Six3 and Six6 Are Jointly Required for the Maintenance of Multipotent Retinal Progenitors through Both Positive and Negative Regulation

Graphical Abstract

Highlights
- Six3 and Six6 are jointly required to maintain multipotent retinal progenitors
- Six3 and Six6 together suppress Wnt/β-catenin signaling
- Six3 and Six6 together maintain the expression of multiple retinogenic factors
- Wnt3a/β-catenin signaling promotes ciliary margin cells at the cost of neuroretina

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In Brief
Gene regulation of multipotent retinal progenitor cells is partially understood. Through genetic, molecular, and transcriptomic characterization of Six3 and Six6 compound null retinas in mice, Diacou et al. demonstrate that Six3 and Six6 jointly suppress Wnt/β-catenin signaling but promote the expression of retinogenic factors to maintain multipotent retinal progenitor cells.

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Six3 and Six6 Are Jointly Required for the Maintenance of Multipotent Retinal Progenitors through Both Positive and Negative Regulation

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SUMMARY

Gene regulation of multipotent neuroretinal progenitors is partially understood. Through characterizing Six3 and Six6 double knockout retinas (DKOs), we demonstrate Six3 and Six6 are jointly required for the maintenance of multipotent neuroretinal progenitors. Phenotypes in DKO s were not found in either Six3 nulls or Six6 nulls. At the far periphery, ciliary margin (CM) markers Otx1 and Cdon together with Wnt3a and Fzd1 were ectopically upregulated, whereas neuroretinal progenitor markers Sox2, Notch1, and Otx2 were absent or reduced. At the mid periphery, multi-lineage differentiation was defective. The gene set jointly regulated by Six3 and Six6 significantly overlapped with the gene networks regulated by WNT3A, CTNNB1, POU4F2, or SOX2. Stimulation of Wnt/β-catenin signaling by either Wnt-3a or a GSK3β inhibitor promoted CM progenitors at the cost of neuroretinal identity at the periphery of eyecups. Therefore, Six3 and Six6 together directly or indirectly suppress Wnt/β-catenin signaling but promote retinogenic factors for the maintenance of multipotent neuroretinal progenitors.

INTRODUCTION

Cell fate specification and maintenance are fundamental for tissue formation in embryogenesis. In murine retinal development, specified early retinal progenitors are marked by the expression of eye field transcription factors, including Six3, Pax6, Rx, Otx2, and Lhx2 in embryonic day 8.0 (E8.0)–8.5 embryos (Liu et al., 2010). From the early retinal progenitors, neuroretinal (NR) progenitors and retinal pigment epithelial (RPE) progenitors, marked by the expression of Vsx2 and Mitf, respectively, are then specified and partitioned into the inner and outer layers of optic cups by E10.5 (Horsford et al., 2005; Liu et al., 2010). At the periphery of the NR abutting RPE in optic cups, ciliary margin (CM) progenitors are specified at E12.5, as indicated by the expression of Otx1 and Cdon (Bélanger et al., 2017; Liu et al., 2007). CM tissue is also found at the transition zone between fibroblast growth factor (FGF)-induced RPE-derived NR tissue and the endogenous RPE in chicks (Dias da Silva et al., 2007). Activation of Wnt/β-catenin signaling is necessary and sufficient for CM specification in chicks and mice (Cho and Cepko, 2006; Esteve et al., 2011; Fotaki et al., 2013; Heavner et al., 2014; Kinoshita et al., 2016; Kubo et al., 2003; Liu et al., 2007). CM progenitors give rise to both retinal neurons and non-pigmented ciliary epithelial cells in mice (Bélanger et al., 2017; Marucci et al., 2016). Despite the advances, it is unclear how NR maintenance and CM specification are coordinated resulting in a tapered CM zone in the peripheral NR.

NR progenitors in optic cups are multipotent, generating retinal neurons and Müller glia in an evolutionary conserved order through coordinated retinal cell differentiation and maintenance of a pool of NR progenitors (Cepko, 2014). Transcription factor Pax6 is required for the multipotency of NR progenitors through direct transcriptional activation of retinogenic bHLH factors (Marquardt et al., 2001). Notably, Pax6 inactivation in peripheral optic cups reveals divergent progenitor pools of NR progenitors, since Pax6 prevents premature activation of a photoreceptor-differentiation pathway at the periphery but regulates the multipotency of NR progenitors in more central regions (Oron-Karni et al., 2008). Intrinsic differences in NR progenitors are also demonstrated by recent fate mapping and live imaging studies (Cepko, 2014). Besides Pax6, Hh signaling regulator Sufu is required to maintain the multipotency and identity of NR progenitors through antagonizing Hh/Gli2 signaling (Cwinn et al., 2011); transcription factor Sox2 is required for NR progenitor competence and NR-CM boundary in a dose-dependent manner (Heavner et al., 2014; Matsushima et al., 2011; Taranova et al., 2006). Despite these findings, it is unclear how regulatory genes for the multipotency of NR progenitors are involved in CM specification.

We have demonstrated that homeodomain transcription factor Six3 is an essential cell-fate determinant in NR specification. In mice, starting at E7.5–8.0, Six3 is expressed at the anterior neural plate, eye field, optic vesicles, and optic cups (Lagutin et al., 2003; Liu et al., 2010). Germline Six3 inactivation ablates both rostral forebrain and eye field following a rostral expansion of Wnt1 (Lagutin et al., 2003). Conditional Six3 inactivation using Rx-Cre ablates NR specification but expands RPE following a rostral expansion of Wnt8b (Liu et al., 2010). Six3 in a small population of progenitors at E8.5 is required for NR specification...
through suppressing Wnt8b, maintaining FGF/ERK signaling, and cell survival (Liu and Cvekl, 2017). Six6, a paralog of Six3, is expressed in optic vesicles starting at E8.5–9.0, a later stage compared to Six3, and is largely co-localized with Six3 in the progenitors in optic vesicles and optic cups (Jean et al., 1999; Liu and Cvekl, 2017). Neither Six3 inactivation at E9.5–10.5 nor Six6 deletion (Li et al., 2002) overtly affect NR specification, but Six3 inactivation at E9.5 together with Six6 deletion ablates NR specification, indicating genetic interactions between Six3 and Six6 in retinal development (Liu and Cvekl, 2017).

To test the hypothesis that Six3 and Six6 together play a role in specified NR progenitors, here we conditionally inactivated Six3 using α-Cre at E10.5 (Marquardt et al., 2001) together with Six6 deletion (DKO; Six3 and Six6 double knockout). Phenotypes in the DKO retinas were not found in either Six3 null or Six6 null retinas: the CM was expanded at the cost of NR progenitors at the far periphery, whereas multi-lineage differentiation was defective at the mid periphery. Through phenotypic analysis of DKO retinas, RNA sequencing (RNA-seq) transcriptome profiling, Ingenuity Pathway Analysis, and stimulation of Wnt/β-catenin signaling in mouse eye cup cultures, we conclude that Six3 and Six6 jointly suppress Wnt/β-catenin signaling but promote retinogenic factors to maintain multipotent NR progenitors in mice.

RESULTS

**Six3 Exhibits in a Gradient in E10.5–E11.5 Neuroretina, and Six3 and Six6 Compound Deletion (DKO) Causes Morphological Abnormalities**

To determine Six3 and Six6 functions after NR specification, we conditionally inactivated Six3 at E10.5 using α-Cre (Marquardt et al., 2001) in combination with Six6 deletion (Li et al., 2002). In E10.5 mouse retina, Six3 protein expression exhibited a gradient along the peripheral-central axis with high levels in central regions (Figure 1A), consistent with a gradient along the peripheral-central axis with high levels in the peripheral retina, resulting in far-peripheral and mid-peripheral regions (arrowhead in Figure 1I; see also Figure 1O for R26R reporter expression for α-Cre, Figures 2D, 3D, and 4D for efficient Six3 deletion in both far- and mid-peripheral regions, and Figures 4H, 4P, and 4X for distinct gene expression profiles in far- and mid-peripheral regions). Interestingly, GFP expression (Cre-IRES-GFP) was expanded centrally in E14.5 DKO retinas compared to that in conditional Six3 null retinas (Figures 1J and 1K). On postnatal day 11, the control, conditional Six3 null (Figures 1L and 1M), and Six6 null retinas (data not shown) were translucent, but DKO retinas were opaque except for in a narrow central band in which R26R reporter expression for α-Cre was absent (Figures 1N and 1O). In addition, DKO retinas displayed severe degenerations at far-peripheral regions (arrowheads in Figure 1N). On sections, DKO retinas lacked a laminar structure and comprised sparsely dispersed cells (Figures 1P–1W; see also Figures 5, S6, and S7 for molecular characterization). Collectively, Six3 deletion at E10.5 using α-Cre together with Six6 deletion drastically disrupts retinal cell differentiation.

