Title: Regulation of Brn3b by Dlx1 and Dlx2 is required for retinal ganglion cell differentiation in the vertebrate retina

Authors: Qi Zhang1,*, Jamie Zagozewski2,&, Shaohong Cheng3,**, Rajiv Dixit5,*, Shunzhen Zhang2, Jimmy de Melo1,&&, Xiujian Mu6,#, William H. Klein6, Nadean L. Brown7, Jeffrey T. Wigle3, Carol Schuurmans5,*, and David D. Eisenstat1,2,3,4,*,##,+

Affiliations: Human Anatomy and Cell Science1, Biochemistry and Medical Genetics2, Pediatrics and Child Health3, Ophthalmology4, University of Manitoba, Winnipeg, Canada; Hotchkiss Brain Institute5, University of Calgary, Canada; Department of Biochemistry and Molecular Biology6, University of Texas M.D. Anderson Cancer Center, Houston, TX; and Department of Cell Biology and Human Anatomy7, University of California, Davis CA.

Current addresses:
*Pathology and Laboratory Medicine, Western University, London, Canada
**VA Medical Center, Loma Linda CA 92357
&Medical Genetics, University of Alberta, Edmonton, Canada
&& Johns Hopkins University School of Medicine, Baltimore MD
#Ophthalmology, University at Buffalo, Buffalo, NY
^Sunnybrook Research Institute, University of Toronto, Toronto, Canada
##Pediatrics, University of Alberta, Edmonton, Canada

*Correspondence should be addressed to eisensta@ualberta.ca

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**Summary statement:**

*Dlx1/2* homeobox genes regulate retinal ganglion cell (RGC) differentiation by directly activating *Brn3b*. Triple knockout mice have near complete RGC loss with a marked increase in amacrine cells.

**Abstract:**

Regulated retinal ganglion cell (RGC) differentiation and axonal guidance is required for a functional visual system. Homeodomain and basic helix loop helix transcription factors are required for retinogenesis, as well as patterning, differentiation and maintenance of specific retinal cell types. We hypothesized that Dlx1/Dlx2 and Brn3b homeobox genes function in parallel intrinsic pathways to determine RGC fate and generated Dlx1/Dlx2/Brn3b triple knockout mice. A more severe retinal phenotype was found in the Dlx1/Dlx2/Brn3b null retinas than predicted by combining features of the Brn3b single and Dlx1/Dlx2 double knockout retinas, including near total RGC loss with a marked increase in amacrine cells in the ganglion cell layer. Furthermore, we discovered that DLX1 and DLX2 function as direct transcriptional activators of Brn3b expression. Knockdown of Dlx2 expression in primary embryonic retinal cultures and Dlx2 gain-of-function in utero strongly support that DLX2 is both necessary and sufficient for Brn3b expression in vivo. We suggest that Atoh7 specifies RGC committed progenitors and that Dlx1/Dlx2 functions both downstream of Atoh7 and in parallel but cooperative pathways involving regulation of Brn3b expression to determine RGC fate.
Six classes of neurons and Müller glial cells are generated from a common retinal progenitor cell (RPC) population, following a precise temporal sequence (Livesey and Cepko, 2001). The first differentiated cells in the mouse are retinal ganglion cells (RGC), followed by horizontal, cone and amacrine cells. Development of rod photoreceptors, bipolar cells and Müller glia extends into the second postnatal week (Cepko et al., 1996).

Transcription factors (TFs) act intrinsically during different stages of retinogenesis. Homeodomain (HD) and basic helix-loop-helix (bHLH) TFs initiate retinal development and maintain RPC (Ohsawa and Kageyama, 2008). Over-expression of seven eye field TFs in *Xenopus* embryos can induce eye-like structures with functional properties (Viczian et al., 2009).

*Atoh7* (*Math5*) is a bHLH TF essential for RPC to become RGCs (Brown et al., 2001; Wang et al., 2001; Yang et al., 2003). *Brn3b (Pou4f1)* genetically downstream of *Atoh7* is required for the terminal differentiation and survival of most RGCs, but not their initial specification (Erkman et al., 1996; Erkman et al., 2000; Gan et al., 1996; Xiang, 1998). *Brn3b* and *Brn3a (Pou4f1)* have overlapping yet distinct roles controlling RGC development and function (Badea et al., 2009). *Isl1*, a LIM-HD TF, under the regulation of ATOH7, defines a distinct but overlapping sub-population of RGCs with *Brn3b* (Mu et al., 2008; Pan et al., 2008). This *Atoh7-Brn3b/Isl1* pathway determines a population of RGCs, whereas other RGCs rely on the Distal-less homeobox genes *Dlx1* and *Dlx2* for their differentiation and survival (de Melo et al., 2005; de Melo et al., 2008).

Retinas from *Dlx1/Dlx2*−/− mice have reduced RGCs due to enhanced apoptosis of late-born RGCs (de Melo et al., 2005). DLX1/DLX2 regulation of the *Dlx5/6* intergenic enhancer and brain derived neurotrophic factor mediated TrkB signalling may contribute to the differentiation and survival of RGCs, respectively (de Melo et al., 2008; Zhou et al., 2004).

DLX2 and BRN3b are expressed in distinct but partly overlapping regions in the retinal neuroepithelium (de Melo et al., 2003). Furthermore, DLX2 and to a lesser extent DLX1, are expressed in cycling as well as postmitotic RPC (Eisenstat et al., 1999). We hypothesized that *Dlx1/Dlx2* and *Brn3b* function in parallel intrinsic pathways to determine RGC fate and
generated Dlx1/Dlx2/Brn3b triple knockout (TKO) mice. We found almost complete RGC loss with a marked increase in amacrine cells in the ganglion cell layer (GCL). DLX1 and DLX2 were also identified as transcriptional activators of Brn3b expression supported by in utero retinal electroporation of Dlx2 and siRNA-mediated knockdown of Dlx2 in primary embryonic retinal cultures. Taken together, Dlx1 and Dlx2 are necessary and sufficient for Brn3b expression during retinal development.

RESULTS

Loss of Dlx1/Dlx2 and Brn3b gene function leads to defective RGC specification

In the Dlx1/Dlx2 DKO there is 33% loss of late-born RGCs at E18.5, whereas Brn3b deletion results in a 60-70% reduction of RGCs in the postnatal retina depending upon the genetic background. However, neither the Dlx1/Dlx2 DKO nor Brn3b single knockouts (SKO) have defects in other retinal cell classes (de Melo et al., 2005; Erkman et al., 1996; Gan et al., 1996). We hypothesized that the TKO retina would have severe abnormalities in RGC differentiation and survival with a significantly reduced GCL. Dlx1/Dlx2/Brn3b TKO mice die shortly after birth at P0. Unexpectedly, the TKO retina showed only a modestly decreased GCL (Fig. 1a A,D) whereas the inner plexiform layer (IPL) separating the GCL and NBL was significantly reduced (p<0.05, Fig. 1a D boxed, E).

