Meis homeobox genes control progenitor competence in the retina

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The vertebrate eye is derived from the neuroepithelium, surface ectoderm, and extracellular mesenchyme. The neuroepithelium forms an optic cup in which the spatial separation of three domains is established, namely, the region of multipotent retinal progenitor cells (RPCs), the ciliary margin zone (CMZ)—which possesses both a neurogenic and nonneurogenic potential—and the optic disk (OD), the interface between the optic stalk and the neuroretina. Here, we show by genetic ablation in the developing optic cup that *Meis1* and *Meis2* homeobox genes function redundantly to maintain the retinal progenitor pool while they simultaneously suppress the expression of genes characteristic of CMZ and OD fates. Furthermore, we demonstrate that Meis transcription factors bind regulatory regions of RPC-, CMZ-, and OD-specific genes, thus providing a mechanistic insight into the Meis-dependent gene regulatory network. Our work uncovers the essential role of *Meis1* and *Meis2* as regulators of cell fate competence, which organize spatial territories in the vertebrate eye.

retina | Meis | development

The retinal neuroepithelium is derived from the anterior region of the embryonic neural tube that coexpresses several eye field transcription factors (1, 2). The first morphological indication of eye development is the formation of the optic vesicle, which is derived from this eye-specified neuroepithelium. The optic vesicle subsequently invaginates in coordination with the lens placode to form a double-layered optic cup, while the inner layer of the neuroepithelium gives rise to the neural retina (3). The peripheral part of the optic cup, called the ciliary margin zone (CMZ), contains both neural retinal and nonneuronal ciliary epithelial progenitor cells. The latter cell population gives rise to the ciliary body and iris, while the former population of CMZ progenitors, being distinct from classic retinal progenitor cells (RPCs), contribute to mammalian retinogenesis (4). RPCs that reside in the inner layer of the optic cup are multipotent and generate six types of neurons, as well as the Müller glia in an evolutionarily conserved time order. RPCs are heterogeneous and change their competence in order to generate specific cell types at the appropriate developmental stages. The temporal identity of RPCs is primarily controlled by intrinsic factors (5–7). Relatively little is known regarding how the transition of RPC competence is controlled; however, previous studies have identified several transcription factors that are involved in this process (8–12). The centrally located part of the optic cup, the optic stalk, is initially formed as a hollow structure that maintains the connection of the optic cup to the diencephalon. Once the axons of retinal ganglion cells (RGCs) reach the optic stalk, they grow into it, forming the optic nerve. The interface between the optic stalk and the retina constitutes the optic disk (OD), which develops from the edges of the optic fissure. OD precursors represent a unique population of Bmp7-dependent $Pax2^+$ cells (13, 14). Progenitor cells located at the OD, the interface between the optic stalk and the neural retina, express multiple axon guidance molecules (15). One such molecule, Netrin-1 (Ntn1), acts via its receptor Dcc on the RGC axons to promote their growth out of the eye (16). Numerous genetic and molecular studies of retina development have focused on various aspects of RPC, CMZ, or OD biology. However, the molecular mechanisms underlying the regionalization of the developing optic cup into CMZ, RPC, and OD zones remain poorly understood.

Meis genes encode the TALE superclass of atypical homeodomain-containing transcription factors. Meis proteins can bind to DNA on their own, or in cooperation with a wide range of transcription factors. For example, Meis proteins heterodimerize with the PBC subclass of TALE proteins and the complex cooperatively associates with DNA along with additional tissue-specific transcription factors, such as Hox transcription factors. It has been proposed that the main function of the TALE complex is to poise regulatory regions for transcription, which is then triggered by the binding of HOX and possibly other transcription factors (17–20). Three Meis homologs—Meis1, Meis2, and Meis3—have been identified in mice (18, 21). Both *Meis1*- and *Meis2*-null mutations are embryonically lethal at midgestation due to hematopoetic and cardiovascular defects (22–24). Recently, *Meis1* and *Meis2* have been shown to act redundantly or functionally compensate for each other in the lens, as expected from the high sequence homology between them (25). During retinal development in mice, both *Meis1* and *Meis2* are expressed in the optic vesicle (26). Previous studies have shown that *Meis1* plays a critical role in early retinal development (18, 22, 27). A *Meis1*-null mutation results in microphthalmia, exhibiting lens vesicle reduction and partial duplication of the retina in mice (22, 27). Furthermore, *Meis1* controls proliferation,

Significance

The retina is derived from the neuroepithelium in the vertebrate. During retinal development, three spatially patterned domains are formed, namely the ciliary margin zone, the optic disc, and a region that contains retinal progenitor cells. Little is known about the mechanisms that regulate this patterning and specification. In this study, we show that retinal regionalization is impaired in the *Meis1* and *Meis2* compound-null retina in mice. By analyzing transcriptomic and epigenomic profiles, we map and characterize the Meis-dependent gene regulatory network within the embryonic retina. We propose that *Meis1* and *Meis2* function redundantly to promote expression of retinal progenitor cell-specific genes, while simultaneously restricting ciliary margin zone- and optic disc-specific genes.

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dorsoventral and ventrodistal patterning of the optic cup in a dose-dependent manner, and also regulates components of the Notch signaling pathway, as well as genes associated with human microphthalmia (26, 27). Meis1-null mutants die between embryonic day (E) 11.5 and E14.5 due to hematopoietic and vascular system defects (22), making it impossible to examine the role of *Meis1* at later stages of retinal development.

In this study, we examined the role of *Meis1* and *Meis2* homeobox genes during retinal development by conditional genetic ablation in the developing optic cup. Our results demonstrate that *Meis1* and *Meis2* are redundantly required for optic cup regionalization by promoting RPC fate, while simultaneously restricting the CMZ and OD ones. By combining chromatin immunoprecipitation-sequencing (ChIP-seq) and bulk RNA-sequencing (RNA-seq) experiments, we identified Meis-dependent target genes that are expressed during optic cup regionalization and early stages of retinal neurogenesis. Furthermore, we identified the transcription factor Lhx2 and members of the basic helix–loop–helix (bHLH) family as the key coregulators involved in the Meis-dependent gene regulatory network that underlie cell fate decisions in the embryonic retina.

Results

***Meis1/2* Deletion Results in Hypocellular Retina due to Prolonged Cell Cycle Length and Increased Cell Death.** To investigate the role of *Meis1* and *Meis2* during the retinal development, we first examined the spatiotemporal Meis1 and Meis2 protein expression. Meis1 was detected throughout the optic cup and maintained both in the neuroblastic layer and in the ganglion cell layer at later stages (*SI Appendix*, Fig. S1). At the optic cup stages, Meis2 was detected in the neuroepithelium. At later stages, the Meis2 protein was strongly expressed in the CMZ, OD, and a subset of cells in the outer neuroblastic layer and ganglion cell layer (*SI Appendix*, Fig. S1). In order to overcome the embryonic lethality of $Meis1^{-/-}$ and $Meis2^{-/-}$ embryos, we generated mice containing a *Meis1* floxed allele ($Meis1^{f/f}$) (*Materials and Methods*) and crossed with $Meis2^{f/f}$ (23) and *mRx-Cre* mice. Cre recombinase is active in the retina-committed progenitor cells from E9.0 onwards (28); however, residual Meis1 and Meis2 proteins were detected by E13.5, probably due to protein stability, which dictates target-inactivation time. These proteins were specifically deleted in the *mRx-Cre*;$Meis1^{f/f}$;$Meis2^{f/f}$ (Meis1/2 conditional knockout [cKO]) retina at E14.5 (*SI Appendix*, Fig. S1). *mRx-Cre* is active in the presumptive retinal pigment epithelium as well; however, Meis proteins did not appear to be efficiently deleted in the tissue and the retinal pigment epithelium was virtually normal (*SI Appendix*, Figs. S1 and S2). To investigate the consequences of *Meis1* and *Meis2* inactivation in RPCs, frontal sections of *Meis1/2* cKO embryos were stained with hematoxylin and eosin. The *Meis1/2* cKO retina was virtually normal at E11.5; however, the retina subsequently became hypocellular and exhibited coloboma (*SI Appendix*, Fig. S1). At postnatal day 1, *Meis1/2* cKO embryos developed a thin retinal layer and displayed dysplasia of the iris and ciliary body, accompanied with aberrant expression of ciliary body and iris-specific genes (*SI Appendix*, Figs. S1 and S2). The *mRx-Cre*;$Meis1^{f/f}$ (*Meis1* cKO) retina became progressively hypocellular at postnatal stages, while the *mRx-Cre*;$Meis2^{f/f}$ (*Meis2* cKO) retina was virtually normal (*SI Appendix*, Fig. S3).

Meis1 regulates proliferation in the retina, at least partially through the down-regulation of *Ccnd1*, which promotes progression through the G1 phase of the cell cycle (26, 27). Consistent with this, we found that Ccnd1 was down-regulated in the *Meis1/2* cKO retina (*SI Appendix*, Fig. S4). RPCs of the $Ccnd1^{-/-}$ retina progress through the cell cycle at a slower rate (29). To determine whether the cell cycle length is altered in the *Meis1/2* cKO retina, we utilized window-labeling based on two distinct thymidine analogs. We labeled S-phase RPCs by administering pregnant dams with sequential BrdU and EdU pulses separated by a 1.5-h interval. The lengths of the whole cell cycle (Tc) and S-phase (Ts) were calculated as previously described (29, 30). The length of the whole cell cycle in the *Meis1/2* cKO retina at E12.5 was 26.5 ± 4.5 h, which was significantly longer than that of the retina in the control (Tc = 17.0 ± 0.9 h), while the length of S-phase was 10.4 ± 1.7 h, which was comparable to that of the control (Ts = 9.4 ± 0.6 h). Similar results were obtained at E14.5 (*SI Appendix*, Fig. S4). At the same time, we observed significantly increased cell death, as assayed by the antibody against cleaved caspase 3 (*SI Appendix*, Fig. S4). Taking these data together, we conclude that the slower rate of proliferation and increased cell death contribute to the hypocellularity of the *Meis1/2* cKO retina.