**NR Progenitors Are Defective, and Retinal Cell Differentiation Is Affected in E14.5 DKO Retinas**

We sought to determine the molecular mechanisms underlying the phenotypes in DKO retinas. In E14.5 DKO retinas, Six3 was efficiently deleted except for in a narrow central region where α-Cre was inactive (Figures 2A–2D). Sox2, a dose-dependent essential regulator of NR progenitor competence and NR-CM boundary, regulates Notch1 expression in the NR (Heavner et al., 2014; Matsushima et al., 2011; Taranova et al., 2006). Sox2 expression was normal in both Six6 null retinas and Six3F/F;α-Cre retinas but was significantly reduced in the peripheral regions of DKO retinas (Figures 2E–2H). Accordingly, Notch1 was nearly absent (Figures 2I–2L), and Hes5 was severely reduced (Figures S2A–S2D) in DKO retinas. Gli1, a target of Shh signaling (Lee et al., 1997), is expressed in NR progenitors but not in the CM. Gli1 is increased in Sufu null retinas in which multipotency of NR progenitors is disrupted (Cwinn et al., 2011). In DKO retinas, Gli1 expression was absent in far-peripheral regions and severely reduced in mid-peripheral regions compared to the control retinas (Figures 2M–2P), indicating phenotype differences between DKO retinas and Sufu null retinas. Ascl1, a bHLH factor that defines a competence-restricted lineage and is required for cell proliferation (Brzezinski et al., 2011; Marquardt et al., 2001), is expressed in NR progenitors but not in the CM. In DKO retinas, Ascl1 was absent in far-peripheral regions and severely reduced in mid-peripheral regions compared to the control retinas (Figures 2Q–2T). Neuron4 and Neuron1 are jointly required for amacrine cell differentiation, and misexpression of these bHLH genes alone induces rod genesis (Ioue et al., 2002). In DKO retinas, Neuron4 expression was severely reduced in far-peripheral regions and reduced in mid-peripheral regions (Figures S2E–S2H). Fzd5, a receptor of Wnt signaling proteins, is expressed in the NR; Fzd5 inactivation in germ-line disrupts optic cup formation (Burns et al., 2008). In DKO retinas, Fzd5 was severely reduced in

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Figure 1. A Gradient of Six3 Expression in E10.5–11.5 Neuroretina and Morphological Abnormalities Caused by Six3 and Six6 Compound Deletion (DKO)

(A and B) Six3 expression displayed a gradient with high levels in central NR (A), whereas Pax6 expression exhibited an inverse gradient (B).

(C) R26R reporter expression for α-Cre was detected in peripheral NR as early as E10.5.

(D and E) Graded Six3 expression remained in E11.5 NR (D). Six3 protein deletion was detected in peripheral NR as early as E11.5 (E).

(F–I) At E14.5, newly differentiated retinal neurons and NR progenitors formed two layers in Six3F/F;Six6+/C0 retinas (dotted line in F), Six3F/F;Six6−/− retinas (G), and Six3F/F;α-Cre;Six6−/− retinas (H). In peripheral DKO retinas, the layer of newly differentiated neurons was absent; an abrupt change in retinal thickness was found, resulting in far- and mid-peripheral regions (arrowhead in I; see also Figures 2D and 3D for efficient Six3 deletion).

(J and K) GFP (Cre-IRES-GFP) expression was expanded in E14.5 DKO retinas (K) compared to that in Six3F/F;α-Cre retinas (J).

(L–N) On postnatal day 11, Six3F/F retinas (L) and Six3F/F;α-Cre retinas (M) were translucent, but DKO retinas were opaque except for in a narrow central band (N). Severe degenerations were found at the far-peripheral regions of DKO retinas (arrowheads in N).

(O) On postnatal day 0, R26R reporter for α-Cre was widely expressed in the retina except for in a narrow central band.

(P–W) On sections, unlike Six3F/F retinas (P and T) and Six3F/F;α-Cre retinas (Q and U) and Six3F/F;α-Cre;Six6+/C0 retinas (R and V), DKO retinas lacked a laminar structure and comprised sparsely dispersed cells (S and W).

Scale bars, 100 μm (A, C, D, F, J, and T), 200 μm (P), 1 mm (L). n = 3 embryos. See also Figures S1–S7.
far-peripheral regions and moderately reduced in mid-peripheral regions (Figures S2I–S2L). Cyclin kinase inhibitor p57 is elevated in Six6 null retinas (Li et al., 2002). In the control retinas, p57 was moderately expressed in the CM but barely expressed in the NR (Figure S2M). In Six3F/F;α-Cre;Six6/− retinas, p57 expression was marginally increased (Figures S2M and S2O). In DKO retinas, p57 expression was ectopically upregulated in far-peripheral regions (Figures S2M and S2P), indicating a CM expansion. Atoh7, a bHLH transcription factor required for retinal ganglion cell differentiation (Brown et al., 1998; Wang et al., 2001), was severely reduced in the peripheral regions of DKO retinas (Figures S3A–S3D). Banded Atoh7 expression in peripheral DKO retinas could be caused by incomplete Six3 deletion and/or represent Atoh7 expression in amacrine cell lineages (Feng et al., 2010), since amacrine cell differentiation was largely unaffected in DKO retinas (see also Figures 4P, 5I, 5L, S5L, S6D, S6H, S6L, and S6P). Neurog2, a NR progenitor marker (Marquardt et al., 2001), was reduced in far-peripheral regions of DKO retinas (Figures S3E–S3H). Fgf15, a marker for NR progenitors (Burns et al., 2008), was also reduced in far-peripheral regions of DKO retinas (Figures S3I–S3L). The reduction in Fgf15 expression explained the posterior expansion of lens epithelium in DKO retinas (Figures 1 I and S2P), since retina-derived FGFs promote lens fiber differentiation (Lovicu and McAvoy, 2005). Collectively, NR progenitors are grossly normal in conditional Six3 null or Six6 null retinas but are strikingly defective in DKO retinas.

To evaluate early retinal differentiation, we examined retinal neurons in E14.5 DKO retinas. Six3 was efficiently deleted in
Six3F/F; Six6+/− retinas and DKO retinas (Figures 3C and 3D). Pou4f2 (also known as Brn3b), a POU domain transcription factor required for retinal ganglion cell development (Gan et al., 1996), was grossly normal in both Six3F/F; Six6+/− and Six3F/F; α-Cre; Six6+/− retinas (Figures 3A–3G). In DKO retinas, however, Pou4f2 was nearly absent in far-peripheral regions and severely reduced and disorganized in mid-peripheral regions (Figure 3H), indicating defective retinal ganglion cell differentiation. Tubb3 and p27 are expressed in early born retinal neurons, including retinal ganglion cells. In DKO retinas, Tubb3 and p27 were severely reduced in far-peripheral regions (Figures 3L and 3P), confirming the defects in early retinal differentiation. Pax6, a transcription factor essential for the multipotency of NR progenitors (Marquardt et al., 2001), was expressed in NR progenitors, CM progenitors (bracket in Figure 3Q), and early-differentiated retinal neurons in the control retinas (Figure 3Q). In DKO retinas, Pax6 expression remained and was even upregulated in far-peripheral regions (bracket in Figures 3Q and 3T), indicating that NR defects in DKO retinas were not caused by Pax6 down-regulation. Collectively, early retinal cell differentiation is largely normal in both Six3F/F; Six6+/− and Six3F/F; α-Cre; Six6+/− retinas but is remarkably defective in DKO retinas.

To assess cell proliferation in DKO retinas, we examined Ccnd1 (also known as cyclin D1) and pH3. Ccnd1 was highly expressed in the NR but marginally expressed in the CM in the control retinas (Figure S4A). Similar Ccnd1 expression was found in both Six3F/F; Six6+/− retinas and Six3F/F; α-Cre; Six6+/− retinas (Figures S4A–S4C). In DKO retinas, Ccnd1 was nearly absent in peripheral NR (Figures S4A and S4D), indicating severe defects in cell proliferation. In the control retinas, pH3 positive cells were found at the apical surface of the NR (Figure S4E). In DKO retinas, pH3 positive cells were reduced in density in far-peripheral regions (Figures S4E–S4H and S4M).
and occasionally mis-localized in mid-peripheral regions (Figure S4H) likely due to the defects in apical junctions (Figures S4O and S4R). Meanwhile, apoptosis was increased in peripheral DKO retinas (Figures S4I–S4L and S4N), likely caused by altered or mixed cell identity.

