Developmental Biology 371 (2012) 280–292

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Id2a functions to limit Notch pathway activity and thereby influence the transition from proliferation to differentiation of retinoblasts during zebrafish retinogenesis

Rosa A. Uribe [a,b], Taejoon Kwon [b,d,e], Edward M. Marcotte [b,d,e], Jeffrey M. Gross [a,b,c,*]

[a] Section of Molecular Cell and Developmental Biology, The University of Texas at Austin, Austin, TX 78712, United States
[b] Institute for Cell and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, United States
[c] Institute for Neuroscience, The University of Texas at Austin, Austin, TX 78712, United States
[d] Center for Systems and Synthetic Biology, The University of Texas at Austin, Austin, TX 78712, United States
[e] Department of Biochemistry, The University of Texas at Austin, Austin, TX 78712, United States

ARTICLE INFO

Article history:
Received 20 June 2012
Received in revised form
16 August 2012
Accepted 28 August 2012
Available online 8 September 2012

Keywords:
Id2a
Id2
Retinogenesis
Notch
Zebrafish

ABSTRACT

During vertebrate retinogenesis, the precise balance between retinoblast proliferation and differentiation is spatially and temporally regulated through a number of intrinsic factors and extrinsic signaling pathways. Moreover, there are complex gene regulatory network interactions between these intrinsic factors and extrinsic pathways, which ultimately function to determine when retinoblasts exit the cell cycle and terminally differentiate. We recently uncovered a cell non-autonomous role for the intrinsic HLH factor, Id2a, in regulating retinoblast proliferation and differentiation, with Id2a-deficient retinae containing an abundance of proliferative retinoblasts and an absence of terminally differentiated retinal neurons and glia. Here, we report that Id2a function is necessary and sufficient to limit Notch pathway activity during retinogenesis. Id2a-deficient retinae possess elevated levels of Notch pathway component gene expression, while retinae overexpressing *id2a* possess reduced expression of Notch pathway component genes. Attenuation of Notch signaling activity by DAPT or by morpholino knockdown of Notch1a is sufficient to rescue both the proliferative and differentiation defects in Id2a-deficient retinae. In addition to regulating Notch pathway activity, through a novel RNA-Seq and differential gene expression analysis of Id2a-deficient retinae, we identify a number of additional intrinsic and extrinsic regulatory pathway components whose expression is regulated by Id2a. These data highlight the integral role played by Id2a in the gene regulatory network governing the transition from retinoblast proliferation to terminal differentiation during vertebrate retinogenesis.

Introduction

The retina is composed of distinct and specialized neuronal and glial cell types that are generated with spatial and temporal precision during embryonic development. The mature retina contains seven differentiated cell types: ganglion cells, amacrine cells, bipolar cells, horizontal cells, rods, cones and Müller glia. These cells are organized into precise laminae—the ganglion cell layer (GCL), inner nuclear layer (INL) and the outer nuclear layer (ONL) (Dowling, 2012). During retinogenesis each retinal cell type develops from a common pool of retinal progenitor cells located in the inner neuroblastic layer of the optic cup. Cell fate specification and commitment mechanisms bias retinal progenitors into distinct cell fates that are executed upon terminal differentiation (reviewed in Zaghloul et al., 2005; Agathocleous and Harris, 2009). Cell fate specification and terminal differentiation are spatially regulated, beginning in the ventral-nasal retina and sweeping around dorsally and temporally in a fan-like fashion (Hu and Easter, 1999; Schmitt and Dowling, 1994). Similarly, temporal components of retinogenesis are highly regulated in all vertebrates where the GCL is the first to differentiate, followed by the INL and then the ONL. Finally, balancing proliferation and differentiation, neurogenic waves in the retina are coupled to intervening phases of proliferation that serve to maintain the retinoblast progenitor population for subsequent waves of neurogenesis (Hu and Easter, 1999). Together, all of these events are exquisitely coordinated in order to generate a mature retina of the proper size and neuronal/glial composition.

From numerous studies, a model has emerged for the specification, commitment and differentiation of retinal cell types that involves both intrinsic and extrinsic factors (Cepko, 1999;

* Correspondening author at: University of Texas at Austin, Section of Molecular Cell and Developmental Biology, 1 University Station, Box C1000, Austin, TX 78712, United States. Fax: 1 512 471 3878.
E-mail address: jmgross@austin.utexas.edu (J.M. Gross).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.08.032