***Meis1* and *Meis2* Are Required for Maintenance of the RPC Expression Program.** To study the role of *Meis1* and *Meis2* in RPC gene expression, we performed bulk RNA-seq of the wild-type and *Meis1/2* cKO embryonic neural retina. We crossed *R26-EYFP* mice with *mRx-Cre* or *mRx-Cre*;$Meis1^{f/f}$;$Meis2^{f/f}$ (Fig. 1*A*) and isolated $EYFP^+$ RPCs and their progeny from E14.5 retina by fluorescence-activated cell sorting. Principal component analysis of gene expression of *Meis1/2*-deficient and control retinae showed that individual samples were clustered according to genotype (Fig. 1*B*). Next, we examined differentially expressed genes between *Meis1/2* cKO and control retina and identified 722 down-regulated genes ($\log_2$FC [fold-change] < −0.55, q-value < 0.05) and 839 up-regulated genes ($\log_2$FC > 0.55, q-value < 0.05) (Fig. 1*C* and Dataset S1).

The inactivation of *Meis1* and *Meis2* resulted in hypocellularity due to the prolonged length of the cell cycle and increased apoptosis. Therefore, we investigated whether the expression of cell cycle regulators was altered in the *Meis1/2* cKO retina. Consistent with our immunohistochemistry data, we found that *Ccnd1* mRNA was significantly down-regulated in the *Meis1/2* cKO retina. The *Meis1/2* cKO retina displays lengthening of the cell cycle time, as observed in the $Ccnd1^{-/-}$ retina (29). However, in contrast to the *Meis1/2* cKO retina, the laminar structure is maintained and proliferating cells persist beyond the normal period of RPC proliferation in the $Ccnd1^{-/-}$ retina (29, 31). Furthermore, transfection with a *Ccnd1* construct only partially rescued the RPC proliferation defect caused by forced expression of a dominant-negative Meis1 construct in the chick retina (26). These studies indicate that *Meis1/2* likely regulate additional cell cycle regulatory genes. Indeed, we found that cell cycle regulators *Myb* and *Plag1* were significantly down-regulated in the *Meis1/2* cKO retina (Dataset S1). Taken together, these results indicate that *Meis1/2* maintain RPC pools by controlling cell cycle regulators.

Clark et al. (12) showed that neuroretinal progenitors go through three distinct stages: neuroepithelial state, in which Notch signaling is essentially absent and the rates of promotion are very low; early-stage primary retinal progenitors, in which Notch signaling is relatively low; and late-stage primary progenitors, where Notch signaling is very high. At E14.5, RPCs are primarily composed of early RPCs and neurogenic RPCs. Therefore, we examined the expression of "canonical" RPC genes, early RPC genes, neurogenic RPC genes, and Notch signaling components. The expression levels of some of the canonical RPC genes, such as *Pax6* and *Vsx2*, which are excluded from the OD, were down-regulated, while the expression of *Rax* and *Six3*, which are expressed in both RPCs and the OD (32), was apparently unchanged. Genes characteristic of early RPCs, such as *Fgf15*, and neurogenic RPCs, such as *Atoh7*, were also significantly down-regulated (Dataset S1). Furthermore, Notch signaling components, such as *Dll1*, *Dll3*, *Dll4*, and *Rbpj*, in addition to Notch targets *Hes* and *Hey*, were down-regulated in the *Meis1/2* cKO retina (Dataset S1). Our results indicate that transition of RPCs' developmental competence was impaired in the *Meis1/2* cKO retina.

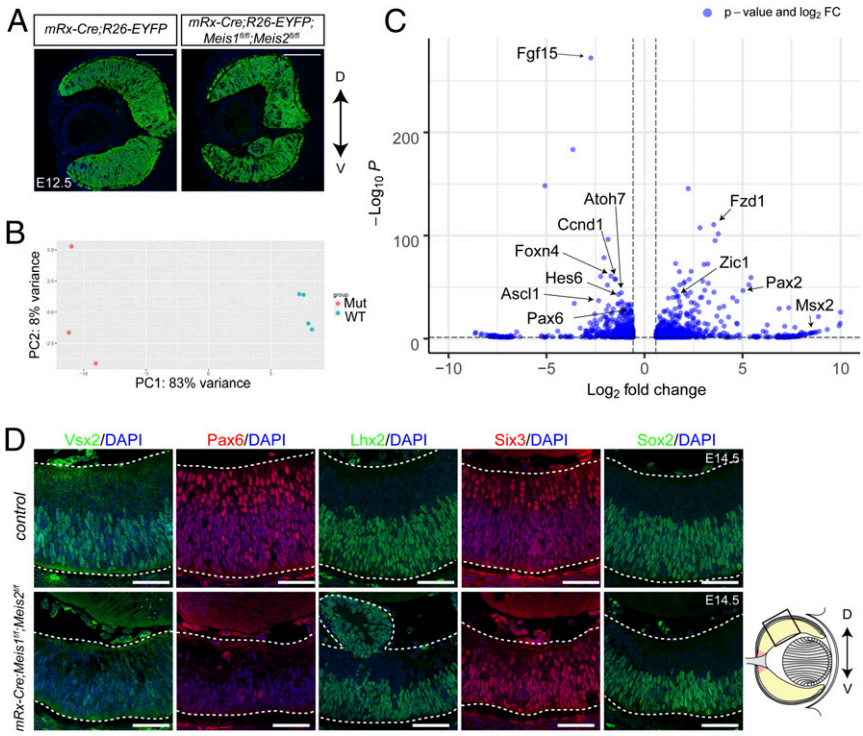

Fig. 1. The effects of *Meis1/2* deletion on the transcriptomes of the RPCs. (*A*) Frontal sections showing EYFP expression of *mRx-Cre;R26-EYFP* and *mRx-Cre;Meis1$^{f/f}$;Meis2$^{f/f}$;R26-EYFP* at E12.5. (Scale bars in *A*, 100 μm.) (*B*) Principal component (PC) analysis of bulk RNA-seq data. Four samples from control embryos and three samples of *Meis1/2* cKO embryos were used for RNA-seq. (*C*) Volcano plot of control versus *Meis1/2 cKO* retina at E14.5. Significantly misregulated genes (P_{adj} < 0.05, |log$_2$FC| > 0.58) are indicated in blue. Selected differentially expressed genes are labeled. (*D*) Immunostaining with Vsx2, Pax6, Lhx2, Six3, and Sox2 antibodies in frontal sections of control and *Meis1/2* cKO embryos at E14.5. Schematic representation of frontal sections of the eyes. The boxed area indicates the area where representative confocal microscopic images were taken. The dorsal-ventral axis is indicated by an arrow. D, dorsal; V, ventral. (Scale bars in *D*, 50 μm.)

To confirm our bulk RNA-seq data, we examined the expression of *Pax6*, *Vsx2*, *Lhx2*, *Six3*, and *Sox2* by immunohistochemistry. Consistent with our bulk RNA-seq data, Pax6 and Vsx2 protein levels were reduced, whereas other canonical RPC-specific transcription factors Six3, Sox2, and Lhx2 were maintained at an apparently normal level in the *Meis1/2* cKO central retina (Fig. 1*D*). Moreover, we found that RGC-, amacrine cell- (AC), and cone photoreceptor cell (CP)-specific genes, such as *Ptf1a*, were also down-regulated in the *Meis1/2* cKO retina (*SI Appendix*, Fig. S5). It is important to note that we did not observe down-regulated expression of *Pax6* and *Vsx2* in the *mRx-Cre;Meis1$^{f/f}$* (*Meis1* cKO) and *mRx-Cre;Meis2$^{f/f}$* (*Meis2* cKO) retinae (*SI Appendix*, Fig. S3), indicating that *Meis1* and *Meis2* function redundantly to control RPC identity. Together, these data show that *Meis1/2* initiates early stages of neurogenesis and developmental competence by promoting Notch signaling, neurogenic bHLH factors, and RPC gene expression.

Meis1/2 Directly Regulate the RPC-Specific Genes. To provide the mechanistic understanding of the genetic program governed by *Meis1* and *Meis2* during retinal neurogenesis, we performed ChIP-seq using chromatin prepared from the neural retina at E14.5. We made use of antibodies that recognize either Meis1 alone, or both Meis1 and Meis2. In accordance with previous reports (19, 27, 33), the majority of Meis binding sites identified in retina samples were located in intergenic and intronic regions (Fig. 2*A*). To study the chromatin status at Meis1/2 and Meis1 target loci, we analyzed the correlation of Meis1/2 ChIP-seq peaks with ATAC-seq peak (34). We found that the presence of Meis-bound regions was frequently associated with open chromatin (Fig. 2*B*).