Taken together, although either conditional Six3 null or Six6 null retinas are grossly normal, DKO retinas display striking defects in NR identity and differentiation.

Figure 4. Loss of NR Identity Is Accompanied by Ectopic Upregulation of Wnt/β-Catenin Signaling and CM Markers in E15.5 DKO Retinas

(A–D) Six3 was expressed in Six3F/F retinas (A), but was efficiently deleted in Six3F/F;α-Cre retinas (B) and DKO retinas (D). Six3 was unaffected in Six6 null retinas (C).

(E–H) Compared to that in Six3F/F retinas (E), Vsx2 was unaffected in Six3F/F;α-Cre retinas (F) and Six3F/F;Six6−/− retinas (G). In DKO retinas, Vsx2 was absent in mid-peripheral regions (H, areas II and IV) but was present in far-peripheral regions (H, areas I and V). Vsx2 remained where Six3 was present (H, area III).

(I–L) Compared to that in Six3F/F retinas (I), Otx2 was unaffected in Six3F/F;α-Cre retinas (J) and Six3F/F;Six6−/− retinas (K). In DKO retinas, Otx2 was nearly absent where Six3 was deleted (L). Quantification represented three eyes (L; ***p < 0.001 in t test).

(M–P) Compared to that in Six3F/F retinas (M), Axin2 was unaffected in Six3F/F;α-Cre retinas (N) and Six3F/F;Six6−/− retinas (O). In DKO retinas, Axin2 was absent (P, area V) or severely reduced (P, areas I) in far-peripheral regions but remained in mid-peripheral regions with disrupted stratification (P, area II).

(Q–T) Compared to that in Six3F/F retinas (Q), Otx1 expression was ectopically upregulated in far-peripheral regions (T, areas I and V).

Scale bars, 200 μm. n = 3 embryos. See also Figure S5.

Loss of NR Identity in DKO Retinas Is Accompanied by Ectopic Upregulation of Wnt/β-Catenin Signaling and CM Markers

We sought to determine whether the defects in NR identity and differentiation in DKO retinas were correlated to cell fate changes. At E15.5, Six3 was efficiently deleted at the peripheral regions in both Six3F/F;α-Cre retinas and DKO retinas (Figures 4A–4D). Six3 was unaffected in Six6 null retinas (Figure 4C), and Six6 was unaffected in conditional Six3 null retinas either (data not shown). Vsx2, a marker for both NR progenitors and CM progenitors (Horsford et al., 2005; Kuwahara et al., 2015), was unaffected in either Six3F/F;α-Cre retinas or Six3F/F;Six6−/− retinas (Figures 4E–4G) but was drastically affected in DKO retinas (Figure 4H). Although Six3 was deleted in both far- and mid-peripheral regions in DKO retinas (Figure 4D), Vsx2 was absent in mid-peripheral regions but remained in far-peripheral regions.
(Figure 4H). Vsx2 expression at the far-peripheral regions in DKO retinas likely represented its expression in the CM, since additional CM markers were also present (see also Figures 4T and 4X). The loss of Vsx2 expression in the mid-peripheral regions indicated defective NR progenitors in DKO retinas. Consistently, multi-lineage retinal differentiation was severely affected. Otx2, a transcription factor required for photoreceptor, bipolar, and horizontal cell development (Nishida et al., 2003; Sato et al., 2007), was unaffected in either Six3F/F;α-Cre retinas or Six3F/F;Six6−/− retinas (Figure 4I–4K) but was nearly absent at both far- and mid-peripheral regions of DKO retinas (Figures 4A, 4L, and 4L'), indicating severe defects in photoreceptor, bipolar, and horizontal cell differentiation. In addition, retinal ganglion cell differentiation was also severely affected. Shh is expressed in retinal ganglion cells and is required for retinal laminar structure (Wang et al., 2002). Shh was unaffected in either Six3F/F;α-Cre retinas or Six3F/F;Six6−/− retinas (Figures S5A–S5C) but was nearly absent in far-peripheral regions and significantly reduced in mid-peripheral regions in DKO retinas (Figures S5A and S5D). Severe reduction in Shh expression (Figure S5D) was consistent with the absence of Gli1 (Figure 2P), a target of Shh signaling (Lee et al., 1997). Defects in retinal ganglion cell differentiation were further confirmed by severe reduction of Pou4f2 at both expression levels and numbers at E14.5 and E15.5 (Figures 3H, S5H, and S5H'). In contrast, amacrine cell differentiation was relatively unaffected in mid-peripheral regions of DKO retinas. Neurod1, a bHLH transcription factor involved in amacrine cell differentiation (Inoue et al., 2002; Marquardt et al., 2001), was unaffected in either Six3F/F;α-Cre, or Six3F/F;Six6−/− retinas (Figures 4M–4O). In DKO retinas, Neurod1 was absent or significantly reduced in far-peripheral regions but remained in mid-peripheral regions with disrupted stratification (Figure 4P). Similar expression changes were found for Foxn4 (Figures S5I–S5L), a transcription factor both...
### Top Upstream Regulators (pad < 0.05, |log2FC| > 1; 333 analysis-ready genes)

| Upstream Regulator | p-value of overlap | Predicted Activation |
|--------------------|-------------------|---------------------|
| WNT3A              | 1.57E-13          |                     |
| POU4F2             | 2.39E-13          |                     |
| CTNNB1             | 4.11E-11          |                     |
| SOX2               | 4.50E-11          |                     |
| beta-estradiol     | 5.76E-11          | Inhibited           |

### Top Diseases and Bio Functions

#### Molecular and Cellular Functions

| Function                     | p-value                  | #Molecules |
|------------------------------|--------------------------|------------|
| Cellular Movement           | 4.05E-05 - 2.54E-18     | 121        |
| Cellular Development        | 4.20E-05 - 4.10E-15     | 158        |
| Cellular Growth and Proliferation | 4.02E-05 - 4.10E-15   | 142        |
| Cell Morphology             | 4.02E-05 - 2.30E-11     | 84         |
| Cell Death and Survival     | 2.56E-05 - 9.43E-11     | 134        |

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**C** Six3F/F, Six6−/−

**D** Six3F/F, Six6−/−

**E** Six3F/F, α-Cre

**F** Six3F/F, α-Cre; Six6−/−

**G** E14.5

**H** Fzd1

**I** Wnt3a

**J** Wnt16

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(legend on next page)
necessary and sufficient for amacrine cell fate but nonredundantly required for horizontal cells (Li et al., 2004). In DKO retinas, the absence or reduction of Otx2, Neurod1, Shh, Pou4f2, and Foxn4 in the far-peripheral regions (Figures 4L, 4P, SSD, SSN, and SSL) confirmed the loss of NR identity; the presence of Neurod1 and Foxn4 (Figures 4P and SSL) but the absence or reduction of Vsx2, Otx2, and Shh (Figures 4H, 4L, and SSD) in the mid-peripheral regions indicates differentiation defects in multiple lineages but amacrine cells.

The loss of NR identity in far-peripheral regions of DKO retinas could be accompanied by a gain of CM marker expression, as indicated by upregulated CM marker p57 (Figure S2P). Activation of Wnt/β-catenin signaling is necessary and sufficient for CM development in mice (Cho and Cepko, 2006; Esteve et al., 2011; Fotaki et al., 2013; Heavner et al., 2014; Liu et al., 2007). Axin2, a readout of Wnt/β-catenin signaling (Jho et al., 2002), was very weakly expressed in the CM in the control retinas (Figure 4Q) and unaffected in either Six3F/F;α-Cre retinas or Six3F/F; Six6−/− retinas (Figures 4R and 4S) but was ectopically upregulated in DKO retinas (Figure 4T). Consistently, Otx1, a CM marker stimulated by Wnt/β-catenin signaling (Liu et al., 2007), was unaffected in either Six3+/F;α-Cre retinas or Six3F/F; Six6−/− retinas (Figures 4U–4W) but ectopically upregulated in DKO retinas (Figures 4U and 4X). Interestingly, Otx1 upregulation was mostly restricted to the far-peripheral regions (Figure 4X) where Neurod1 and Foxn4 were largely absent (Figures 4P and SSL). In Six3F/F;α-Cre retinas, Cre expression was widely restricted in the peripheral retinas at E12.5 but restricted to CMs at E15.5 (Figures S5Q and S5R). In DKO retinas, however, Cre expression was continuously found in wide areas at E15.5 (Figures S5S and S5R). In DKO retinas, however, Cre expression was continuously found in wide areas at E15.5 (Figures S5Q and S5S), consistent with the expression pattern of GFP (Cre-IRES-GFP) at E14.5 (Figures 1J and 1K). Importantly, upregulated Otx1 was found in Cre-positive Six3-negative cells in which Axin2 was also upregulated but NR markers Otx2, Shh, and Pou4f2 were absent or severely reduced (Figures 4D, 4L, 4P, 4T, and 4X; Figures SSD, SSH, SSL, SSR, and SST), indicating that ectopic Otx1 expression was accompanied by ectopic upregulation of Wnt signaling and loss of NR identity in DKO cell population. Collectively, DKO retinal cells in far-peripheral regions gained CM cell fate at the cost of NR identity, whereas DKO retinal cells in mid-peripheral regions maintained NR fate but lost multipotency.