Perron and Harris, 2000; Harada et al., 2007; Agathocleous and Harris, 2009). Specification events serve to bias a group of competent cells toward a limited number of fates, whereas commitment events restrict a group of specified cells toward a single cell fate, enabling differentiation of specialized cell types to then occur. Intrinsically, specification events are primarily mediated by transcription factors such as Pax6, Six3b and Foxn4 (Harada et al., 2007; Luo et al., 2012; Zaghloul et al., 2005). Cell commitment to a neuronal lineage then requires the expression and function of neurogenic family transcription factors such as NeuroD4, Atoh7, Ap2α and NeuroD (Bassett et al., 2012; Luo et al., 2012; Zaghloul et al., 2005). Finally, differentiation involves the activity of additional factors such as the Histone modifying factors Brg-1 and Histone deacetylase1(Gregg et al., 2003; Yamaguchi et al., 2005). Extrinsically, the Notch, Sonic Hedgehog, Fibroblast growth factor and Wnt signaling pathways have all been shown to influence proliferation of the retinal progenitor cell population as well as the specification, commitment and differentiation of neuronal lineages within the vertebrate retina (Cepko, 1999; Martinez-Morales et al., 2005; Murali et al., 2005; Zhang and Yang, 2001). Gene regulatory network interactions between intrinsic factors and extrinsic pathways are complex; extrinsic signaling pathway activity often leads to changes in the expression or distribution of the intrinsic factors, while intrinsic regulators are capable of modulating the extrinsic signaling pathways (Luo et al., 2012; Yamaguchi et al., 2005).

Previously, we identified the intrinsic factor Id2a as a regulator of zebrafish retinal development (Uribe and Gross, 2010). Embryos in which Id2a expression was blocked by morpholino-mediated knockdown (“Id2a-deficient”) were microphthalmic, yet retinal progenitors remained proliferative and failed to efficiently exit the cell cycle or terminally differentiate in Id2a-deficient retinae. Id2a’s role in both of these processes was cell non-autonomous, indicating that Id2a function was required upstream of extrinsic regulators of retinal development. In the present study, we explore the cell non-autonomous role of Id2a further, and present data demonstrating that Id2a function is necessary and sufficient to limit Notch pathway activity during retinogenesis, thereby modulating retinoblast proliferation and terminal differentiation. Utilizing a novel, unbiased RNA-seq approach, we also identified additional gene products whose expression is dependent on Id2a. These data position Id2a as a key factor in the gene regulatory network regulating proliferation and differentiation during retinogenesis and identify a number of pathways influenced by Id2a during retinal development.

Materials and methods

Fish care

Zebrafish (*Danio rerio*) were maintained at 28.5 °C on a 14 h light/10 h dark cycle. Animals were treated in accordance with University of Texas at Austin IACUC provisions.

Morpholino and mRNA injections

All morpholinos and mRNAs were co-injected with fluorescein dextran (Invitrogen) at the 1–4 cell stage as a lineage tracer to ensure ubiquitous distribution/expression. The following morpholinos were used: Id2aMO (5′-GCCTTCATGTTGACAGCAGGATTTC-3′) (Uribe and Gross, 2010), Id2a mismatch morpholino (Id2aMM) (5′-GCGTTGATGTTCACAGCACGAATTC-3′) (Uribe and Gross, 2010) and Notch1aMO (5′-GAAACGGTTCATAACTCCGCCTCGG-3′) (Lorent et al., 2004; Bill et al., 2008; Ishitani et al., 2010). The knockdown efficacy of Id2aMO was reported in Uribe and Gross (2010). For *gfp* and *id2a* mRNA injections, pCS2-id2a and pCS2-gfp (Uribe and Gross, 2010) were linearized and capped mRNAs were transcribed using the mMessage kit (Ambion). 100 pg of *id2a* mRNA was injected into embryos for overexpression experiments.

Retinal RNA extraction and purification

From Id2aMM and Id2aMO injected embryos, 50 whole retinae (lenses removed) were dissected between 48 and 50 hpf in sterile Ringer’s solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM HEPES, pH 7.2). Dissected retinae from each condition were immediately pooled and dissolved in 1 mL of Trizol reagent (Invitrogen) and briefly vortexed at room temperature. Total retinal RNA was purified by the addition of 200 mL of chloroform, the samples were vortexed for 1 min and then centrifuged for 5 min, 16,000 g at 4 °C. The resulting aqueous layer was transferred to a new tube, mixed with an equal volume of RNase-free 70% ethanol, loaded into an RNeasy MiniElute kit column (Qiagen) and centrifuged for 15 s, 8000 g at 4 °C. The column was washed twice with 700 mL of RPE buffer supplied in the RNeasy MiniElute kit; samples were centrifuged for 30 s, 8000 g at 4 °C in between each wash. The column was given a final wash with 700 mL of RNase-free 80% ethanol and centrifuged for 2 min, 8000 g 4 °C. To remove all residual ethanol from the column, it was centrifuged at top speed for 5 min and the RNA was eluted with 16 mL of RNase-free water. Resulting RNA was subjected to RNA integrity analyses using an Aligent BioAnalyzer, and only samples having an RNA integrity number (RIN) of 9 or higher were used for subsequent RNA-sequencing analyses or quantitative real time PCR.

