

RESEARCH ARTICLE

Sip1 regulates the generation of the inner nuclear layer retinal cell lineages in mammals

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ABSTRACT

The transcription factor Sip1 (Zeb2) plays multiple roles during CNS development from early acquisition of neural fate to cortical neurogenesis and gliogenesis. In humans, SIP1 (ZEB2) haploinsufficiency leads to Mowat-Wilson syndrome, a complex congenital anomaly including intellectual disability, epilepsy and Hirschsprung disease. Here we uncover the role of Sip1 in retinogenesis. Somatic deletion of Sip1 from mouse retinal progenitors primarily affects the generation of inner nuclear layer cell types, resulting in complete loss of horizontal cells and reduced numbers of amacrine and bipolar cells, while the number of Muller glia is increased. Molecular analysis places Sip1 downstream of the eye field transcription factor Pax6 and upstream of Ptf1a in the gene network required for generating the horizontal and amacrine lineages. Intriguingly, characterization of differentiation dynamics reveals that Sip1 has a role in promoting the timely differentiation of retinal interneurons, assuring generation of the proper number of the diverse neuronal and glial cell subtypes that constitute the functional retina in mammals.

KEY WORDS: Sip1, Zeb2, Zfhx1b, Differentiation, Neurogenesis, Progenitor, Retina, Retinogenesis

INTRODUCTION

The generation of correct numbers of each neural population within the CNS relies on coordinating the processes of proliferation and differentiation. The murine neural retina (NR) is an attractive model with which to study mechanisms of cell number and fate control as it is well characterized in terms of cellular composition and the temporal order of cell fate acquisition (Jeon et al., 1998; Masland, 2001; Reese, 2011). The NR consists of three cell layers: the cone and rod photoreceptors are located in the photoreceptor layer; the horizontal (HC), bipolar (BP) and amacrine (AC) interneurons and the Muller glia populate the inner nuclear layer (INL); and the retinal

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ganglion cells (RGCs), which extend axons to the brain, are located in the ganglion cell layer (GCL). These different classes of retinal cell types are generated in vertebrates in a partly overlapping, yet stereotypic pattern from a pool of proliferating retinal progenitor cells (RPCs). RGCs, cones, HCs and ACs are formed first, whereas the rods, BP and Muller cells appear later in development (Young, 1985).

Studies of retinal cell lineages, as well as heterochronic cell mixing experiments, revealed that the multipotent RPCs undergo a gradual change in their intrinsic composition that influences their probability of acquiring a specific cell fate (Belliveau et al., 2000; Cepko, 2014; Cepko et al., 1996; Gomes et al., 2011; Hafler et al., 2012; He et al., 2012; Rapaport et al., 2001). This shift in differentiation competence is mediated by multiple transcription factors (TFs) and signaling pathways (Xiang, 2013). Although some TFs, such as the eye field TF Pax6, are required for the differentiation potential of all RPCs (Shaham et al., 2012), recent studies emphasize the heterogeneity in the gene expression profile of different RPCs (Cepko, 2014; Trimarchi et al., 2008). It has been shown that different TFs function in restricted subsets of RPCs and govern the differentiation of specific cell types. For example, Foxn4 is required in progenitors for the generation of the HC and AC lineages (Li et al., 2004), whereas Olig2 biases RPCs toward producing photoreceptors, HCs and ACs (Hafler et al., 2012). Members of another group of TFs are subsequently upregulated following cell cycle exit and are required to execute specific terminal differentiation programs, such as Atoh7 for RGC and Ptf1a for HC and AC differentiation (Brown et al., 2001; Brzezinski et al., 2012; Fujitani et al., 2006; Furukawa et al., 1997; Lelièvre et al., 2011; Nakhai et al., 2007; Wang et al., 2001).

Recent reports have revealed the pleotropic activity of Smadinteracting protein 1 (Sip1; also known as Zeb2 or Zfhx1b) in forebrain development. Sip1 is necessary for the fate and guided migration of cortical interneurons, for the correct timing of cortical neurogenesis and gliogenesis, as well as for hippocampus development (McKinsey et al., 2013; Miquelajauregui et al., 2007; Seuntjens et al., 2009; van den Berghe et al., 2013). Sip1, which together with Zeb1 forms the zinc finger homeobox (ZFHX) TF family, contains a Smad-interacting domain and a homeodomain-like DNA-binding domain flanked by two DNA-binding zinc finger clusters (Remacle et al., 1999; Verschueren et al., 1999). Both ZFHX proteins seem to function as both transcriptional repressors and activators.

Consistent with its roles in neural development, in humans SIP1 (ZEB2) haploinsufficiency leads to Mowat-Wilson syndrome, a condition characterized by multiple congenital defects, including microcephaly and intellectual disability (Cacheux et al., 2001; Mowat et al., 1998; Wakamatsu et al., 2001). Several studies link Sip1 to the development and function of the eye: ocular coloboma has been reported in a carrier of a missense mutation in SIP1, a genome-wide association study has implicated the involvement of SIP1 in high myopia in humans, and in the mouse Sip1 is required for normal lens

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development (Gregory-Evans et al., 2004; Khor et al., 2013; Manthey et al., 2014; Yoshimoto et al., 2005). Considering these important roles of Sip1 in neurogenesis and eye development we aimed in this study to uncover its contributions to retinogenesis.

RESULTS

Pax6 regulates expression of $\mathit{Sip1}$ in the developing mouse retina

The eye field TF Pax6 is essential for the generation of most retinal cell types and functions upstream of multiple TFs known to regulate cell proliferation and cell cycle exit of RPCs. Recently, *Sip1* was

identified as one of 316 genes downregulated in Pax6-deficient RPCs (Farhy et al., 2013). Downregulation of Sip1 expression in $Pax6^{loxp/loxp}$; α -Cre retinae was validated at the protein and mRNA levels (Fig. 1A-F). Considering its documented roles in nervous system development we examined Sip1 function in retinogenesis, aiming to decipher its contribution to the complex activity of Pax6.

Sip1 is required for the generation of HCs and for normal numbers of INL cell types

Although Sip1 expression has been reported in the mammalian retina, its precise spatiotemporal expression pattern and role in retinogenesis

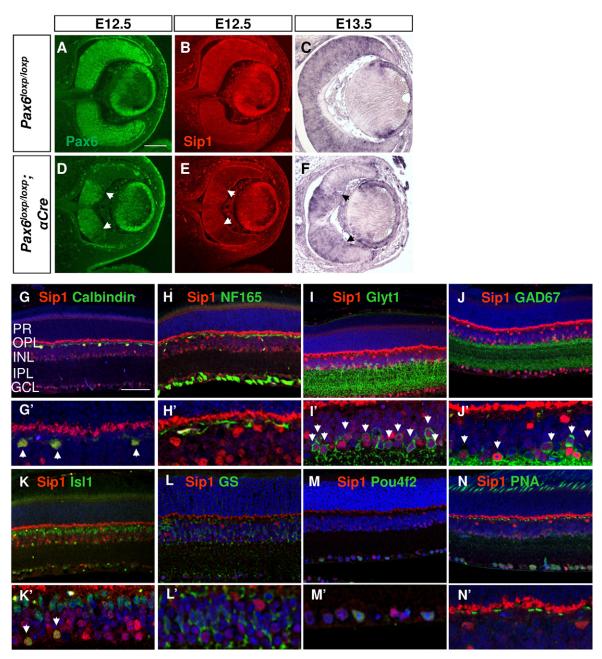


Fig. 1. Sip1 protein expression pattern in the mouse retina. (A-F) The expression in control (A-C) and $Pax6^{loxp/loxp}$;α-Cre (D-F) retina of Pax6 (A,D) and Sip1 (B,E) proteins detected by IIF at E12.5 and Sip1 transcript detected by ISH at E13.5 (C,F). The Pax6 mutation area is marked by arrows. (G-N) P14 $Sip1^{+/+}$ retina was co-labeled with antibodies for Sip1 and the following markers: calbindin for cell bodies of HCs (G), NF165 for HC projections (H), Glyt1 for glycinergic ACs (I), Gad67 for GABAergic ACs (J), Isl1 for BP cells and cholinergic ACs (K), glutamine synthetase (GS) for Muller glia (L), Pou4f2 for RGCs (M) and PNA for cone photoreceptors (N). Retinal layers are magnified in G′-N′, where arrows indicate double labeled cells. PR, photoreceptors; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar in A: 10 μm for A-F; in G: 50 μm for G-N.