Postnatal DKO Retinas Lack a Laminar Structure and Comprise Only a Subpopulation of Amacrine Cells and Infiltrated Astrocytes

To determine the outcomes of defective retinal differentiation in DKO retinas, we analyzed retinal cell types on postnatal day 11, a stage when retinal cell fate determination has completed. Six3 was efficiently deleted (Figures 5A–5F). Retinal cell types were unaffected in Six3F/F;α-Cre;Six6−/− retinas, although Six3F/F;α-Cre;Six6−/− retinas were thinner. In DKO retinas, however, Rho and cone arrestin, markers for rod and cone photoreceptors, respectively, were absent (Figures 5F and 5L), indicating the absence of photoreceptors in DKO retinas. Similarly, bipolar, horizontal, and photoreceptor marker Otx2, retinal ganglion cell marker Pou4f2, and horizontal cell marker neurofilament (medium polypeptide) were absent in DKO retinas (Figures 5L, 5O, and 5R). In areas where residual Six3 remained in DKO retinas, Rho and Otx2 remained (arrows in Figures 5F and 5L), indicating that the phenotypes highly depended on Six3 deletion. In contrast, amacrine cell markers syntaxin and Pax6 were widely expressed in DKOs (Figures 5I and 5L). Furthermore, amacrine cell markers Calretinin, GAD65, GABA, and TH were widely found (Figures S6D, S6H, S6L, and S6P), but GlyT1 and ChAT were rarely found in DKO retinas (Figures S6T and S6X), indicating only a subpopulation of amacrine cells persisted in postnatal DKO retinas. In addition to the defects in retinal neurons, Müller glia was absent in DKO retinas, as indicated by the absence of Lhx2 and Glul (Figures S7A–S7D). Furthermore, DKO retinas were widely infiltrated by Gfap-positive astrocytes (Figures S7E–S7H). Collectively, postnatal DKO retinas lack a laminar structure and comprise only a subpopulation of amacrine cells and infiltrated astrocytes, confirming the defects in multi-lineage retinal differentiation.

The Gene Set Jointly Regulated by Six3 and Six6 Highly Overlaps with the Gene Networks Regulated by WNT3A, CTNNB1, POU4F2, or SOX2

We sought to identify the gene set jointly regulated by Six3 and Six6 in NR progenitors using RNA-seq assay. The whole DKO retinas driven by α-Cre are not ideal for bulk RNA-seq, since the differentially expressed genes (DEGs) in the Cre-positive peripheral NR will be averaged out by the gene expression in the Cre-negative central NR. To overcome the drawback of α-Cre, we utilized CAGGCre-ERTM to ubiquitously delete Six3 at E10.5 to generate E13.5 Six3F/F;CAGGCre-ERTM;Six6−/− compound null retinas. Notably, compound Six3 and Six6 deletion driven by either CAGGCre-ERTM or α-Cre led to consistent phenotypes (data not shown). Transcriptome comparison between three DKO and three wild-type (WT) using DESeq2 identified 800 DEGs in E13.5 DKO (Table S1, padj < 0.05). Expression changes in DEGs were highly correlated with the efficiency of Six3 deletion, since relatively less Six3 deletion in one DKO sample (DKO_rh021) caused reduced levels of expression changes (Figure 6A; data not shown). Visual inspection of the plotted heatmap...
found that one WT sample slightly deviated from the other two in the WT group (Figure 6A). Therefore, we performed an additional transcriptome comparison between two DKO samples and two WT samples after removing the deviated ones, resulting in 1152 DEGs (padj < 0.05). Combining the two lists of DEGs, 1321 DEGs in total were identified in E13.5 DKO retinas driven by CAGGCre-ERTM (Table S2, padj < 0.05), covering 15 out of 24 previously identified DEGs in E14.5–15.5 DKO retinas driven by a-Cre (Figures 2, 3, 4, 6A, and S2–S5). The list in Table S1 is stringent, whereas the list in Table S2 identifies additional DEGs, e.g., Pou4f2 and Neurod1 as confirmed in Figure 3H and Figure 4P, respectively, indicating a value of performing the second comparison. Importantly, RNA-seq assay identified additional DEGs that were functionally relevant, e.g., upregulated Fzd1, Wnt3a, Wnt16, and Cdon; all the four DEGs were validated in DKO retinas (Figures 6A, 6C–6N, and 7A–7D). In addition, Wnt3a, Fzd1, Cdon, Otx1, and Wnt16 were closely clustered in the heatmap, suggesting their close functional relationship. Furthermore, upregulation of Fzd1, Axin2, Otx1, and Neurod1 expression in E15.5 DKO retinas in Figures 4P and 4X).

Figure 7. Stimulation of Wnt/β-Catenin Signaling by Either Wnt-3a or a GSK3β Inhibitor Promotes CM Cell Fate at the Cost of NR Identity at the Periphery of Mouse Eyecups

(A–D) CM marker Cdon was expanded in E14.5 DKO retinas (D) compared to that in Six3+/− retinas (A), Six3−/−; α-Cre retinas (B), and Six3−/−; β-Cre; Six6+/− retinas (C). (E–H) Compared to vehicle controls (E), Wnt-3a (G) or GSK3β inhibitor CHIR99021 (H), but not Wnt-16b (F), expanded the size of CM (Cdon-positive Tubb3-negative) at the cost of NR identity at the periphery of mouse eyecups. (I–L) A working model. At E10.5–11.5, both Six3 and Six6 expression displayed a gradient along peripheral-central axis (I). In most-peripheral regions, low levels of Six3 and Six6 permitted Wnt/β-catenin signaling, leading to CM specification (blue in (K), an illustration of Otx1 expression in E15.5 control retina in Figure 4U). Moving centrally in the neuroretina, high levels of Six3 and Six6 protected multipotent NR progenitors directly or indirectly through (1) suppressing Wnt/β-catenin signaling and (2) maintaining the expression of retinogenic factors. When Six3 was deleted in E10.5 neuroretina using α-Cre in combination with Six6 deletion (J), the suppression of Wnt/β-catenin signaling was removed and the expression of retinogenic factors was disrupted. Retinal cells in far-peripheral regions gained CM identity at the expense of NR progenitors under the stimulation of Wnt/β-catenin signaling, whereas retinal cells in mid-peripheral regions maintained NR identity but lost multipotency (L, an illustration of Otx1 and Neurod1 expression in E15.5 DKO retinas in Figures 4P and 4X).

Scale bars, 100 μm (A), 50 μm (E). n = 3 retinas.
the gene set jointly regulated by Six3 and Six6 highly overlapped with the GRNs regulated by WNT3A, Pou4f2, CTNNB1, or Sox2. In the 333 analysis-ready DEGs, 116 and 132 DEGs were in WNT3A- and CTNNB1-regulated GRNs, respectively, suggesting the involvement of Wnt/β-catenin signaling under the regulation of Six3 and Six6 joint functions. Consistently, KEGG pathway analysis indicated that DEGs in Wnt signaling pathway had the highest percentage (data not shown). In support of the upstream regulator analysis, several components of Wnt/β-catenin signaling, i.e., Axin2, Fzd1, Wnt3a, and Wnt16, were significantly upregulated (Figures 4Q–4T and 6C–6N), whereas Sox2 and Pou4f2 were downregulated in DKO retinas (Figures 2E–2H, 3E–3H, and S5E–S5H). Interestingly, Axin2 and Fzd1 were upregulated more widely than Wnt3a and Wnt16 (Figures 4T, 6F, 6J, and 6N), indicating complex regulation of Wnt/β-catenin signaling by Six3 and Six6 joint functions. IPA also identified the following top five molecular and cellular functions of the DEGs: cellular movement, cellular development, cellular growth and proliferation, cell morphology, cell death, and cell survival (Figure 6B), which were highly consistent with marker analysis (Figures 2, 3, 4, and S2–S5). Collectively, transcriptome profiling of DKO retinas highly indicates that Six3 and Six6 jointly regulate a GRN involving Wnt/β-catenin signaling, Sox2, and Pou4f2 in retinal cell differentiation.

