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 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 Smad-interacting 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; Miquelajaregui et al., 2007; Seuntjens et al., 2009; van den Berge 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 formation.
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 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 Pax6loxp/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...
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 Gly1 (Scler99') ACs, respectively (Fig. 1H-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 Pou42' RGCs (Fig. 1M,M'). Sip1 levels were detected in 36.5±13% of the Pou42' RGCs (Fig. 1M,M'). Sip1 was also detected in displaced ACs located in the GCL, while very low levels were detected in 36.5±13% of the Pou42' RGCs (Fig. 1M,M').

To determine the role of Sip1 in retinogenesis we conditionally mutated the Sip1lox/lox allele using the α-Cre transgenic line (Higashi et al., 2002; Marquardt et al., 2001). Sip1 loss was evident in the Sip1lox/lox;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 Sip1lox/lox;α-Cre embryos in comparison to control littermates (Fig. S4I, n=3 controls and 5 mutants, P=0.009). At P14 the thickness of Sip1lox/lox;α-Cre retinas was reduced by 30% compared with the control (Fig. 2B,F,J, 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).

We next determined changes in the number of each INL cell type, as this cellular layer was most affected. Notably, Sip1lox/lox;α-Cre retinæ were completely devoid of HCs as calbindin and NF165 were not detected (Fig. 2B,F,J, Fig. 3E,J). We quantified, by detection of cell-specific markers, the number of Prox1+ rod photoreceptors that can be detected by a standard ERG protocol. The morphological defects associated with Sip1-deficient retinæ suggested that retinal function might also be perturbed. Electoretinogram (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 was 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 Sip1lox/lox;α-Cre retinae at E15.5 (Fig. S4B,F,J, n=3, P=0.39), E18.5 (Fig. S4C,G,J, n=3, P=0.39) and P0 (Fig. S4D,H,J, n=3, P=0.139).

To determine whether the ablation of Sip1 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 (Dyer 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,H,J, 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 proneural 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). During the early stages of retinogenesis [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. The morphological defects associated with Sip1-deficient 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.
HCs and ACs, we examined possible change in their expression following Sip1 loss.

During normal retinogenesis, Ptf1a+ and Ap2α+ 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±16.4% of control values (Fig. 4F, Fig. S5A, n=3, P=0.007), and Ap2α was not detected at this stage (Fig. 4G). Consistent with the above, Ap2β, which is normally detected in the NB 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). Ap2α and Ap2β+ 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.
The increase in the number of Pou4f2+ cells in the NBL at E16.5 differentiate into RGCs (Fujitani et al., 2006; Nakhai et al., 2007). normally activate 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. (E,J) IIF was used to detect Prox1 and NF165 proteins in control (E) and cells at P0 (Fig. 6A,B). The retinae of the Sip1loxp/loxp; Isl1, which is normally detected in the BP precursors populating the retinae, we characterized the dynamics of the differentiation of the RPCs, which are supposed to differentiate into AC and HC precursors, remain undifferentiated. The mean number of BrdU+ 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 at E16.5 did not show a significant reduction in the number of Ap2β+ BrdU+ cells in Sip1lox/lox;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+ cells at E14.5 and determined the percentage of BrdU+ 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 Ptf1a 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 at E15.5, suggesting that they had exited the cell cycle during the 24 h following the BrdU incorporation. The proportion of 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
In order to assess the possibility that Sip1 directly binds the Ptf1α enhancer in the mouse embryonic retina we scanned for Sip1−Cre in vivo conservation by PhyloP (100 vertebrates basewise) (Remacle et al., 1999) in the enhancer sequence. Putative sites were then sorted according to their evolutionary conservation using the ‘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 Ptf1α enhancer (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 Ptf1α 3′ enhancer.

In order to assess the possibility that Sip1 directly binds the Ptf1α 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 ‘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 Ptf1α 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 Ptf1α 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 Ptf1α in the Sip1loxp/loxp,α-Cre retina, it seems that Sip1 acts as a transcriptional activator of Ptf1α 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 Sip1loxp/loxp,α-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.
By contrast, Ptf1a, Ap2α and Ap2β, 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 Sip1lox/lox;α-Cre retinas 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.
between E14.5 and E15.5 in the Sip1^{loxp/loxp};\alpha-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).
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 Ptf1a 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 Ptf1a. 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 Ptf1a, that promote AC and HC differentiation.
promoting the timely differentiation of Ptf1a+, Ap2αβ+ precursor cells into RGCs because of the reduced Ptf1a. As a result, the generation of Prox1+ HC precursors 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 the generation of Prox1+ 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 and differentiate into Muller glia at the end of retinogenesis. An opposite role has been proposed for Notch pathway genes and Sip1, which suggests that Sip1 antagonizes Muller glia generation. Alternatively, Sip1 might actively repress glial fate.

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 Ptf1aβ and is required for the peak of generation of HCs. At later stages, Sip1 might be required for the generation of Prox1+ HC precursors. When Sip1 is mutated (B), the generation of Ptf1aβ+ precursor cells is delayed as some of them fail to properly exit the cell cycle. This results in a decrease in the numbers of HCs and in a complete disappearance of HCs. Some of these cells might transdifferentiate into RGCs because of the reduced Ptf1aβ.

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

Sip1 mutant retinas 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 retinas, 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 retinas 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 retinas (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.

Table 1. ISCEV standard protocol for full-field electroretinography

<table>
<thead>
<tr>
<th>Step</th>
<th>ERG test session</th>
<th>Flash intensity (cd·s/m²)</th>
<th>Number of flashes averaged</th>
<th>Interval (s)</th>
<th>Background light</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Dark adaptation (overnight)</td>
<td>0.01</td>
<td>10</td>
<td>2</td>
<td>Off</td>
</tr>
<tr>
<td>1</td>
<td>Rod responses</td>
<td>3</td>
<td>4</td>
<td>10</td>
<td>Off</td>
</tr>
<tr>
<td>2</td>
<td>Standard intensity mixed rod-cone responses</td>
<td>10</td>
<td>4</td>
<td>20</td>
<td>Off</td>
</tr>
<tr>
<td>3</td>
<td>High intensity mixed rod-cone response</td>
<td>32</td>
<td>32</td>
<td>0.5</td>
<td>On</td>
</tr>
<tr>
<td>4</td>
<td>High intensity cone responses</td>
<td>10</td>
<td>32</td>
<td>0.5</td>
<td>On</td>
</tr>
</tbody>
</table>
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 intraocularly 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 µg luciferase reporter vector with either the S′ 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 µg; 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

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Supplementary information
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