Few RGCs were detected in the TKO (Fig. 1b D,H) with ~95% reduction of BRN3A+ RGCs in the TKO (78.5±14) compared to wild-type (2701±148) (p<0.01, n=4) and ~80% loss of ISL1+ cells in the TKO (p<0.01, n=4) (Fig. 1b M,N). Decreased BRN3A+ cells (37%) and ISL1+ cells (40%) were also observed in Dlx1/Dlx2 DKO retinas (Fig. 1b C,G,M,N). However, RGC loss in Brn3b null retinas (64% of BRN3A cells, 56% of ISL1 cells) at E18.5 (Fig. 1b B,F,M,N) did not reach the 70% loss previously reported (Erkman et al., 1996; Gan et al., 1999; Gan et al., 1996). At E16.5, both BRN3A+ and ISL1+ RGC were diminished in Brn3b SKO, Dlx1/Dlx2 DKO and TKO retinas. At E13.5, ISL1 was used to detect RGC due to low BRN3a expression with 82% reduction of ISL1+ expression, but only in the TKO (p<0.01, n=4) (Fig. 1b I-L,O). Similar reductions of ISL+ RGCs were observed in E12.5 TKO retinas (Fig. S2). Dlx1/Dlx2 and Brn3b may have redundant functions during early
retinogenesis, since neither knockout mouse demonstrated defective early retinal differentiation.

**Increased amacrine cells in the Dlx1Dlx2/Brn3b null ganglion cell layer**

Amacrine, horizontal and cone cells all have overlapping birthdates with RGC. PAX6+ cells located in the inner NBL were counted as amacrine cells. No significant difference was observed (Fig. 2A-D,Q). PAX6 is expressed in both RGC and displaced amacrine cells in the GCL at E18.5 (Belecky-Adams et al., 1997; de Melo et al., 2003; Inoue et al., 2002). PAX6+ cells in the GCL of Brn3b SKO and Dlx1/Dlx2 DKO were reduced (Fig. 2B,C) due to RGC loss (de Melo et al., 2005; Gan et al., 1996). However, in the TKO GCL, there was only minimal PAX6+ cell reduction (Fig. 2D), supporting more displaced amacrine cells in the TKO GCL. Syntaxin is present in all amacrine cells but not RGCs (Barnstable et al., 1985). TKO GCL cells were only partially reduced (Fig. 2H). A significant 1.8 fold increase of syntaxin+ cells was observed in the TKO GCL (1761±122) compared with wild-type (930±72) (p<0.01, n=4) (Fig. 2E-H,R). However, syntaxin+ cells in Brn3b SKO and Dlx1/Dlx2 DKO GCL were not significantly altered.

No significant abnormalities in horizontal cell number or position were observed in TKO retinas (Fig. 2I-L, S). Most cone photoreceptors are born prenatally in mice, but many markers are not detected until P5 (Chow et al., 2001). Recoverin is expressed by cones and cone bipolar cells from E17.5 (Milam et al., 1993). No abnormalities in recoverin+ cell position (Fig. 2M-P) or number (Fig. 2T) were identified.

To determine the identity of displaced amacrine cells of TKO retinas, we cultured E18.5 TKO and wild-type (WT) retina explants for 7 days in vitro (DIV7). GABAergic and glycinergic cells represent almost 90% of amacrine cells (MacNeil and Masland, 1998). Glutamic acid decarboxylase (GAD) isoforms, GAD65 and GAD67, were similarly expressed in the IPL and GCL of WT and TKO DIV7 retinas (Fig. 3A, B). Starburst cholinergic amacrine cells (expressing choline acetyltransferase, ChAT) are early born GABAergic amacrine cells (Voinescu et al., 2009). Compared to WT littermates (Fig. 3E), more ChAT+ cells are observed in the TKO GCL (98.4±9.7 versus 59.5±3.4, p<0.01, n=4) (Fig. 3F). However, there was no difference in the expression of a glycinergic amacrine cell
marker, glycine transporter (GlyT1) (Fig. 3G, H). Similarly, there was no difference in expression of the bHLH NEUROD1 across the four genotypes (Fig. S3). Upregulation of ChAT but not GlyT1 in TKO retinas is consistent with increased early born amacrine cells.

Birth-dating experiments labelled RPC with a single BrdU pulse in pregnant animals at E12.5, E13.5 or E16.5. Most E12.5 and E13.5 birth-dated cells were located in the GCL, without differences in quantity or spatial expression between TKO and WT controls (Fig. 4A,B,E,F; data not shown). Few E16.5 birth-dated cells migrated to the GCL; most remain in the NBL (Fig. 4C,D,G,H). BrdU+ cell numbers in the GCL were similar between WT and TKO in the E12.5, E13.5 and E16.5 birth-dated retinas (Fig. 4I). Early migration of RPC to the GCL was unaffected in the TKO. Few cells were co-labelled by BRN3a and BrdU in E12.5 birth-dated TKO retinas (14±1 of mutants versus 199±10 of WT, p<0.005, n=4; Fig. 4A,B,J). However, more cells were co-labelled with syntaxin and BrdU in the GCL (Fig. 4E,F,K). Similar results were observed in E13.5 as well as E16.5 birth-dated mutants (Fig. 4C,D,G,H,J,K; data not shown). Hence, in TKO retinas, most progenitors that exit mitosis migrate to the GCL and express amacrine rather than RGC markers.

Increased apoptosis and abnormal cell division in Dlx1/Dlx2/Brn3b null retinas

At E13.5, the TKO had a significant 4-fold increase in apoptotic cells (70.5±11 of mutants versus 15.5±7 of WT, p<0.01, n=4; Fig. 5A,B,G). However, for E16.5 and E18.5 TKO, apoptotic cell numbers were similar to WT (Fig. 5G). In the TKO at E13.5, the majority of caspase-3+ cells were confined to the inner retina, where prospective RGCs are located. Unlike the Brn3b SKO or Dlx1/Dlx2 DKO, enhanced apoptosis later than E13.5 was not detected in the TKO.

TKO retinas displayed a significant reduction (46% and 41%) in M-phase cells at E16.5 (Fig. 5D,H) and E18.5, respectively (Fig. 5H). Similarly, fewer S-phase cells were detected in E16.5 and E18.5 mutants (data not shown). Flow cytometry yielded concordant results at E16.5 and E18.5, with significantly reduced proportions of cells in S phase (p=0.005, n=3) but changes in G2/M and G1/G0 phases were not significant (Fig. 5E,F,I; data not shown).
**Expression of BRN3b and DLX2 in Atoh7 (Math5) null retinas**

Math5/DLX2 co-expressing cells are present at E11.5 when DLX2 is first detected (Fig. 6 A-C), extending to E13.5 (Fig. 6 D-F), but co-expression is absent at E18.5 (Fig. 6 E16.5 G-I; E18.5 J-K). Math5 expression was unaffected in the Brn3b SKO, Dlx1/Dlx2 DKO and the TKO (Fig. 7a). BRN3b expression was severely reduced in the Math5-/- retina at E13.5 (Fig. 7b G, H) and E16.5 (Fig. 7b I, J). However, DLX2 expression was significantly reduced at E13.5 (p=0.01, Fig. 7b A, B, E) but not significantly in the Math5 null GCL at E16.5, a time when Math5 expression is down-regulated (p=0.09, Fig. 7b C, D, F). Interestingly, of the few BRN3b+ cells remaining in the Math5-/- GCL at E16.5, a proportion co-express DLX2 (data not shown), supporting a role for DLX2 in promoting or maintaining Brn3b expression in the absence of Math5 function.