**Stimulation of Wnt/β-Catenin Signaling by Either Wnt-3a or a GSK3β Inhibitor Promotes CM Cell Fate at the Cost of NR Identity at the Periphery of Mouse Eyecups**

To determine the functional consequences of upregulated Wnt3a, Wnt16, and Wnt/β-catenin signaling in DKO retinas, we examined the CM in mouse eyecup cultures supplemented with recombinant Wnt-3a, Wnt-16b, or a GSK3β inhibitor CHIR99021. Cdon, a CM marker (Belanger et al., 2017), was significantly upregulated in DKO retinas (Figures 6A and 7A–7D). In the mouse eyecup cultures treated with vehicles, the CM was found in Cdon-positive and Tubb3-negative areas (Figure 7E). In mouse eyecup cultures treated with Wnt-16b, the CM size was comparable to that in the vehicle controls (Figure 7F). In the eyecups treated by Wnt-3a, however, the length of CM (Cdon-positive Tubb3-negative) was much longer than that in the vehicle controls (Figures 7E and 7G). Similarly, the CM was also longer in the eyecups treated with a GSK3β inhibitor CHIR99021 (Figures 7E and 7H). Interestingly, in the eyecups treated with either Wnt-3a or CHIR99021, CM expansion was restricted to the periphery of mouse eyecups (Figures 7E, 7G, and 7H). In the expanded CM, Six3 expression was reduced (data not shown). Collectively, activation of Wnt/β-catenin signaling by either Wnt-3a or a GSK3β inhibitor promotes CM cell fate at the cost of NR identity at the periphery of mouse eyecups.

**DISCUSSION**

Through molecular characterization of DKO retinas, transcriptome profiling, IPA, and manipulation of Wnt/β-catenin signaling in mouse eyecup cultures, we have demonstrated that Six3 and Six6 together are essential for maintaining a pool of multipotent NR progenitors at least through two molecular mechanisms. Six3 and Six6 jointly suppress Wnt/β-catenin signaling, since an inverse relationship between Six3/Six6 joint functions and Wnt/β-catenin signaling was found. Six3 and Six6 together also maintain the expression of multiple retinogenic regulators, including Sox2, Notch1, Hes5, Shh, Gil1, bHLH transcription factors, Fgf15, Pou4f2, and Otx2. Thus, Six3 and Six6 are jointly required for the maintenance of multipotent NR progenitors through both negative and positive regulation.

Six3, together with Six6 at later stages, is an essential a cell-fate determinant to protect NR progenitors from adopting alternative cell fates at least through suppressing Wnt/β-catenin signaling. Six3 initially represses Wnt1 to promote rostral forebrain and eye field precursors against caudal forebrain cell fate (Lagutin et al., 2003). Next, Six3 represses Wnt8b to promote NR progenitors against RPE progenitor cell fate (Liu et al., 2010). Here, we have demonstrated that Six3 and Six6 together suppress Wnt/β-catenin signaling to protect multipotent NR progenitors against CM progenitor cell fate, because (1) Wnt3a, Fzd1, Axin2, and Wnt16 were ectopically upregulated in DKO retinas; (2) the gene set jointly regulated by Six3 and Six6 highly overlapped with the GRNs regulated by WNT3A or CTNNB1; and (3) stimulation of Wnt/β-catenin signaling by either Wnt-3a or a GSK3β inhibitor promoted CM progenitors at the cost of NR identity at the periphery of mouse eyecups. In DKO retinas, Fzd1 and Axin2 were upregulated widely, whereas Wnt3a and Wnt16 were upregulated in patches, suggesting complex suppression of Wnt/β-catenin signaling by Six3/Six6 joint functions. Simultaneous upregulation of Fzd1 and Wnt3a in DKO retinas would synergize to stimulate Wnt/β-catenin signaling, since Wnt3a interacts with Fzd1 in signal transduction (Vologhoshenkov et al., 2017). The association between upregulated Wnt/β-catenin signaling and CM markers in DKO retinas is well consistent with the model that activation of Wnt/β-catenin signaling is necessary and sufficient for CM development in chicks and mice (Cho and Cepko, 2006; Esteve et al., 2011; Fotaki et al., 2013; Heavner et al., 2014; Kinoshita et al., 2016; Kubo et al., 2003; Liu et al., 2007). Importantly, only far-peripheral NR adopted CM cell fate at the cost of NR identity in response to either joint Six3/Six6 deletion in vivo or stimulation of Wnt/β-catenin signaling in vitro. Relatedly, Wnt/β-catenin signaling and CM specification were normally found at the most-peripheral NR where both Six3 and Six6 were weakly expressed, and stimulation of Wnt/β-catenin signaling in mouse eyecups did not affect Six3 expression in central NR. Collectively, low levels of Six3 and Six6 permit Wnt/β-catenin signaling resulting in CM specification at the most-peripheral NR, whereas high levels of Six3 and Six6 jointly suppress Wnt/β-catenin signaling to protect multipotent NR progenitors against CM progenitor cell fate in the central NR. Six3 and Six6 together constitute an additional regulatory program required for the multipotency of NR progenitors. Previous studies demonstrate that Pax6, Sufu, and Sox2 also regulate multipotent NR progenitors (Cwinn et al., 2011; Heavner et al., 2014; Marquardt et al., 2001; Matsushima et al., 2011; Taranova et al., 2006). Although amacrine cells are exclusively found in Pax6 null, Sufu null, and DKO retinas at postnatal stages, the positions of these amacrine cells and the underlying molecular mechanisms differ in the mutant mice. In Pax6 null and Sufu null retinas, amacrine cells were found in far-peripheral regions (Cwinn et al., 2011; Marquardt et al., 2001), whereas amacrine
cells were found in mid-peripheral regions of DKO retinas. In embryonic DKO retinas, Pax6 expression remained and Gli1 expression was nearly absent, indicating that the phenotypes in DKO retinas were not mediated by either Pax6 loss as seen in Pax6 null retinas or by increased Gli1 as seen in Sufu null retinas. Reduced Sox2 expression in DKO retinas contributed to the downregulation of NR progenitor markers, e.g., Notch1, Hes5, and Gli1, but amacrine cells were found in DKO retinas but not in Sox2 null retinas, indicating that the pathways regulated by Six3/Six6 joint functions and Sox2 differ in some aspects. In embryonic DKO retinas, the severe reduction or absence of Atoh7 (Wang et al., 2001), Pou4f2 (Gan et al., 1996), and Shh/Gli1 (Wang et al., 2002) resulted in defects in retinal ganglion cell differentiation; the loss of Otx2 (Nishida et al., 2003; Sato et al., 2007) disrupted photoreceptor, bipolar, and horizontal cell differentiation; the sustained expression of Neurod1 (Inoue et al., 2002) and Foxn4 (Li et al., 2004) suggested largely unaffected amacrine cell differentiation. Cell type analysis in postnatal DKO retinas confirmed those embryonic alterations. The lack of a laminar structure in DKO retinas is probably due to apical junction defects mediated by upregulated Wnt/β-catenin signaling, since continuous Wnt/β-catenin signaling leads to neuroepithelial aberrations via the accumulation of aPKC at the apical pole (Herrera et al., 2014). Collectively, Six3 and Six6 jointly regulate the expression of multiple retinogenic factors essential for the multipotency of NR progenitors.

CM expansion at the far periphery and multipotency loss at the mid periphery in DKO retinas imply intrinsic differences in NR progenitors along peripheral-central axis. Similarly, in mouse eyecup cultures, the CM was expanded only at the periphery when Wnt/β-catenin signaling was stimulated by either Wnt-3a or GSK3β inhibitor CHIR99021. These findings indicate that NR progenitors at the periphery, but not at the center, had the competence to adopt CM cell fate at the expense of NR progenitors in response to either simultaneous Six3 and Six6 deletion or activated Wnt/β-catenin signaling. Moving centrally, NR progenitors maintained neural identity but lost multipotency when both Six3 and Six6 were deleted. Consistent with our findings, a divergence of NR progenitors in response to Pax6 deletion (using α-Cre) was described (Oron-Karni et al., 2008). Further analysis will determine how the region-specific phenotypes formed in DKO retinas.