remain unknown (Grabitz and Duncan, 2012). We employed indirect immunofluorescence analysis (IIF) to characterize the distribution of Sip1 protein during retinal development (Fig. 1). Following the completion of retinal differentiation at postnatal day (P) 14 (Voinescu et al., 2009), Sip1 was detected in all HCs based on colocalization with calbindin and NF165 (Nefm) (Fig. 1G-H'). Sip1 was also detected in 56.3±11.9% and 62.1±6.8% of Gad67 (Gad1)⁺ and Glyt1 (Slc6a9)⁺ ACs, respectively (Fig. 1I-J', Fig. S1A, n=3). All of the cholinergic ACs appeared to contain Sip1, as shown by colocalization with Isl1 (Fig. 1K,K'). The BP (Isl1⁺) and Muller glia (glutamine synthetase⁺) cells did not detectably contain Sip1 (Fig. 1K-L'). Sip1 was also detected in displaced ACs located in the GCL, while very low levels were detected in 36.5±13% of the Pou4f2⁺ RGCs (Fig. 1M,M', Fig. S1B, n=3). In addition to the nuclear localization of Sip1 in some of the cell types, we detected Sip1 in the outer plexiform layer (OPL), probably in rod terminals as the cone pedicle marker PNA was not colocalized with this synaptic staining (Fig. 1N,N'). This synaptic staining is reminiscent of that of its interacting partner Ctbp2 and might reflect a role for Sip1 in ribbon synapses (Schmitz et al., 2000; van Grunsven et al., 2003). During the early stages of retinal development [embryonic day (E) 12.5], Sip1 protein was detected in both the nuclei and cytoplasm of proliferating progenitors in the neuroblastic layer (NBL) (Fig. 2A), and an increase in nuclear staining was evident from ~E15.5 (not shown) in cells located towards the differentiated cell layer, possibly suggesting that these are postmitotic precursors.

To determine the role of Sip1 in retinogenesis we conditionally mutated the $Sip1^{loxp}$ allele using the α -Cre transgenic line (Higashi et al., 2002; Marquardt et al., 2001). Sip1 loss was evident in the $Sip 1^{loxp/loxp}$; α -Cre embryos at E12.5 in the distal optic cup (OC) (Fig. 2E). In accordance, at E15.5 quantitative PCR (qPCR) analysis showed Sip1 mRNA levels to be decreased by 27% in whole eyes of Sip1^{loxp/loxp};α-Cre embryos in comparison to control littermates (Fig. S4J, n=3 controls and 5 mutants, P=0.009). At P14 the thickness of $Sip1^{loxp/loxp}$; α -Cre retinae was reduced by 30% as compared with the control (Fig. 2B,F,I, Fig. S1H, n=4, P=0.0017), although overall lamination was unaffected. Most of this reduction could be attributed to reduction in the OPL and inner plexiform layer (IPL). The OPL was reduced by 89.75%, while the IPL was reduced by 56.6% (Fig. 2F,I, Fig. S1C,D, n=4, P=0.0018). The thickness of the INL was also reduced by 28% (Fig. 2F,I, Fig. S1E, n=4, P=0.004). We did not detect a significant change in the width of the GCL or the photoreceptor layer (Fig. 2I, Fig. S1F,G).

To determine whether the reduction in the number of $Pax6^+$ ACs was due to a reduction in a specific AC subtype, we quantified the number of GABAergic and glycinergic ACs by counting $Pax6^+$ cells that were positive for Gad67 or Glyt1. This revealed a 47% and 29% reduction, respectively, in the number of GABAergic (Fig. 2J, n=4, P=0.002) and glycinergic (Fig. 2J, n=4, P=0.019) ACs. Thus,

the early born GABAergic ACs seemed to be more severely affected by *Sip1* removal than the late born glycinergic ACs.

Photoreceptor function is intact but BP cell function is lost in Sip1-deficient retinae

The morphological defects associated with Sip1-deficient retinae suggested that retinal function might also be perturbed. Electroretinogram (ERG) recordings were conducted to study electrophysiological function in Sip1-deficient retina, in particular the function of the photoreceptors that seemed morphologically and molecularly intact despite Sip1 loss.

The b-wave amplitudes of rod, mixed rod-cone and cone pathway responses were significantly reduced in *Sip1* mutants, but there were no significant differences between groups in the a-wave amplitude of most responses (Fig. 2K,L, Fig. S3). The b-wave is generated primarily by BP cells (Mojumder et al., 2008). Therefore, these results were consistent with the alterations in *Sip1* mutant retinae, including the disruption in the OPL, which outputs to the BPs, and the reduction in BP numbers. Since the amplitudes of a-waves, which are generated by photoreceptors (Robson and Frishman, 2014), were mostly unaffected in Sip1-deficient retinae (Fig. 2L), it is likely that the Sip1 expression detected in the rod photoreceptor terminals is not essential for any electrophysiological function of the rod photoreceptors that can be detected by a standard ERG protocol.

Sip1 is required for the generation of HC precursors

The reduction in the numbers of ACs and BPs and the HC loss were probably not due to selective cell death as we did not detect any significant differences in the expression of cleaved caspase 3 (cCasp-3) in control and $Sip1^{loxp/loxp}$; α -Cre retinae at E15.5 (Fig. S4B,F,I, n=3, P=0.39), E18.5 (Fig. S4C,G,I, n=3, P=0.39) and P0 (Fig. S4D,H,I, n=3, P=0.139).

To determine whether the ablation of Sip 1 affects the specification or differentiation of early stage RPCs, we characterized the expression pattern of several genes associated with the generation of the AC and HC lineages at E14.5 (Fig. 3). Two mediators of Foxn4 activity that are expressed at this stage and associated with the generation of HCs and ACs are the bHLH TFs Neurod1 and Neurod4 (Math3) (Li et al., 2004), while Prox1 acts downstream of Foxn4 and is involved in HC differentiation (Dver et al., 2003). In situ hybridization (ISH) and qPCR analyses in whole eyes detected reduction in Sip1 expression (Fig. 3A,F, Fig. S4J), whereas we did not detect a change in the expression of Foxn4, Neurod4 and Neurod1 transcripts in Sip1-deficient eyes as compared with the control (Fig. 3B-D,G-I, Fig. S4J, n=3 controls and 5 mutants; P=0.16, P=0.26 and P=0.11, respectively). However, the IIF signal of Prox1 and NF165 proteins, which are normally detected in HC precursors (Dyer et al., 2003; Shaw and Weber, 1984) (Fig. 3E), was diminished (Fig. 3J). These findings suggest that Sip1 functions either downstream of, or in parallel to, Foxn4 and the proneuronal genes and upstream of Prox1 in the generation of the HC lineage.

Sip1 controls the onset of expression of INL precursor genes during early stages of retinogenesis

In the mammalian retina, Ptf1a is essential for the generation of HCs and most of the ACs (Fujitani et al., 2006; Nakhai et al., 2007). Prox1 protein is severely reduced in Ptf1a-deficient retinae, suggesting that Ptf1a acts upstream of Prox1 in the generation of HCs (Fujitani et al., 2006). In addition to Ptf1a, the TFs Ap2 α (Tfap2 α) and Ap2 β (Tfap2 β) are expressed and function in HC and AC types (Bassett et al., 2007, 2012). Considering the importance of these factors to the generation of

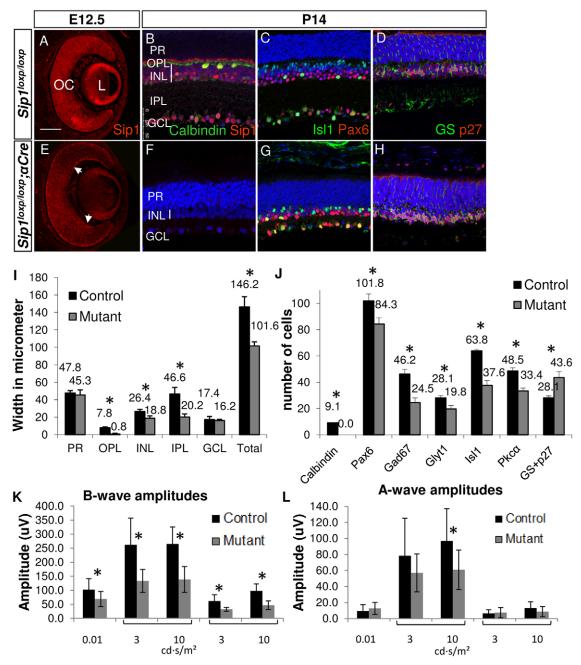


Fig. 2. Somatic mutation of Sip1 in the embryonic retina reduces the number of INL neurons. (A-H) Control (A-D) and $Sip1^{loxp/loxp}$;α-Cre (E-H) E12.5 (A,E) and P14 (B-D,F-H) retinae were analyzed by IIF for detection of Sip1 (A,E), calbindin and Sip1 (B,F), Isl1 and Pax6 (C,G) and GS and p27Kip1 (D,H). Arrows (E) demarcate the border of the Sip1-deficient area. (I) The mean width of the different retinal layers was measured in DAPI-stained sections (n=4). (J) The mean number of the different INL cell types was counted using retinal sections stained for the following markers: calbindin for HCs, Pax6 for all ACs, Gad67 for GABAergic ACs, Glyt1 for glycinergic ACs, Isl1 and PKCα for BP cells, and GS and P27Kip1 for Muller glia. n=3 for PKCα and n=4 for the other markers. (K,L) Mean±s.d. a- and b-wave amplitudes of control (n=5) and $Sip1^{loxp/loxp}$;α-Cre (n=11) mice. x-axis shows stimulus intensities. *p<0.05. Scale bar in A: 100 μm in A,E; in B: 50 μm in B-D,F-H.

