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A Conserved Regulatory Logic Controls Temporal Identity in Mouse Neural Progenitors

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SUMMARY

Neural progenitors alter their output over time to generate different types of neurons and glia in specific chronological sequences, but this process remains poorly understood in vertebrates. Here we show that *Casz1*, the vertebrate ortholog of the *Drosophila* temporal identity factor *castor*, controls the production of mid-/late-born neurons in the murine retina. Casz1 is expressed from mid/late stages in retinal progenitor cells (RPCs), and conditional deletion of *Casz1* increases production of early-born retinal neurons at the expense of later-born fates, whereas precocious misexpression of *Casz1* has the opposite effect. In both cases, cell proliferation is unaffected, indicating that *Casz1* does not control the timing of cell birth but instead biases RPC output directly. Just as *Drosophila castor* lies downstream of the early temporal identity factor *hunchback*, we find that the *hunchback* ortholog *Ikzf1* represses Casz1. These results uncover a conserved strategy regulating temporal identity transitions from flies to mammals.

In Brief

How neural progenitors alter their output over time remains unclear in vertebrates. Mattar et al. identify Casz1 as a key regulator of temporal progression in retinal progenitors and provide evidence for conservation of the transcriptional cascade strategy used in *Drosophila* neuroblasts.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.12.052.

AUTHOR CONTRIBUTIONS

P.M. generated alleles, constructs, and performed and analyzed all experiments. J.E. and S.B. generated antibodies, supplied constructs, contributed to the design of the study, and helped revise the manuscript. P.M. and M.C. designed the experiments and wrote the manuscript.

INTRODUCTION

In the developing nervous system, various neuronal and glial cell types are generated from multipotent progenitor cells in a specific chronological sequence. Key to this process is the ability of progenitors to change their "temporal identity" as development proceeds, such that they gain or lose the competence to generate specific cell types at the appropriate developmental stage (Kohwi and Doe, 2013; Li et al., 2013a; Livesey and Cepko, 2001; Molyneaux et al., 2007). The alteration of progenitor output over time is a classical feature of the developing vertebrate retina, in which multipotent RPCs give rise to elaborate lineages consisting of many different classes of neurons and one glial cell type in a specific order. Cone photoreceptor, horizontal, and ganglion cells are mostly generated in the earliest phase of retinogenesis, closely followed by amacrine cells, and later by rod photoreceptor, bipolar, and Müller glial cells (Rapaport et al., 2004; Young, 1985). While the mechanisms regulating the production of each individual fate upon cell-cycle exit are beginning to be elucidated, how exactly RPCs change their identity over time remains poorly understood (Cepko, 2014).

Mechanistically, regulation of temporal identity in neural progenitors is best understood in invertebrate systems. In *Drosophila* neuroblast lineages, several transcription factor cascades have been shown to alter temporal identity over developmental time (Baumgardt et al., 2009; Bayraktar and Doe, 2013; Li et al., 2013a, 2013b; Maurange et al., 2008; Pearson and Doe, 2003). Among the best characterized of these cascades operates during motoneuron production, in which sequential expression of the transcription factors *hunchback* (*hb*), *Krüppel* (*Kr*), *pdm1/2*, and *castor* (*cas*) is necessary and sufficient to specify fates at a given neuroblast division and/or to regulate the progression of the cascade (Cleary and Doe, 2006; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Pearson and Doe, 2003).

We have shown previously that the zinc finger transcription factor *Ikzf1* (*Ikaros/Znfn1a1*), an ortholog of *Drosophila hb*, is necessary and sufficient to confer early temporal identity in RPCs (Elliott et al., 2008). It remains unknown, however, what might confer later temporal identity in RPCs, and whether transcription factor cascades similarly to those used in flies might constitute a general mechanism regulating temporal patterning in vertebrates. A mouse gene homologous to *Drosophila cas*, named *Casz1*, is expressed in the retina (Blackshaw et al., 2004), but the function of *Casz1* in the vertebrate nervous system has yet to be examined. Here, we demonstrate that *Casz1* lies downstream of *Ikzf1* and is essential for conferring mid/late temporal identity in murine RPCs, suggesting conservation of the temporal identity cascade used in flies.

RESULTS

An Ortholog of *Drosophila castor* Is Expressed in Mid-Stage RPCs

Through genome homology searches, we found that a single gene homologous to the *Drosophila* temporal identify factor *cas* exists in vertebrates, named *Casz1*. Like *cas*, *Casz1* encodes multiple unusual C2H2C2H2 zinc finger domains (Figure 1A). The *Casz1* locus generates two alternative transcripts, *Casz1v1* and *Casz1v2*, which share an identical N-

terminal cluster of four highly conserved C2H2C2H2 domains and a fifth more divergent domain. *Casz1v1* additionally contains six divergent C2H2C2H2 motifs (Figure 1A). Thus, since *Casz1* is the definitive ortholog of *Drosophila cas*, and its function has not been explored in the nervous system, we hypothesized that it might play an analogous role in regulating temporal identity in mouse RPCs.