**DLX1/DLX2 regulate Brn3b by specific binding to the Brn3b promoter in vivo**

As development proceeds, more BRN3b cells express DLX2, and from E16.5, all BRN3b+ cells co-express DLX2 (Fig. S1). We postulated that DLX1 and/or DLX2 regulate Brn3b transcription during retinogenesis. We used chromatin immunoprecipitation (ChIP) of embryonic retina and specific antibodies to detect DLX proteins localized to the Brn3b promoter region in vivo. Candidate regions were selected based on groups of putative TAAT/ATTA homeodomain consensus DNA-binding motifs. We focused on a region of the Brn3b promoter 2,263 bp upstream of the start codon. ChIP was performed by using PFA cross-linked cells prepared from E16.5 retina and hindbrain used as a negative tissue control (Zhou et al., 2004). For PCR analysis we designated seven fragments as 3bP1-7. Only site 3bP4 was amplified from DLX1 or DLX2 immunoprecipitated DNA, supporting DLX1 and DLX2 occupancy of this Brn3b promoter region in E16.5 retina (Fig. 8a A, B).
Radiolabeled 3bP4 oligonucleotide fragments were incubated with recombinant DLX1/DLX2 proteins; we observed two specific protein-DNA band shifts as DLX1-3bP4 and DLX2-3bP4 complexes in electrophoretic mobility shift assays (EMSA) (Fig. 8a C, lanes 2,7). These bands were competitively inhibited by unlabeled 3bP4 probe (Fig. 8a C, lanes 3,8), and were “supershifted” by addition of specific DLX1 or DLX2 antibodies (Fig. 8a C, lanes 4,9). IgG was used as a control antibody (Fig. 8a C, lanes 5,10).

We then performed transient co-transfection and site-directed mutagenesis assays. A plasmid expressing Dlx1, Dlx2, or both was co-transfected into HEK293 cells with a vector in which the Brn3b 3bP4 promoter region drives reporter gene expression. DLX1 and DLX2 co-transfection resulted in 2.1 and 2.5 fold increases of luciferase activity, respectively (Fig. 8b). Co-transfection of Dlx1 with Dlx2 yielded similar results to Dlx2 co-transfection alone (Fig. S4). Mutations of any of the three candidate binding motifs within the 3bP4 promoter region significantly reduced luciferase activity by DLX1 and DLX2 in vitro, suggesting that DLX1 or DLX2 activation of 3bP4 promoter expression may occur via any one of these binding sites.

**Brn3b expression is decreased by Dlx2 knockdown in primary embryonic retinal cultures**

Primary cultures of WT E14.5 retina were transiently transfected with siRNA targeting the Dlx2 coding sequence or a scrambled control siRNA (de Melo et al., 2008). There was efficient knockdown of Dlx2 mRNA, with a concomitant decrease of Brn3b mRNA expression, compared to the transfection of control siRNA and untreated cells (Fig. 8c). Taken together with the RGC phenotype of the Dlx1/Dlx2 DKO (de Melo et al., 2005), these knockdown experiments support that Dlx2 function is necessary for Brn3b expression in situ.
**Gain of Dlx2 function in utero results in ectopic Brn3b expression in vivo**

Gain-of-function assays in retinal explants are compromised by the loss of trophic support of RGCs due to optic nerve transection during tissue preparation (de Melo et al., 2008). Subsequently, we ectopically expressed Dlx2 in the intact embryo. E14.5 retinas were electroporated in utero with pCIG2-mCherry-Dlx2 (Fig. 9A-G) or pCIG2-mCherry control plasmids (Fig. 9H-J) then harvested at E18. In pCIG2-mCherry-Dlx2 electroporated retinas, mCherry epifluorescence reflects ectopic DLX2 expression in the outer NBL (arrows in Fig. 9C,D) with ectopic BRN3b expression in the outer NBL (arrows in Fig. 9F,G). Endogenous DLX2 and BRN3b expression was also detected in the GCL and INL (stars in Fig. 9C,D,F,G,I,J). No ectopic BRN3b was detected in the control NBL (Fig. 9I,J). Similar results were obtained from additional experimental (3) and control (2) electroporated retinas. To distinguish from endogenous DLX2 or BRN3b expression, cell counting was performed on mCherry+ cells located in outer NBL but not the GCL. 84% mCherry+ cells express DLX2 in Dlx2 electroporated retinas. 12% of these mCherry+ cells co-express BRN3b in Dlx2 but not in control electroporated retinal patches in vivo. p<0.01, n=4 (Fig. 9K). These in utero gain of function experiments support that exogenous Dlx2 gene function is sufficient for Brn3b expression in the embryonic retina in vivo.

**Regulation of Dlx2 expression by BRN3B is not mediated by direct interactions with Dlx1/Dlx2 regulatory regions in vivo**

Brn3b negatively regulates Dlx1 and Dlx2 in the embryonic retina (Mu et al., 2005; Qiu et al., 2008). We observed a transient increase in DLX2 expression at E13.5 but not at E11.5 or E16.5 in the Brn3b SKO retina (Fig. S6). Since RGC apoptosis occurs in Brn3b mutants at ~E15.5, the initial increase in DLX2 expression could be lost due to the ensuing cell death. Repression of Dlx2 by BRN3b could occur earlier, during the peak of early-born RGC differentiation regulated by Atoh7-Brn3b. This repression is removed with diminishing Atoh7 expression after E16.5 (Brown et al., 1998).
We tested whether this negative regulation could be due to occupancy of Dlx1/Dlx2 regulatory regions by BRN3b. Cis-acting elements of the Dlx1/Dlx2 bigenic cluster include two intergenic enhancers, I12b and I12a, and two upstream regulatory elements (URE), URE1 and URE2 (Ghanem et al., 2003; Ghanem et al., 2007) (Fig. S7). These elements were examined for consensus Pou4f2 DNA-binding sites [5’- (A/G)TTAATGAG(C/T)-3’] (Xiang et al., 1995); two putative binding sites are in URE2 with one site in I12b. Two putative Pou4f2 binding sites were also found in Dlx1 exon 3 and Dlx2 exon 2. BRN3b directly regulates expression of Eomes, a T-box TF (Mao et al., 2008a). BRN3b occupancy was not detected at any of these four Dlx1/Dlx2 cis-regulatory regions, although we replicated binding to the Eomes promoter (Fig. S7). Hence, repression of Dlx1/Dlx2 expression by BRN3b is likely mediated by protein-protein interactions (Feng et al., 2011) rather than transcriptional regulation.

**DISCUSSION**

*Downstream of Atoh7, Dlx1/Dlx2 and Brn3b are necessary for RGC specification*

Neither Dlx1/Dlx2 nor Brn3b is necessary for the initiation of RGC differentiation. Expression of TFs positioned upstream of Dlx1/Dlx2 or Brn3b, such as Atoh7 and Vsx2 (Chx10), is required for the competence of RPC to initiate expression of Brn3b and Dlx1/Dlx2 for terminal differentiation, maturation and survival of RGC (Wang et al., 2001; Yang et al., 2003). DLX1/DLX2 expression originates in Chx10+ cells (de Melo et al., 2003); similarly, using lineage tracing in the zebrafish retina, Ath5 expressing progenitors originate from Vsx2+ RPC (Jusuf et al., 2011; Vitorino et al., 2009).

The observed 70% loss of RGCs in Brn3b KO mice occurs at E15.5 or later, whereas 33% loss of RGCs in the Dlx1/Dlx2 DKO mice is detected by E18.5 (de Melo et al., 2005; Gan et al., 1999). Unlike these “late” RGC losses, we found that the TKO resulted in a dramatic reduction of RGCs by E12.5. Although most E12.5 and E13.5 birth-dated retinal cells were in the GCL, few expressed RGC differentiation markers in the TKO. In addition to increased apoptosis in the TKO, decreased proliferation as demonstrated by decreased staining for phospho-H3 (M-phase marker) and reduced S-phase (by FACS analysis) could
also contribute to the observed dramatic RGC loss. Potentially, this cell cycle phenotype in the TKO could be due to increased expression of cell cycle regulators such as p53, CDKN1B (p27) or CDKN1C (p57). Hence, without affecting early migration of RPC to the nascent GCL, combined loss of Dlx1/Dlx2 and Brn3b function severely impaired RGC genesis, proliferation, differentiation and survival.