HCs and ACs, we examined possible change in their expression following Sip1 loss.

During normal retinogenesis, Ptf1a⁺ and Ap2 α ⁺ cells are detected in the peripheral OC at E14.5 (Fig. 4A,B). By contrast, in Sip1-deficient retinae Ptf1a⁺ cell numbers were reduced to $40\pm16.4\%$ of control values (Fig. 4F, Fig. S5A, n=3, P=0.007), and Ap2 α was not detected at this stage (Fig. 4G). Consistent with the above, Ap2 β , which is normally detected in the NBL at E16.5 (Fig. 4C), was detected in only a few cells of the *Sip1* mutant OC (Fig. 4H). Despite the marked

reduction in AC precursors at E14.5 and E16.5, at later stages (E18.5) partial recovery in the number of Ptf1a⁺ cells in Sip1 mutant retina was observed. The Sip1 mutant retinae contained 67±9.67% of the number of Ptf1a⁺ cells detected in the control (Fig. S5B, n=3, P=0.026). The increase in the proportion of the Ptf1a⁺ cells at E18.5 in comparison to E14.5 was statistically significant (n=3, P=0.034). Ap2o+ and Ap2o+ cells were also detected in Sip1 mutant retinae at this stage (Fig. 4E,J; not shown). These results suggest a delayed onset of expression of the AC and HC postmitotic precursor genes in Sip1-deficient progenitors.

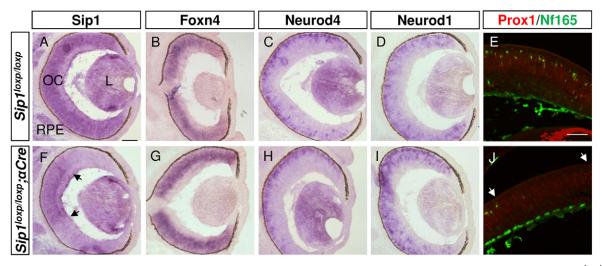


Fig. 3. HC and AC progenitor specification genes are expressed normally, whereas postmitotic HC precursors are absent in E14.5 *Sip1*^{loxp/loxp};α-Cre retina. (A-D,F-I) ISH was performed on E14.5 control and *Sip1*^{loxp/loxp};α-Cre retinae for detection of *Sip1* (A,F), *Foxn4* (B,G), *Neurod4* (C,H) and *Neurod1* (D,I). (E,J) IIF was used to detect Prox1 and NF165 proteins in control (E) and *Sip1*^{loxp/loxp};α-Cre (J) retinae. Arrows mark the border of the Sip1-deficient area in the retina. L, lens; OC, optic cup; RPE, retinal pigmented epithelium. Scale bar in A: 100 μm in A-D,G-I; in E: 10 μm in E,J.

To determine whether the generation of RGCs and photoreceptor precursors, which are also early born cell types, is also delayed in $Sip 1^{loxp/loxp}$; α -Cre retinae we examined the expression of Pou4f2 and the photoreceptor differentiation marker Crx (Fig. 5A,B,D,E). No obvious alterations were observed in the presence of either proteins following Sip1 loss at E13.5. However, at E16.5 we detected a significant increase in the number of Pou4f2⁺ cells in the NBL (Fig. 5C,F,G, Fig. S5C, n=3, P=0.04). It was previously reported that in Ptf1a knockout retinae \sim 30% of the cells that normally activate Ptf1a expression undergo a switch in cell fate and differentiate into RGCs (Fujitani et al., 2006; Nakhai et al., 2007). The increase in the number of Pou4f2⁺ cells in the NBL at E16.5 might be the result of HC/AC precursors undergoing a fate switch upon Ptf1a reduction.

Because the number of BP cells also decreased in Sip1-deficient retinae, we characterized the dynamics of the differentiation of BP cells, which are generated at postnatal stages (Young, 1985). We monitored the generation of these precursors by the presence of Isl1, which is normally detected in the BP precursors populating the apical side of the INL (P7, P9, Fig. 5H,I). By contrast, in the Sip1-deficient OC, the number of Isl1⁺ BPs was reduced by 83% compared with the control (Fig. 5J, Fig. S5E,F, *n*=3). At P9 the number of Isl1⁺ BPs in *Sip1* mutant retinae seemed to increase (Fig. 5K), and by P14 the mutant retinae contained almost 59% of the number of Isl1⁺ BPs found in the control (Fig. 2J, Fig. S5F). These results suggested that Sip1 plays a role in the differentiation timing of both the embryonically generated AC and postnatally generated BP interneurons.

To determine whether the observed delay in the generation of HC/AC precursors genuinely reflects a delay in their generation rather than a delay in the expression of specific TFs, we performed a BrdU birthdating analysis of Ap2 β ⁺ ACs and HCs by pulse labeling at E13.5, E14.5 and E16.5 followed by analysis of BrdU⁺ and Ap2 β ⁺ cells at P0 (Fig. 6A,B). The retinae of the *Sip1loxp/loxp;\alpha-Cre* pups labeled at E13.5 and E14.5 contained approximately half as many cells positive for both BrdU and Ap2 β than controls (Fig. 6C, Fig. S6A,B; n=3, P=0.028 for E13.5 and n=4, P=0.025 for E14.5). These results indicated that fewer Ap2 β ⁺ ACs and HCs were generated during the early stages of retinogenesis. By contrast,

birthdating analysis at E16.5 did not show a significant reduction in the number of Ap2 β^+ BrdU⁺ cells in $Sip1^{loxp/loxp}$; α -Cre as compared with control retinae (Fig. 6C, Fig. S6C, n=3, P=0.109). The recovery in the generation of AC precursors at E16.5 corresponded with the increase in the number of Ptf1a⁺ cells detected in Sip1-deficient retinae at E18.5.

We further determined birth dates of RGCs and the early born photoreceptors that are generated in parallel to ACs and HCs. The birthdating analyses of RGCs was conducted by detection of BrdU⁺ Pou4f2⁺ cells at P0, following BrdU labeling at E13.5, E14.5 and E16.5. This analysis did not reveal a statistically significant change in the number of RGCs generated in the *Sip1* mutant retina at the three stages (Fig. S7A,C-E). We did not detect any significant change in the number of photoreceptors generated at E14.5 (Fig. S7B,F).

Since we observed a delay in the generation of ACs, it is possible that some of the cells that failed to express Ptfla on time failed to properly exit the cell cycle and remained undifferentiated for a longer period of time. To identify the proportion of cells that properly exit the cell cycle at E14.5, we pulse labeled cycling cells with BrdU at E14.5 and determined the percentage of BrdU⁺ Ki67⁻ cells among BrdU⁺ cells 24 h after injection (E15.5, Fig. 6D,E). In control retinae, 24.8±1.4% of the cells that incorporated BrdU at E14.5 did not express Ki67 at E15.5, suggesting that they had exited the cell cycle during the 24 h following the BrdU incorporation. By contrast, in Sip1-deficient retinae, 19.9±1.6% of the cells that incorporated BrdU at E14.5 did not express Ki67 24 h later (Fig. 6F, Fig. S8A, n=3, P=0.008). This suggests that without Sip1 some of the RPCs, which are supposed to differentiate into AC and HC precursors, remain undifferentiated. The mean number of BrdU⁺ cells was similar in the mutant and the control retinae (247.3±55.5 and 248.8±25.3, respectively), suggesting that there is no alteration in the number of cells entering S-phase.