As described above, the expression of multiple cell cycle regulators was down-regulated in the *Meis1/2* cKO retina. We, therefore, investigated whether Meis1/2 directly interacts with the regulatory regions of the corresponding genes. We found that Meis transcription factors bind to the regulatory regions of several cell proliferation-related genes, most notably *Ccnd1*, *Myb*, and *Plag1*, which were down-regulated in the *Meis1/2* cKO retina (*SI Appendix*, Fig. S6), indicating that *Meis1* and *Meis2* directly regulate genes that are involved in cell cycle progression.

Our bulk RNA-seq data demonstrated that *Meis1* and *Meis2* are required for the maintenance of the RPC-specific gene-expression program. Hence, we next investigated whether the regulatory regions of RPC-specific genes down-regulated in the *Meis1/2* cKO retina are bound by Meis1/2 in vivo. Indeed, we identified Meis1/2 interaction with several loci encoding RPC-specifying factors, early RPC, or neurogenic RPC markers (Fig. 2*C* and *SI Appendix*, Fig. S7). Interestingly, Meis1/2-bound regions were also found at the loci of late RPC-specific genes *Nfia* and *Nfib*, which are not expressed in the E14.5 retina (*SI Appendix*, Fig. S8). In addition, *Nfib* was 1.95-fold up-regulated in the *Meis1/2* cKO retina (Dataset S1). Thus, Meis1/2 might premark retinal-specific enhancers of late-expressing genes or they may repress late RPC-specific genes at early stages.

In order to provide further evidence that Meis1/2 directly regulate the expression of RPC-specific genes, we determined whether the Meis-bound regions are located within the previously identified enhancers of the corresponding genes. Meis1/2 occupancy regions correlated with a well-characterized set of RPC enhancers. For example, at the *Pax6* locus, we found that Meis transcription factors were bound to SIMO, HS234, and HS5 enhancers within the downstream regulatory region, as well as the

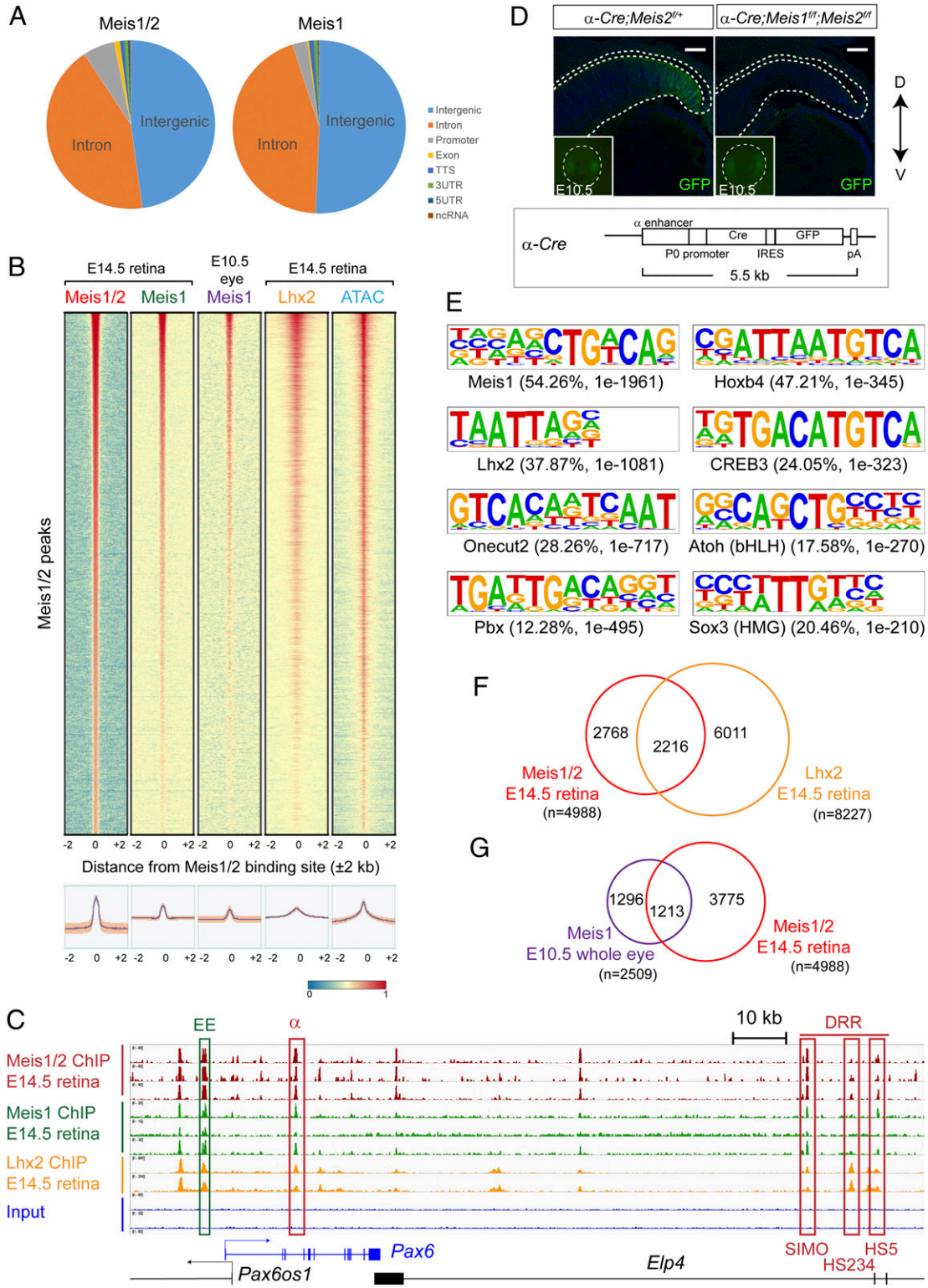

Fig. 2. *Meis1/2* directly regulate RPC-specific genes in concert with *Lhx2*. (*A*) Distribution of E14.5 retina Meis1/2 and Meis1 ChIP-seq peaks in the genome. (*B*) Density plots showing the distribution of Meis1 binding within the E10.5 whole eye [GSE62786 (27)], Meis1/2, Meis1 (E-MTAB-10112, present study), Lhx2 binding [GSE99818 (49)], and open chromatin region [GSE87064 (34)] in E14.5 neural retina at Meis1/2-bound regions (±2 kb from binding sites). The color scale shows the intensity of the distribution signal. The plotted lines were aligned according to E14.5 Meis1/2 ChIP-seq peaks. (*C*) ChIP-seq showing Meis1/2, Meis1, or Lhx2 binding in the E14.5 retina at *Pax6*. ChIP-seq peaks located within previously identified enhancers and promoters of *Pax6* (downstream regulatory region [DRR] and α) are indicated by red boxes. (*D*) Frontal sections of E14.5 *α-Cre;Meis2*$^{f/+}$ (control) and *α-Cre;Meis1*$^{f/f}$*;Meis2*$^{f/f}$ embryos showing GFP signal. *Insets* show GFP signals from the heads of E10.5 embryos. The areas around the eye are shown in the *Insets* (Microscopic field: 400 × 400 μm). Schematic representation of *α-Cre* transgene. (Scale bar: 50 μm.) (*E*) DNA-binding motifs found in the Meis1/2-bound region by Homer. Percentage of target sequences with the motif and *P* value are indicated. (*F*) Venn diagram showing overlapping binding regions shared between Meis1/2 (E-MTAB-10112, present study) and Lhx2 [GSE99818 (48)] in E14.5 retina. (*G*) Venn diagram showing overlapping binding regions shared between Meis1/2 in E14.5 retina (E-MTAB-10112, present study) and Meis1 in E10.5 whole eye (27).

α-enhancer, all of which have been shown to drive *PAX6* expression in the developing retina (Fig. 2*C*) (35–37). To assess whether the α-enhancer is responsive to *Meis1* and *Meis2*, we crossed *Meis1$^{f/f}$;Meis2$^{f/f}$* with *α-Cre* mice. In this Cre line, *cre* and *gfp* expression is under the control of the α-enhancer, which is active in the retina as early as E10.5 (38) (Fig. 2*D*). GFP signal was detected both in the *α-Cre;Meis2$^{f/+}$* and *α-Cre;Meis1$^{f/f}$;Meis2$^{f/f}$* retinae at E10.5. At E14.5, GFP^{+} cells were present in the peripheral retina of *α-Cre;Meis2$^{f/+}$* mice but not in the *α-Cre;Meis1$^{f/f}$;Meis2$^{f/f}$* retina (Fig. 2*D*), indicating that the α-enhancer is under the control of Meis1/2 transcription factors. In addition to *Pax6* enhancers, we found that Meis transcription factors were bound to the regions corresponding to the U9 chicken *SOX2* enhancer (39), *Six3* promoter (40), CR4 enhancer of *Foxn4* (41), and CNS enhancer of *Ascl1* (42), which are active in the embryonic retina, and Atoh7 remote shadow enhancer (43, 44), which controls Atoh7 expression in the retina (*SI Appendix*, Fig. S7). In addition to RPC-specific genes, Meis1/2 ChIP-seq peaks were found at the loci of genes characteristic of the RGC, AC, and CP (*SI Appendix*, Fig. S5). These observations strongly indicate that Meis1/2 directly regulate a defined set of transcription factor genes previously implicated in RPC development and retinal differentiation.