RNA sequencing

RNA sequencing was performed using the Illumina HiSeq 2000 at the Genome Sequencing and Analysis Facility, UT Austin. Two biological replicates were performed for Id2aMM control and Id2aMO knockdown conditions. A starting amount of 1 microgram of total retinal RNA per condition was used to generate cDNA libraries using the TruSeq RNA Sample Prep kit (Illumina). Sequencing results have been submitted to the Gene Expression Omnibus database (Accession number: GSE38786).

Short read mapping and differential expression analysis

RNA-seq reads were mapped to all known 48,636 *D. rerio* cDNA sequences (EnsEMBL release 66) using bowtie (version 0.12.7) (Langmead et al., 2009), with the option to report all alignments per read. Approximately 46–52% of total reads (43–57 million reads) were mapped to the cDNA database. While this approach can inflate absolute abundance estimation for transcript isoforms or paralogous genes in the genome by counting shared reads among them multiple times, it should not introduce significant bias in relative abundance analysis performed in this study, mainly because the identical cDNA database is used in a comparison between two samples (Id2aMM vs. Id2aMO). The number of reads per each transcript was normalized with total number of mapped reads and transcript length, converting all raw read counts to ‘number of reads per 1000 bases per 100 million reads’ (RPK100M). ‘100 million reads’ was chosen as a normalization factor, instead of the conventional 1 million reads (RPKM) (Mortazavi et al., 2008), to generate positive values of $\log_{10}$-transformed normalized values. Transcripts without any mapped reads in more than two biological samples were discarded, then mapped transcripts were rounded to an integer using DESeq package (version 1.6.1) (Anders and Huber, 2010) to identify differentially expressed genes between Id2aMM and Id2aMO

samples. Genes showing greater than a 2-fold change and less than a 0.05 adjusted *p*-value between Id2aMM and Id2aMO samples were defined as differentially expressed (DE) genes.

Functional enrichment analysis

DAVID (version 6.7) (Huang da et al., 2009a,b) was used for functional enrichment analysis. Genes identified in this experiment were used as background (Supplemental Table 1) and tested for which functional groups are enriched in either up-regulated genes or downregulated genes in Id2aMO compared to Id2aMM (Supplemental Table 2). Out of 24,506 background genes (43,155 transcripts), 18,232 genes were successfully submitted to the DAVID server as background. Similarly, 720 out of 871 down-regulated genes and 312 out of 384 up-regulated genes were used in this test. The Gene Ontology (GO) terms in three categories, InterPro protein domain and KEGG pathways with at least three member were considered, and enriched functional categories were determined by <0.05 adjusted *p*-value (Benjamini) (Supplemental Tables 3 and 4).

Quantitative RT-PCR (qRT-PCR)

The following qPCR primers were used: *tubulin, alpha 1* 5′-TGGAGCCCACTGTCATTGATG-3′ and 5′-CAGACAGTTTGCGAACCCTATCT-3′ *hes6* 5′-CGAAGGAGGCTGACAGTGTG-3′ and 5′-ATTCGAGGAGGTGGTTCAGC-3′, *her4.1* 5′-CACTGGATCAATCAGCAGCA-3′ and 5′-TCTCGGCGGATCTTCTCC-3′, *dla* 5′-GATCGACCACTGCTCTTCCA-3′ and 5′-TTGGCAAGGGTACATCGAAC-3′, *ascl1a* 5′-GGGCTCATACGACCCTCTGA-3′ and 5′-TCCCAAGCGAGTGCTGATATTT-3′, *notch1a* 5′-ACATCACCCTTCCAGCAGTC-3′ and 5′-AGGCTTCCCTAAACCCTGAA-3′, *cdk6* 5′-CCTACTTCGTTTCCTGTCCTTC-3′ and 5′-TACAGGCTCTTGCTGGTGCT-3′, *skp2* 5′-AAAGCACACCGAGTCTTCGT-3′ and 5′-CAGAGAGCACCAATCCTTCA-3′, *cdkn1a* 5′-AGAAGAGCAGCGAGCTGAAG-3′ and 5′-TAGACGCTTCTTGGCTTGGT-3′, *fzd6* 5′-GCAGTGTGAGCGACTGGAG-3′ and 5′-TTAGCTGGAGCGGACACC-3′, *rspo1* 5′-GATCCCATGCAAAGGAGAAA-3′ and 5′-GTGGTGGACCGGTTAGTGTT-3′, *tcf7l1b* 5′-CTCACCTCGTCCCTCATCAT-3′ and 5′-GGCCTGGAGACTTCGTGTTA-3′. Real time PCR experiments were performed in triplicate (three biological replicates) using Power SYBER Green PCR Master Mix (Applied Biosystems) and an Applied Biosystems 7900HT real time PCR machine. Real time PCR data was analyzed using the Comparative C_t method (Schmittgen and Livak, 2008). Statistical significance was determined using a Student's *T*-test (GraphPad Prism).