Sip1 directly regulates Ptf1a in the mouse retina

Although mostly known as a repressor of transcription, Sip1 can also activate transcription in both oligodendrocyte precursors and cortical neurons (Srivatsa et al., 2015; van den Berghe et al., 2013; Weng et al., 2012). Since Ptf1a is the most upstream member of the HC/AC differentiation network and is downregulated in the

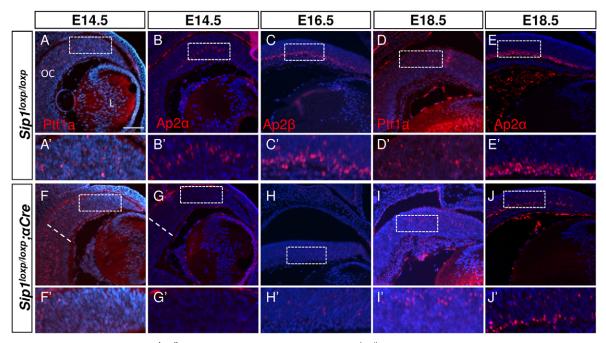


Fig. 4. AC differentiation is delayed in $Sip1^{loxp/loxp}$; α -Cre retinae. Control (A-E) and $Sip1^{loxp/loxp}$; α -Cre (F-J) were analyzed by IIF for detection of Ptf1a (A,D,F,I), Ap2 α (B,E,G,J) or Ap2 β (C,H). The boxed regions are magnified in A'-J'. Dashed lines indicate the border of the Sip1-deficient area. Blue, DAPI. OC, optic cup. Scale bar: 100 μ m.

 $Sip1^{loxp/loxp}$; α -Cre retina, we examined the possibility that Sip1 directly regulates Ptf1a in the developing retina.

Ptf1a expression in the developing mouse retina has been shown to be regulated by two enhancers: a 2.3 kb sequence upstream of Ptf1a (the 5' enhancer) and a 12 kb sequence just downstream of the Ptf1a gene (the 3' enhancer) (Meredith et al., 2009) (Fig. 7A). The regulation of these two regions by Sip1 was examined by luciferase assay in HeLa cells. Whereas Sip1 protein was not able to increase luciferase activity derived from the 5' enhancer, it was able to significantly increase that derived from the 3' enhancer by 2.5-fold (Fig. 7B, Fig. S8B,C, P=0.0008, n=3), suggesting that Sip1 can activate transcription controlled by the Ptf1a 3' enhancer.

In order to assess the possibility that Sip1 directly binds the *Ptf1a* 3' enhancer in the mouse embryonic retina we scanned for Sip1 binding sites [5'-CACCT(G)-3' (Remacle et al., 1999)] in the enhancer sequence. Putative sites were then sorted according to their evolutionary conservation using the '100 vertebrates basewise conservation by PhyloP' tool from the UCSC genome browser (Kent et al., 2002). Interestingly, the four most highly conserved Sip1 binding sites were located in a 1.1 kb area located 3.3 kb from the 3' end of the *Ptf1a* gene (Fig. 7A,C).

Chromatin immunoprecipitation (ChIP) on E16.5 whole eyes was used to assess whether Sip1 protein binds directly to any of these sites. qPCR analysis revealed that two of the Sip1 binding sites (sites 1 and 2) are significantly enriched when precipitated with the Sip1 antibody as compared with the IgG control (Fig. 7C, Fig. S8D,E, n=4; P=0.02 and 0.037, respectively), suggesting that Sip1 directly binds the Ptf1a 3′ enhancer in vivo. By contrast, sites 3 and 4 were not significantly enriched in the Sip1 immunoprecipitation (Fig. 7C, Fig. S8F,G; n=4, P=0.15 for site 3 and n=3, P=0.17 for site 4), further confirming the specificity.

Taken together with the results of the luciferase experiment and the reduction of Ptf1a in the $Sip1^{loxp/loxp}$; α -Cre retina, it seems that Sip1 acts as a transcriptional activator of Ptf1a in the embryonic eye.

DISCUSSION

This study reveals that Sip1 is an important member of the gene network governed by Pax6 that contributes to the intrinsic heterogeneity and multipotency of RPCs, and uncovers a unique and novel role for Sip1 in ensuring the genesis, in the correct proportions, of multiple retinal cell types destined to the INL fate (Fig. 8). Interestingly, our analysis of cell cycle dynamics and timing of onset of expression of AC and HC precursor genes suggests that Sip1 controls the number of INL cell types at least partly by ensuring the timely differentiation of specific cell types.

The present data coupled with our earlier studies (Farhy et al., 2013) show that expression of Sip1 requires Pax6. Interestingly, multiple candidate Pax6 binding sites are present close to one of the Sip1 transcription start sites. Electrophoretic mobility shift assay results show that Pax6 is able to bind six of these binding sites (data not shown), suggesting that Pax6 might be a direct regulator of Sip1 expression. In regard to this, the complexity of the Sip1 regulatory regions should be considered as the mouse gene has more than one promoter (Nelles et al., 2003) and both the human and mouse genes are flanked by a 3 Mb gene desert that contains many binding sites and histone signatures. Understanding the control of Sip1 expression and the possible role of Pax6 therein will require further research.

Sip1 is a key regulator of the HC and AC lineages

The HC and AC lineages depend on a common set of TFs, leading to the view that the two lineages share a common precursor (termed here the HC/AC precursor; Bassett et al., 2012; Fujitani et al., 2006; Li et al., 2004; Xiang, 2013). A few TFs are known to be specific to each of the lineages. Prox1, for example, is required and sufficient to induce the HC but not AC lineage (Dyer et al., 2003). The most dramatic phenotype of Sip 1loxp/loxp; a-Cre retinae is the complete absence of HCs. In Sip1 mutants, we did not detect any change in the expression patterns of Foxn4, Neurod1 or Neurod4, suggesting that Sip1 functions downstream of, or parallel to, these regulators

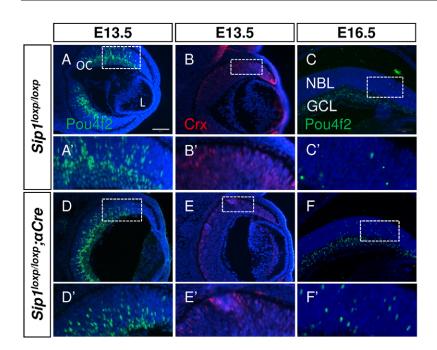
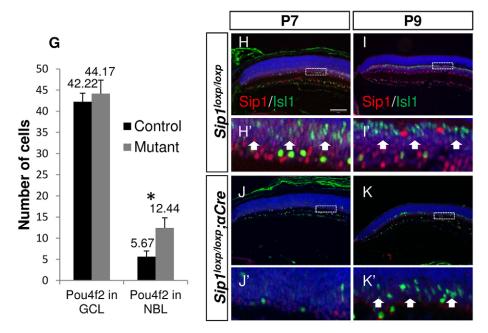


Fig. 5. The effect of Sip1 mutation on RGCs, photoreceptors and BP cells. (A-F) Control (A-C) and Sip1^{loxp/loxp};α-Cre (D-F) retinae were analyzed by IIF for detection of Pou4f2 at E13.5 (A,D), Crx at E13.5 (B,E) and Pou4f2 at E16.5 (C,F). Boxed regions are magnified in A'-F'. (G) The mean number of Pou4f2⁺ cells in the GCL and in the neuroblastic layer (NBL) in a 130 µm field of retina was quantified (n=3). (H-K) Control (H,I) and Sip1^{loxp/loxp};α-Cre (J,K) retinae were analyzed for detection of Sip1 and Isl1 at P7 (H,J) and P9 (I,K). Boxed regions are magnified in H'-K', where arrows mark Isl1 + cells. *P<0.05. GCL, ganglion cell layer; L, lens; OC, optic cup. Scale bar in A: 100 µm for A-F; in H: $100 \mu m$ for H-K.



(Fig. 8). By contrast, Ptf1a, Ap2 α and Ap2 β , which are key factors in HC/AC precursors, were substantially reduced at E14.5, while Prox1⁺ cells were not detected in the mutant retina. Luciferase and ChIP analyses also suggest Sip1 to be a direct activator of *Ptf1a*. These results reveal Sip1 as a key component of the regulatory cascade required for both AC and HC development, with its most prominent effect on HCs (Fig. 8).