In order to examine if Meis transcription factors bind to the same loci in the neuroepithelium at E10.5 and in the RPC at E14.5, we analyzed whether E14.5 retina Meis1/2 ChIP-seq peaks were colocalized with E10.5 whole-eye Meis1 ChIP-seq peaks (27). Only 24% of E14.5 retina Meis1/2 ChIP-seq peaks colocalized with E10.5 whole-eye Meis1 ChIP-seq peaks (Fig. 2*G*), which was likely due to the distinct transcriptional profiles observed between the neuroepithelium and RPCs. Both E10.5 whole-eye Meis1 ChIP-seq and E14.5 retina Meis1/2 ChIP-seq peaks were observed at *Six3*, which is expressed in both the neuroepithelium and RPC. In contrast, E14.5 retina Meis1/2 ChIP-seq peaks were observed at neurogenic RPC genes, such as *Atoh7*, and late RPC-specific genes, such as *Nfia*, as well as genes that are required for retinal differentiation, such as *Prdm1* (*SI Appendix*, Figs. S5, S7, and S8).

***Meis1* and *Meis2* Regulate the RPC-Specific Genes in Cooperation with *Lhx2*.** Meis proteins can bind DNA directly or in cooperation with a diverse range of transcription factors, most notably with other homeodomain-containing proteins but also members of more distant transcription factor families (17, 18). For example, Hox transcription factors were shown to choose particular Meis-bound enhancers, which stabilize Meis binding to generate a functional output of enhancer activity (45). Partners of *Meis1/2* in the embryonic retina are currently unknown. To identify transcription factors that potentially cooperate with Meis1/2, we performed de novo motif analysis by Homer using the whole-genome set of retina-specific Meis1/2-bound sites. As expected, the most enriched motif was the Meis1 motif itself (54.3% of the Meis1/2 ChIP-seq peaks contained the Meis consensus; P = 1E-1961) (Fig. 2*E*). With the exception of Meis1, the DNA regions bound by Meis1/2 were enriched with motifs of the homeobox transcription factor Lhx2, TALE class homeobox transcription factor Pbx3, Onecut2 (Hnf6b), CREB3, bHLH transcription factor Atoh1, and SRY-related HMG Box transcription factor Sox3 (Fig. 2*E*). Sox1, Sox2, and Sox3 belong to the SoxB1 subgroup. One member of the group compensates for the loss of another and shares the same binding motif (46). bHLH transcription factors, such as Atoh7 and Ascl1, which are expressed in RPCs, bind to the same E-box motif as Atoh1 (the CAGCTG motif) (47, 48), suggesting that these transcription factors may cooperate with Meis1/2. Only half of Meis1/2-bound regulatory regions in the retina contained the Meis motif, which indicated that Meis1/2 must bind DNA through noncanonical motifs in cooperation with other transcription factors. Interestingly, when analyzed in silico, 37.5% of Meis1/2 ChIP-seq peaks contained the Lhx2 binding motif (P value = 1E-1081) (Fig. 2*E*). Lhx2 was previously shown to primarily bind to open chromatin regions of early RPC genes (49).

We hypothesized that *Meis1/2* cooperate with Lhx2 to regulate RPC-related genes. We therefore analyzed whether Meis1/2 ChIP-seq peaks were colocalized with Lhx2 ChIP-seq peaks (49). We found that Meis1/2 ChIP-seq peaks frequently colocalized with Lhx2 ChIP-seq peaks and that 44.6% of the Meis1/2 binding regions were also occupied by Lhx2 (Fig. 2*F*). We further tested the statistical significance for the spatial concordance of Meis1/2 ChIP-seq sites with Lhx2 motifs and Lhx2 ChIP-seq sites using a sliding window approach (*Materials and Methods*). We found significant enrichment of the Lhx2 motif at Meis1/2 ChIP-seq sites (Fisher's exact test, P < 2.2E-16, odds ratio = 1.89, 95% CI 1.79 to 1.98). Similarly, cooccurrence of Meis1/2 ChIP-seq peaks and Lhx2 ChIP-seq peaks was highly significant (Fisher's exact test, P < 2.2E-16, odds ratio = 146.1, 95% CI 137.9 to 153.4). Thus, the cooccupancy of Lhx2 and Meis1/2 at this frequency appeared unlikely to occur by chance. Meis1/2 and Lhx2 cooccupy the retinal specific enhancers of canonical RPC genes, such as *Pax6*, and neurogenic RPC genes, such as *Foxn4* and *Atoh7* (Fig. 2*C* and *SI Appendix*, Fig. S7). Lhx2 expression in RPCs is required for maintaining the optic cup identity and its morphogenesis and for RPC competence transition. The *Lhx2* inactivation causes the reduction of the RPC population and alters the RPC competence (50, 51), which in fact resembled the phenotype observed in the *Meis1/2* cKO retina. Taken together, our data indicate that *Meis1/2* functions in cooperation with *Lhx2* to regulate RPC-specific genes.

There were two regions that contained Meis1/2 and Lhx2 binding sites at −27 kb and −38 kb upstream of the transcription start site of *Vsx2* (Fig. 3*A*), designated *cis*-regulatory module 1 and 2 (CRM1 and CRM2). CRM2 was located within the 32-kb Vsx2 core regulatory circuit superenhancer (52). CRM1 and CRM2 were found to be evolutionarily conserved among chicken, mouse, and human and contained Meis1/2 and Lhx2 motifs (*SI Appendix*, Fig. S9). Furthermore, two open chromatin regions were present at the Vsx2 locus in the retina at E14.5 and were found to be associated with CRM1 and CRM2 (Fig. 3*A*). *Vsx2* was down-regulated in both Meis1/2 cKO and Lhx2 cKO retinae (Fig. 1) (51), suggesting that Meis1/2 and Lhx2 regulates the expression of *Vsx2* through these enhancers. In order to examine the functionality of the CRMs, we deleted these CRMs in mice using CRISPR/Cas9 (*Materials and Methods* and Fig. 3*B*). *Vsx2ΔCRM1*, *Vsx2ΔCRM2*, and *Vsx2ΔCRM1&2* embryos showed a microphthalmic phenotype as observed in the *Vsx2*-null mutant (53), although *Vsx2ΔCRM2* displayed a less severe phenotype (Fig. 3*B*). *Vsx2* was down-regulated in *Vsx2ΔCRM2*, while its expression was abolished in *Vsx2ΔCRM1* and *Vsx2ΔCRM1&2* retinae (Fig. 3*B*). Both Meis1/2 and Lhx2 were bound to CRM1 and CRM2 and *Vsx2* was down-regulated in the *Lhx2* cKO as well as the *Meis1/2* cKO retina; thus, *Meis* and *Lhx2* are likely to cooperatively regulate *Vsx2* expression through the CRMs.

***Meis* Deficiency Results in Expansion of the CMZ at the Expense of the Neural Retina.** By analyzing the bulk RNA-seq data from wild-type and *Meis1/2* cKO retinae, we found that CMZ-specific genes, such as *Otx1*, were up-regulated in *Meis1/2* cKO mice (*SI Appendix*, Fig. S10). To confirm bulk RNA-seq data, we analyzed the expression of CMZ markers *Cdo*, *Zic1*, *Msx1*, *Aqp1*, and *FoxP2* (4, 54) by immunohistochemistry. CMZ is composed of the proximal and distal parts, which are distinguished by the expression of *Msx1* and *Aqp1*, respectively, whereas *Cdo* marks the entire CMZ (4).

In control mice, *Cdo* and *Zic1* were restricted to the peripheral retina and *FoxP2* was present in the CMZ, as well as in the RGC subset. In the *Meis1/2* cKO retina, the expression of Cdo, Zic1, and FoxP2 proteins was expanded toward the central part of the