In situ hybridization

Hybridizations using digoxigenin labeled antisense RNA probes were performed essentially as described (Jowett and Lettice, 1994). The cDNA clones containing *dla* and *her4.1* were purchased from ZIRC (Eugene, OR) and cDNA clones containing *ascl1a* and *notch1a* were kindly provided by Bruce Riley (Texas A&M University). For comparisons between samples (e.g., control and experimental samples), in situs were developed in parallel, using identical reagents, probes, etc., and reactions were stopped at the same time for both samples.

Immunohistochemistry

Immunohistochemistry was performed as described in (Uribe and Gross, 2007). The following antibodies and dilutions were used: anti-pH3 (1:250) (06-570, Millipore), anti-BrdU (1:250) (ab6326, Abcam), zpr1 (1:200) (ZIRC), 5e11 (1:100) (gift of Dr. James Fadool, Florida State University) and goat anti-mouse, anti-rabbit or anti-rat secondary antibodies (1:200, Jackson ImmunoResearch). Nuclei were counterstained with SytoxGreen (1:10,000; Molecular Probes). For BrdU analyses, dried cryosections were first rehydrated in PBST, incubated in 4 N HCl for 12 min at 37 °C and then washed three times in PBST before incubation in block.

DAPT treatment

Insolution™ γ-Secretase Inhibitor 1× DAPT Stock solution (Calbiochem) (25 mM DAPT dissolved in DMSO) or DMSO alone was used for all experiments. Embryos were incubated in fish water supplemented with either 50 μM DAPT or DMSO as a control. After incubation, embryos were extensively washed in fish water prior to fixation in 4% PFA.

Results

Id2a-deficient (Id2aMO-injected) retinoblasts remain proliferative and fail to terminally differentiate and these defects result from a cell-non autonomous function of Id2a, indicating that Id2a acts upstream of extrinsic regulators of retina development (Uribe and Gross, 2010). These phenotypes are reminiscent of the effects of sustained Notch pathway activity on retinal development and therefore, we hypothesized that Notch pathway activity may be regulated by Id2a. To begin to test this hypothesis, Notch pathway activity was globally inhibited in Id2a-deficient embryos using the gamma-secretase inhibitor, DAPT (Geling et al., 2002). Embryos treated with DAPT exhibited phenotypes characteristic of Notch inhibition, i.e., disrupted somite morphology (Geling et al., 2002; Amsterdam et al., 2004; van Eeden et al., 1996; *data not shown*), and, in the retina, an expansion of RGCs (Supp. Fig. 1; Bernardos et al., 2005). Id2aMM and Id2aMO injected embryos were treated with DMSO (vehicle control) or DAPT from 28–48 hpf, subjected to a 30 min BrdU pulse at 48 hpf, and then immediately sacrificed and processed for BrdU immunohistochemistry. As expected, Id2aMM control embryos treated with DMSO contained a low proportion of BrdU-positive cells in their retinae (15%; Fig. 1F), and these BrdU-positive cells were localized largely at the marginal zones (Fig. 1A). DMSO-treated Id2aMO retinae possessed a significantly higher proportion of BrdU-positive cells (53%, $p=0.0004$), and these were distributed ectopically throughout the central retina (Fig. 1B and F). Id2aMO retinae also contained significantly fewer cells than Id2aMM retinae, as previously shown (Fig. 1E) ($p=.03$) (Uribe and Gross, 2010). Treatment of Id2aMM embryos with DAPT decreased retinal cell number slightly, likely due to elevated apoptosis (Bernardos et al., 2005), but DAPT had no effect on BrdU incorporation (Fig. 1C and F). Treatment of Id2aMO embryos with DAPT, however, rescued proliferation defects (Fig. 1D and F); Id2aMO retinae displayed a significant reduction in BrdU-positive cells when compared to DMSO controls (28%; $p=.0014$), and proliferative cells were largely limited to the retinal periphery as in Id2a-MM controls (Fig. 1D and F). In addition, total retinal cell number was significantly rescued ($p=0.04$), and comparable to that of Id2aMM-DAPT embryos (Fig. 1E).

Given that inhibition of the Notch pathway rescued proliferation defects in Id2a-deficient retinae, we were curious if inhibition would also be sufficient to restore the presence of terminally differentiated neurons in Id2a-deficient retinae. To assay terminal differentiation, Id2aMM and Id2aMO embryos were treated with DMSO or DAPT from 28–72 hpf, and at 72 hpf, immunohistochemistry for 5e11 was used to assay for the presence of terminally differentiated amacrine cells (Hyatt et al., 1996; Fadool et al., 1999), or for Zpr1, which detects red/green cone cells (Larison and BreMiller, 1990). As expected, DMSO-treated Id2aMM retinae differentiated amacrine cells and red/green cones