Ptf1a is thought to be upregulated immediately following cell cycle exit and to be necessary for the generation of HCs and ACs (Fujitani et al., 2006; Nakhai et al., 2007). Although our results suggest that Sip1 directly promotes expression of the Ptf1a gene in the retina, the generation of Ptf1a⁺ cells in the Sip1loxp/loxp;α-Cre retinae is not terminated but rather its onset seems to be delayed. In accordance, most of the ACs are maintained despite Sip1 loss, and only the HCs are completely missing. This differential response might relate to the developmental stages at which Sip1 is most

required for the activation of *Ptf1a* within the precursors. Since HCs are generated in a narrow time window during early retinogenesis (Rapaport et al., 2004), one intriguing possibility is that due to the delay in their generation there are simply not enough Ptf1a⁺ precursors during the time of HC generation, thereby resulting in an absence of HCs, whereas ACs are also generated at later stages when other factors are able to compensate for Sip1 loss. The fact that GABAergic ACs were more severely reduced than glycinergic ACs in the *Sip1* mutants also supports this hypothesis as they are also generated in an earlier time window (Voinescu et al., 2009). It should be further considered that, similar to Prox1 (Dyer et al., 2003), Sip1 might have two different roles – one in the generation of the AC and HC precursors and another in the final specification and differentiation of HCs.

Our BrdU pulse-chase experiment suggests that there is a decrease in the number of ACs/HCs that properly exit the cell cycle

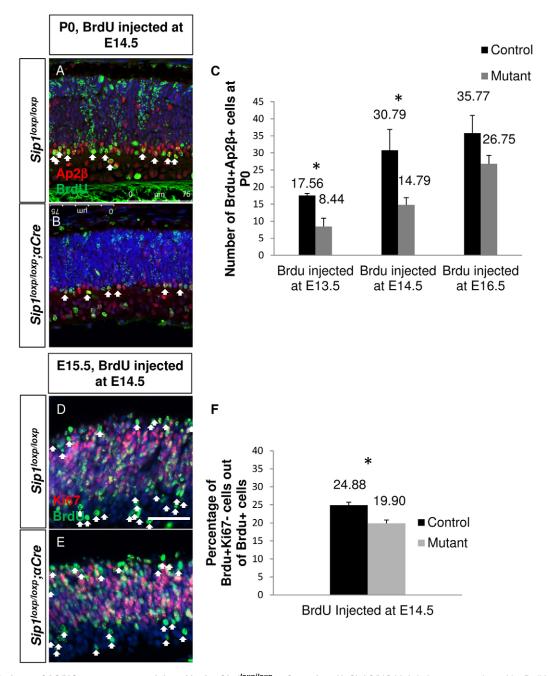


Fig. 6. The birth dates of AC/HC precursors are delayed in the $Sip1^{loxp/loxp}$;α-Cre retina. (A-C) AC/HC birthdating was conducted by BrdU pulse labeling at E13.5, E14.5 or E16.5 and analysis at P0 by detection of BrdU⁺ Ap2β⁺ cells. (A,B) An example for control and mutant retinae injected with BrdU at E14.5 is presented. BrdU⁺ Ap2β⁺ cells are marked by arrows. (C) The mean number of BrdU⁺ Ap2β⁺ cells is presented for each time point (n=3 for E13.5 and E16.5, n=4 for E14.5). (D-F) For total cell cycle exit analysis control and $Sip1^{loxp/loxp}$;α-Cre embryos were injected with BrdU at E14.5 and analyzed at E15.5 by detection of BrdU and Ki67. (D,E) An example for control and mutant retinae is presented. BrdU⁺ Ki67⁻ cells are marked by arrows. (F) The mean percentage of BrdU⁺ Ki67⁻ cells among all BrdU⁺ cells was calculated (n=3). *P<0.05. Scale bar in A: 75 μm in A,B; in D: 50 μm in D,E.

between E14.5 and E15.5 in the $Sip1^{loxp/loxp}$; α -Cre retina. Sip1 has been suggested to promote cell cycle exit in the squamous cell carcinoma line A431 (Mejlvang et al., 2007), although it is not essential for proper cell cycle length and exit in the embryonic cortex (Seuntjens et al., 2009). We cannot exclude the possibility that Sip1 is directly involved with the cell cycle mechanism of RPCs destined to become HCs and ACs. Alternatively, the absence of cues needed for proper differentiation, such as Ptf1a, might result in cells not properly exiting the cell cycle and dwelling for a longer period of time as RPCs. Indeed, misexpression of Ptf1a in the chick retina

results in a decrease in the number of cells in S and M phases (Lelièvre et al., 2011). It is thus possible that Sip1 affects cell cycle exit by promoting *Ptf1a* expression.

It is intriguing that the decrease in cell cycle exit detected in the *Sip1* mutants did not result in a hypercellular retina. This might be explained by the small size of the subpopulation affected by Sip1. Another possibility is that the cells that seem to fail to exit the cell cycle properly do not actually continue to proliferate. It has been shown that genes directing the differentiation of RPCs can also decrease the expression of cell cycle genes (Lacomme et al., 2012).

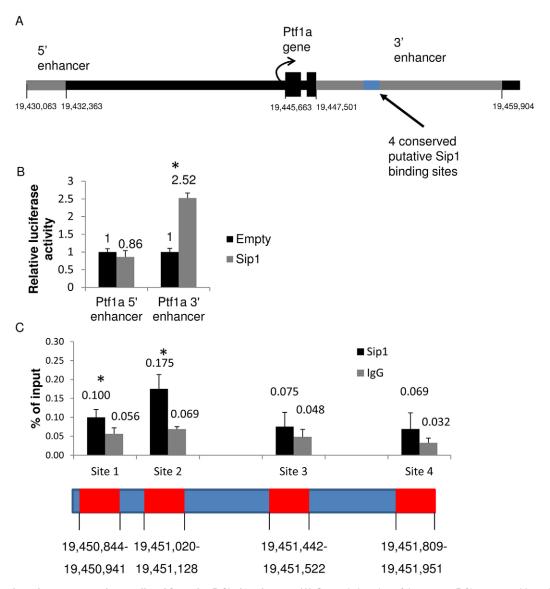


Fig. 7. Sip1 directly activates expression mediated from the *Ptf1a* 3' enhancer. (A) Genomic location of the mouse *Ptf1a* gene and its enhancers on chromosome 2. The 5' and 3' enhancers are marked in gray and genomic coordinates are designated based on the UCSC genome browser. (B) Luciferase assay was used to assess whether Sip1 protein can activate expression through each of the *Ptf1a* enhancers (*n*=3). The results are presented as mean fold change relative to the control. (C) ChIP using Sip1 or IgG antibodies was performed on chromatin extracted from E16.5 eyes. The mean percentage of input immunoprecipitated by the different antibodies at the four Sip1 binding sites in the 3' enhancer was quantified by qPCR (*n*=4, except *n*=3 for site 4). A scheme of the genomic area containing the four Sip1 binding sites in the 3' enhancer and their location on chromosome 2 is shown beneath. **P*<0.05.

It is thus possible that Sip1⁻ RPCs that fail to express differentiation markers upon cell cycle withdrawal retain the expression of some cell cycle determinants, such as Ki67, and therefore were not detected in our cell cycle exit assay. Alternatively, some of these undifferentiated RPCs might go through apoptosis in small numbers over a long time span and thus might not be detected.

Sip1 as a general regulator of the number of INL cells

Sip1 appears to represent one of the first examples of a TF that is specifically required for generating the correct number of all three INL neural types. The generation of Isl1⁺ BP cells was delayed in a manner similar to that observed for Ptf1a at earlier stages, suggesting that events similar to the delay in early retinogenesis also occur during postnatal stages.

The similarity of the phenotypes observed in the differentiation of AC/HC precursors and in the differentiation of BPs suggests that

common mechanisms involving Sip1 are at work in the generation of the three INL interneuron types. Considering that Ptfla is not involved in the differentiation of BPs, this suggests that Sip1 promotes the differentiation of INL interneurons by means other than the activation of Ptfla. Indeed, Sip1 was also suggested to promote differentiation and maturation in several other populations of progenitors (Conidi et al., 2011; Goossens et al., 2011; Omilusik et al., 2015; van Helden et al., 2015; Weng et al., 2012), suggesting a more general role for Sip1 in progenitor differentiation. In oligodendrocytes, Sip1 was suggested to play a dual role in differentiation as it was found to repress genes that act as inhibitors of differentiation, such as Id2 and Hes1, while activating the expression of genes related to oligodendrocyte differentiation, such as Mbp and Mag (Weng et al., 2012). Considering this, it is possible that Sip1 has a similar role in RPCs, activating specific differentiation genes, such as Ptfla, that promote AC and HC

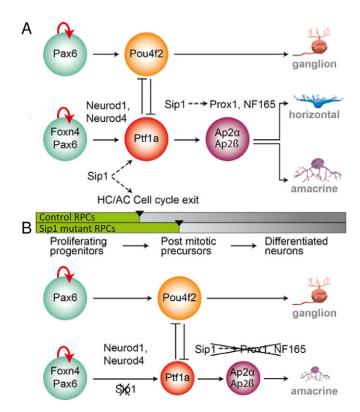


Fig. 8. Model for the roles of Sip1 in the differentiation of early born retinal lineages. Early progenitor cells expressing Pax6 and Foxn4 have the competence to generate ACs and HCs. During normal retinogenesis (A), Sip1 functions downstream to Pax6 and downstream or parallel to Foxn4, Neurod1 and Neurod4. Sip1 promotes the transcription of Ptf1a and is required for promoting the timely differentiation of $Ptf1a^{+}$, $Ap2\alpha^{+}$ and $Ap2\beta^{+}$ precursor cells during the peak of generation of HCs. At later stages, Sip1 might be required for the generation of $Ptf1a^{+}$ HC precursors. When Sip1 is mutated (B), the generation of $Ptf1a^{+}$ precursor cells is delayed as some of them fail to properly exit the cell cycle. This results in a decrease in the number of ACs and in a complete disappearance of HCs. Some of these cells might transdifferentiate into RGCs because of the reduced Ptf1a.

differentiation while repressing inhibitors of differentiation that also affect other INL cell types.

