5000 in T-TBS buffer. After washing, the blots were then scanned using Li-COR Odyssey IR Imager. Densitometry of bands was performed in ImageJ. The densitometry for each band was subtracted to the background and normalized with GAPDH densitometry from the same sample.

**Real-time quantitative PCR**

RNA was isolated from 3–6 control and Chbh1Cbh2 cKO retinas using TRIZOL reagent (Gibco life technologies), according to the manufacturer manual, and after the final precipitation dissolved in RNase-free water. After genomic DNA degradation with RNase-free DNase I (New England Biolabs), 1 µg of total RNA was reverse transcribed into first-strand cDNA with Superscript III Plus RNase H-Reverse Transcriptase (Invitrogen) and 50 ng random hexamer primers, during 30 min at 50°C in a total volume of 20 µl. To the resulting cDNA sample, 14 µl of 10 mM Tris, 1 mM EDTA was added. From all samples, a 1:20 dilution was made and used for qPCR analysis. For this analysis, primer pairs were designed with a melting temperature of 60–62°C, giving rise to an amplicon of 80–110 bp. Real-time qPCR was based on the real-time monitoring of SYBR Green I dye fluorescence on a ABI Prism 7300 Sequence Detection System (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). The PCR conditions were as follows: 12.5 µl SYBR Green PCR 2× mastermix (Applied Biosystems), 20 pmol of primers, and 2 µl of the diluted cDNA (ca 3 ng total RNA input). An initial step of 50°C for 2 min was used for AmpErase incubation followed by 15 min at 95°C to inactivate AmpErase and to activate the AmpliTaq. Cycling conditions were as follows: melting step at 95°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 40 cycles. At the end of the PCR run, a dissociation curve was determined by ramping the temperature of the sample from 60 to 95°C while continuously collecting fluorescence data. Non template controls were included for each primer pair to check for any significant levels of contaminants. Values were normalized by the mean of the 3 reference genes hypoxanthine-guanine phosphoribosyltransferase, elongation factor 1-a and ribosomal protein S27a.

**Statistical analysis**

Normality of the distribution was tested by Kolmogorov-Smirnov test. Statistical significance was calculated by using t-test of 3–5 independent retinas (20 sections)/genotype/age. Values are expressed as mean ± s.e.m. Values of *P<0.05, **P<0.01, ***P<0.001 were considered to be statistically significant. Calculations and graphs were generated using GraphPad Prism 5.

**Supporting Information**

**Figure S1** Retinal activity in Chbh1Cbh2 mutant retinas is severely impaired, (A–C) Single-flash electrotretinogram age series in double heterozygote Chbh1+/−Cbh2+/− cKO (blue), Chbh1−/−Cbh2 cKO (purple) and Chbh1Cbh2 cKO mice (red) at 1M (A), 3M (B) and 6M (C). Scotopic b-wave amplitudes were plotted as a function of the logarithm of the flash intensity. Boxes indicate the 25% and 75% quartile range, whiskers indicate the 5% and 95% quantiles, and solid lines connect the medians of the data. In affected Chbh1−/−
~Crb2 cKO and Crb1Crb2 cKO mice, the b-wave amplitude was already considerably reduced at the age of 1M under both scotopic and photopic conditions, and declined even at 3M and 6M to a flat electroretinogram.

(TIF)

**Figure S2** In vivo retinal imaging in Cbx1Cbx2 cKO mice. Cbx1Cbx2 cKO mice were examined with scanning laser ophthalmoscopy (A,D,G), spectral domain optical coherence tomography (B,C,E,F,H,I) at the age of 1M (A–C), 3M (D–F) and 6M (G–I). Due to the fact that younger mice from this genotype did not show morphological alterations (data not shown), 12M Cbx1Cbx2 cKO were used as controls, and even here no abnormalities were found neither in the native fundus image, nor in the autofluorescence or the retinal vasculature (Figure 3). The retinal organization was also unaffected, as observed by optical coherence tomography analysis (Figure 3). Cbx1Cbx2 cKO animals already at 1M showed a spotty fundus, as well as several degeneration sites represented by the presence of fluorescent material detectable at 488 nm (A). In the optical coherence tomography analysis, a decrease in the retinal thickness was observed as well as a wavy appearance of the outer plexiform layer together with the formation of structures like rosettes located in the outer nuclear layer (B,C). At 3M, the retinal thickness was further decreased, specially at the level of the outer nuclear layer (E,F). In the autofluorescence image, many hyper and hypo fluorescent regions as well as several vascular changes indicating neovascularization processes were observed (D). Six month old individuals presented a more severe degeneration ascertained by scanning laser ophthalmoscopy (G) and optical coherence tomography (H,I). Abbreviations: AF, autofluorescence; d, dorsal; FA, fluorescein angiography; RF, red free; v, ventral.