In Isl1/Brn3b DKO mice, there was severe RGC loss but normal RGC genesis before E13.5, and with Brn3b, Isl1 was shown to define a distinct, yet overlapping RGC sub-population under the regulation of ATOH7 (Mu et al., 2008; Pan et al., 2008). Almost complete RGC loss as well as severe losses of other retinal cell types resulted in Atoh7/Brn3b DKO mice (Moshiri et al., 2008). Based upon co-expression of DLX2 with Math5 or BRN3B as early as E11.5, reduced DLX2 expression in the Atoh7 KO at E13.5 (Fig. 7) and report of a Dlx regulatory region as a direct ATH5 target in the chick retina (Del Bene et al., 2007), we propose that Atoh7-Brn3b/Isl1 and Atoh7-Dlx1/Dlx2-Brn3b regulate parallel transcriptional pathways for RGC differentiation (Fig. 10). Math5/DLX2 co-expressing RPC could later co-express DLX2/BRN3B. Furthermore, downregulation of DLX2 by BRN3B in some RGC progenitors may be a mechanism underlying RGC sub-type specification. Lineage tracing and evaluation of cadherin 6 (De la Huerta et al., 2012) and other markers will contribute towards identifying a role for specific RGC subtypes. Deletion of both Isl1 and Brn3b would not affect RPC competence to become RGC, since Dlx1/Dlx2 would be available for promoting RGC differentiation. However, deletion of Dlx1/Dlx2 and Brn3b would block two of the more dominant pathways for RGC differentiation and survival. The few remaining RGC in Dlx1/Dlx2/Brn3b compound mutants could derive from the small population of Isl1 expressing progenitors (Fig. 1b H), which are distinct from those expressing Brn3b (Moshiri et al., 2008), although ISL1 also co-labels ChAT+ amacrine cells. Interestingly, these early born cholinergic amacrine cells also derive from Ath5-lineage cells (Jusuf et al., 2012). ISL1 and BRN3B can cooperate to specify RGC cell fate (Li et al., 2014; Wu et al., 2015).

In addition to early RGC loss, we found a significant increase in dislocated amacrine cells in the E18.5 TKO GCL. This result was unexpected, since only RGCs are affected by deletion of either Dlx1/Dlx2 or Brn3b (de Melo et al., 2005; Erkman et al., 1996; Gan et al., 1996). A similar phenotype was reported in Atoh7 mutants, with increased cones, bipolar and
Müller cells and increased starburst amacrine cells in the GCL (Brown et al., 2001; Wang et al., 2001). *Atoh7*, expressed in RPC, promotes RGC specification by driving cell cycle exit and repressing non-RGC specifying TFs (Brown et al., 1998; Brown et al., 2001; He et al., 2012; Jusuf et al., 2012; Le et al., 2006; Mu et al., 2008). However, lineage tracing of *Atoh7*+ cells in the mouse also suggests a more indirect role for *Atoh7* in RGC specification (Brzezinski et al., 2012).

Amacrine cell specification in TKO retinas might result from a different mechanism than the *Atoh7* mutants. There was preserved expression of *Atoh7* and NEUROD1 in the Brn3b, Dlx1/Dlx2 and triple KO mice. RPC may accumulate more than one competency by expressing “early” factors, such as *Atoh7* or *Neurod1*. Using lineage tracing, *Atoh7* expression is associated with RGC, photoreceptors, horizontal, and amacrine cells (Yang et al., 2003). Cells with restricted developmental potentials migrate properly and are further specified to differentiate under the regulation of factors expressed later in retinogenesis. In this case, both *Dlx1/Dlx2* and *Brn3b* could act downstream of *Atoh7* promoting the terminal differentiation of RGCs (Fig. 10). Without *Dlx1/Dlx2* and *Brn3b*, cells restricted to RGC and amacrine cell fates migrate to the GCL but are unable to terminally differentiate into RGC and either undergo apoptosis or differentiate into amacrine cells.

Interestingly, there is an increase in ISL+ cholinergic amacrine cells in a *Math5-Cre* knockin mouse (Feng et al., 2010). Similarly, the *Barhl2* KO retina demonstrates a 2-fold increase in ChAT+ amacrine cells and 35% RGC cell death (Ding et al., 2009). A *Neurod1* knock-in at the *Atoh7* locus re-established RGC gene expression, specification and optic nerve formation, without over-producing amacrine cells (Mao et al., 2008b). RPC single cell gene-expression profiles showed significant levels of *Atoh7*, *Neurog2* and *Neurod1* in individual RPCs (Trimarchi et al., 2008). Hence, “early” factors, including *Atoh7* and *Neurod1*, advance RPC to more narrowly defined but overlapping competencies, whereas, “late” factors, such as *Dlx1/Dlx2* and *Brn3b*, promote these restricted-potential RPC towards more specialized fates.
**Dlx1/Dlx2 and Brn3b function in co-regulatory genetic pathways**

Spatiotemporal DLX2 and BRN3b expression studies supported a second, later phase of Brn3b expression regulated by DLX1/DLX2. Both DLX1 and DLX2 bind to a specific Brn3b promoter region in situ and activate its expression in vitro. Knockdown of Dlx2 in primary cultures resulted in decreased expression of Brn3b consistent with reduced BRN3b expression in the Dlx1/Dlx2 DKO (de Melo et al., 2005). Ectopic expression of BRN3b occurred following in utero retinal electroporation of Dlx2. These observations strongly support the direct regulation by DLX1 and DLX2 of Brn3b transcription in vivo. Hence, Dlx1 and/or Dlx2 are necessary and sufficient for Brn3b expression during retinal development.

Loss of Dlx1/Dlx2 function results in reduced expression of Pou3f1 and Pou3f4 in the embryonic forebrain (Anderson et al., 1997b) suggesting that DLX1/DLX2 could regulate other Class III POU HD TFs besides Brn3b, such as Brn3a or Brn3c (Pou4f3) in the developing retina and might further explain the severe retinal phenotype observed in the TKO. Loss of Dlx1 and/or Dlx2 function could reduce transcription of all Brn3 genes in the retina, therefore blocking the functional redundancy of Brn3 family members (Pan et al., 2005).

Taken together, the mutual regulatory interactions between Dlx1/Dlx2 and Brn3b and the more severe phenotype observed in the TKO suggests that Atoh7-Dlx1/Dlx2-Brn3b and Atoh7-Brn3b/Isl1 are two parallel but cross-regulatory genetic pathways in retinogenesis (Fig. 10). Characterization of the Dlx1 and Dlx2 transcriptomes will further contribute to understanding their role in retinal development.