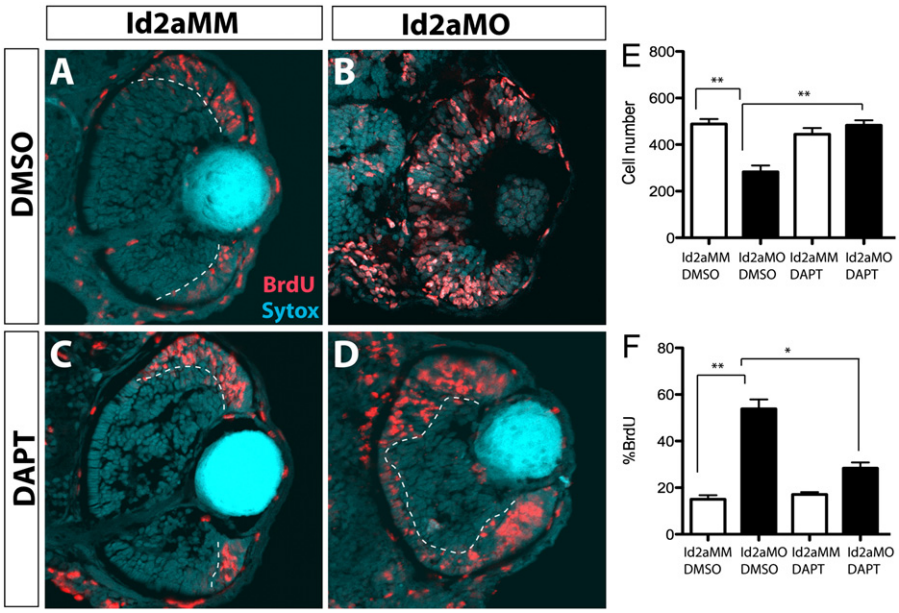

Fig. 1. DAPT inhibition of the Notch pathway rescues proliferation defects in Id2a-deficient retinae. (A)–(D) Id2aMM and Id2aMO embryos treated with either (A) and (B) DMSO or (C) and (D) DAPT from 28–48 hpf and pulsed with BrdU for 30 min prior to fixation. Transverse retinal sections showing BrdU-positive cells at 48 hpf (red). Quantification of average cell number per retinal section (E) and the proportion of BrdU-positive cells per retinal section (F) at 48 hpf. Nuclei are stained with Sytox-green (cyan). Dorsal is up in all images. Error bars represent SEM, $n=9$; $^{*}p<0.05$, $^{**}p<0.005$.

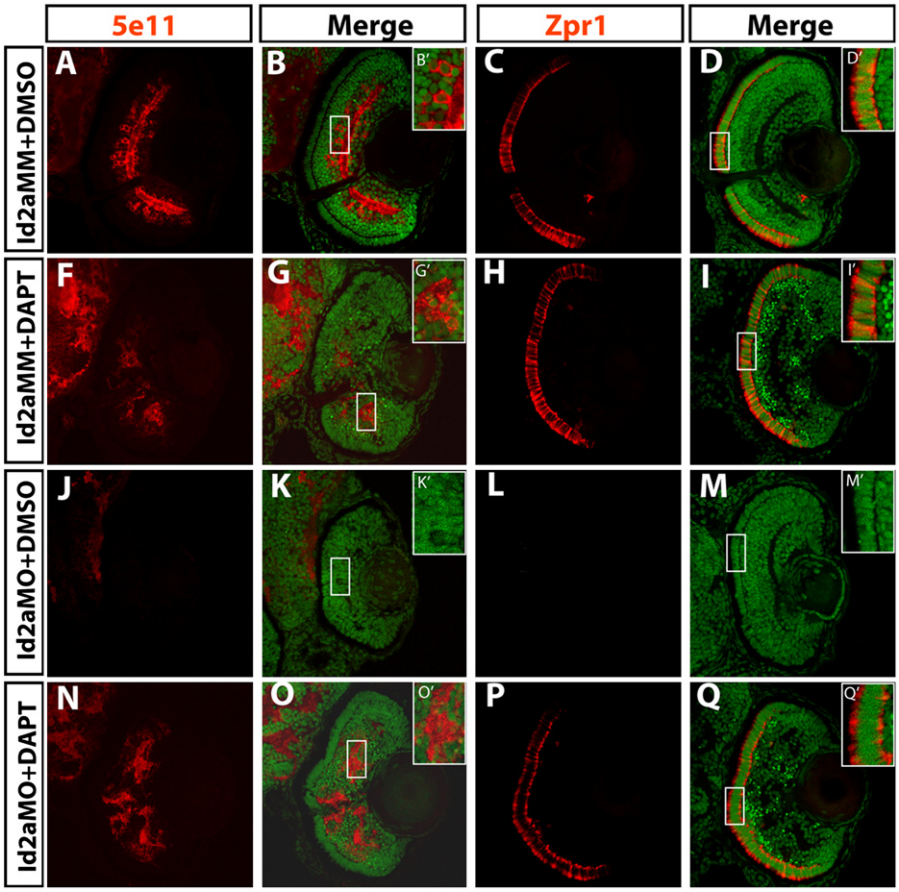

Fig. 2. DAPT inhibition of the Notch pathway rescues terminal differentiation in Id2a-deficient retinae. Transverse retinal sections at 72 hpf from Id2aMM-DMSO (A)–(D), Id2aMM-DAPT (F)–(I), Id2aMO-DMSO (J)–(M) and Id2aMO-DAPT (N)–(Q) embryos. Embryos were treated with DMSO or DAPT from 28–72 hpf. Amacrine cells marked by 5e11 (A), (F), (J) and (N) and red/green cones marked by Zpr1 (C), (H), (L) and (P) fail to differentiate in Id2aMO retinas treated with DMSO (J), while in Id2aMO retinas treated with DAPT, both amacrine cells and red/green cones are detected (N) and (P). Merged images show co-staining of retinal marker (red) and nuclei (Sytox-green; green). Dorsal is up in all images.