Sip1 mutant retinae also contained an increased number of Muller glia, which suggests that Sip1 antagonizes Muller glia generation. One possible hypothesis is that since fewer BP cells are differentiated in Sip1 mutant retinae, there are more cells that are able to differentiate into Muller glia at the end of retinogenesis. An opposite role has been proposed for Notch pathway genes and Rax, which are also required for RPC proliferation (Jadhav et al., 2009). Alternatively, Sip1 might actively repress glial fate.

The massive reduction in the plexiform layers observed in *Sip1* mutants is likely to reflect the role of Sip1 in controlling the numbers of INL cells, as a similar collapse of plexiform layers was observed in *Foxn4* and *Lim1* mutant retinae in which HCs and ACs

are affected (Keeley et al., 2013; Li et al., 2004). The reduction in b-wave amplitudes observed in our ERG recordings is consistent with the collapse of the OPL and the reduction in the number of BPs. It should be noted that different strains of mice present large variations in the numbers and ratios of different retinal cell types, yet all have functional retinae (Keeley et al., 2014). Considering this, it is possible that the collapse of the OPL and the disruption in retinal function observed following Sip1 loss are mediated not only by regulation of INL cell numbers but also by additional roles of Sip1 in the physiology of the differentiated retinal neurons.

MATERIALS AND METHODS

Mouse lines

 $Sip1^{loxp}$, $Pax6^{loxp}$ and α -Cre were described previously (Ashery-Padan et al., 2000; Higashi et al., 2002; Marquardt et al., 2001). Sip1 and Pax6 mutant mice were of a mixed genetic background. ICR mice (Harlan Laboratories, Jerusalem, Israel) were used for the ChIP analysis. All animal work was conducted according to national and international guidelines and approved by the Tel Aviv University Institutional Animal Care and Use Committee (IACUC permit M12098).

Electroretinogram

Recordings were conducted in 11 $Sip1^{loxp/loxp}$; α -Cre and five control 3-month-old littermates as controls. Animals were dark adapted overnight, were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg) and their pupils were dilated with 0.5% tropicamide at least 15 min before recording. To improve conduction, recorded eyes were kept moist with a drop of 1.4% hydroxymethylcellulose. Signals were recorded using a 0.25 mm gold wire loop. Subcutaneous needles served as reference and ground electrodes, and were placed at the temporal canthus of the ipsilateral eye and the base of the tail, respectively, and impedance maintained at <5 k Ω . Both eyes were recorded in random order. During recording of the first eye, dark adaptation of the second eye was maintained by patching it with a dark cover.

Recordings were conducted using a handheld multi-species electroretinography system (HMsERG, Ocuscience, Henderson, NV, USA) with a bandpass of 0.3-300 Hz. Background adaptation light and stimuli were delivered using the system's handheld mini-Ganzfeld. Rod, mixed rod-cone and cone pathway responses were recorded using the International Society for Clinical Electrophysiology of Vision (ISCEV) protocol (Table 1) (McCulloch et al., 2015).

Amplitudes of a- and b-waves were measured from baseline to the first trough and from that trough to the next positive peak, respectively.

Immunofluorescence, in situ hybridization and cell quantifications

IIF was performed as described previously (Oron-Karni et al., 2008). Antibodies are listed in Table S1.

ISH analysis was performed on frozen sections as described previously (Oron-Karni et al., 2008). Probes used for ISH were Foxn4 (Li et al., 2004), Neurod1, Neurod4 (Inoue et al., 2002; Takebayashi et al., 1997) and Sip1 (Maruhashi et al., 2005). Slides were viewed on an Olympus BX61 fluorescence microscope or a Leica SP5 410 laser-scanning confocal microscope. For cell quantification, the number of cells expressing each marker was counted and normalized to the area of the analyzed images

Table 1. ISCEV standard protocol for full-field electroretinography

Step	ERG test session	Flash intensity (cd×s/m²)	Number of flashes averaged	Interval (s)	Background light
0	Dark adaptation (overnight)				
1	Rod responses	0.01	10	2	Off
2	Standard intensity mixed rod-cone responses	3	4	10	Off
3	High intensity mixed rod-cone response	10	4	20	Off
0	Light adaptation (10 min at 30 cd/m ²)				
4	Standard intensity cone responses	3	32	0.5	On
5	High intensity cone responses	10	32	0.5	On

based on DAPI staining. The counts from each eye were the average of at least three sections.

RNA extraction and qPCR analysis

RNA was extracted from whole eyes of E15.5 embryos using the RNeasy Mini Kit (Qiagen). qPCR analysis was performed as described previously (Raviv et al., 2014). Results were calibrated relative to expression levels of *Actb*. Primers are described in Table S2.

Analysis of cell cycle exit

Cell cycle exit analysis was performed as described previously (Farhy et al., 2013). BrdU was administrated at E14.5 and retinae dissected at E15.5. At least three sections from three different mice were counted for each group.

Birthdating analysis

5-bromo-2'-deoxyuridine (BrdU; 140 µg/g body weight, Sigma) was injected intraperitoneally into pregnant females at E13.5, E14.5 or E16.5. Retinae were harvested at P0. Sections were stained for BrdU incorporation and for a cell type-specific marker using IIF. No fewer than three sections from three different mice were counted for BrdU $^{+}$ marker $^{+}$ cells.

Luciferase reporter assay

Reporter assays were performed in HeLa cells as described previously (Raviv et al., 2014). Cells were transfected with 1 μ g luciferase reporter vector with either the 5′ or 3′ Ptf1a enhancer, 50 ng of an expression vector carrying the Sip1 ORF or empty vector, and 10 ng normalizing vector (pRL-TK). Cells were not tested for contamination.

Chromatin immunoprecipitation

Embryonic eyes were dissected in HBSS and dissociated by 30 min incubation with papain. Cross-linking, DNA shearing and immunoprecipitations were performed as previously described (Sailaja et al., 2012). Rabbit anti-Sip1 antibody or normal rabbit IgG (both $1.6\,\mu g$; Table S1) were used for the immunoprecipitations. Enrichment levels were quantified by qPCR.

Statistical analysis

Statistical analysis was conducted in Microsoft Excel using Student's *t*-test. Error bars in all figures represent standard error unless stated otherwise.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.M.-L., P.O.-G. and R.A.-P. conceived and designed the experiments. Y.M.-L., P.O.-G., Q.X., R.E.-E., S.P.-H., A.C. and C.F. performed the experiments. Y.M.-L., P.O.-G., Q.X., R.E.-E., R.O., A.C. and R.A.-P. analyzed the data. Y.H., T.V.d.P., H.K. and D.H. provided essential reagents. Y.M.-L., P.O.-G., R.O. and R.A.-P. wrote the manuscript.