We first studied *Casz1* transcript and protein expression during retinogenesis. Casz1 was barely detectable during early stages of retinogenesis but increased between embryonic day 14.5 (E14.5) and E16.5 (Figures 1B and 1C; Figures S1A and S1B). During intermediate stages of retinogenesis (E16.5–postnatal day 0 [P0]), Casz1 was almost completely restricted to the progenitor layer (Figures 1C–1E) but became more prominent in differentiating photoreceptors by P4 (Figure 1F; Figure S1) and remained expressed in adult rods and cones (Figure 1G; Figures S1A and S1B). Interestingly, no difference in the expression pattern of *Casz1v1* and *Casz1v2* was noted at any stage (Figure S1B). Co-stainings at E16.5 and P0 revealed that Casz1 is expressed in almost all proliferating Vsx2+/Ki67+ RPCs during mid-phase retinogenesis (Figures 1H–1O). A few Casz1+;Ki67 — cells were also found, however, suggesting Casz1 expression in differentiating cells. Accordingly, Otx2, a marker of committed rod photoreceptor precursors during postnatal stages of retinogenesis, was extensively co-expressed with Casz1 at the apical margin of the progenitor layer (Figures 1P–1S). Conversely, precursors for early-born cell types, such as Lhx1+ horizontal cells and Pou4f+ (Brn3) RGCs did not express Casz1 (data not shown). Finally, by P4, Casz1 was restricted to Otx2+ rod precursors and was undetectable in Ki67+/Otx2 — RPCs (Figures 1T–1W). In summary, Casz1 protein is expressed from mid/late stages in RPCs and is maintained in differentiating rod precursors and mature rods. Additionally, Casz1 is found in postmitotic early-born cones and a small subset of GABAergic amacrine cells (Figure S2), suggesting other functions of Casz1 in terminally differentiated neurons. These results indicate a high correlation between mid/late RPC temporal identity and Casz1 expression.

Casz1 Is Required to Suppress the Production of Early-Born Retinal Neurons

To elucidate the function of *Casz1* in the developing murine retina, we generated *Casz1* mouse mutant alleles. $Casz1^{SALacZ}$ is predicted to be a null allele by causing premature transcription arrest before the zinc finger domains (Figure 2A). Accordingly, no viable $Casz1^{SALacZ/SALacZ}$ homozygotes were found in E10.5 litters (wild-type: 10/33; $Casz1^{SALacZ/+}$: 21/33; $Casz1^{SALacZ/SALacZ}$: 0/33) or after birth (n = 0/115), consistent with the previously reported essential role for *Casz1* in heart and vascular system development (Charpentier et al., 2013; Christine and Conlon, 2008; Liu et al., 2014). Taking advantage of the $Casz1^{SALacZ/+}$ allele, we studied the general expression pattern of the *Casz1* locus in developing embryos. In the retina, α-Gal was expressed in a pattern consistent with what we observed by in situ hybridization (Figures S1A and S1B), while whole embryo staining revealed β-Gal expression in the developing heart and arteries, as well as the dorsal neural tube and somatic sensory neurons (Figures S1C–S1E).

Since *Casz1* deletion is lethal prior to eye formation, we converted $Casz1^{SALacZ}$ into a conditional $Casz1^{Flox}$ allele using the FRT/flipase system in vivo. To monitor Cre-mediated recombination, we additionally introduced a reporter allele (*R26-EYFP*). Since our goal was

to study the role of *Casz1* in the development of individual lineages, we first inactivated *Casz1* clonally in RPCs using retroviral vector-mediated Cre expression. We infected E13.5 *Casz1*$^{Flox/+}$; *R26-EYFP* or *Casz1*$^{Flox/Flox}$; *R26-EYFP* littermate retinal explants and analyzed the size and composition of resulting viral heterozygote (*v-Het*) or conditional knockout (*v-cKO*) clones 20 days later, when retinal development is complete (Figure 2E). Strikingly, we found that early-born fates such as horizontal, amacrine, and cone cells were significantly overrepresented in *v-cKO* clones, whereas mid-/late-born rods were significantly reduced (Figures 2F–2J; Tables S1 and S2). In contrast, later-born bipolar cells were not affected and Müller glia were increased. It is therefore likely that many *Casz1 cKO* RPCs that fail to adopt the rod fate default to Müller glia, as this is the latest fate in the temporal sequence. These results suggest that *Casz1* suppresses early temporal competence as well as progression into the final temporal state, which is consistent with our finding that RPCs do not express Casz1 during the latest stages of retinogenesis (Figures 1T–1W). Importantly, the overall size distribution of *v-cKO* clones was unchanged compared to controls (Figure 2K), indicating that *Casz1* does not function by regulating the timing of cell-cycle exit or cell death.