normally (Fig. 2A–D). DAPT-treatment resulted in elevated cell death and retinal lamination defects in these embryos, but this did not prevent the terminal differentiation of amacrine cells or red/green cones (Fig. 2F–I). Also as expected, Id2aMO retinae treated with DMSO failed to differentiate amacrine cells and red/green cones (Fig. 2J–M); however, when treated with DAPT, Id2aMO retinae exhibited a striking rescue of neuronal differentiation; both amacrine cells and red/green cones were present (Fig. 2N–Q). As with DAPT-treated controls, lamination was abnormal in retinae from DAPT treated Id2aMO embryos, but amacrine cells and cone photoreceptors nonetheless terminally differentiated. These data indicate that inhibition of Notch pathway activity is sufficient to rescue proliferation and terminal differentiation defects resulting from a loss of Id2a function and suggests that elevated Notch pathway activity could underlie some of the cell non-autonomous effects of Id2a loss of function on retinogenesis.

To determine if Id2a levels influence Notch pathway gene expression, quantitative real-time PCR (qRT-PCR) was used to quantify the expression of Notch pathway genes in Id2aMM and Id2aMO retinae. Here, retinae were isolated from Id2aMM and Id2aMO embryos at 48 hpf and the expression levels of *achaete-scute complex-like1a* (*ascl1a*), *notch1a* (*n1a*), *deltaA* (*dla*), *deltaC* (*dlc*), *hairy-related 4.1* (*her4.1*) and *hairy and enhancer of split 6* (*hes6*) was assessed. In Id2aMO retinae, the expression of each of these factors was elevated over that in Id2aMM retinae; the Notch ligand genes *dla* and *dlc*; the Notch receptor gene *n1a*; and the Notch pathway target genes *ascl1a, her4.1* and *hes6* were all significantly upregulated in retinae lacking Id2a (Fig. 3A). Moreover, in situ hybridization for *ascl1a*, *n1a* and *dla* demonstrated expanded expression domains at 48 hpf in Id2aMO retinae when compared with Id2aMM controls, which only exhibited expression of each of these genes within the ciliary marginal zones of the retina (Fig. 3B). These qRT-PCR and in situ hybridization data, in conjunction with the results of DAPT inhibition experiments, support a model in which Notch pathway gene expression is modulated by Id2a.

To directly examine epistatic interactions between Id2a and specific Notch pathway components, we utilized co-knockdown rescue experiments to determine if, in the absence of Id2a, removal of a specific Notch pathway component would also rescue proliferation and/or terminal differentiation of retinal neurons. We chose to focus on Notch1a, as its expression was elevated in Id2a-deficient retinae (Fig. 3). Zebrafish possess two paralogues (N1a and N1b) and during retinal neurogenesis, *n1a* expression is concentrated near the apical surface of the retina (adjacent to the retinal pigmented epithelium), where Notch signaling activity is predominately active (Del Bene et al., 2008). Cell–cell interactions involving Delta ligands and Notch receptors activate the canonical Notch signaling pathway—this then up regulates the expression of members of the hairy and enhancer of split (Hes) family, which serve to inhibit the expression of proneural genes to inhibit differentiation (Bae et al., 2000; Perron and Harris, 2000). Morpholino knockdown of N1a alone reduced the expression domain of the Notch reporter gene *her4.1* at 24 hpf (Supp. Fig. 2), and N1a morphants exhibited abnormal tail somite morphology at 28 hpf (Supp. Fig. 2), phenocopying defects resulting from DAPT-mediated Notch pathway inhibition (Geling et al., 2002) and those observed in *notch1a* mutants (van Eeden et al., 1996; Amsterdam et al., 2004).