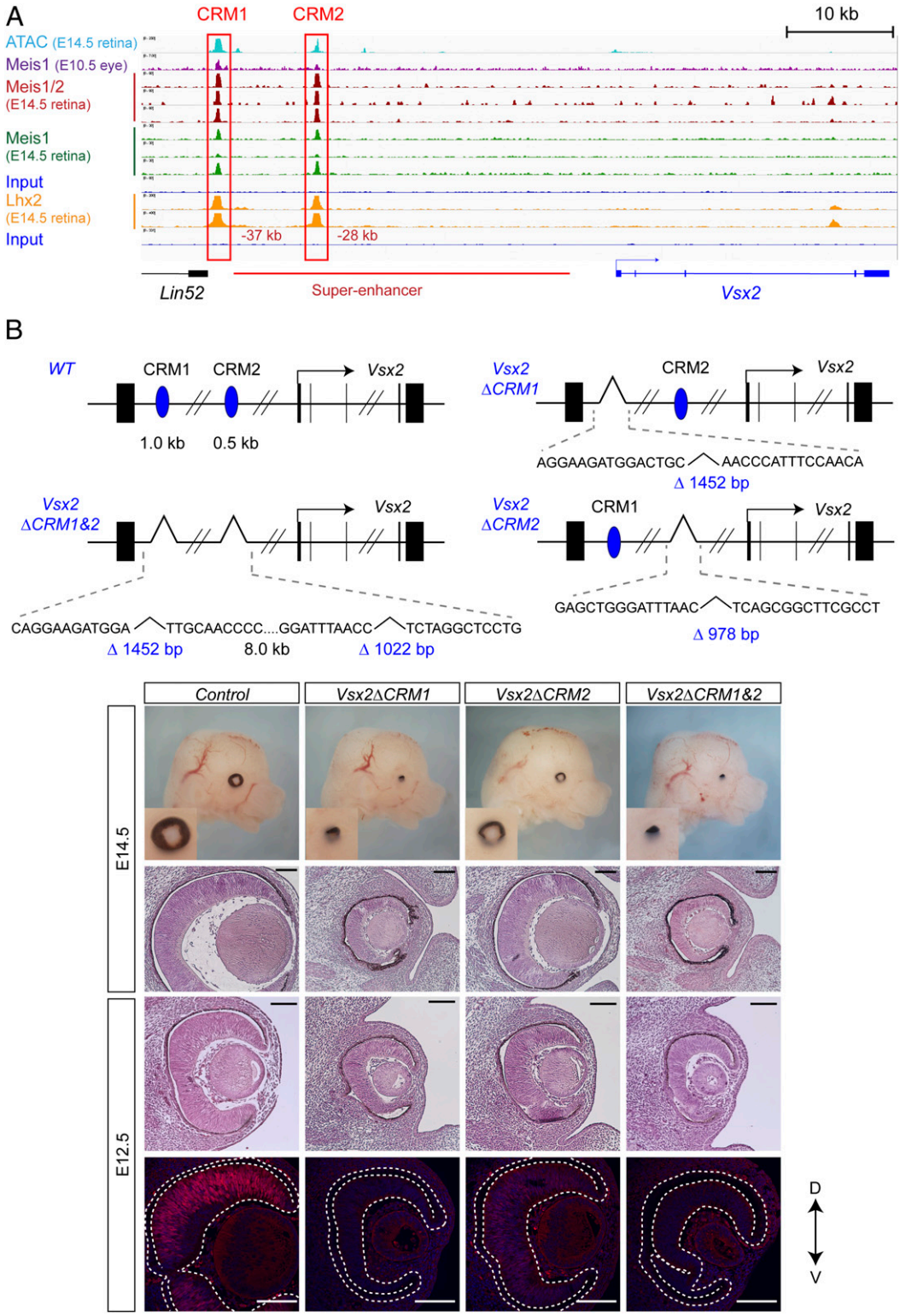

Fig. 3. Meis1/2 regulate *Vsx2* expression through the CRM1 and 2. (*A*) ChIP-seq showing Meis1/2, Meis1 (E-MTAB-10112, present study), Lhx2 binding [GSE99818 (49)], and ATAC-seq [GSE87064 (34)] in the E14.5 retina and Meis1 binding in the E10.5 whole eye [GSE62786 (27)] at *Vsx2*. Previously identified Vsx2 superenhancer (52) is indicated by a red line. (*B*) Schematic representation of *Vsx2ΔCRM1*, *Vsx2ΔCRM2*, and *Vsx2ΔCRM1&2*. CRM1 and CRM2 are depicted by blue ovals. The size of deletions and sequences across the deletion junctions are indicated in the panel. Haematoxylin and eosin-stained frontal sections, immunostaining with Vsx2 antibody in frontal sections, and whole head of control and *Vsx2ΔCRM1*, *Vsx2ΔCRM2*, and *Vsx2ΔCRM1&2* at indicated stages. (Scales bars, 100 μm.)

retina. We confirmed that FoxP2$^+$ cells in the expanded CMZ area were not colocalized with Brn3a, which excludes the possibility that FoxP2$^+$ cells in the CMZ were RGCs. In addition to Cdo, Zic1, and FoxP2, Msx1$^+$ cells were also located more centrally in the *Meis1/2* cKO retina compared to the control, whereas expression of Apq1 was apparently unchanged (Fig. 4 *A*–*H*′ and

SI Appendix, Fig. S11). These results indicate that the proximal CMZ is expanded while the distal CMZ is unaffected. Although the CMZ markers were centrally expanded in the *Meis1/2* cKO retina, they were not detected in the central retina at E14.5. Notably, the expression of Cdo, Msx1, and Aqp1 proteins was not changed in either *Meis1* cKO or *Meis2* cKO (*SI Appendix*, Fig. S11). In addition, hematoxylin and eosin staining demonstrated that the structure of the CMZ was not altered in the *Meis1*- and *Meis2*-deficient retina (*SI Appendix*, Fig. S11). These data indicate that *Meis1* and *Meis2* function redundantly to restrict the CMZ area.

Meis Proteins Restrict CMZ by Modulating Wnt/β-Catenin and Bmp Signaling in the Retinal Periphery. The precise regulation of Wnt/β-catenin and Bmp signaling activity is essential for determining the boundary between the neural retina and CMZ (55, 56). We analyzed the expression of Wnt/β-catenin and Bmp signaling components. Multiple Bmp ligands, such as *Bmp2*, its receptor *Bmpr1b*, Wnt ligands including *Wnt7b*, and Wnt receptors, such as *Fzd1*, were up-regulated in the *Meis1/2* cKO retina (*SI Appendix*, Fig. S10). In order to investigate whether the up-regulation of Bmp ligands causes the activation of Bmp signaling in the *Meis1/2* cKO retina, we performed immunohistochemistry with antiphosphorylated Smad1/5. Phosphorylated Smad1/5 was restricted to the peripheral region in the control retina, while it was detected more centrally in the *Meis1/2* cKO retina (Fig. 4 *I–J′*), correlating with the observed expansion of CMZ marker genes. To examine the activity of Wnt/β-catenin signaling, we analyzed the expression of *Lef1*, which is directly regulated by this signaling in the CMZ (55). We observed that Lef1 expression was expanded centrally in the *Meis1/2* cKO (Fig. 4 *K–L′*), correlating with the observed expansion of CMZ marker genes and Bmp signaling activity.

We next investigated if Meis transcription factors could directly regulate genes encoding some of the components of Wnt/β-catenin and Bmp signaling pathways. Indeed, by analyzing the ChIP-seq data, we identified Meis occupancy within the putative regulatory regions of *Wnt7b* and *Bmp4* (*SI Appendix*, Fig. S12). In addition, we also found that Meis transcription factors were bound to the loci of CMZ-specific genes, such as *Cdo* and *FoxP2* (*SI Appendix*, Fig. S12). Since *Six3/6* and *Sox2* are known to suppress Wnt/β-catenin signaling in the retina in order to maintain the pool of RPCs (57–59), we analyzed the expression of *Sox2* and *Six3* in the *Meis1/2* cKO retina. We found that Sox2 and Six3 were maintained at normal levels in the central retina; however, they were down-regulated in the peripheral retina of *Meis1/2* cKO embryos (Fig. 4 *M–P′*), suggesting that *Meis1/2* may be required for *Sox2* and *Six3* expression. It has been shown that Meis1 interacts with Sox2 (60). Furthermore, the homeodomain core motif, which can be bound with Six3 (61) and Sox motifs, are enriched in the Meis1/2 ChIP-seq peaks (Fig. 2*E*). We hypothesized that *Six3* and *Sox2* suppress CMZ-specific genes and Wnt/β-catenin signaling regulators in concert with *Meis1/2*. We therefore analyzed whether the Meis1/2-bound regions of these genes contained a Sox2 and Six3 consensus. We confirmed that these motifs are present within Meis1/2-bound enhancers of CMZ-specific genes and Wnt/β-catenin signaling regulators (*SI Appendix*, Fig. S12), suggesting that *Meis1/2* cooperatively restrict the CMZ fate by inhibiting Wnt/β-catenin signaling components in concert with *Sox2* and *Six3*.

Taken together, our data suggest that *Meis1* and *Meis2* in the retina periphery regulate the activities of Bmp and Wnt/β-catenin signaling pathways in order to maintain the population of RPCs.

***Meis* Deficiency Results in Expansion of the OD at the Expense of the Neural Retina.** Surprisingly, by searching the RNA-seq data, we found that OD marker *Pax2* was up-regulated 32-fold in the E14.5 *Meis1/2* cKO retina compared to the control. We first validated the mRNA data by performing immunohistochemistry for the Pax2 protein. We found that in the *Meis1/2* cKO retina,