Loss-of-function studies previously described (Liu and Cvekl, 2017) and here indicate that Six3 and Six6 jointly regulate retinal differentiation. Given that Six3 and Six6 are largely co-localized and display high similarity in their homeodomain and six domains, Six3 and Six6 may share gene targets and/or interacting proteins. If true, the lack of overt phenotypes in either Six3 null (deletion at E9.5–10.5) or Six6 null retinas is due to functional compensation, whereas the ablation of NR specification caused by Six3 deletion at E8.0–8.5 (Liu et al., 2010) is because of a lack of compensation due to a late onset of Six6 expression. It will be interesting to determine how much Six6 will compensate for the loss of Six3 when Six6 is knocked into Six3 locus. Alternatively, Six3 and Six6 may have unique gene targets and/or interacting proteins, and their downstream effectors synergize in retinal cell differentiation. Since the exact phenotypes in DKO retinas have not been described in any single mutant mouse retinas, it is conceivable that Six3 and Six6 jointly regulate unknown or multiple factors in retinal cell differentiation, which makes it difficult to pinpoint candidates for testing. Further studies will elucidate how Six3 and Six6 together suppress Wnt/β-catenin signaling but promote retinogenic factors to maintain multipotent NR progenitors in mouse embryonic retinas.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and four tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.10.106.

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AUTHOR CONTRIBUTIONS

R.D. performed and analyzed experiments; Y.Z. analyzed RNA-seq data under the supervision of D.Z.; A.C. edited the manuscript; and W.L. designed, performed, and analyzed experiments and wrote the manuscript. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Goat polyclonal anti-Choline Acetyltransferase | Millipore | Cat#AB144P; RRID:AB_2079751 |
| Rabbit polyclonal anti-Cone Arrestin | Millipore | Cat#AB15282; RRID:AB_11210270 |
| Mouse monoclonal anti-GAD65 | Fisher Scientific | Cat#BD559931; RRID:AB_397380 |
| Rabbit polyclonal anti-GABA | Sigma | Cat#A2052; RRID:AB_477652 |
| Rabbit polyclonal anti-GFAP | Sigma | Cat#G9269; RRID:AB_477035 |
| Mouse monoclonal anti-Glutamine Synthetase (also known as Glu) | BD Bioscience | Cat#610517; RRID:AB_2313837 |
| Goal polyclonal anti-Glycine Transporter 1 | Millipore | Cat#AB1770; RRID:AB_90893 |
| Rat monoclonal anti-Laminin-1 A&B | Millipore | Cat#MAB1904; RRID:AB_2133631 |
| Goal polyclonal anti-Lhx2 | Santa Cruz | Cat#sc-19344; RRID:AB_2135660 |
| Rabbit polyclonal anti-Neurofilament M | Millipore | Cat#AB1987; RRID:AB_91201 |
| Goat polyclonal anti-Otx2 | R&D Systems | Cat#AF1979; RRID:AB_2157172 |
| Rabbit polyclonal anti-p27 | Santa Cruz | Cat#sc776; RRID:AB_2276045 |
| Rabbit polyclonal anti-Pax6 | Covance | Cat# PRB-278P, RRID:AB_291612 |
| Rabbit polyclonal anti-ph3 | Upstate | Cat#06-570; RRID:AB_310177 |
| Rabbit polyclonal anti-PRKC zeta (Prkcz) | Santa Cruz | Cat#sc-216; RRID:AB_2300359 |
| Mouse monoclonal anti-Rhodopsin (Rho 4D2) | Abcam | Cat#ab98887; RRID:AB_10696805 |
| Rabbit polyclonal anti-Six3 | Rockland | Cat#600-401-A26; RRID:AB_11180063 |
| Rabbit polyclonal anti-Sox2 | Chemicon | Cat#AB5770 |
| Rabbit polyclonal anti-Syntaxin | Sigma | Cat#S0664; RRID:AB_10641968 |
| Rabbit polyclonal anti-Tyrosine Hydroxylase | Millipore | Cat#B9880; RRID:AB_2313773 |
| Sheep monoclonal anti-Vsx2 | BabCo | Cat#MR203818 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Recombinant Human Wnt-3a Protein | R&D Systems | Cat# 5036-WN-010 |
| Recombinant Human Wnt-16b Protein | R&D Systems | Cat# 7790-WN |
| CHIR99021 | TOCRIS | Cat#4423 |
| **Deposited Data** | | |
| RNA-seq of mouse embryonic retinas at E13.5 | This paper | GEO: GSE115258 |
| **Experimental Models: Organisms/Strains** | | |
| Mouse: Six3<sup>F<sup>+</sup></sup> | Guillermo Oliver: Liu et al., 2006 | N/A |
| Mouse: Six6<sup>−/−</sup> | Michael Rosenfeld: Li et al., 2002 | N/A |
| Mouse: α-Cre | Peter Gruss: Marquardt et al., 2001 | N/A |
| Mouse: CAGGCre-ERTM<sup>TM</sup> | The Jackson Laboratory | JAX: 004682 |
| **Recombinant DNA** | | |
| Fzd1 (NM_021457) Mouse Tagged ORF Clone | Origene | Cat#MR209705 |
| Wnt3a (NM_009522) Mouse Tagged ORF Clone | Origene | Cat#MR227165 |
| Wnt16 (NM_053116) Mouse Tagged ORF Clone | Origene | Cat#MR222522 |
| Hes1 (NM_008235) Mouse Tagged ORF Clone | Origene | Cat#MR203818 |
| Ascl1 template plasmid | Marquardt et al., 2001 | N/A |
| Atoh7 template plasmid | Brown et al., 1998 | N/A |
| Axin2 template plasmid | Jho et al., 2002 | N/A |
| Fgf15 template plasmid | Burns et al., 2008 | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents generated in this study should be directed to and will be fulfilled by the Lead Contact, Wei Liu (Wei.Liu@einstein.yu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

The mice were housed in the Animal Institute in Albert Einstein College of Medicine using a standard husbandry. Six3\textsuperscript{F/F} (Liu et al., 2006), Six6\textsuperscript{+/−} (Li et al., 2002), Pax6\textsuperscript{α-Cre} (Marquardt et al., 2001), and CAGGCre-ERT\textsuperscript{M} mice (Hayashi and McMahon, 2002) were maintained in the NMRI background and genotyped using methods as previously described. Six3\textsuperscript{F/F}; α-Cre embryos and mice were generated by crossing female Six3\textsuperscript{F/F} or Six3\textsuperscript{F/−} mice with male Six3\textsuperscript{F/−}; α-Cre mice. Six3\textsuperscript{F/F}; α-Cre; Six6\textsuperscript{−/−} embryos and mice were generated by crossing female Six3\textsuperscript{F/F}; Six6\textsuperscript{−/−} mice with male Six3\textsuperscript{F/F}; α-Cre; Six6\textsuperscript{−/−} mice. For the generation of Six3\textsuperscript{F/F}; CAGGCre-ERT\textsuperscript{M}; Six6\textsuperscript{−/−} embryos, Six3\textsuperscript{F/F}; Six6\textsuperscript{−/−} pregnant dams crossed with male Six3\textsuperscript{F/F}; CAGGCre-ERT\textsuperscript{M}; Six6\textsuperscript{−/−} mice were administered with Tamoxifen using intraperitoneal injections (3 mg in corn oil per mouse each time) on E10.5 and E11.5. Embryos and postnatal mice with the same genotype, the same age were allocated into experimental groups. Retinal phenotypes in DKO retinas were always correlated to the efficiency of conditional Six3-deletion. Six3\textsuperscript{F/F} retinas, α-Cre retinas, and Six3\textsuperscript{F/F}; Six6\textsuperscript{−/−} retinas were indistinguishable in the assays described here, and were used as controls for DKO retinas. Littermates were used when possible. The age of embryos and mice were reported in each analysis. Since gender as variables for retinal cell differentiation have never been reported in literature, embryos and mice with both genders were used indistinguishably in our studies. A minimum of three embryos in each group was analyzed. Animal experiments were approved by the Animal Care and Use Committees in Albert Einstein College of Medicine.