**METHODS**

**Animal and Tissue Preparation**

Animal studies were in accordance with guidelines established by the Canadian Council on Animal Care. Dlx1/Dlx2 DKO mice were generated as previously described (Anderson et al., 1997a; Qiu et al., 1997). Brn3b SKO mice (provided by Dr. W. Klein) were maintained on a CD1 background. The Dlx1/Dlx2-/- Brn3b-/- compound heterozygous line from crossing Dlx1/Dlx2-/- heterozygotes with Brn3b-/- heterozygotes was used to generate Dlx1/Dlx2/Brn3b triple homozygous null (TKO) mice. For comparative studies, all TKO
mice were paired with $Dlx1/Dlx2^{+/+}Brn3b^{--}$ (SKO), $Dlx1/Dlx2^{+/+}Brn3b^{++}$ (DKO) and $Dlx1/Dlx2^{+/+}Brn3b^{++}$ (all genetic heterozygotes are WT) littermate controls. Genotyping was performed as previously described (Gan et al., 1996; Qiu et al., 1997). Embryonic age was determined by the day of appearance of the vaginal plug (E0.5). E16.5 and E18.5 eyes were dissected from embryos while E13.5 eyes were left in situ prior to brief fixation in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Tissues were sectioned coronally at 12 μm thickness.

**Histology, Immunofluorescence (IF), TUNEL Assay, and in situ Hybridization**

Histological and IF staining on cryosections was performed as described (de Melo et al., 2005). Primary antibodies used were: mouse anti-BrdU (1:200, Chemicon), mouse anti-BRN3a (1:200, Santa Cruz), goat anti-BRN3b (1:200, Santa Cruz), rabbit anti-Caspase-3 (1:500, Cell Signaling Technologies), rabbit anti-DLX2 (1:400, C199 affinity purified), mouse anti-ISLET-1 (1:600, DSHB, U. Iowa), rabbit anti-phosphohistone H3 (1:1000, Upstate), rabbit anti-PROX1 (1:500, Chemicon), rabbit anti-PAX6 (1:800, Covance), and mouse anti-Syntaxin (1:6000, Sigma). Secondary antibodies and fluorescent tertiary molecules used were: FITC-conjugated goat anti-rabbit (1:200), Biotin-SP-conjugated goat anti-rabbit (1:200), Biotin-SP-conjugated goat anti-mouse (1:200) (Jackson Immunoresearch), Streptavidin conjugated Oregon Green-488 (1:200) and Streptavidin conjugated Texas Red (1:200) (Molecular Probes). Negative controls omitted the primary antibody. TUNEL staining used the In Situ Cell Death Detection Kit, TMR Red (Roche Diagnostics). Non-radioactive digoxigenin in situ RNA hybridization was performed as described previously (de Melo et al., 2005).

**Retinal explant and amacrine subtype analysis**

Retinas were dissected from E18.5 TKO and WT littermates and transferred onto Millicell-CM cell culture insert with 0.4 μM filters (Millipore). After removing the lens and the hyaloid vessels, retinas were flattened and cultured at 37°C with 5% CO$_2$ in a humidified incubator. Explant culture media contained 50% high glucose MEM, 25% Hank’s solution, 25% horse serum, 200μM L-glutamine, 6.75mg/ml glucose, 2.5mM HEPES buffer solution
and 1% penicillin/streptomycin. After 7 DIV, explants were fixed in 4% PFA for 30 minutes and sectioned. ChAT+ cells were counted at 120μm intervals and results were pooled.

**Pulse Labeling and Birthdating**
For pulse labeling, BrdU (100μg/g) was injected intraperitoneally (i.p.) to pregnant dams 1 hour before euthanasia. Sections were incubated in 50% formamide/2X SSC for 2 hr at 65°C and 2N HCL for 30 min at 37°C, followed by 0.1 M Sodium Borate for 10 min at room temperature (RT).

**Propidium Iodide (PI) Staining and Cell Cycle Analysis**
Retinas were dissociated into single cell suspensions in 1X PBS and fixed in 70% Ethanol overnight at 4°C. Cells were resuspended in 250μl PI (50μg/ml) and 1μl RNase (20μg/ml), and incubated for 15 min at RT. Flow cytometry was performed on a FACSCalibur apparatus (Becton Dickinson), and analyzed using BD CellQuest Pro Version 3.5 software.

**Cell Counting and Statistical Analysis**
Cell counts were performed on paired WT and mutant retinas (de Melo et al., 2005). For RGCs, BRN3a+ and ISL1+ cells located in the GCL were counted for E16.5 and E18.5 retinas; only ISL1 was used to count RGCs at E13.5. Only PAX6+ cells in the inner NBL were counted as amacrine cells; Syntaxin+/DAPI+ cells in the GCL were counted as dislocated amacrine cells; and PROX1+ cells located in the outer NBL were counted as horizontal cells. For BrdU birthdating studies, the BRN3a+/BrdU+ cells represented RGCs born at the time of BrdU injection; Syntaxin+/BrdU+ cells in GCL represent dislocated amacrine cells born at the time of the BrdU pulse. Statistical analyses were performed with SPSS software; the paired T-test was used; p-values < 0.05 were considered significant.

**Microscopy and Imaging**
Images were acquired using an Olympus BX51 microscope with a SPOT 1.3.0 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) or an Olympus DP70 digital camera. Fluorescent images were acquired using an Olympus IX81 inverted microscope with a
 Fluoview FV500 confocal laser scanning system (Olympus Optical Co., Tokyo, Japan). Images were processed using Adobe Photoshop software (Adobe Systems) for presentation.

**Chromatin Immunoprecipitation and Electrophoretic Mobility Shift Assays**

E16.5 retina tissues were isolated for ChIP assays as described previously (Zhou et al., 2004). Oligonucleotide primers for PCR amplification were designed according to the *Brn*3b gene promoter sequence (MGI 102524). The primer sets were: S4F, 5’-GTTCAGCAGACTGTTGCCAC-3’ and S4R, 5’-TCCTTCCTCACTCAACACTGAG-3’. The target region (S4-pro) is 318 bp and located at position 81,326,124-81,326,442, on mouse chromosome 8, within the 5’ proximal promoter 2.6 Kbp upstream of the *Brn*3b transcription start site. Genomic DNA from the E16.5 mouse embryo tail was used as a positive control. PCR products were purified for TOPO TA cloning (Invitrogen, Burlington, ON) and sequenced.

For EMSA, the S4-pro region was excised from the pCR2.1-TOPO vector (Invitrogen) with EcoR1, and labeled with α-[^32]P-dATP (Perkin Elmer) using the Klenow (large) Fragment of DNA PolII (Invitrogen). The binding reaction mixture contained labeled probes (90,000 cpm), binding buffer (20% glycerol, 5mM MgCl₂, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl and 50mM Tris-HCl pH7.5), 1μg poly-dI-dC, and purified recombinant DLX1 or DLX2 protein, and was incubated for 30 min. at RT. 100-fold excess unlabeled probes were used for “cold competition” experiments; rabbit polyclonal DLX1 and DLX2 antibodies were used for “supershift” experiments; and a rabbit polyclonal antibody to mouse IgG (Jackson Immunoresearch) was used as a negative antibody control.

**Cell Culture and Reporter Gene Assays**

HEK-293 cells were grown in alpha DMEM (Gibco) with 10% FBS at 37°C with 5% CO₂. 24 hours prior to transfection, cells were seeded at a density of 1x10⁷ per 36 mm² dish. Lipofectamine 2000 reagent (Invitrogen) was used for transient transfection. Reporter plasmids were constructed by inserting the 318 bp S4-pro fragment into the pGL3-basic vector (Promega). Putative DLX DNA binding sites in S4-pro were deleted respectively by site-directed mutagenesis using the Quick Change Kit (Stratagene); mutations (Δ1, Δ2 and Δ3)
were verified by DNA sequencing. Effector plasmids expressing *Dlx1* and *Dlx2* genes were constructed by insertion of a PCR amplified 790 bp *Dlx1* cDNA and 1020 bp *Dlx2* cDNA (Dr. J. Rubenstein, UCSF) into the pcDNA3 vector (Invitrogen). The plasmid pRSV-β-gal (Promega) was co-transfected to assess transfection efficiency.