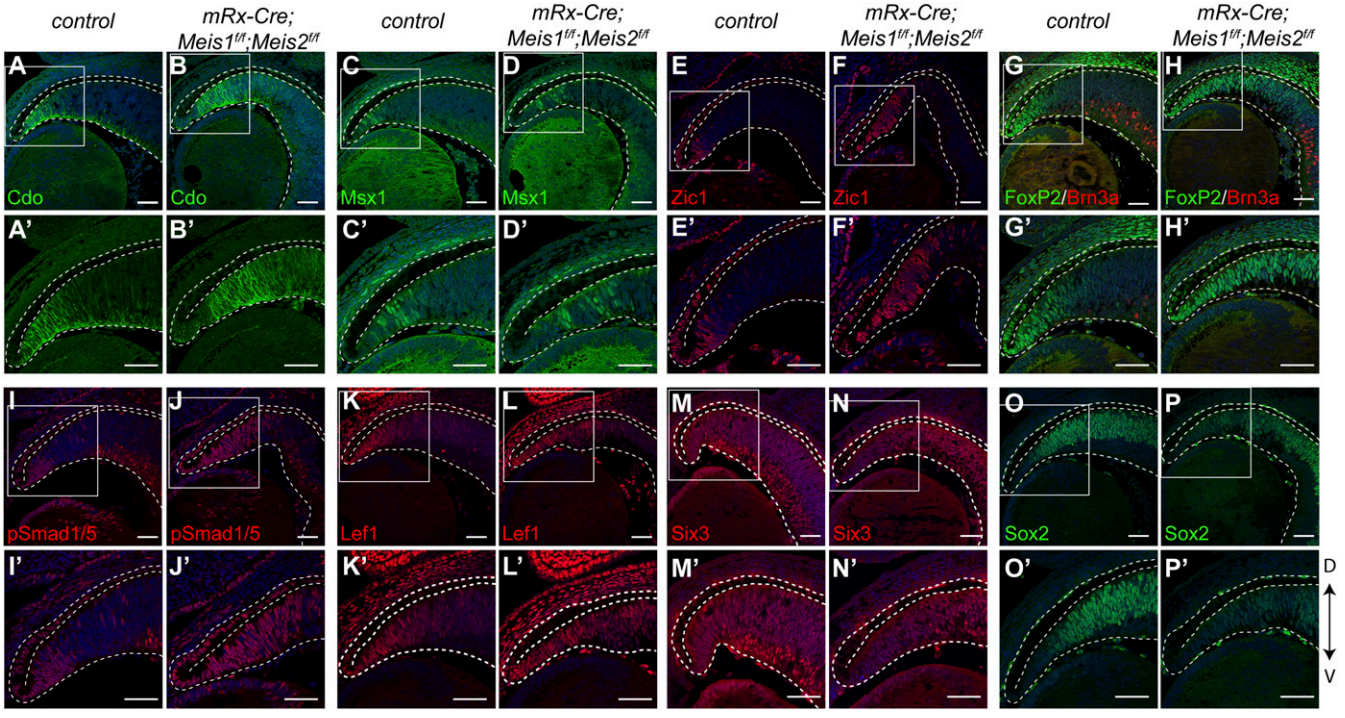

Fig. 4. *Meis1/2* deletion results in expansion of the CMZ. (*A–P′*) Immunostaining with Cdo, Msx1, Zic1, FoxP2, Brn3a, phosphorylated Smad1/5, Lef1, Six3, and Sox2 antibodies in frontal sections of control and *Meis1/2* cKO embryos at E14.5. Magnified views indicated by the white boxed areas of top images are shown below. The dorsal-ventral axis is indicated by an arrow. (Scale bars, 50 μm.)

the Pax2 protein was ectopically expressed (Fig. 5 *A*–*C*), most likely at the expense of typical RPC markers *Vsx2*. The expression of *Pax6* is normally excluded from the OD, and the optic stalk/optic cup boundary is established by reciprocal transcriptional repression of *Pax2* and *Pax6* (62). To determine whether *Pax2* up-regulation in the *Meis1/2* cKO retina is mediated by *Pax6* down-regulation, we crossed *mRx-Cre* with *Pax6*$^{f/f}$ mice to inactivate *Pax6* in RPCs and analyzed the expression of *Pax2*. At E13.5, the Pax2 protein was ectopically expressed in the ventral *mRx-Cre;Pax6*$^{f/f}$ retina (Fig. 5 *D* and *E*), which indicates that *Meis1/2* may restrict the expression of *Pax2* to the OD, at least in some regions, through the action of *Pax6*. Since *Pax2* is required for OD formation, we next analyzed the expression of additional genes that are characteristic of the OD fate. Interestingly, we found that several OD-specific genes were up-regulated in the *Meis1/2* cKO retina (Fig. 5*F*) (32, 63–68). Furthermore, we found that Meis1/2 directly bound to the putative regulatory regions of the up-regulated OD-specific genes, such as *Ntn1* and *Epha4* (Fig. 5*G*). From these results, we conclude that *Meis1* and *Meis2* are required to suppress the OD fate in the developing retina.

Discussion

Here we have proposed, based on the genetic manipulation of mice, that *Meis1* and *Meis2* function redundantly to promote the expression of RPC-specific genes, while they simultaneously restrict CMZ- and OD-specific genes (Fig. 6). The molecular mechanism underlying this phenomenon likely involves direct DNA binding of Meis1/2, as well as temporally dependent and cell-type–specific cooperation with other transcription factors. To this end, we demonstrated that Meis transcription factors bind the regulatory regions of RPC-, CMZ-, and OD-specific genes and act in cooperation with other transcription factors, such as Lhx2, bHLH, Six3, and Sox2, depending on the context. Meis1/2 are expressed throughout the retina and Meis1/2-bound regulatory regions contain motifs of multiple classes of transcription factors. Thus, Meis1/2 likely cooperate with additional RPC-, CMZ-, and OD-specific transcription factors to control gene expression. Indeed, previous studies have indicated that the outputs of Meis1/2-bound regulatory regions are tightly controlled by other tissue-specific transcription factors, such as Hox (19, 45, 69). These tissue-specific transcription factors determine the functional outputs of the enhancers and promoters of Meis target genes. For example, during branchial arch development, Meis1 binds to the regulatory regions that are common to all branchial arches; however, only a subset of them is activated in a tissue-specific manner. In the secondary branchial arch, Hoxa1 is recruited to Meis1-premarked enhancers that contain Hox motifs, which facilitates Meis binding to Hoxa1 target genes. The increased Meis1 binding subsequently leads to gene activation (19). Although Hox genes are not expressed in the retina, it is reasonable to assume that one of the modes of action by which transcription factors cooperate with *Meis1/2* in the retinal tissue may also involve increased DNA binding. This view is supported by the fact that only about one half of the retina-specific Meis1/2 ChIP-seq peaks contained the Meis consensus logo, indicating that the other half of ChIP-seq peaks represent indirect Meis1/2 DNA binding, or contain a relaxed consensus predisposed to regulatory mechanisms, such as increased DNA-binding affinity mediated by cooperating factors.

RPCs are heterogeneous and change their developmental competence over time in order to generate different types of neurons and Müller glia (5). Primary immature RPCs harbor competence to give rise to all types of retinal cells. Early RPCs mostly generate early-born cell-type RGCs, ACs, horizontal cells, and CPs, while late-born cell types, bipolar cells and Müller glia, originate from late RPCs. The temporal identity of RPCs is primarily controlled by intrinsic signals, including transcription factors. However, intrinsic factors that govern the RPC competence transition to the neurogenic RPC are poorly understood (5, 7). In this study, we suggest that *Meis1/2* transcriptional factors play a pivotal role in this process. The neurogenic RPC-specific genes are down-regulated in *Meis1/2* cKO, suggesting that RPC competence is impaired in the *Meis1/2* cKO retina. Our data also indicate that Meis1/2 regulate RPC-specific genes directly through the set of previously well-characterized retina-specific enhancers. Global analysis of transcription factor occupancy provided strong evidence for the cooperation of Meis1/2 with the homeodomain-containing transcription factor, Lhx2, to control RPC-specific genes. *Lhx2* allows the establishment of open chromatin regions in the vicinity (49), indicating that *Lhx2* controls chromatin accessibility at Meis1/Lhx2 binding sites.

Meis1/2-bound regulatory regions are also highly enriched with the motif of bHLH transcription factors, which are known to control the competence of RPCs. Thus, our observations indicate that *Meis1/2* cooperate with the bHLH transcription factors and act as intrinsic factors to control the RPCs' competence by directly regulating the expression of transcription factors that influence the temporal identity of RPCs. Among RPC-specific regulators, Sox2 has been shown to interact with Meis transcription factors (60). Meis1/2-bound enhancers of CMZ-specific genes, such as Cdo and FoxP2, contain Sox2 and Six3 binding motifs. TALE factors are known to access the regulatory regions to poise them for later activation. For example, the TALE complex occupies the *hoxb1a* promoter already during early blastula stages when the expression of hoxb1a is inhibited. At the gastrula stage, Hoxb1b is recruited to the TALE complex at the *hoxb1a* promoter, which triggers the expression of *hoxb1a* (20). We have shown that Meis1/2 bind to late RPC-specific genes, such as *Nfia* and *Nfib*, in the retina at E14.5, when these genes are not yet expressed in the RPCs. Thus, *Meis1/2* probably premarks or supresses the regulatory regions of late RPC-specific genes in the RPCs to ensure the expression of these genes at the appropriate stage of retinal development. These observations have lead us to propose that RPC-specific transcription factors—including Lhx2, bHLH, Sox2, Six3, and Nfia/Nfib—determine the output of Meis1/2-premarked enhancers within the retina, paralleling a mechanism that has been previously observed in other biological contexts. Further studies are nevertheless necessary for the identification of additional transcription factors that determine the functional outputs of Meis1/2-bound regulatory regions.

Meis1/2 and *Pax6* are coexpressed in the vertebrate retina during embryogenesis. In *Drosophila*, the *Pax6* homolog *ey*, *Meis* homolog *hth*, and *Tzhz* homolog *tsh* function as a complex in eye development (70). In vertebrates, Meis2 directly interacts with Pax6 and Dlx2 to functionally cooperate during subventricular zone neurogenesis (71). The Pax6/Meis interaction may represent an evolutionarily conserved mechanism.