METHOD DETAILS

Immunohistochemistry and in situ hybridization

Embryos were fixed in 4% paraformaldehyde overnight at 4°C, cryopreserved in 30% sucrose, embedded in OCT (Tissue Tek), and stored in −80°C until further use. Transverse frozen sections (10 μm) were used for immunohistochemistry and in situ hybridization using standard protocols. For immunohistochemistry, antigen was first recognized by primary antibodies, and then visualized using...
Alexa Fluor 488- or Cy3.5-conjugated secondary antibodies under Zeiss AxioObserver Z1 microscope. These primary antibodies are used: Choline Acetyltransferase (Millipore AB144P, 1:400), Cone Arrestin (Millipore AB15282, 1:5000), GAD65 (Fisher Scientific BDBS59931, 1:200), GABA (Sigma A2052, 1:100), GFAP (Sigma G9269, 1:4000), Glu (also known as glutamine synthetase, BD Bioscience #610517, 1:500), Glycine Transporter 1 (Millipore AB1770, 1:5000), Laminin-1 A&B (Millipore MAB1904, 1:500), Lhx2 (Santa Cruz sc-19344, 1:1600), Neurofilament M (Millipore AB1987, 1:500), Otx2 (R&D Systems AF1979, 1:1500), p27 (Santa Cruz sc-776, 1:200), Pax6 (Covance #PRB-278P, 1:500), pH3 (Upstate #06-570, 1:250), Prkcz (also known as PKC ε, Santa Cruz sc-216, 1:400), Pou4f2 (also known as Bm3b, Santa Cruz sc-6026, 1:1000), Rho (Abcam ab98887, 1:500), Six3 (Rockland #600-401-A26, 1:500), Sox2 (Chemicon #AB8757, 1:1500), Syntaxin (Sigma #S0664, 1:1000), Tyrosine Hydroxylase (Millipore AB152, 1:400), Tubb3 (BabCO MMS435P clone Tuj1, 1:500), and Vspx2 (Abcam #AB9016, 1:500).

To prepare DIG-labeled RNA probes for *in situ* hybridization, cDNA of mouse genes (Origene) were subcloned into pBluescript plasmid. The resulting template plasmids were linearized for antisense direction, and *in vitro* transcribed using DIG RNA Labeling Kit (Roche #11277073910). Template plasmids were also requested from other laboratories. The *in situ* hybridization probes are: Ascl1 (Xba/SPE), Atoh7 (Asc1/T7), Axin2 (NotI/T3), CyclinD1 (NarI/T7), FGF15 (NotI/T7), Foxn4 (BglII/T3), Fzd5 (NotI/T7), Fzd1 (BamHI/T3), Gli1 (NotI/T3), Hes1 (EcoRI/T3), Hes5 (BamHI/T7), Neurod1 (XhoI/T7), Neuod4 (XhoI/T3), Neurog2 (NotI/T7), Notch1 (Cia/T7), Otx1 (Ndel/T7), p57 (SalI/T3), Shh (HindIII/T3), Six3F/F; Six6+/–, Six6+/+; ear (Sp6), Sox2 (Chemicon #AB5770, 1:800), Syntaxin (Sigma #S0664, 1:1000), Tyrosine Hydroxylase (Millipore AB152, 1:400), Tubb3 (BabCO MMS435P clone Tuj1, 1:500), and Vspx2 (Abcam #AB9016, 1:500).

E12.5 eyecups containing the neuroretina and lens were dissected out from wild-type NMRI embryos, rinsed, and placed into a 24-well plate on an orbital shaker for 6-day culture (37°C, 5% CO2). The culture medium (DMEM/F12, 8% FBS, 2% B27, and PenStrep) was supplemented with recombinant human Wnt-3a (250 ng/mL, R&D Systems 5036-WN-010), Wnt-16b (250 ng/ml, R&D Systems 7790-WN-025), a GSK3β inhibitor CHIR99021 (3 μM, TOCRIS), or vehicles (0.1% BSA or DMSO), with a medium change on day 3. After the culture, the eyecups were fixed in 4% PFA in PBS for 2 hr at room temperature, and then processed for cryosections. Three eyecups in each group were analyzed.

**Transcriptome profiling using RNA-seq**

Six3F/F; Six6+/– dams crossed with Six3F/F; CAGGCre-ERTM; Six6+/– male mice were intraperitoneally injected with Tamoxifen (0.15 mL of 20 mg/mL stock solution in corn oil per mouse for each injection) at E10.5 (approximately 2pm) and E11.5 (approximately 9 am). One pair of retinas from each E13.5 embryo (noon) were dissected out in ice-cold PBS, rinsed, and then stored individually in 100 μL of RNAlater (Ambion) in an Eppendorf tube overnight at 4 °C. After RNAlater was removed, the samples were stored at −80 °C until purification.

The retinas with the following genotypes were used for RNA isolation using the RNAsesy Micro Kit (QIAGEN) after genotyping: 1) WT (Six3F/F; Six6+/–); 2) Six3KO (Six3F/F; CAGGCre-ERTM); 3) Six6KO (Six3F/F; Six6+/–); 4) DKO (Six3F/F; CAGGCre-ERTM, Six6+/–). For each genotype group, RNA from one pair of retinas was individually isolated, with three pairs of retinas in total. The retinas were homogenized with an electric pellet pestle motor in 500uL of Qiazol. Total RNA was purified using micro columns, treated with RNase-free DNase set (QIAGEN), and eluted in 14uL of RNase-free water. The quantity and quality of RNA were verified using Qubit fluorometer (Thermo Fisher Scientific) and Bioanalyzer (Agilent Genomics), respectively.

Total RNA (181.2-792 ng for each sample, RIN > 9) was used for library preparation using KAPA RNA HyperPrep Kit with RiboseErase (HM) (Kapa Biosystems). Sequencing was run on Illumina HiSeq 2500 in 100-bp single-end high-output mode in the Einstein Epigenomics Core Facility. About 30 million reads were generated for each sample. Each genotype group initially had three biological replicates, but one Six6KO replicate was later removed due to over duplication. After trimming adaptor with Trim Galore (v. 0.3.7), RNA-seq reads were aligned back to mouse genome mm10 using Tophat (v. 2.0.13) (Trapnell et al., 2009). The number of reads mapped back to each gene was calculated with HTseq (v.0.6.1) (Anders et al., 2015) using Refseq gene annotation (downloaded from the UCSC genome browser in 03/17). The Cuffdiff in Cufflinks package (v.2.2.1) (Trapnell et al., 2010) was used to generate FPKM values. We focused on 13498 genes that have FPKM value > 1 in at least one of samples. Deseq2 (Love et al., 2014) was used to determine the differentially expressed genes (DEGs) with FDR (padj) less than 0.05 as a cutoff unless it is stated otherwise. For visualization, heatmap was plotted using the normalized counts generated by DESeq2. For functional annotation of the DEGs from the comparison between three DKO and three WT samples (padj < 0.05, log2FC > 1, 333 analysis-ready DEGs), Ingenuity Pathway Analysis (Krämer et al., 2014) was used.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Cell counting**

Transverse frozen sections (10 μm) at levels close to optic nerve were used for either immunostaining or TUNEL assay (ApoTag Kit, Chemicon). The numbers of positive cells (pH3 positive cells, TUNEL positive cells, Otx2 positive cells, and Pou4f2 positive cells) in the marked peripheral areas were counted, and the lengths of the marked apical surface were measured using ImageJ (Schneider et al., 2012). The density of positive cells was expressed as the number of positive cells per 1 mm of marked apical length per section. Retinas from three wild-type and three DKO embryos were used for cell counting.
**Statistical analysis**

*p value* of Student’s *t* test (two tailed distribution, equal variance), determined by Microsoft Excel for Mac 2011 software, was used to compare the two means for wild-type and DKO retinas in the density of pH3 positive cells, TUNEL positive cells, Otx2 positive cells, and Pou4f2 positive cells, with *p* < 0.001 considered statistical significance.

**DATA AND SOFTWARE AVAILABILITY**

**Data deposit**
The accession number for the RNA-seq data reported in this paper is GEO: GSE115258. ([https://www.ncbi.nlm.nih.gov/geo](https://www.ncbi.nlm.nih.gov/geo)).

**Software availability**
The software used for RNA-seq analysis in this study is available using the URLs listed in Key Resources Table.
Supplemental Information

Six3 and Six6 Are Jointly Required for the
Maintenance of Multipotent Retinal Progenitors
through Both Positive and Negative Regulation

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Inventory of Supplemental Information

1. Supplemental figures: Fig. S1-S7
2. Supplemental tables: Tab. S1-S4
Supplemental figure legends

A) Six3
B) Six3<sup>-/-</sup>; α-Cre
C) α-Cre
D) Six3<sup>-/-</sup>; α-Cre

E) Atoh7
F) Neurog2
G) Ascl1
H) Rax
I) p27
J) pH3

Legend:
- A) Six3
- B) Six3<sup>-/-</sup>; α-Cre
- C) α-Cre
- D) Six3<sup>-/-</sup>; α-Cre
- E) Atoh7
- F) Neurog2
- G) Ascl1
- H) Rax
- I) p27
- J) pH3
**Fig. S1.** *Six3*-deletion using α-Cre at E10.5 does not cause overt phenotypes at E14.5. Related to Fig. 1. (n = 3 embryos). 