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Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.136101.supplemental

References

- Ashery-Padan, R., Marquardt, T., Zhou, X. and Gruss, P. (2000). Pax6 activity in the lens primordium is required for lens formation and for correct placement of a single retina in the eye. *Genes Dev.* 14, 2701-2711.
- Bassett, E. A., Pontoriero, G. F., Feng, W., Marquardt, T., Fini, M. E., Williams, T. and West-Mays, J. A. (2007). Conditional deletion of activating protein 2alpha (AP-2alpha) in the developing retina demonstrates non-cell-autonomous roles for AP-2alpha in optic cup development. *Mol. Cell. Biol.* 27, 7497-7510.
- Bassett, E. A., Korol, A., Deschamps, P. A., Buettner, R., Wallace, V. A., Williams, T. and West-Mays, J. A. (2012). Overlapping expression patterns and redundant roles for AP-2 transcription factors in the developing mammalian retina. *Dev. Dyn.* 241, 814-829.
- Belliveau, M. J., Young, T. L. and Cepko, C. L. (2000). Late retinal progenitor cells show intrinsic limitations in the production of cell types and the kinetics of opsin synthesis. *J. Neurosci.* 20, 2247-2254.
- Brown, N. L., Patel, S., Brzezinski, J. and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. *Development* 128, 2497-2508.
- Brzezinski, J. A., Prasov, L. and Glaser, T. (2012). Math5 defines the ganglion cell competence state in a subpopulation of retinal progenitor cells exiting the cell cycle. *Dev. Biol.* 365, 395-413.
- Cacheux, V., Moal, F. D.-L., Kääriäinen, H., Bondurand, N., Rintala, R., Boissier, B., Wilson, M., Mowat, D. and Goossens, M. (2001). Loss-of-function mutations in SIP1 Smad interacting protein 1 result in a syndromic Hirschsprung disease. *Hum. Mol. Genet.* **10**, 1503-1510.
- Cepko, C. (2014). Intrinsically different retinal progenitor cells produce specific types of progeny. Nat. Rev. Neurosci. 15, 615-627.
- Cepko, C. L., Austin, C. P., Yang, X., Alexiades, M. and Ezzeddine, D. (1996). Cell fate determination in the vertebrate retina. *Proc. Natl. Acad. Sci. USA* 93, 589-595.
- Conidi, A., Cazzola, S., Beets, K., Coddens, K., Collart, C., Cornelis, F., Cox, L., Joke, D., Dobreva, M. P., Dries, R. et al. (2011). Few Smad proteins and many Smad-interacting proteins yield multiple functions and action modes in TGFβ/BMP signaling in vivo. *Cytokine Growth Factor Rev.* 22, 287-300.
- Dyer, M. A., Livesey, F. J., Cepko, C. L. and Oliver, G. (2003). Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat. Genet.* 34, 53-58.
- Farhy, C., Elgart, M., Shapira, Z., Oron-Karni, V., Yaron, O., Menuchin, Y., Rechavi, G. and Ashery-Padan, R. (2013). Pax6 is required for normal cell-cycle exit and the differentiation kinetics of retinal progenitor cells. *PLoS ONE* 8, e76489.
- Fujitani, Y., Fujitani, S., Luo, H., Qiu, F., Burlison, J., Long, Q., Kawaguchi, Y., Edlund, H., MacDonald, R. J., Furukawa, T. et al. (2006). Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. *Development* 133, 4439-4450.
- Furukawa, T., Morrow, E. M. and Cepko, C. L. (1997). Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* **91**, 531-541.
- Gomes, F. L. A. F., Zhang, G., Carbonell, F., Correa, J. A., Harris, W. A., Simons, B. D. and Cayouette, M. (2011). Reconstruction of rat retinal progenitor cell lineages in vitro reveals a surprising degree of stochasticity in cell fate decisions. Development 138, 227-235.
- Goossens, S., Janzen, V., Bartunkova, S., Yokomizo, T., Drogat, B., Crisan, M., Haigh, K., Seuntjens, E., Umans, L., Riedt, T. et al. (2011). The EMT regulator Zeb2/Sip1 is essential for murine embryonic hematopoietic stem/progenitor cell differentiation and mobilization. *Blood* 117, 5620-5630.
- Grabitz, A. L. and Duncan, M. K. (2012). Focus on molecules: Smad Interacting Protein 1 (Sip1, ZEB2, ZFHX1B). *Exp. Eye Res.* **101**, 105-106.
- Gregory-Evans, C. Y., Vieira, H., Dalton, R., Adams, G. G. W., Salt, A. and Gregory-Evans, K. (2004). Ocular coloboma and high myopia with Hirschsprung disease associated with a novel ZFHX1B missense mutation and trisomy 21. *Am. J. Med. Genet.* **131A**, 86-90.
- Hafler, B. P., Surzenko, N., Beier, K. T., Punzo, C., Trimarchi, J. M., Kong, J. H. and Cepko, C. L. (2012). Transcription factor Olig2 defines subpopulations of retinal progenitor cells biased toward specific cell fates. *Proc. Natl. Acad. Sci. USA* 109, 7882-7887.
- He, J., Zhang, G., Almeida, A. D., Cayouette, M., Simons, B. D. and Harris, W. A. (2012). How variable clones build an invariant retina. *Neuron* 75, 786-798.
- Higashi, Y., Maruhashi, M., Nelles, L., Van de Putte, T., Verschueren, K., Miyoshi, T., Yoshimoto, A., Kondoh, H. and Huylebroeck, D. (2002).

- Generation of the floxed allele of the SIP1 (Smad-interacting protein 1) gene for Cre-mediated conditional knockout in the mouse. *Genesis* **32**, 82-84.
- Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J. E. and Kageyama, R. (2002).
 Math3 and NeuroD regulate amacrine cell fate specification in the retina.
 Development 129, 831-842.
- Jadhav, A. P., Roesch, K. and Cepko, C. L. (2009). Development and neurogenic potential of Müller glial cells in the vertebrate retina. *Prog. Retin. Eye Res.* 28, 249-262.
- Jeon, C.-J., Strettoi, E. and Masland, R. H. (1998). The major cell populations of the mouse retina. *J. Neurosci.* **18**, 8936-8946.
- Keeley, P. W., Luna, G., Fariss, R. N., Skyles, K. A., Madsen, N. R., Raven, M. A., Poché, R. A., Swindell, E. C., Jamrich, M., Oh, E. C. et al. (2013). Development and plasticity of outer retinal circuitry following genetic removal of horizontal cells. *J. Neurosci.* 33, 17847-17862.
- Keeley, P. W., Whitney, I. E., Madsen, N. R., St John, A. J., Borhanian, S., Leong, S. A., Williams, R. W. and Reese, B. E. (2014). Independent genomic control of neuronal number across retinal cell types. *Dev. Cell* 30, 103-109.
- Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M. and Haussler, D. (2002). The human genome browser at UCSC. Genome Res. 12, 996-1006.
- Khor, C. C., Miyake, M., Chen, L. J., Shi, Y., Barathi, V. A., Qiao, F., Nakata, I., Yamashiro, K., Zhou, X., Tam, P. O. S. et al. (2013). Genome-wide association study identifies ZFHX1B as a susceptibility locus for severe myopia. *Hum. Mol. Genet.* 22, 5288-5294.
- Lacomme, M., Liaubet, L., Pituello, F. and Bel-Vialar, S. (2012). NEUROG2 drives cell cycle exit of neuronal precursors by specifically repressing a subset of cyclins acting at the G1 and S phases of the cell cycle. *Mol. Cell. Biol.* 32, 2596-2607.
- Lelièvre, E. C., Lek, M., Boije, H., Houille-Vernes, L., Brajeul, V., Slembrouck, A., Roger, J. E., Sahel, J. A., Matter, J. M., Sennlaub, F. et al. (2011). Ptf1a/Rbpj complex inhibits ganglion cell fate and drives the specification of all horizontal cell subtypes in the chick retina. *Dev. Biol.* 358, 296-308.
- Li, S., Mo, Z., Yang, X., Price, S. M., Shen, M. M. and Xiang, M. (2004). Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. Neuron 43, 795-807.
- Manthey, A. L., Lachke, S. A., FitzGerald, P. G., Mason, R. W., Scheiblin, D. A., McDonald, J. H. and Duncan, M. K. (2014). Loss of Sip1 leads to migration defects and retention of ectodermal markers during lens development. *Mech. Dev.* 131, 86-110.
- Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F. and Gruss, P. (2001). Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105, 43-55.
- Maruhashi, M., Van De Putte, T., Huylebroeck, D., Kondoh, H. and Higashi, Y. (2005). Involvement of SIP1 in positioning of somite boundaries in the mouse embryo. *Dev. Dyn.* **234**, 332-338.
- Masland, R. (2001). Neuronal diversity in the retina. Curr. Opin. Neurobiol. 11, 431-436.
- McCulloch, D. L., Marmor, M. F., Brigel, M. G., Hamilton, R., Holder, G. E., Tzekov, R. and Bach, M. (2015). ISCEV standard for full-field clinical electroretinography (2015 update). *Doc. Ophthalmol.* **130**, 1-12.
- McKinsey, G. L., Lindtner, S., Trzcinski, B., Visel, A., Pennacchio, L. A., Huylebroeck, D., Higashi, Y. and Rubenstein, J. L. R. (2013). Dlx1&2dependent expression of Zfhx1b (Sip1, Zeb2) regulates the fate switch between cortical and striatal interneurons. *Neuron* 77, 83-98.
- Mejlvang, J., Kriajevska, M., Vandewalle, C., Chernova, T., Sayan, A. E., Berx, G., Mellon, J. K. and Tulchinsky, E. (2007). Direct repression of cyclin D1 by SIP1 attenuates cell cycle progression in cells undergoing an epithelial mesenchymal transition. *Mol. Biol. Cell* 18, 4615-4624.
- Meredith, D. M., Masui, T., Swift, G. H., MacDonald, R. J. and Johnson, J. E. (2009). Multiple transcriptional mechanisms control Ptf1a levels during neural development including autoregulation by the PTF1-J complex. *J. Neurosci.* 29, 11139-11148
- Miquelajauregui, A., Van de Putte, T., Polyakov, A., Nityanandam, A., Boppana, S., Seuntjens, E., Karabinos, A., Higashi, Y., Huylebroeck, D. and Tarabykin, V. (2007). Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation. *Proc. Natl. Acad. Sci. USA* 104, 12919-12924.
- Mojumder, D. K., Sherry, D. M. and Frishman, L. J. (2008). Contribution of voltage-gated sodium channels to the b-wave of the mammalian flash electroretinogram. *J. Physiol.* **586**, 2551-2580.
- Mowat, D. R., Croaker, G. D., Cass, D. T., Kerr, B. A., Chaitow, J., Adès, L. C., Chia, N. L. and Wilson, M. J. (1998). Hirschsprung disease, microcephaly, mental retardation, and characteristic facial features: delineation of a new syndrome and identification of a locus at chromosome 2q22-q23. *J. Med. Genet.* 35, 617-623.
- Nakhai, H., Sel, S., Favor, J., Mendoza-Torres, L., Paulsen, F., Duncker, G. I. W. and Schmid, R. M. (2007). Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. *Development* **134**, 1151-1160.