We next wanted to study the impact of *Casz1* inactivation on overall retinal development in vivo. To do this, we crossed the α-*Pax6::Cre* mouse line, which expresses Cre recombinase in peripheral RPCs from E10.5 (Marquardt et al., 2001), into the *Casz1*$^{Flox/Flox}$*; R26-EYFP* background to generate retina-specific *Casz1* conditional knockouts (α-*cKO*) (Figure 2A). As expected, α-*cKO* animals were viable in normal Mendelian ratios, and Casz1 protein was lost in almost all EYFP+ cells (Figures 2B–2D), validating the knockout strategy and confirming the specificity of our antibodies. Consistent with the clonal analysis, adult-staged (~P40–P80) α-*cKO* animals had fewer rod photoreceptor cells in the peripheral retina where Cre is expressed (control: 68.93% ± 0.63% of total cells, n = 6; α-*cKO*: 60.08% ± 0.08%, n = 6; $p < 0.0001$; Figure S3). The fraction of inner nuclear layer (INL) cells, which includes both interneurons and Müller glia, was concomitantly increased (control: 26.28% ± 0.47%, n = 6; α-*cKO* INL: 33.07% ± 0.42%, n = 6; $p = 0.0013$; Figure S3). More specifically, early-born fates like amacrines, cones, as well as ganglion cells, which could not be studied in the *v-cKO* clones because they rapidly degenerate in explants, were all increased in P0 or P21 α-*cKO* retinas in vivo (Table S3; Figure S3). Thus, cell fate alterations in α-*cKO* retinas are consistent with what we observed upon clonal inactivation of *Casz1* (increased early fates at the expense of mid-/late-born rods), albeit less severe. It is well-known, however, that multiple pathways can compensate for changes in cell proportions in the retina in vivo, including feedback and apoptosis-mediated mechanisms (Brzezinski et al., 2010; Close et al., 2005; Dyer and Cepko, 2000; Kim et al., 2005; Wang et al., 2005), and it is likely that such compensatory mechanisms are operating when *Casz1* is inactivated in a large population of cells, as in the α-*cKO* retinas. Interestingly, in much older (~P230) α-*cKO* retinas, we noted extensive degeneration of the photoreceptor layer (Figures S3G and S3H), suggesting an additional role for Casz1 in the maintenance of mature photoreceptor cells.

Precocious Misexpression of *Casz1* Promotes Mid-/Late-Born Neuronal Fates

To test whether *Casz1* is sufficient to promote the generation of mid-/late-born fates, we used retroviral vectors to misexpress each isoform of *Casz1* in RPCs at E13.5, a stage prior

to the upregulation of *Casz1* expression. Three weeks later, *Casz1v1*-overexpressing clones contained normal proportions of rods but nearly double the number of bipolar cells compared to controls (Figures 3A–3D and 3G; Table S1). *Casz1v2*, in contrast, had no effect on bipolar cell production but significantly increased rod photoreceptor production (Figures 3E–3G; Tables S1 and S2). The increase in mid-/late-born rods and bipolars came both at the expense of early fates, such as amacrines, horizontals, and cones and also at the expense of the last-born Müller glial cells (Figure 3G). As observed upon *Casz1* inactivation, we found no effect on clone size distribution after overexpressing either *Casz1* isoform (Figure 3H), reinforcing the idea that *Casz1* alters RPC output independently of proliferation or apoptosis.

Casz1 Lies Downstream of *Ikzf1*

In *Drosophila*, *hb* acts as a repressor to suppress *cas* expression and, conversely, when *hb* is converted to an obligatory activator by fusing it to the VP16 activation domain, it upregulates *cas* expression (Tran et al., 2010). We therefore wondered whether *Ikzf1* might similarly regulate *Casz1* expression. To test this idea, we generated *Ikzf1* and *Ikzf1-VP16* constructs and first transfected them into P0 RPCs to test their effect on fate. After 14 days, RPCs transfected with *Ikzf1* generated an unusually large number of early-born Pax6+ amacrine/horizontal cells and few rods, as previously reported (Elliott et al., 2008). In contrast, *Ikzf1-VP16* appeared to behave as a dominant negative, as transfected RPCs generated primarily latest-born Ccnd3+ Müller glia (Figures 4A–4H).

To determine whether Ikzf1 regulates endogenous Casz1 expression in mouse RPCs, we transfected *Ikzf1* or *Ikzf1-VP16* and studied Casz1 expression levels in transfected cells. As predicted, *Ikzf1* led to a significant repression of Casz1, whereas *Ikzf1-VP16* upregulated Casz1 expression relative to untransfected neighboring cells (Figures 4I–4O). To determine whether this regulation was direct, we screened the *Casz1* locus for *cis* regulatory modules (CRMs) that might mediate the transcriptional effects of Ikzf1 (Figures S4A–S4G). To do this, we examined various published chromatin immunoprecipitation sequencing (ChIP-seq) datasets for transcription factors implicated in retinal development, reasoning that consistent ChIP-seq peaks in *Casz1* would correspond to potential regulatory regions. In this way, we identified CRM1 and CRM2, located within intron 2 and 3 of *Casz1*, respectively (Figures S4A–S4D). Both CRMs contained conserved Crx ChIP-seq peaks (Corbo et al., 2010), and CRM2 additionally contained conserved binding sites for Sox2 and Otx2 (Buecker et al., 2014; Lodato et al., 2013). To study the activity of these CRMs in RPCs, we generated CRM::mGFP reporter constructs, as well as an ~550 bp *Casz1* promoter::EGFP construct (PR::EGFP). These CRM constructs, as well as control GFP constructs, were electroporated in the retina at P0, along with mCherry to monitor transfection efficiency. Both CMR2::mGFP and PR::EGFP were able to drive GFP expression in a pattern similar to Casz1 (Figures S4H–S4K), while CRM1::mGFP or control vectors did not yield any GFP expression (Figures S4L–S4N). Interestingly, both CRM2::mGFP and PR::EGFP constructs were strongly repressed by Ikzf1 in RPCs, but neither element was activated by Ikzf1-VP16 (Figures S4O–S4T). Since *Ikzf1-VP16* upregulates Casz1, but cannot affect *Ikzf1*-sensitive regulatory elements, we conclude that *Ikzf1* and *Ikzf1-VP16* regulatory activities are probably routed independently and thus indirectly. Together, these results indicate that *Ikzf1*

sits upstream of *Casz1* in a functionally similar epistatic relationship to that of *hb* and *cas* in *Drosophila*.