To first determine if elevated Notch1a expression contributes to the proliferation defects in Id2a-deficient retinae, co-knockdown of Id2a and Notch1a (Id2aMO/N1aMO) was performed, and resulting retinae were examined at 48 hpf for the presence of pH3, a marker of late G2/M. As shown previously (Uribe and Gross, 2010), Id2aMO retinae contained fewer pH3-positive cells when compared to

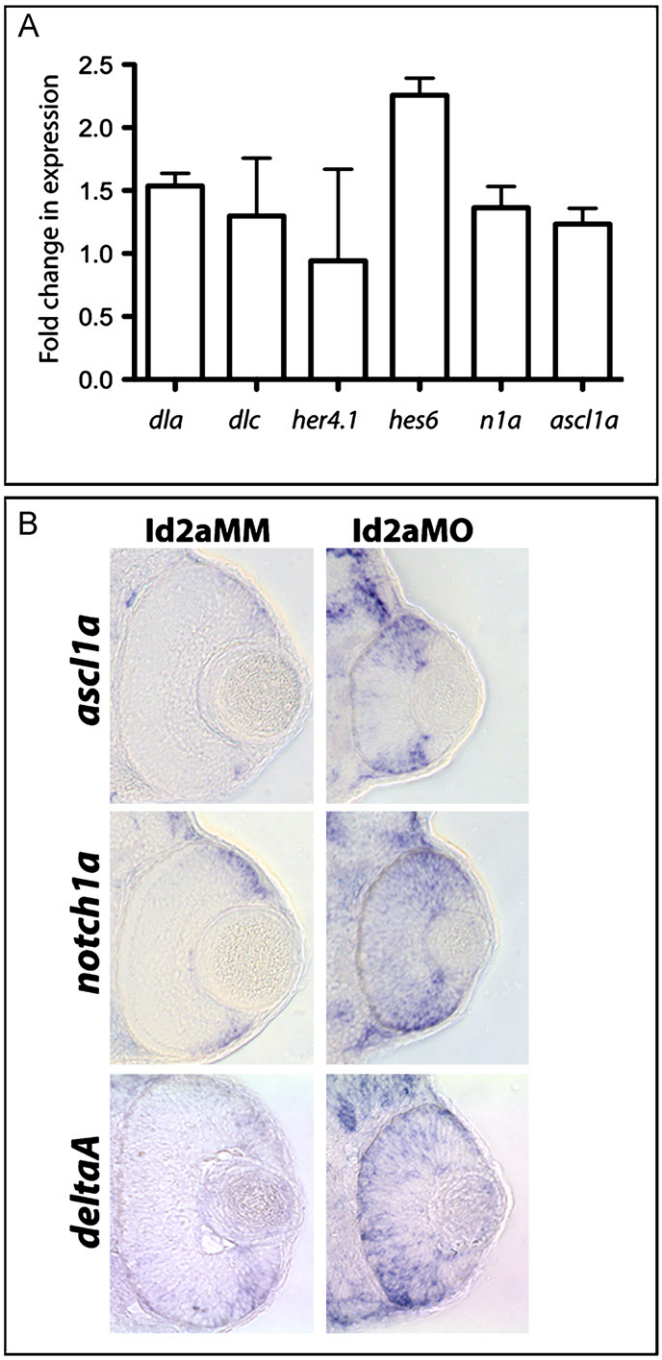

Fig. 3. Notch pathway component gene expression is upregulated in Id2a-deficient retinae. (A) qRT-PCR quantification of 48 hpf *dla*, *dlc*, *her4.1*, *hes6*, *n1a* and *ascl1a* levels in Id2aMO and Id2aMM retinae. Transcript levels were normalized to *tubulin, alpha 1* and the fold-change in expression in Id2aMO vs. Id2aMM presented. Error bars represent SEM, $^{**}p<0.05$, $n=3$ biological replicates. (B) Transverse sections reveal the expression domains for *ascl1a*, *notch1a* and *deltaA* in Id2aMM and Id2aMO embryos at 48 hpf following in situ hybridization ($n=6$ sectioned embryos/condition).

Id2aMM retinae (Fig. 4A, B and F $p=0.015$) and they were microphthalmic, containing significantly fewer cells than controls (Fig. 4E; $p=0.001$). N1aMO retinae exhibited no significant difference in the percentage of pH3-positive cells when compared to Id2aMM control (Fig. 4C and F, $p=0.62$). In the double knockdown condition, Id2aMO/N1aMO retinae recovered pH3 levels to that of Id2aMM controls, exhibiting a significant increase in the percentage of pH3-positive cells over that in Id2aMO retinae alone