**Primary Embryonic Retinal Cell Culture and siRNA Transfection**

E14.5 CD-1 mice retinas were dissociated with Accumax (Sigma-Aldrich) and cultured on poly-L-lysine and laminin-coated 6-well plates. Cells were cultured in DMEM/F12 medium supplemented with HEPES, 0.1% BSA, 1% FBS, N2 and B27 (Invitrogen), at 37°C with 5% CO2 for 24 hours before interfering RNA (siRNA) transfection. Two duplex siRNAs were designed to target mouse *Dlx2* coding sequence and a control siRNA was synthesized to scrambled *Dlx2* coding sequence (Invitrogen). siRNAs were prepared and transfected as previously described (de Melo et al., 2008). RNA was isolated 48hrs after transfection.

**Real-time quantitative PCR analysis**

RNA was extracted from cultured retinal cells with TRIzol (Invitrogen), and treated with DNase (Sigma). 1μg RNA was used to synthesize cDNA with SuperScript II reverse transcriptase (Invitrogen). 1μl cDNA was used as a template for gene amplification. Real-time quantitative PCR was performed using the iCycler iQ™ system (Bio-RAD). mRNA copy numbers were calculated utilizing standard curves generated by using plasmids containing the target sequences. *Gapdh* was used to normalize *Dlx2* and *Brn3b* mRNA expression levels. Primers were: *Dlx2*-F: 5’ cca aac tca ggt caa aat ctg 3’; *Dlx2*-R: 5’ tta gaa aat cgt ccc cgc g 3’; *Brn3b*-F: 5’ gtc ggc agc ctc ctc ttc ttc tgt g; *Brn3b*-R: 5’ gct tct cgc ggt ggg att tct cag; *Gapdh*-F: 5’ ctc atg acc aca gtc cat gc 3’; *Gapdh*-R: 5’ cac att ggg ggg tag gaa cac 3’.

**In utero electroporation**

Timed pregnant C57/BL6 mice were used for in utero electroporation experiments. *Dlx2* cDNAs were cloned into pCIG2, an expression vector containing a CMV/β-actin enhancer/promoter and an IRES-mCherry cassette. Endotoxin-free plasmid DNA for
electroporation was generated using a column-based purification system (Qiagen; Mississauga, ON). *In utero* electroporation was performed as described (Dixit et al., 2011; Langevin et al., 2007; Mattar et al., 2008). Injection of E13.5 or E14.5 retinas took advantage of the pigmented retinal epithelium (RPE) for targeting; embryos developed until E18.5. Electroporated eyes were identified by mCherry epifluorescence.
ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Q.Z. performed experiments with contributions from J.Z., S.C., R.D. C.S., S.Z., and J.d.M. X.M and W.K. provided Brn3b SKO mice and technical advice. N.B. provided Math5 KO tissues and technical advice. Q.Z. and D.D.E. conceived the experiments; Q.Z., J.Z., J.T.W. and D.D.E. analyzed the data and wrote the manuscript.
REFERENCES


Ding, Q., Chen, H., Xie, X., Libby, R. T., Tian, N. and Gan, L. (2009). BARHL2 differentially regulates the