Marcos et al. (27) have shown that *Meis1* regulates components of the Notch signaling pathway and genes associated with human microphthalmia in the lens and neuroepithelium by ChIP-seq and bulk RNA-seq with E10.5 whole eye. In this study, we investigated the Meis gene regulatory network (GRN) in RPCs by ChIP-seq and bulk RNA-seq with E14.5 neural retina. At E10.5 (i.e., prior to the onset of neurogenesis), the presumptive retina is primarily composed of neuroepithelium, while the neural retina at E14.5 is mainly formed of RPCs and the genesis of early retinal cell types has been initiated (5). Single-cell RNA-seq analysis of retinal development shows a clear transcriptional distinction between the neuroepithelium and RPCs (12). Thus, Meis-dependent GRN is expected to reflect transcriptional changes. Indeed, 51% of E10.5 whole-eye Meis1 ChIP-seq peaks and 76% of E14.5 retina Meis1/2 ChIP-seq peaks were unique at each stage. Meis proteins bind to the genes expressed in both the neuroepithelium and RPCs at E10.5 as well as E14.5. However, only E14.5 retinal samples allowed the identification of Meis1/2 ChIP-seq peaks at neurogenic RPC genes, late RPC-specific genes, or genes required for retinal differentiation. Analysis of our

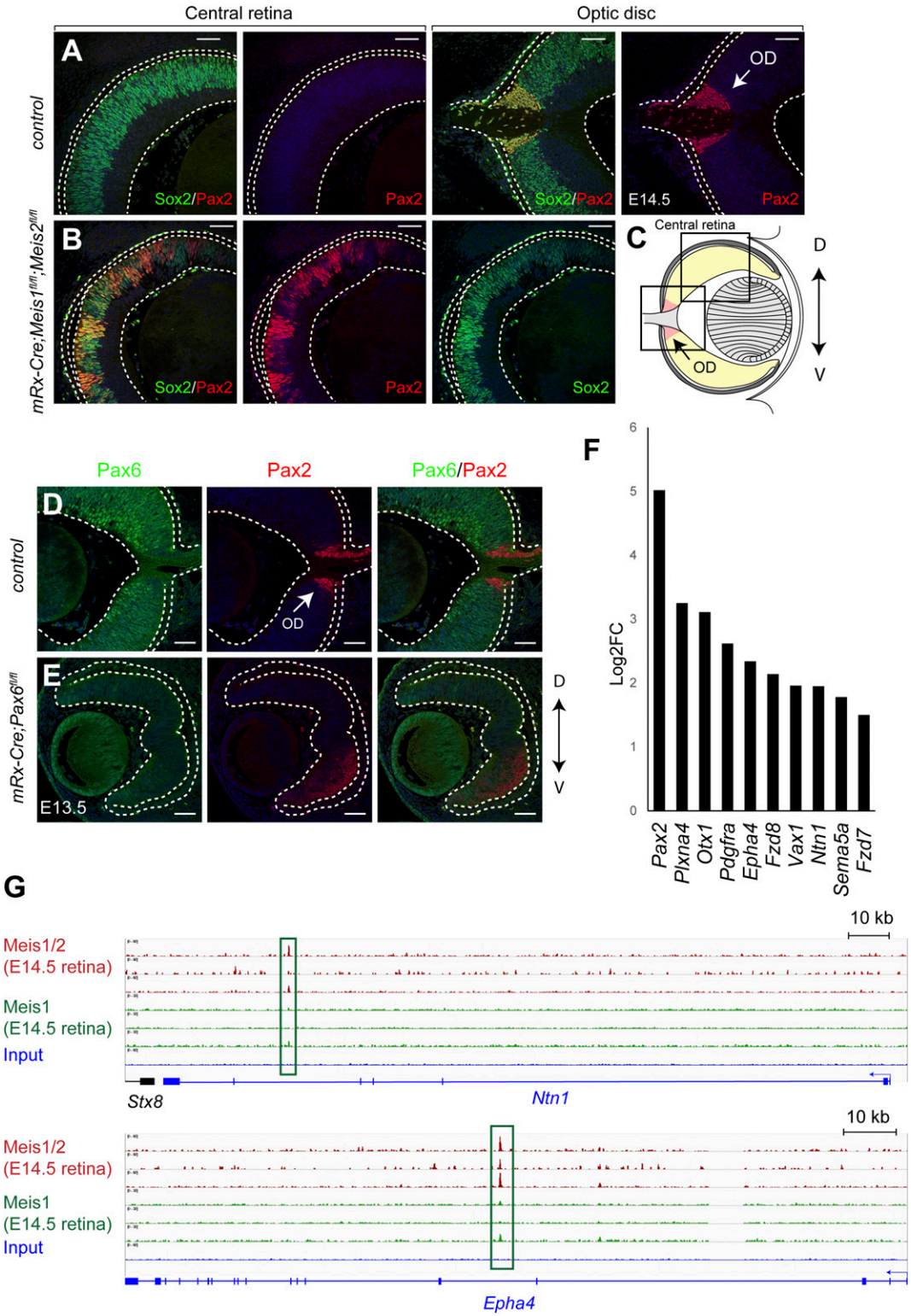

Fig. 5. *Meis1/2* deletion results in expansion of the optic disk. (*A* and *B*) Immunostaining with Pax2 and Sox2 antibodies in frontal sections of control and *Meis1/2* cKO embryos at E14.5. (*C*) Schematic representation of a frontal section of the eye. Boxed areas indicate the central retina and the OD that are shown in *A* and *B*. (*D* and *E*) Immunostaining with Pax6 and Pax2 antibodies in frontal sections of control and *Pax6* cKO embryos at E13.5. (*F*) OD-specific genes that are up-regulated in the *Meis1/2* cKO ($log_2FC > 0.58$, q-value < 0.05). (*G*) ChIP-seq showing Meis1/2 or Meis1 binding the loci of *Ntn1* and *Epha4*. Arrows indicate the OD. The dorsal-ventral axis is indicated by an arrow. (Scale bars, 50 μm.)

current and previous data allowed us to reveal a more comprehensive Meis-dependent GRN in retinal development during embryogenesis.

Our data strongly suggest that *Meis1/2* function in cooperation with *Lhx2* to regulate RPC-specific genes. Lhx2 binding motifs are highly enriched within Meis1/2 ChIP-seq peaks and about

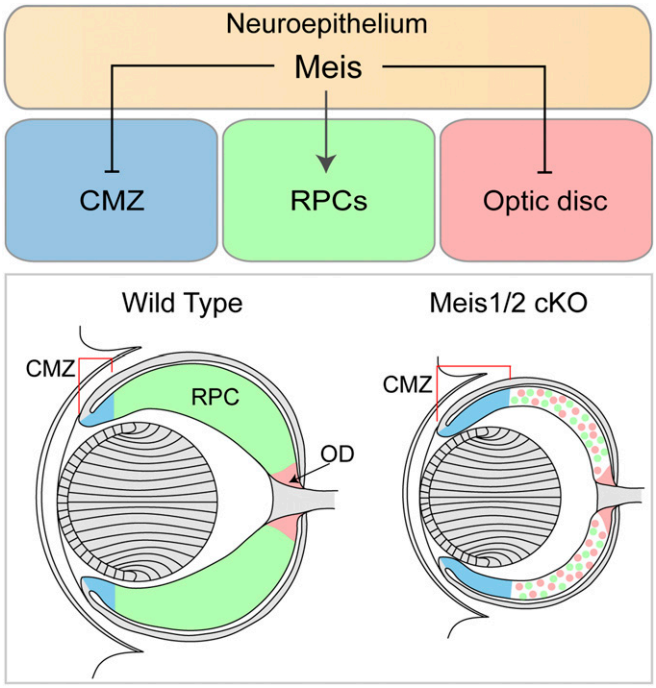

Fig. 6. Schematic representation of the retinal phenotype in the *Meis1/2*-deficient retina. *Meis1* and *Meis2* redundantly promote RPC-specific genes and simultaneously restrict CMZ and OD fate. Inactivation of *Meis1* and *Meis2* in the retina results in expansion of CMZ and OD at the expense of RPC.

half of Meis1/2 in vivo binding sites are in close vicinity of experimentally identified Lhx2 binding sites. As observed in *Meis1/2* cKO, *Lhx2* cKO embryos display an RPC proliferation defect and a substantially reduced expression of RPC-specific genes, such as *Vsx2* and *Foxn4*, and a range of genes selectively expressed in the differentiating retinal cells (49, 51). Both Meis1/2 and Lhx2 bind the *Vsx2* enhancers CRM1 and CRM2, which are required for the expression of *Vsx2* (Fig. 3). Vsx2 is down-regulated in the *Meis1/2* cKO retina even though Lhx2 is present (Fig. 1), suggesting that Lhx2-mediated expression of *Vsx2* requires Meis1/2. *Lhx2* cKO embryos do not exhibit the OD/CMZ expansion phenotype but, rather, the up-regulation of a set of genes that are normally expressed in the thalamic eminence and anterodorsal hypothalamus (51). *Chx10-Cre;Lhx2*$^{f/f}$ mice show an early arrest in lens fiber development due to the down-regulation of Fgfs in the retina (72). In contrast, the lens is smaller but no defects in lens fiber differentiation are evident in *Meis1/2* cKO mice (Fig. 1 and *SI Appendix*). *Fgf3*, *Fgf9*, and *Fgf15* are down-regulated in the *Chx10-Cre;Lhx2*$^{f/f}$ retina (72). *Fgf3/15* were down-regulated, however, *Fgf9* expression was unchanged in the *Meis1/2*-deficient retina (Dataset S1). In addition, *Fgf8* was up-regulated in the *Meis1/2* cKO retina (Dataset S1). Presumably, down-regulation of *Fgf3/15* is compensated by *Fgf8/9*, which maintains development of the lens.

mRx-Cre;Meis1$^{f/f}$ displayed a distinct retinal phenotype that was observed in *Meis1*-deficient mice, probably due to delayed depletion of Meis1 protein. In addition, a truncated Meis1 protein that has been predicted to act as a dominant-negative form is produced in *Meis1*$^{-/-}$ mice (22). In another *Meis1*-deficient mouse model, *Meis1a*^{ER}, a Meis1–ERT2 fusion protein, is present in the cytoplasm (24). The phenotypic discrepancies in eye development among *Meis1*-deficient mice indicates the potential effects of Meis1–ERT2 or truncated Meis1 protein in vivo (22, 24, 27). No truncated Meis1 protein was detected in *mRx-Cre;Meis1*$^{f/f}$*;Meis1*$^{f/f}$, suggesting that *Meis1*$^{f/f}$ would represent a true loss-of-function allele, albeit with a delayed time course.