DISCUSSION

There has been much progress over the past few years to identify factors that promote the production of individual neural cell types, but how exactly the intrinsic potential of neural progenitor cells change over time to regulate histogenesis remains unclear. In this study, we identify the zinc finger protein Casz1 as an important regulator of temporal identity in mouse RPCs. We find that Casz1 is expressed in mid-stage RPCs and is necessary and sufficient to regulate the balance of early- versus late-born cell types in the developing retina. Moreover, we demonstrate that *Ikzf1* regulates Casz1 expression, suggesting that the regulatory logic of the fly *hb* >>> *cas* temporal identity cascade is conserved in mice.

In both vertebrates and invertebrates, neural progenitors use time as a cue to alter their output. Reconstruction of randomly sampled vertebrate RPC lineages, both in clonal culture or in vivo, did not reveal deterministic behavior and instead suggested a model in which RPCs divide or differentiate into specific cell types according to biased probabilities that change over time but remain fixed for multiple cell generations (Gomes et al., 2011; He et al., 2012). The molecular effectors that change biases over time to produce mostly early- or late-born fates at the appropriate stage, however, have remained unknown. We propose that Ikzf1 and Casz1 have this role in mammalian RPCs (Figure 4P). Such a model is consistent with observations that loss or gain of function of either *Ikzf1* or *Casz1* alters the balance of early- versus later-born cell types without affecting lineage size, and that loss of either gene leads to a decrease but not a complete absence of cell types associated to a given temporal window. Additionally, ectopic expression of Ikzf1 or Casz1 increases the propensity to generate early- or mid-born cell types, respectively, but does not completely abolish the competence to produce cell types associated to a different temporal window. This suggests that Ikzf1 and Casz1 function non-deterministically to confer specific temporal identities to RPCs during which they are more or less biased to give rise to specific combinations of cell types: early fates during the Ikzf1 expression window and later fates during the Casz1 expression window.

Our data suggest that Casz1 confers a mid-phase temporal identity to RPCs. While our preconception was that Casz1 should control late temporal competence, the upregulation of Casz1 in RPCs occurs considerably earlier than would be expected. Casz1 upregulation coincides with the onset of (1) the decline in early fate production and (2) rod generation (Rapaport et al., 2004). While rods, bipolars, and Müllers are all generated from RPCs during the Casz1 expression window, rod production peaks toward the end of the Casz1 expression window, whereas bipolar and Müller cell production peaks several days after. Casz1-mediated suppression of Müller production is therefore consistent with Casz1 suppressing both the early and latest temporal identities. Overexpression of Casz1 probably expands the mid temporal window into the late Müller-producing window, thereby causing a decrease in Müller glia. Conversely, inactivation of *Casz1* most likely triggers the late temporal window to start earlier than normal, thereby leading to an overproduction of Müller glia (Figure 4P). It remains unclear, however, why bipolar cells are not suppressed upon

Casz1 inactivation. One potential explanation is that Casz1 alters the balance between early- and late-born bipolar cell subtypes, as there is evidence for temporal regulation of subtype production (Morrow et al., 2008). Another possibility is that the actions of Casz1 are confined solely to neurogenesis and/or that the tendency of Müller glia to be produced at the end of RPC lineages accounts for the observed difference. Future experiments will be required to distinguish between these possibilities.

An interesting observation in this study is the differential effects of Casz1 isoforms. At present, we have only a preliminary understanding of why Casz1v1 and Casz1v2 achieve different effects on cell fate. We found in pilot experiments that the genes most differentially regulated by the two isoforms are *Ascl1* and *Vsx2*, two determinants of bipolar cells. *Ascl1* was repressed in cells overexpressing Casz1v2, while *Vsx2* was upregulated in cells overexpressing Casz1v1 (data not shown), suggesting a potential explanation, but the data remain to be corroborated and validated functionally.

While our data do not completely exclude the possibility that Casz1 acts as a traditional fate determinant, we suggest that Casz1 is a bona fide temporal identity factor since (1) Casz1 is expressed in virtually all RPCs within its expression window, whereas the expression of fate determinants tends to be restricted to specific committed precursors, (2) Casz1 does not affect proliferation of RPCs, implying that it does not determine a specific cell identity, whereas fate determinants usually trigger cell-cycle exit to induce differentiation, and (3) Casz1 overexpression promotes rods and bipolar cells, rather than a single cell type. These features are highly reminiscent of the regulation of temporal competence by *Drosophila cas*, suggesting functional and perhaps mechanistic conservation.