**Fig. 1. Severe RGC loss in Dlx1/Dlx2^+/^-Brn3b^-/- retinas.** (a A-E) There is a reduced GCL in mutant retinas (D). Compared to wild-type (A), the GCL of the Dlx1/Dlx2^+/^-Brn3b^-/- single (B), Dlx1/Dlx2^+/^-Brn3b^-/- double (C), and Dlx1/Dlx2^+/^-Brn3b^-/- triple (D) KO retinas appear reduced, but no significant difference can be distinguished among the mutants. Near fusion of the GCL and NBL is evident in the TKO (D insert, arrow) with significant reduction of the IPL (E). Inserts show boxed regions at a higher magnification. * marks the optic nerve head. (b A-H) Immunostaining with RGC markers BRN3a and ISL1 shows decreased RGCs in all mutant retinas at E18.5 but is more severe in the TKO (D, H arrow). (I-L) Significant loss of ISL1+ RGC is only observed in TKO retinas at E13.5. (M-N) At E18.5, a more severe RGC loss is detected in the TKO (95% reduced BRN3a+ and 84% reduced ISL1+ cells). (O) At E13.5, 81% ISL1+ RGC loss only occurs in TKO retinas. Histograms represent the mean ± SD, n=4, *p<0.01. L, lens; NBL, neuroblastic layer; R, retina. Scale bars: 50μm in a D; 25μm in insert; 20μm in b H (applies to A-H); 50μm in L.
Fig. 2. Increased amacrine cells are located in the Dlx1/Dlx2<sup>−/−</sup>Brn3b<sup>−/−</sup> ganglion cell layer. (A-D, Q) No significant differences in PAX6+ amacrine cells are identified in the inner NBL among the different mutants (arrow, Q). However, more PAX6+ cells are evident in the TKO GCL (D, asterisks). (E-H, R) Syntaxin immunostaining reveals increased number of displaced amacrine cells in the TKO GCL (H, arrow), with an 1.8 fold increase of syntaxin+ amacrine cells (R). (I-L, S) The horizontal cell marker NF165 is expressed in normal number (S) and position in the TKO (arrow). (M-P, T) Recoverin expression is not significantly different between the mutants and wild-type. Histograms represent the mean ± SD, n=4, *: p<0.01. Scale bar: 20μm
Fig. 3. More cholinergic amacrine cells are located in the GCL of TKO retinal explants. (A-D) At 7 DIV, GAD65 and GAD67 are highly expressed in the IPL, as well as GCL and inner INL of both wild-type and TKO retinas (A-D arrow heads). (E-F) More ChAT expressing cells are located in the TKO GCL than in wild-type retina (E, F arrow heads). (G, H) GlyT1 expression is restricted to the INL of both WT and TKO retinas (G, H arrow heads). Scale bar: 20μm.
Fig. 4. More retinal progenitors adopt an amacrine cell fate in the $\text{Dlx1}/\text{Dlx2}^{-/-}\text{Brn3b}^{-/-}$ mutant GCL (A-B, E-F) BrdU pulse labeling at E12.5 and co-expression with BRN3a or Syntaxin reveal few RGC in the TKO (A, B, arrow); more cells differentiate as amacrine cells and migrate to the GCL (E, F, arrow). Most E12.5 BrdU pulse labeled cells are located in the GCL (B”, F”). (C-D, G-H) BrdU pulses at E16.5 identify more displaced amacrine cells in the TKO, although most BrdU+ cells remain in the NBL. (I-K) E12.5, E13.5 and E16.5 birth-dated cells identified similar quantities of BrdU+ cells in the GCL of WT and TKO retinas (I). At all stages tested, RPC of the TKO prefer an amacrine rather than RGC cell fate in GCL (J, K). Histograms represent the mean ± SD, n=4, *: p<0.005, #: p<0.05. Scale bar: 20μm
Fig. 5. Combined loss of Dlx1/Dlx2 and Brn3b results in enhanced apoptosis and abnormal cell proliferation. (A, B, G) Cleaved-caspase-3 immunostaining shows a 4-fold increase of apoptotic cells in E13.5 TKO retinas (B, G). There is no significant difference in apoptosis at E16.5 and E18.5 (G). (C-D, H) Anti-phospho-histone H3 quantification revealed decreased M-phase cells at E16.5 and E18.5 in the TKO (D, D’, H) but not at E13.5 (H). C’ and D’ show higher magnification of the boxed regions in C and D. (E, F, I) There are lower percentages of S-phase (P=0.005) and G2/M phase (NS) cells at E16.5 in the TKO (n=3). Histograms represent the mean ± SD, n=4, *: p<0.01. Scale bars: 50μm in B; 25μm in D.
Fig. 6. *Math5* mRNA and DLX2 protein are co-expressed in early but not in late embryonic stages (A). (A-C) *Math5* mRNA and DLX2 protein are expressed in the central E11.5 retina. (D-F) At E13.5, *Math5* mRNA is expressed throughout the retina, except the inner central region; *Math5*+/DLX2+ cells are located in the middle layer. *Math5*-/DLX2+ cells are easily detected in the inner central retina, where differentiated cells are located. *Math5*+/DLX2- cells are restricted to the outmost proliferating zone. (G-I) *Math5* expression
is down-regulated at E16.5 (G, arrow heads). DLX2 is strongly expressed in the GCL and inner NBL (H, arrows). Double positive cells can be detected in the NBL (I insert, arrows). (H-L) At E18.5, Math5 is localized proximal to the ciliary margin (J, arrow heads). DLX2 expression is restricted in the GCL and inner NBL (K, arrows), without double positive cells.
Fig. 7. (a) *Math5 (Atoh7)* expression is not affected by the combined deletion of *Dlx1/Dlx2* and *Brn3b*. *In situ* hybridization at E13.5 shows *Math5* expression throughout the proliferating zone of developing retina, except for the ciliary margin. No difference was detected between WT and the genetic mutants. Scale bar: 40μm. (b) *DLX2* and *BRN3b* expression is down-regulated in *Math5* null retinas at E13.5. (A-F) *DLX2* expression is significantly reduced in the *Math5 KO* at E13.5 (A,B,E) but not at E16.5 (C,D,F). (G-J) *BRN3b* is severely reduced in the *Math5* KO retina at both E13.5 (G,H) and E16.5 (I,J). Scale bar: 100μm.
**Fig. 8. DLX1 and DLX2 regulate Brn3b expression.** (a) DLX1 and DLX2 proteins bind to the Brn3b promoter region. (A) Brn3b has candidate homeodomain binding sites located within the 5′-promoter region (denoted 3bP4) (A, underlined). (B) ChIP identified specific Brn3b promoter DNA sequences occupied by DLX1/DLX2 in vivo (lanes 1, 2). E16.5 hindbrain was a negative tissue control. Control ChIP was performed without primary antibodies (lane 3). Genomic DNA was used as positive input (lane 4). (C) Using recombinant (r) DLX1 and DLX2 proteins, EMSA demonstrated direct and specific in vitro binding of DLX1/DLX2 proteins to the Brn3b promoter region (3bP4) identified by ChIP in situ. Radiolabeled 3bP4 oligonucleotides, when incubated alone migrate as free probe (lanes 1, 6). rDLX1 or rDLX2 proteins form complexes with labeled 3bP4 probe and are shifted (lanes 2, 7 arrows, respectively). Specific DLX1/DLX2 antibodies when incubated with rDLX1/rDLX2 proteins and labeled 3bP4 probe, form supershifted bands (lanes 4, 9 arrowheads, respectively). Excess unlabeled probe was used for cold competition (lanes 3, 8). Antibody to mouse IgG was used as a control without supershifts (lanes 5, 10). (b) DLX1/DLX2 activate Brn3b transcription in vitro. Co-transfection of either Dlx1 or Dlx2 with the Brn3b promoter region 3bP4 reporter construct activated luciferase activity. Mutations of any of the three candidate binding motifs significantly reduced the activation of reporter gene activity by DLX1 and DLX2. *: P<0.05, **: P<0.01. (c) Knockdown of Dlx2 expression in primary embryonic retinal cultures at E14.5 results in decreased Brn3b expression. (A) RT-PCR shows reduced expression of Dlx2 and Brn3b mRNA in the Dlx2 siRNA transfected cells, compared to untreated and control siRNA transfected cells. (B,C) Quantitative real-time RT-PCR demonstrated efficient knockdown of Dlx2 expression using specific siRNA but not with control siRNA. Dlx2 knockdown results in a significant reduction of Brn3b expression. (* p<0.05).
Fig. 9. In utero electroporation of Dlx2 results in ectopic Brn3b expression in vivo. E14.5 retinas electroporated with pCIG2-mCherry-Dlx2 (A-G) and pCIG2-mCherry control plasmids (H-J) were harvested at E18. In pCIG2-mCherry-Dlx2 electroporated retinas, mCherry epifluorescence replicated ectopic DLX2 expression in the outer NBL (arrows in C, D) resulting in ectopic BRN3b expression in the outer NBL (arrows in F, G); results are quantified in (K). Endogenous DLX2 and BRN3b expression was also detected in the GCL and INL (stars in C, D, F, G, I, J). No ectopic BRN3b expression was detected in control electroporated NBL (I, J). Scale bars: 50μm in A; 20μm in B (applies to B-J).
Fig. 10. A model for transcriptional regulation of RGC differentiation.

(A) Loss of DLX2 expressing cells in E13 Math5 KO supports Dlx1/Dlx2 participation in a regulatory pathway downstream of Math5, where along with Brn3b and Isl1, Dlx1/Dlx2 then co-regulates the genes required for RGC differentiation and survival. In addition, cross-regulation of these factors is observed. Loss of Dlx2, Brn3b or Isl1 individually does not affect initial RGC genesis, suggesting that a combination/co-regulation of these factors and/or other unidentified factors are responsible for RGC fate specification. In later development, loss of Dlx2, Brn3b and Isl1 all result in increased RGC death, supporting the requirement of these factors for RGC survival by controlling the expression of genes necessary for this process including transcriptional control of Brn3a by DLX2 (Zhang and Eisenstat, unpublished observations), ISL1 and BRN3b (Pan et al., 2008) and DLX2 regulation of TrkB (de Melo et al., 2008). (B) Math5 expression begins at ~E11, preceding Dlx1/Dlx2 and Brn3b. Math5 and Dlx1 expression are both down-regulated after E16.5 with...
neither detected after P0. *Dlx2* and *Brn3b* expression starts at E11.5 and persists to adulthood. Solid lines, established regulation; broken lines, proposed regulation.
SUPPLEMENTARY FIGURES