(TIF)

**Figure S3** Loss of Crumbs complex and adherens junctions, ectopic synapses and cell death in Cbx1Cbx2 cKO retina. Confocal immunohistofluorescent representative pictures of CRB1 and CRB2, adherens junction marker (Nectin1), Crumbs complex members (PALSL1 and MUPP1), OPL ribbon synapse markers (PSD95 and PKCα for bipolar cells) in control (left panel) and Cbx1Cbx2 cKO (right panel) retinas at P14 (A–D). Adherens junctions and CRB complex proteins were totally absent in the subapical region, except in photoreceptor rosettes which contained few wild type cells still expressing CRB2 in Cbx1Cbx2 cKO (A–B; white arrowheads). The synapses between photoreceptor and bipolar cells were rods (Rhodopsin) at P14 and mainly bipolar cells in control (left panel) and Cbx1Cbx2 cKO (right panel) retinas at P14 (E–F). A) and apoptotic cells immunostained with cleaved caspase 3 (B) were quantified from E15.5 to P5 in 10–15 representative pictures of whole retinas from 3–5 control and Cbx1Cbx2 cKO (F) retinas. The number of apoptotic cells immunostained with cleaved caspase 3 (B) were quantified from E15.5 to P5 in 10–15 representative pictures of whole retinas from 3–5 control and Cbx1Cbx2 cKO (F) retinas. The number of mitotic and apoptotic cells at E17.5. At P5 an increase in the number of mitotic and a decrease in the number of apoptotic cells are observed. Mitotic cells were immunostained with pH3 immunostaining (M-phase) and Ki67 immunostaining (M, G2, S and late G1 phases) and counterstained with DAPI in representative pictures of control (left panel) and Cbx1Cbx2 cKO (right panel) retinas at E17.5 (C) and in the center of the retina at P5 (D). The mitotic cells displayed an aberrant distribution in Cbx1Cbx2 cKO retinas, especially the M-phase cells in E17.5 retina, which in the control localized at the outer limiting membrane whereas in Cbx1Cbx2 cKO retina these cells had a scattered distribution throughout the whole neuroblast layer (C). At P5 in the centre of the retina few Ki67+ cells were detected, whereas in Cbx1Cbx2 cKO retina many cells were still dividing especially in M-phase (D).

(TIF)
profiles of control and Cbh1Gbh2 CKO retinal cells at E17.5 (G) based on Ki67 labelling and DNA content. The number of total proliferating cells using Ki67 immunostaining is increased by a factor two in Cbh1Gbh2 CKO retinas at P5 (H). Scale bar: 50 µm. ***P<0.001.

**Figure S7** CRB1 and CRB2 act on proliferative signalling pathways. Representative pictures of P120-catenin (A) and P1. Representative Western Blot of YAP, pYAP, Notch pathways. Representative pictures of P120-catenin (A), of the retinal progenitors (colocalization with Chx10 subapical region adjacent to adherens junctions and in the nuclei of the retinal progenitors (colocalization with Chx10 CRE-GFP) whereas YAP was found only in ectopic rosette structures (white arrowheads). YAP protein was normally localized at the subapical region adjacent to adherens junctions and in the nuclei of the retinal progenitors (colocalization with Chx10 CRE-GFP) whereas YAP was found only in ectopic rosette structures (white arrowheads) similarly to the catenins and in the cytoplasm of the retinal progenitors in Cbh1Gbh2 CKO retinas. Quantification of the number of phospho-ribosomal protein positive cells (D) showed no difference between control and Cbh1Gbh2 CKO retinas at E17.5 and P1. Representative Western Blot of YAP, pYAP, Notch intracellular domain (NICD), Kaiso, β-catenin (β-catenin), P120-catenin (P120-catenin), actin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at E17.5 (E) and P1 (F). Scale bar: 50 µm. Values are presented as mean ± s.e.m.

**Table S1** Antibody list and dilution used for immunohistochemistry.

**Text S1** Detailed Materials and Methods of In vivo analysis, immunohistochemical analysis and flow cytometry.

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**Author Contributions**

Conceived and designed the experiments: LPP JW MWS ALB. Performed the experiments: LPP CHA PMQ RMV JR NW SCB GH VS. Analyzed the data: LPP CHA PMQ RMV DMSL JJD JW NT. Contributed reagents/materials/analysis tools: BH. Wrote the paper: LPP JW.

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