Our finding that Ikzf1 negatively regulates Casz1 expression provides a mechanism by which the appropriate sequential expression of each temporal identity factor is controlled in RPCs, analogous to the logic used to control temporal identity in fly neuroblasts. Previous functional analysis of *Ikzf1* in ret-inogenesis revealed that *Ikzf1* is necessary and sufficient to confer early competence to RPCs, but it remained unclear whether this biasing activity represented true conservation of the *Drosophila* temporal fate determination system or was merely coincidence. In this study, we show that *Casz1* acts downstream of *Ikzf1* in RPCs to promote later neuronal fates. Moreover, just as repressor activities of hb are required for its temporal functions, we show that Ikzf1 similarly acts as a repressor, since Ikzf1-VP16 acts oppositely to overproduce late fates. Nonetheless, the effect of Ikzf1-VP16 on late fate production is different than that of ectopic Casz1 expression. While Ikzf1-VP16-mediated induction of Casz1 might contribute to these effects, the observed overproduction of Müller glia along with our failure to observe sustained Casz1 induction at later time points (data not shown) suggests that Ikzf1-VP16 regulates additional target genes and/or accelerates RPC maturation into the terminal Casz1-negative state.

Thus, an analogous transcription factor cascade appears to control temporal competence during vertebrate CNS development. However, our observations suggest that there are also important differences between the murine and fly temporal cascades, including that (1) murine temporal factors act as biasing factors to repress or promote certain fates without completely abolishing the competence to produce fates associated to other temporal

windows, whereas *Drosophila* factors appear to act more deterministically to suppress alternative fates entirely, (2) murine factors regulate competence windows but do not entirely control them, and (3) interactions between mouse cascade members appear to be much less rigid than their *Drosophila* counterparts. However, it must be emphasized that other factors may lie in between *Ikzf1* and *Casz1* in the mouse cascade. Orthologs of *pdm* in mice, for example, belong to a family of Pou-domain-containing transcription factors (Pou2f family) that have not been studied in the retina, and there are hundreds of putative *Kr* orthologs. It will be important to determine whether these candidates are part of the same temporal cascade as Ikzf1 and Casz1.

Since *Ikzf1* was recently shown to control early-born neuronal fates in the cerebral cortex (Alsiö et al., 2013), and *Casz1* is expressed in other regions of the nervous system such as the dorsal spinal cord and medial telencephalon (Liu et al., 2014; Figure S1), it will be interesting to determine whether this system is generally used to control temporal patterning in other regions of the nervous system.

EXPERIMENTAL PROCEDURES

Animals

All animal work was carried out in accordance with the guidelines of the Cana-dian Council on Animal Care and the IRCM animal care committee. *Casz1* alleles were generated from the HEPD0679_6_F09 Eucomm ES cell line (Skarnes et al., 2011) using standard procedures. CD1 and C57BL/6J mice were from Taconic. The following alleles were genotyped according to the supplier's protocol: *Rosa26Flpo* and *R26-Stop-EYFP* (Jackson Labs), α-*Pax6 enhancer::Cre* (Marquardt et al., 2001). For timed matings, E0.5 was considered to be the morning on which the vaginal plug was observed. Genotyping primers are shown in a Table in Supplemental Experimental Procedures.

Explant Transfection/Transduction

Retinal electroporation and retroviral transduction were performed as reported (Kechad et al., 2012) with modifications and constructs described in detail in the Supplemental Experimental Procedures.

Histology

Immunohistochemistry was performed as per Elliott et al. (2008) with modifications and antibodies described in detail in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Casz1 expression is upregulated in mid-/late-stage retinal progenitors
- Casz1 is required to suppress early-born retinal fate production
- Ectopic expression of Casz1 promotes mid-/late-born neuronal fates
- Casz1 is repressed by Ikzf1, analogously to *Drosophila castor* by hunchback