(A, B) *Six3* was efficiently deleted in peripheral retinas. 

(C-L) NR progenitor markers Pax6, Atoh7, Neurog2, Ascl1, and Rax were unaffected in *Six3*-deficient retinas. 

(M, N) Cyclin kinase inhibitor p27, a marker for newly differentiated neurons, was unaffected in *Six3*-deficient retinas. 

(O, P) pH3 immunostaining was unaffected in *Six3*-deficient retinas. Scale bars, 100 µm.
Fig. S2. Hes5, Neurod4, and Fzd5 are downregulated whereas p57 is ectopically upregulated in E14.5 DKO retinas. Related to Fig. 2. (n = 3 embryos). (A-D) Hes5
were unaffected in Six3\textsuperscript{F/}; Six6\textsuperscript{+/−} retinas, slightly reduced in Six3\textsuperscript{F/+}; α-Cre; Six6\textsuperscript{−/−} retinas, but significantly reduced in the far peripheral regions of DKO retinas (brackets in H). (E-H) NR progenitor marker Neurod4 was unaffected in either Six3\textsuperscript{F/}; Six6\textsuperscript{+/−} retinas or Six3\textsuperscript{F/+}; α-Cre; Six6\textsuperscript{−/−} retinas. In DKO retinas, Neurod4 was severely reduced in far peripheral regions (areas I and V), and moderately reduced in mid peripheral regions (areas II). (I-L) NR progenitor marker Fzd5 was unaffected in Six3\textsuperscript{F/}; Six6\textsuperscript{+/−} retinas, slightly reduced in Six3\textsuperscript{F/+}; α-Cre; Six6\textsuperscript{−/−} retinas. In DKO retinas, Fzd5 was significantly reduced in both far and mid peripheral regions (areas I and II). (M-P) In Six3\textsuperscript{F/F}; Six6\textsuperscript{+/−} control retinas, p57 was moderately expressed in the CM and marginally expressed in the NR. p57 was unaffected in Six3\textsuperscript{F/}; Six6\textsuperscript{+/−} retinas, marginally increased in Six3\textsuperscript{F/+}; α-Cre; Six6\textsuperscript{−/−} NR, but was ectopically upregulated in DKO retinas (brackets in M, P). Scale bar, 100 µm.
Fig. S3. Atoh7, Neurog2, and Fgf15 are affected in E14.5 DKO retinas. Related to Fig. 2. (n = 3 embryos). (A-D) Atoh7 was unaffected in Six3F/F; Six6−/− retinas and Six3F/F; α-Cre; Six6+/− retinas, but was significantly reduced in both far and mid peripheral regions of DKO retinas. (E-L) Neurog2 and Fgf15 was unaffected in Six3F/F; Six6−/− retinas and Six3F/F; α-Cre; Six6+/− retinas, but was significantly reduced in the far peripheral regions of DKO retinas. Scale bar, 100 µm.
Fig. S4. Proliferation is decreased and apoptosis is increased in E14.5 DKO retinas. Related to Figs. 2, 3. (n = 3 embryos). (A-H) In Six3\textasciitilde F/F, Six6\textasciitilde control retinas, proliferation marker Ccnd1 was found in the NR, but not in the CM (A). Ccnd1 was unaffected in Six3\textasciitilde F/F, Six6\textasciitilde \textasciitilde retinas, slightly reduced in Six3\textasciitilde F/F; α-Cre; Six6\textasciitilde \textasciitilde retinas, but nearly absent in the peripheral regions of DKO retinas (D). (E-H) In DKO retinas, pH3 positive cells was reduced in density in far peripheral regions (dashed lines in H), and occasionally mis-localized in mid peripheral regions (arrowhead in H). The red dots outline the basal surface of DKO retinas (H). (I-L) Apoptosis was rare in both Six3\textasciitilde F/F, Six6\textasciitilde \textasciitilde retinas and Six3\textasciitilde F/F, Six6\textasciitilde \textasciitilde retinas, slightly increased in Six3\textasciitilde F/F; α-Cre; Six6\textasciitilde \textasciitilde retinas, but significantly increased in the far peripheral regions of DKO retinas (L). (M, N) Quantification for pH3 and TUNEL positive cells, respectively. pH3 index was significantly increased, but TUNEL index was significantly decreased when DKO retinas were compared to Six3\textasciitilde F/F, Six6\textasciitilde \textasciitilde control retinas (n = 3 embryos, p < 0.001). (O-R) Apical junctions were defective in E15.5 DKO retinas. Scale bars, 100 μm (A, E, I), 50 μm (O).
Fig. S5. Shh, Pou4f2, Foxn4, and Pax6 are affected in E15.5 DKO retinas. Related to Fig. 4. (n = 3 embryos). (A-D) Shh was unaffected in either Six3^{F/F}; α-Cre retinas or Six3^{F/F}; Six6^{-/-} retinas. In DKO retinas, Shh was nearly absent in far peripheral regions (areas I and V), and significantly reduced in mid peripheral regions (areas II and IV). (E-H) Pou4f2 was unaffected in either Six3^{F/F}; α-Cre retinas or Six3^{F/F}; Six6^{-/-} retinas. In DKO retinas, Pou4f2 was nearly absent in far peripheral regions, and significantly reduced in both expression levels and numbers in mid peripheral regions. Quantification (H') represents three eyes (**, p<0.005 for t-test). (I-L) Foxn4 was unaffected in either Six3^{F/F}; α-Cre retinas or Six3^{F/F}; Six6^{-/-} retinas. In DKO retinas, Foxn4 was nearly absent in far peripheral regions (areas I and V), and disorganized in mid peripheral regions (areas II and IV). (M-P) Pax6 expression was unaffected in either Six3^{F/F}; α-Cre retinas or Six3^{F/F}; Six6^{-/-} retinas, but increased in the far peripheral regions of DKO retinas. (Q-T) In Six3^{F/F}; α-Cre retinas, Cre expression was widely found in the peripheral retinas at E12.5, but restricted to ciliary margins at E15.5 (Q, R; compare Cre expression in panel R to Otx1 expression in Fig. 4U). In DKO retinas, however, Cre expression was continuously found in wide areas at E15.5: at high levels in area I and at moderate levels in area II (T). Cre and Otx1 were co-localized in area I (T; compare Cre expression in panel T to Otx1 expression in Fig. 4X). Scale bars, 200 µm (A), 100 µm (Q, R).
Fig. S6. DKO retinas on postnatal day 11 comprise a subpopulation of amacrine cells. Related Fig. 5. (n = 3 retinas). (A-X) Six3\textsuperscript{F/F}; α-Cre retinas and Six3\textsuperscript{F/F}; α-Cre; Six6\textsuperscript{+/−} retinas expressed amacrine cell markers similarly to Six3\textsuperscript{F/F} control retinas. In contrast, DKO retinas widely expressed amacrine cell markers Calretinin, GAD65, Pax6, GABA, and Tyrosine Hydroxylase (TH) (A-P), but did not expressed amacrine cell
markers GlyT1 and ChAT (Q-X). DKO retinas rarely expressed retinal ganglion cell and amacrine cell marker Tubb3 (A-D), and did not expressed Müller glial marker Lhx2 (M-P). Scale bar, 100 µm.
Fig. S7. DKO retinas on postnatal day 11 lack Müller glia and are infiltrated with astrocytes. Related to Fig. 5. (n = 3 retinas). (A-H) Similar to Six3^F/F retinas, Six3^F/F; α-Cre retinas and Six3^F/F; α-Cre; Six6^+/- retinas expressed Müller glial markers Glul and Lhx2 (A-D). In addition, astrocyte marker Gfap was restricted to the inner limiting membrane (E-G). In contrast, DKO retinas did not express Glul and Lhx2, but were widely infiltrated with Gfap positive astrocytes (D, H). Scale bar, 100 µm.
Legends for supplemental tables

Table S1. A list of DEGs resulting from the comparison between three DKO s and three WTs using padj < 0.05 as a cutoff. Related to Fig. 6.

Table S2. A combined list of DEGs resulting from the comparison between three DKO s and three WTs and the comparison between two DKO s and two WTs using padj < 0.05 as a cutoff. Related to Fig. 6.

Table S3. A list of DEGs resulting from the comparison between three Six3KO s and three WTs using padj < 0.05 as a cutoff. Related to Fig. 6.

Table S4. A list of DEGs resulting from the comparison between two Six6KO s and three WTs using padj < 0.05 as a cutoff. Related to Fig. 6.