- Nelles, L., Van de Putte, T., van Grunsven, L., Huylebroeck, D. and Verschueren, K. (2003). Organization of the mouse Zfhx1b gene encoding the two-handed zinc finger repressor Smad-interacting protein-1. *Genomics* 82, 460-469.
- Omilusik, K. D., Best, J. A., Yu, B., Goossens, S., Weidemann, A., Nguyen, J. V., Seuntjens, E., Stryjewska, A., Zweier, C., Roychoudhuri, R. et al. (2015). Transcriptional repressor ZEB2 promotes terminal differentiation of CD8+ effector and memory T cell populations during infection. J. Exp. Med. 212, 2027-2039.
- Oron-Karni, V., Farhy, C., Elgart, M., Marquardt, T., Remizova, L., Yaron, O., Xie, Q., Cvekl, A. and Ashery-Padan, R. (2008). Dual requirement for Pax6 in retinal progenitor cells. *Development* 135, 4037-4047.
- Rapaport, D. H., Patheal, S. L. and Harris, W. A. (2001). Cellular competence plays a role in photoreceptor differentiation in the developing Xenopus retina. *J. Neurobiol.* **49**, 129-141.
- Rapaport, D. H., Wong, L. L., Wood, E. D., Yasumura, D. and LaVail, M. M. (2004). Timing and topography of cell genesis in the rat retina. *J. Comp. Neurol.* 474, 304-324.
- Raviv, S., Bharti, K., Rencus-Lazar, S., Cohen-Tayar, Y., Schyr, R., Evantal, N., Meshorer, E., Zilberberg, A., Idelson, M., Reubinoff, B. et al. (2014). PAX6 regulates melanogenesis in the retinal pigmented epithelium through feedforward regulatory interactions with MITF. PLoS Genet. 10, e1004360.
- Reese, B. E. (2011). Development of the retina and optic pathway. Vision Res. 51, 613-632.
- Remacle, J. E., Kraft, H., Lerchner, W., Wuytens, G., Collart, C., Verschueren, K., Smith, J. C. and Huylebroeck, D. (1999). New mode of DNA binding of multizinc finger transcription factors: deltaEF1 family members bind with two hands to two target sites. *EMBO J.* **18**, 5073-5084.
- Robson, J. G. and Frishman, L. J. (2014). The rod-driven a-wave of the dark-adapted mammalian electroretinogram. *Prog. Retin. Eye Res.* **39**, 1-22.
- Sailaja, B. S., Takizawa, T. and Meshorer, E. (2012). Chromatin immunoprecipitation in mouse hippocampal cells and tissues. *Methods Mol. Biol.* 809, 353-364.
- Schmitz, F., Königstorfer, A. and Südhof, T. C. (2000). RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron* **28**, 857-872.
- Seuntjens, E., Nityanandam, A., Miquelajauregui, A., Debruyn, J., Stryjewska, A., Goebbels, S., Nave, K.-A., Huylebroeck, D. and Tarabykin, V. (2009). Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. *Nat. Neurosci.* 12, 1373-1380.
- Shaham, O., Menuchin, Y., Farhy, C. and Ashery-Padan, R. (2012). Pax6: a multi-level regulator of ocular development. *Prog. Retin. Eye Res.* **31**, 351-376.
- Shaw, G. and Weber, K. (1984). The intermediate filament complement of the retina: a comparison between different mammalian species. Eur. J. Cell Biol. 33, 95-104.
- Srivatsa, S., Parthasarathy, S., Molnár, Z. and Tarabykin, V. (2015). Sip1 downstream Effector ninein controls neocortical axonal growth, ipsilateral branching, and microtubule growth and stability. *Neuron* 85, 998-1012.
- Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakanishi, S., Asashima, M. and Kageyama, R. (1997). Conversion of ectoderm into a neural fate by ATH-3, a vertebrate basic helix-loop-helix gene homologous to Drosophila proneural gene atonal. *EMBO J.* **16**, 384-395.
- Trimarchi, J. M., Stadler, M. B. and Cepko, C. L. (2008). Individual retinal progenitor cells display extensive heterogeneity of gene expression. PLoS ONE 3, e1588
- van den Berghe, V., Stappers, E., Vandesande, B., Dimidschstein, J., Kroes, R., Francis, A., Conidi, A., Lesage, F., Dries, R., Cazzola, S. et al. (2013). Directed migration of cortical interneurons depends on the cell-autonomous action of Sip1. *Neuron* 77, 70-82.
- van Grunsven, L. A., Michiels, C., Van de Putte, T., Nelles, L., Wuytens, G., Verschueren, K. and Huylebroeck, D. (2003). Interaction between Smadinteracting protein-1 and the corepressor C-terminal binding protein is dispensable for transcriptional repression of E-cadherin. J. Biol. Chem. 278, 26135-26145.
- van Helden, M. J., Goossens, S., Daussy, C., Mathieu, A.-L., Faure, F., Marçais, A., Vandamme, N., Farla, N., Mayol, K., Viel, S. et al. (2015). Terminal NK cell maturation is controlled by concerted actions of T-bet and Zeb2 and is essential for melanoma rejection. *J. Exp. Med.* 212, 2015-2025.
- Verschueren, K., Remacle, J. E., Collart, C., Kraft, H., Baker, B. S., Tylzanowski, P., Nelles, L., Wuytens, G., Su, M.-T., Bodmer, R. et al. (1999). SIP1, a novel zinc finger / homeodomain repressor, interacts with Smad proteins and binds to 5-CACCT sequences in candidate target genes. *J. Biol. Chem.* 274, 20489-20498.
- Voinescu, P. E., Kay, J. N. and Sanes, J. R. (2009). Birthdays of retinal amacrine cell subtypes are systematically related to their molecular identity and soma position. J. Comp. Neurol. 517, 737-750.
- Wakamatsu, N., Yamada, Y., Yamada, K., Ono, T., Nomura, N., Taniguchi, H., Kitoh, H., Mutoh, N., Yamanaka, T., Mushiake, K. et al. (2001). Mutations in SIP1, encoding Smad interacting protein-1, cause a form of Hirschsprung disease. Nat. Genet. 27, 369-370.

- Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. *Genes Dev.* 15, 24-29.
- Weng, Q., Chen, Y., Wang, H., Xu, X., Yang, B., He, Q., Shou, W., Chen, Y., Higashi, Y., van den Berghe, V. et al. (2012). Dual-mode modulation of Smad signaling by Smad-interacting protein Sip1 is required for myelination in the central nervous system. *Neuron* 73, 713-728.
- Xiang, M. (2013). Intrinsic control of mammalian retinogenesis. *Cell. Mol. Life Sci.* **70**, 2519-2532.
- Yoshimoto, A., Saigou, Y., Higashi, Y. and Kondoh, H. (2005). Regulation of ocular lens development by Smad-interacting protein 1 involving Foxe3 activation. *Development* 132, 4437-4448.
- Young, R. W. (1985). Cell differentiation in the retina of the mouse. *Anat. Rec.* **212**, 199-205.