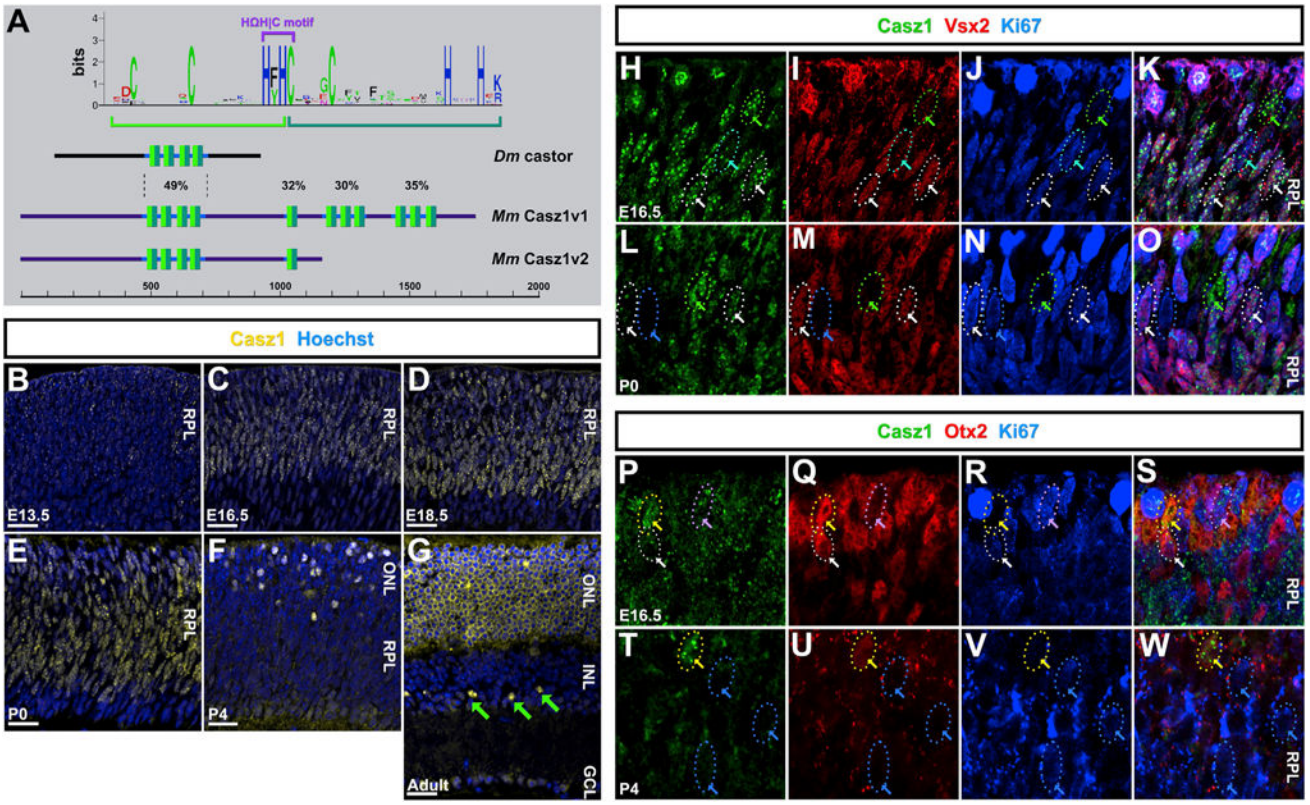

Figure 1. Mouse Casz1 Is the Ortholog of *Drosophila castor* and Is Expressed in RPCs during Mid-Retinogenesis

(A) Meme analysis (Bailey et al., 2009) of murine and *Drosophila* castor proteins identifies a signature HΩH|C motif, where "Ω" represents a bulky amino acid residue, and "|" divides the 2 zinc finger repeats of the domain (green brackets). Murine Casz1 isoforms contain 11 (Casz1v1) or 5 (Casz1v2) such zinc finger repeats (green boxes). (B–G) Spatiotemporal expression profile of Casz1 during retinogenesis. Arrows in (G) indicate rare amacrine cells that also express Casz1. (H–W) Co-expression of Casz1 (green) and Ki67 (blue) with the RPC marker Vsx2 (red, H–O), or the photoreceptor precursor marker Otx2 (red, P–W), at E16.5 or P0 as indicated. Arrow/circle colors refer to the markers expressed by the indicated cell. Scale bar, 20 μm. RPL, retinal progenitor layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