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Development 144: doi:10.1242/dev.142042: Supplementary information
Fig. S1. DLX2 and BRN3b co-expression pattern from E11.5 to E18.5. (A-C) Both DLX2 and BRN3b are first detected at E11.5, and show a high-dorsal-to-low-ventral expression gradient. DLX2 expression is more dispersed than BRN3b. Only a few double positive cells are located in the central inner retina (arrow), while most cells are either DLX2 (asterisks) or BRN3b (arrow head) single positive. (D-F) In contrast to the restricted central inner expression of BRN3b, DLX2 is expressed throughout the E13.5 retina. More cells are co-labeled by DLX2 and BRN3b (arrow) than in E11.5 retina. (G-I) DLX2 expression is primarily localized to the inner retina at E16.5. Most BRN3b+ cells are in the GCL and co-express DLX2 (arrow). (J-L) At E18.5, DLX2 expression is primarily localized to the GCL and inner NBL, whereas BRN3b is expressed only in the GCL. BRN3b+/DLX2+ cells are observed in the GCL, with very few BRN3b single + cells (arrow head). Some DLX2 single + cells are still distinguished in the GCL and inner NBL (asterisks). R, Retina; L, Lens; GCL, ganglion cell layer; NBL, neuroblastic layer. Scale bar: 50μm.
Fig. S2. RGC loss occurs in E12.5 Dlx1/Dlx2\(^{-/-}\)/Brn3b\(^{-/-}\) retinas. Immunostaining with RGCs specific marker ISL1 shows decreased RGC numbers in Dlx1/Dlx2\(^{-/-}\)/Brn3b\(^{-/-}\) retinas at E12.5.
Fig. S3. NeuroD expression is unaffected by the absence of Dlx1/2 and Brn3b.
E18.5 retinas showed two populations of NeuroD expressing cells. Cells located in the outer most region of NBL express NeuroD robustly. Scattered NeuroD positive cells are also detected in the inner layer of NBL, but not in GCL at this developmental stage. In terms of cell distribution and cell numbers, no significant differences are observed between wild-type, $Dlx1/Dlx2^{+/+} Brn3b^{+/+}$, $Dlx1/Dlx2^{+/+} Brn3b^{+/+}$ and $Dlx1/Dlx2^{+/+} Brn3b^{+/+}$ mutants. GCL, ganglion cell layer; NBL, neuroblastic layer. Scale bar: 20μm.
Fig. S4. *Dlx1* or *Dlx2* co-transfection activates expression of a *Brn3b* promoter reporter construct *in vitro*. However, co-transfection of both Dlx1 or Dlx2 expression constructs is neither synergistic nor additive. These results are similar to prior results obtained with the *Dlx5/Dlx6* intergenic enhancer (Zhou et al., 2004) and other DLX1/DLX2 transcriptional targets (data not shown).
Fig. S5. BrdU pulse chase experiments identify proportions of RGC born at E12.5, E13.5 and E16.5

The BrdU birthdating experiments are also presented to show the proportion of RGCs out of the total BrdU labelled cell populations in wild-type (Fig. S5 panels A-I) and Brn3b/Dlx1/Dlx2 triple KO (Fig. S5 panels J-R) retinas. E12.5 (panels A-C, J-L), E13.5 (panels D-F, M-O) and E16.5 (panels G-I, P-R) birthdated cell populations and the labelled RGC proportion of these cells were counted at E18.5. For E12.5 birthdated cells, 22.5% of the progenitors differentiated to RGC in WT, 1.4% in TKO (panel S); for E13.5 birthdated cells: 24% and 1%, respectively (panel T); for E16.5 birthdated cells: 13.2% and 0.9% respectively (panel U).
Fig. S6. *Brn3b transiently represses DLX2 expression at E13.5.* (A-F) Retinal sections from wild-type and *Brn3b*−/− mice were immunostained with DLX2 antibody at E11.5, E13.5 and E16.5. There is a significant increase of DLX2 expression in *Brn3b*-null retinas at E13.5, but not at E11.5 or E16.5. (G) Quantification of DLX2 positive cells reveals a 1.5 fold increase in E13.5 *Brn3b*-null retina compared to wild-type. No significant difference was detected between wild-type and *Brn3b* mutants at E11.5 and E16.5. R, Retina; L, Lens; GCL, ganglion cell layer; NBL, neuroblastic layer. Scale bar: 50μm.
Fig S7. BRN3b does not bind to *Dlx1/Dlx2* cis-regulatory sequences *in situ*. Chromatin immunoprecipitation (ChIP) assays using E16.5 retina and BRN3b antibody followed by PCR identified the previously characterized BRN3b transcriptional target *Eomes* (right upper panel). However, none of the four specific *Dlx1/Dlx2* cis-regulatory sequences (left panel, URE2, I12b, *Dlx1* exon 3, *Dlx2* exon 3) with candidate consensus BRN3b DNA binding motifs were bound to BRN *in situ* (right lower panels). The numbers underneath the arrows indicate the number of *Brn3b* DNA binding sites in that region. E16.5 hindbrain was used as negative control because this tissue does not express DLX1 or DLX2 proteins. Control immunoprecipitations were performed without primary antibodies and demonstrated no bands. Genomic DNA was used as positive input. R, Retina; H, hindbrain; G, genomic DNA; +, with BRN3b antibody; -, without BRN3b antibody.
SUPPLEMENTARY METHODS

BRN3b Chromatin Immunoprecipitation and Oligonucleotide primer design

Brn3b/Pou4f2 consensus DNA-binding site: 5’- (A/G)TTATGAG(C/T)-3’.

Eomesodermin (Eomes) primers:
Eo3b007, 5-GACCAACTTGCCACAAAAAACCC-3
Eo3b008, 5-CTGAACAGGCTTGCTGCATGCTC-3

URE2

GCCCGTGCTCCTCTAAATCAGATACCTACCAGACACATTTAGGAGACCGCAGCCATCCA
ACCTCTAATTCTGTTACCUGGCATAATGTCGTTTTGTTTTCTGTTTTGTCCCC
ATTACACCCAGTAACAGGCGATATGCTCTCTTGCTTCGAGCAAGTGGCGCAT
GCCTCCATGGTGCGGCCCATCTTCTGGCTGATGCGATTTGGTGTCACCGAACAATCC
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I12b

AGCGGTCCTGTGAGTGGTAAGGCTCTATTGTTGGGGAGAGCCTGCGCTTTTGAGCAT
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TCTGAGGTCCGGAATACGAGATAGACTCTGGTGTTATTAAATCAAGAAAAATGGCGACAA
AGTAGAAATGAAATGCCTGGCC

Dlx1 exon 3

AGGCGGTCTTTAGTTAGGGCTCTATTATAATTAAAGCAGTTCATTTTTTTAAAAATGT
TAATCTCAATATAGGCCTAATTAATACGCTCTTGTACTGACAGAGCGAGTTCTCATAAATAT
CTGATTCAAGATTTTCATAATGAGTATATTAATTTAACTATGAATAATCTAAAGGTGCTATATTTAAACAATACCTCATTATAATGATTTAATACTGATTTTCGAATTTATATTGTCTTAACTAATTGTCACATAGAAAACACAACCTTTCTTTATGTATGAGTCTGTAATGGCAAAATGCAATTTTGGAATTTTTTTCCCTTGTTCAAAAATAATGTGAACCTCATTAAAAACACTTCTGAAATAGGTTACACACAGCTTAATGATTATCAAAATGACTCTTTTCTGCAAAAAAAGACCCCAAAAGTG

**Dlx2 exon 3**

CTGCTCCCAYCCTCTACTAATGGAACCTTCTTGTGTTAGATACATAGTGTCGGAGATTACCTACCTTGTGAGGCAAGAAGCCTACATCATCCTAGACCTGAGTCGGTCACCTCATTAAACCTAGCTTTCTGTCCAAGCCCTTTTACCCTCATTCTTAGCTGTAGGAATGATGTTGGATATAGAATGGACAGAGGGAACAAAGGCAAAGCCAGATCTCTTGTGTTAAGAGAGGCTGGTGGTTATGTTCTAGGAGGTGTGGTGAG

**References**