By E11.5, dorsal-ventral and distal-proximal patterning of the optic cup is disrupted in *Meis1*-deficient mice *Meis1a*^{ER}, which is accompanied with abnormal expression of *Pax6*, *Pax2*, *Otx1*, *Tbx5*, and *Vax2* (27). Furthermore, proliferation is reduced and the area in which Tuj1, Isl1/2, and Otx2 are expressed is less than that found in the control, suggesting that Meis1 is required for retinal differentiation and proliferation (27). *Meis1*-deficient mice display phenotypes both in the lens and in the retina (22, 27), which adds a complication to the study of the possible function in the retina using these embryos. For example, the signals from the lens has been shown to influence retinal development by controlling CMZ identity and the initial direction of RGC axon outgrowth (73, 74). In order to overcome early embryonic lethality and provide further insight into retinal differentiation, as well as CMZ development, the conditional deletion of *Meis* genes in the retina is required. Meis1 and Meis2 proteins were depleted at E14.5 in Meis1/2 cKO mutants, thus the optic cup patterning would be presumably maintained by the presence of these proteins at earlier stages. It is complicated to study dorsal-ventral and distal-proximal patterning at later stages due to the OD/CMZ expansion phenotype in the Meis1/2 cKO. In addition, our bulk RNA-seq data did not provide clear evidence of the patterning defect. We did not observe the OD/CMZ expansion phenotype in *mRx-Cre;Meis1*$^{f/f}$ and *mRx-Cre;Meis2*$^{f/f}$. These data combined show that *Meis1* controls optic cup patterning at early stages of retinal development, and at later stages *Meis1* and *Meis2* jointly control the regionalization of CMZ/OD/RPC in the retina.

CMZ/OD expansion in the *Meis1/2* cKO may reflect a direct role of *Meis1/2* in suppression of OD/CMZ fate, or the phenotype might arise indirectly from the depletion of RPCs in the proliferative zone. We are in favor of the first model. Multiple transcription factors, such as *Lhx2*, *Vsx2*, *Hes1*, *Sox2*, and *Pax6* are required for RPC maintenance but inactivation of these genes does not result in OD/CMZ expansion (38, 51, 75, 76). A *Vsx2*-null mutation results in transdifferentiation of the neural retina into pigmented cells (75, 76). *Pax6* inactivation in RPCs leads to exclusive generation of ACs (38). The optic cup/stalk boundary is disrupted in the *Hes1* cKO (77). Only CMZ is expanded at the expense of the RPC in the *Sox2* cKO and *Six3/6* cKO embryos (57, 59). Therefore, simple depletion of RPCs probably does not correlate with CMZ/OD expansion. Our Meis ChIP-seq was performed using E14.5 neural retina, which is primarily composed of RPCs. CMZ and OD represent a small proportion of the neural retinal cell population. Therefore, Meis1/2 ChIP-seq peaks found at CMZ- and OD-specific genes probably represent Meis1/2 binding sites in RPCs. The binding of Meis does not induce transcription of its target genes. Meis proteins act as a hub, which assists combinatorial assembly of other transcription factors, which define transcriptional output of selected enhancers (45). *Meis1/2* appear to cooperate with other retina-specific transcription factors in order to maintain the RPC pool and simultaneously suppress CMZ/OD fate in RPCs.

The molecular mechanisms underlying regionalization of the developing optic cup into CMZ, RPC, and OD zones remain poorly understood. Nevertheless, the involvement of signaling pathways has been well documented. OD development is controlled by Bmp, Shh, and Fzd signaling. In *Bmp7*$^{-/-}$ and *Smad4*$^{-/-}$ retinae, the OD-specific factors *Pax2* and *Ntn1* are lost (13, 78, 79). In addition, the Bmp receptor, *Bmp1b*, is required for RGC axon guidance to the OD (79). *Shh* is absent in the OD, however it is expressed in RGCs. Shh derived from these cells modulates the size of the Pax2^{+} cell population in the OD (67). We found that *Bmp7*, its receptor *Bmpr1b*, *Fzd8*, and *Fzd5* were up-regulated in the *Meis1/2* cKO retina, suggesting that *Meis1/2* maintain the boundary between the OD and neural retina, at

least in part, by controlling the expression of these signaling components. CMZ specification requires Wnt/β-catenin and BMP signaling activities (55, 56). Wnt/β-catenin signaling is antagonized by *Sox2* and *Six3*/*Six6* to suppress CMZ fate. *Sox2* and *Six3*/*Six6* inactivation results in the expansion of CMZ at the expense of the neural retina, accompanied with ectopic activation of Wnt/β-catenin signaling (57–59). We have shown that *Sox2* and *Six3* are down-regulated and Wnt/β-catenin signaling is aberrantly activated in the peripheral retina of the *Meis1/2* cKO retina. Our ChIP-seq analysis revealed that Meis1/2 is bound to the *Six3* promoter and *Sox2* enhancers, which were described to drive *Six3* and *Sox2* expression in the retina. In addition, several Wnt/β-catenin signaling components are expressed in the developing retina and especially *Tcf7*, *Fzd1*, and *Fzd7* are enriched in the CMZ. Furthermore, *Fzd1* and *Tcf7* are also significantly up-regulated in the *Six3*/*Six6*- and *Sox2*-deficient retina, respectively (57, 58). In combination, the available data strongly suggest that *Meis1/2* regulate *Six3* and *Sox2* expression and suppresses CMZ fate in cooperation with *Six3/6* and *Sox2*.

Single-cell RNA-seq analysis of the developing mouse retina has revealed that CMZ-enriched genes, such as *Msx1*, are also expressed in the early neuroepithelium (12). This result raises two major possible explanations regarding why CMZ-specific genes are up-regulated in the *Meis1/2* cKO retina. First, the failure of RPC competence transition from the optic cup or optic vesicle neuroepithelium is responsible for the elevated expression of CMZ-like markers. Second, the up-regulation of CMZ markers is due to the expansion of the CMZ territory. At present, we prefer the latter possibility. In our conditional mouse model, Meis1 and Meis2 proteins are depleted only by E14.5, when the transition from the neuroepithelium should already have been completed. Canonical RPC genes *Pax6* and *Vsx2*, which are also expressed in the early neuroepithelium, are down-regulated in the *Meis1/2* cKO retina. Furthermore, Wnt/β-catenin signaling is not active in the early neuroepithelium and becomes active subsequently in the CMZ, where this signaling controls the transcription of CMZ-specific genes (80). In the *Meis1/2* cKO retina, Wnt/β-catenin signaling is aberrantly activated in the peripheral retina. The retinal explant culture propagated in the presence of Wnt3a and GSK3β inhibitor shows up-regulation of CMZ/specific genes in the peripheral retina but not in the central retina (57). Thus, it appears that only the RPCs located in the peripheral retina are competent to adopt the CMZ fate (57). Despite the deletion of *Meis1/2* throughout the entire neural retina, the expansion of CMZ-specific genes was restricted to the peripheral retina in the *Meis1/2* cKO. These observations favor the possibility that the state of the *Meis1/2* cKO retina is compatible with that of the CMZ rather than the early neuroepithelium.

Nevertheless, we cannot currently exclude the scenario in which *Meis1* and *Meis2* control the competence transition from the early neuroepithelium. First of all, both *Meis1* and *Meis2* are expressed in the neuroepithelium. Although the complete depletion of Meis proteins was typically achieved by E14.5, when the transition from the neuroepithelium should have already been completed, *mRx-Cre* is active in the eye-specified neuroepithelim from E8.5 onwards and we have observed patches of diminished levels of Meis proteins as early as E11.5. We have shown that Meis1/2 directly bind regulatory regions of CMZ-specific genes; however, it remains elusive whether these putative enhancers are active in the early neuroepithelim, CMZ, or both. Further studies are highly warranted to clarify whether *Meis1/2* facilitate competence transition from the neuroepithelium.

Materials and Methods

All experiments with mice were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and national and institutional guidelines. Animal care and experimental procedures were approved by the Animal Care Committee of the Institute of Molecular Genetics. *Vsx2 ΔCRM1*, *Vsx2 ΔCRM2*, and *Vsx2 ΔCRM1&2* mice were generated using the CRISPR/Cas9 approach. Details are described in *SI Appendix*. Immunofluorescence, ChIP-seq, and bulk RNA-seq were performed according to published protocols. More detail regarding specific experimental procedures, a list of antibodies, and primers are provided in the *SI Appendix*.

Data Availability. RNA-seq and ChIP-seq data have been deposited in the ArrayExpress database (accession nos. E-MTAB-9143 and E-MTAB-10112). Dataset S1 is available as part of the submission. All other study data are included in the article and supporting information.

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