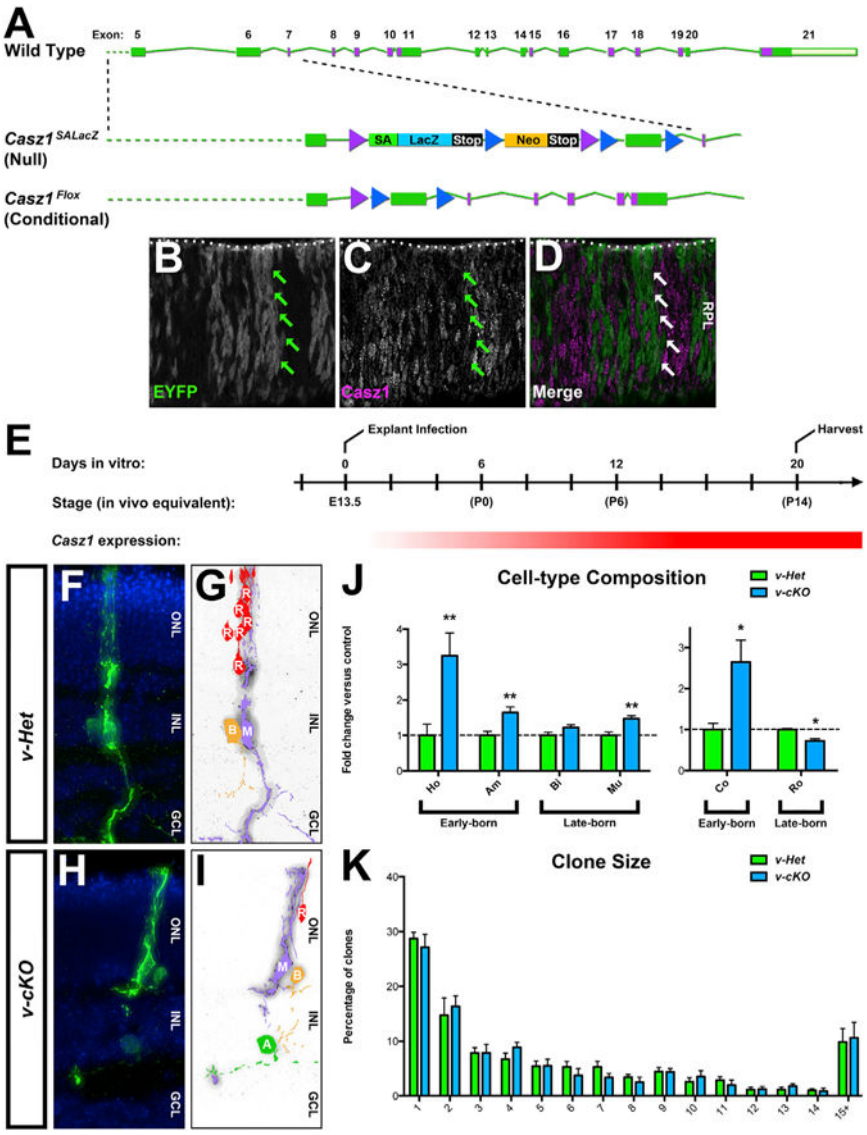

Figure 2. Conditional Inactivation of Casz1 in RPCs Increases Early-Born Neuron Production at the Expense of Mid-/Late-Born Neuronal Fates

(A) Schematic of *Casz1* alleles. Exons are shown as green boxes, zinc finger domains as purple boxes, loxP sites as blue triangles, and FRT sites as purple triangles. (B–D) Immunostaining shows loss of Casz1 protein (purple) in EYFP+ α-*cKO* cells (arrows). (E) Strategy for clonal inactivation of *Casz1* in RPCs. (F–I) Examples of viral heterozygote control (*v-Het*) or conditional knockout (*v-cKO*) clones obtained 20 days after infection. Images of EYFP-positive clones (F and H) or camera-lucida drawing (G and I) are shown. R, rod; A, amacrine; B, bipolar; M, Müller. (J) Quantification of clonal composition expressed as fold change over control (dotted line). (K) Clone size distribution in *v-Het* and *v-cKO* clones. Bar graphs show mean ± SEM. See also Tables S1 and S2 for statistical details.

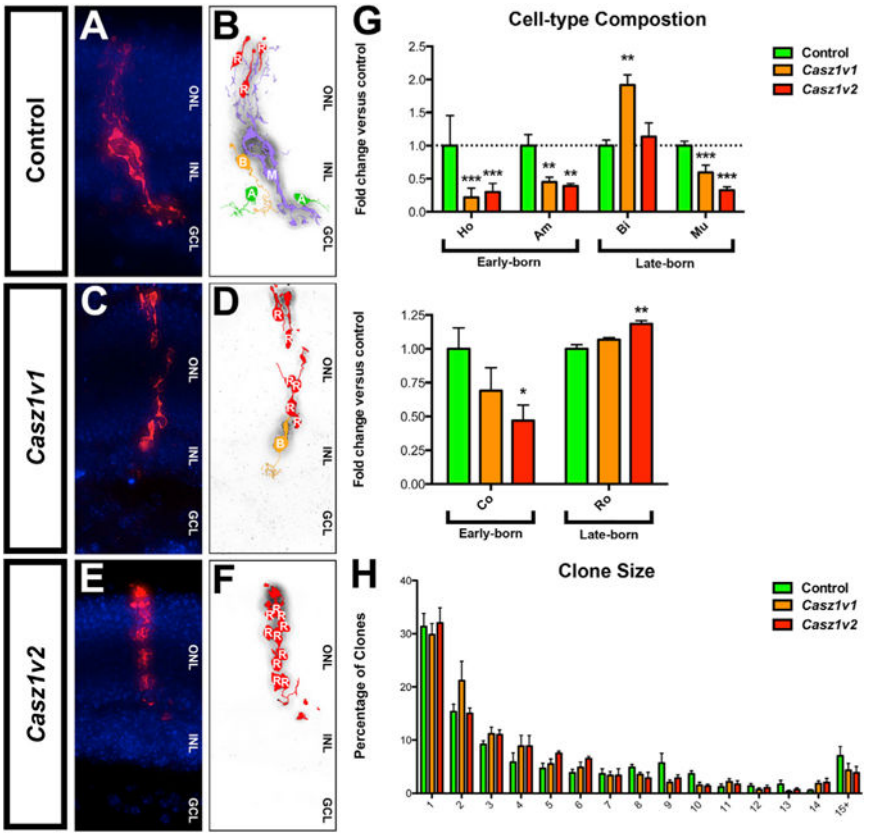

Figure 3. Heterochronic Expression of *Casz1* in Early RPCs Increases Mid-/Late-Born Neuron Production

(A–F) Examples of clones obtained after infection of retinal explants at E13.5 with retroviral vectors expressing GFP alone (Control: A and B), or GFP and Casz1v1 (C and D) or Casz1v2 (E and F). (B), (D), and (F) show camera lucida drawings of the GFP-expressing cells in (A), (C), and (E). R, rod; A, amacrine; B, bipolar; M, Müller. (G) Quantification of clonal composition expressed as fold change over control. (H) Clone size distribution. Bar graphs show mean ± SEM. See also Tables S1 and S2 for statistical details.

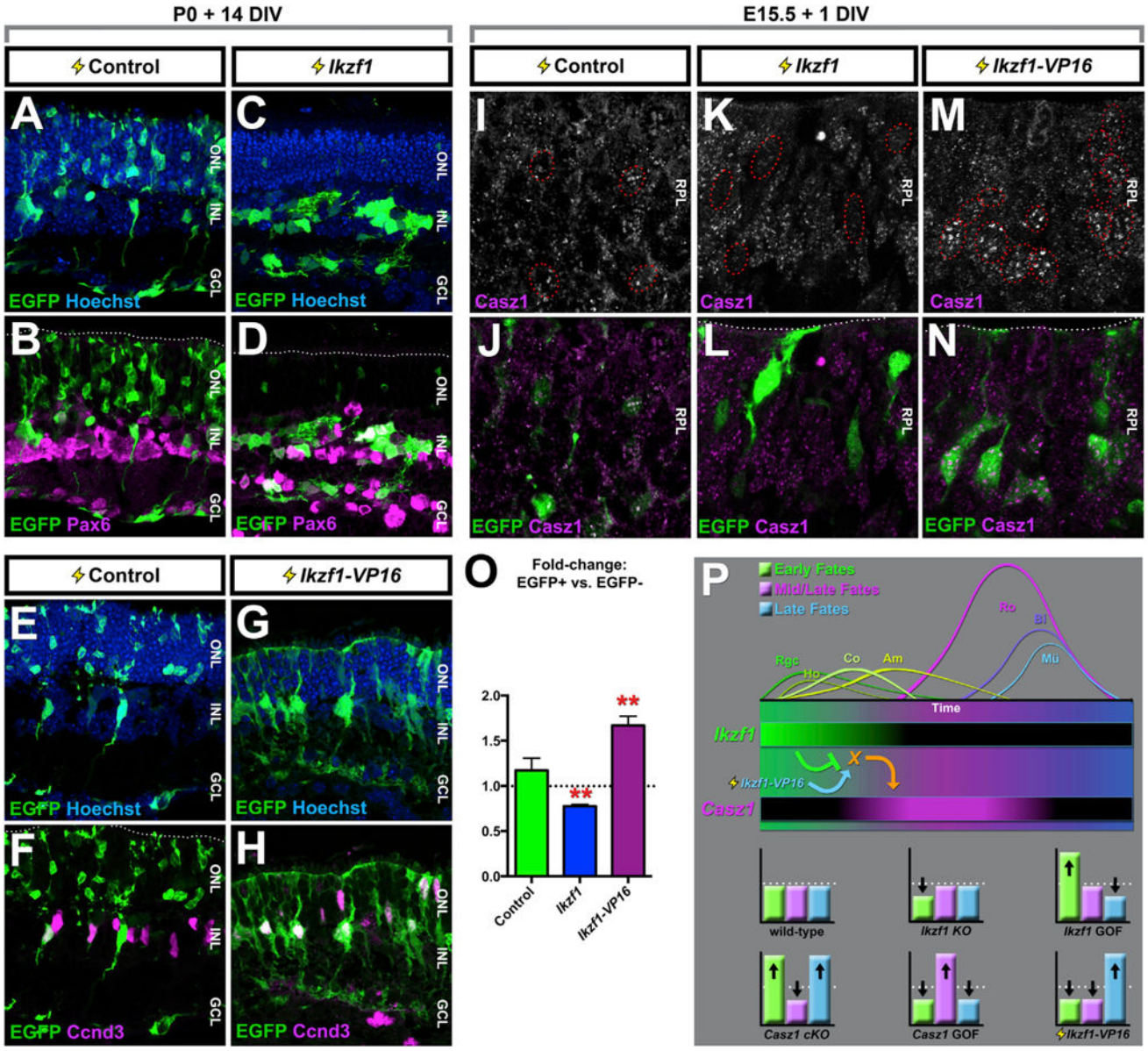

Figure 4. ***Ikzf1*** **Represses** ***Casz1*** **in RPCs**

(A–H) Retinal explants transfected 14 days earlier with constructs expressing EGFP alone (Control), Ikzf1-IRES2-EGFP (*Ikzf1*), or Ikzf1-VP16-IRES2-EGFP (*Ikzf1-VP16*), stained for the early-born cell-type marker Pax6 (B and D), or the late-born Müller cell marker Ccnd3 (F and H). (I–N) Casz1 staining 24 hr after transfection of E15.5 explants with constructs expressing EGFP alone (Control), *Ikzf1*, or *Ikzf1-VP16*. (O) Fluorescence intensity measurements of Casz1 signal in Control, *Ikzf1*-, or *Ikzf1-VP16*-expressing cells relative to adjacent untransfected cells. **p < 0.01. Graph shows mean ± SEM. (P) Model of temporal control of RPC competence modified from birthdating data by (Rapaport et al., 2004). RPCs are multipotent, but their probability to give rise to early-, mid-, or late-born cell types changes over time. *Ikzf1* and *Casz1* manipulations bias RPC production of early (green), mid/late (magenta), and late fates (blue). Ikzf1 is expressed in early RPCs, promotes

early-born fates (Elliott et al., 2008), and suppresses Casz1 indirectly (via factor X), while Casz1 is expressed in mid-/late-phase RPCs and increases the probability of generating of mid-/late-stage neurons. Ikzf1-VP16 acts oppositely to Ikzf1 and promotes late fates.