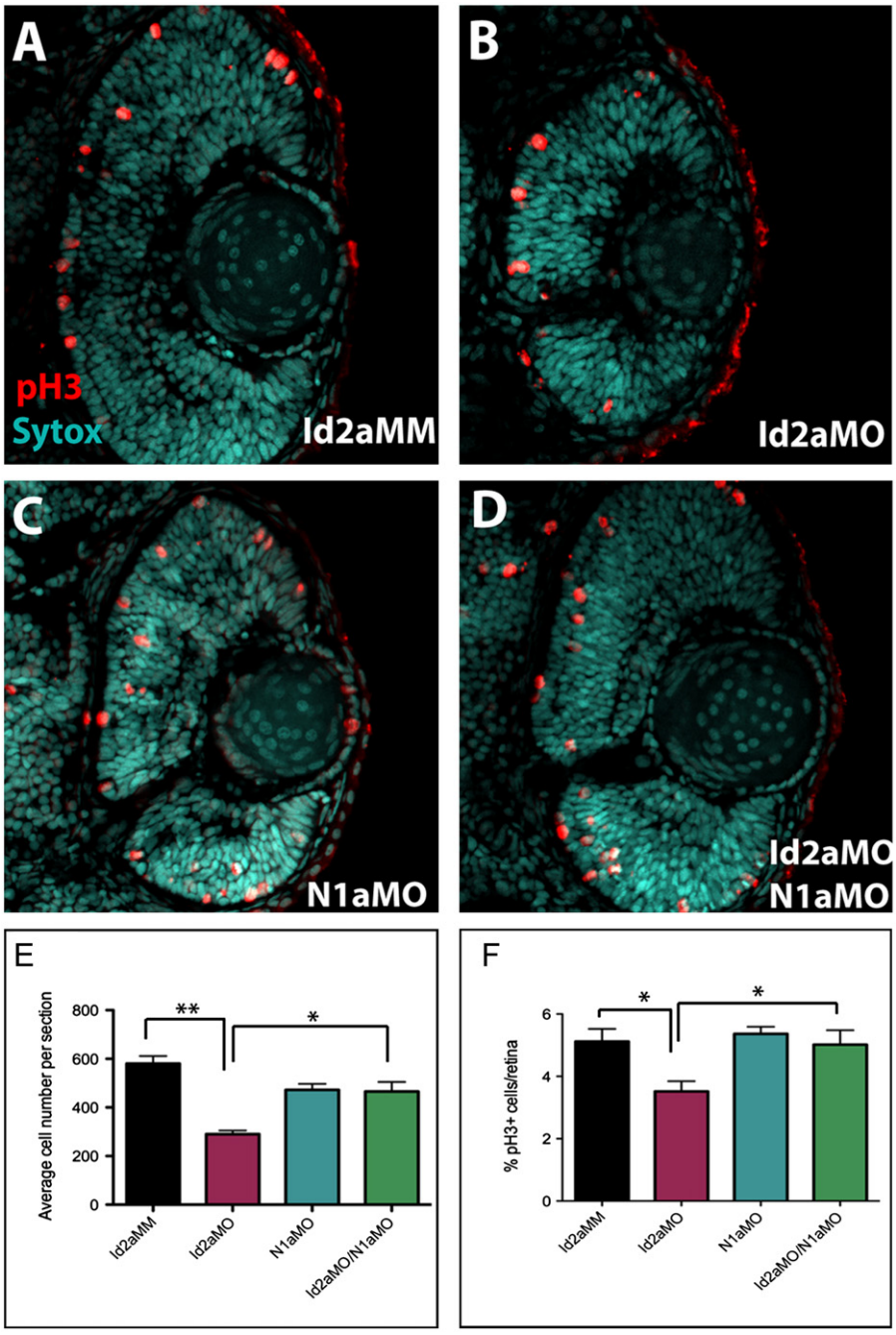

Fig. 4. Knockdown of Notch1a rescues retinoblast proliferation defects in Id2a-deficient retinae. (A)–(D) pH3 immunohistochemistry was used to quantify retinoblasts in late G2/M in transverse retinal sections from (A) Id2aMM, (B) Id2aMO, (C) N1aMO and (D) Id2aMO/N1aMO embryos at 48 hpf. Average total cell number per retinal section was determined for each condition (E), as well as the average percentage of pH3-positive cells per retinal section (F). Dorsal is up in all images. Error bars represent SEM, $n=9$; $^{*}p<0.05$, $^{**}p<0.005$.

(Fig. 4D and F; $p=0.02$). Furthermore, average total cell number in Id2aMO/N1aMO retinae was significantly higher than that in Id2a morphants (Fig. 4E; $p=.003$).

We next investigated if terminal differentiation could also be rescued in Id2aMO/N1aMO knockdown retinae. As above, immunohistochemistry was utilized to assay for the presence of differentiated amacrine cells and red/green cones. As expected at 72 hpf, Id2aMO retinae lacked amacrine cells and red/green cones in comparison to Id2aMM controls (Fig. 5A–H), while terminal differentiation of amacrine cells and red/green cones appeared unaffected in N1aMO retinae (Fig. 5I–L, Gross et al., 2005). Examination of terminal differentiation in Id2aMO/N1aMO retinae revealed a striking rescue of both amacrine cells and red/green cones (Fig. 5M–P), indicating that a reduction in Notch pathway activity, via loss of Notch1a, is sufficient to restore the presence of terminally differentiated neurons in an Id2a-deficient retina.

That Notch pathway genes were upregulated in Id2a-defcient retinae and pharmacological or N1aMO-mediated inhibition of Notch pathway activity rescued proliferation and terminal differentiation defects in Id2a morphants suggests that Id2a activity is necessary to limit Notch pathway activity during retinal development. To further test this model, we next determined if Id2a was sufficient to restrict the expression of Notch pathway genes

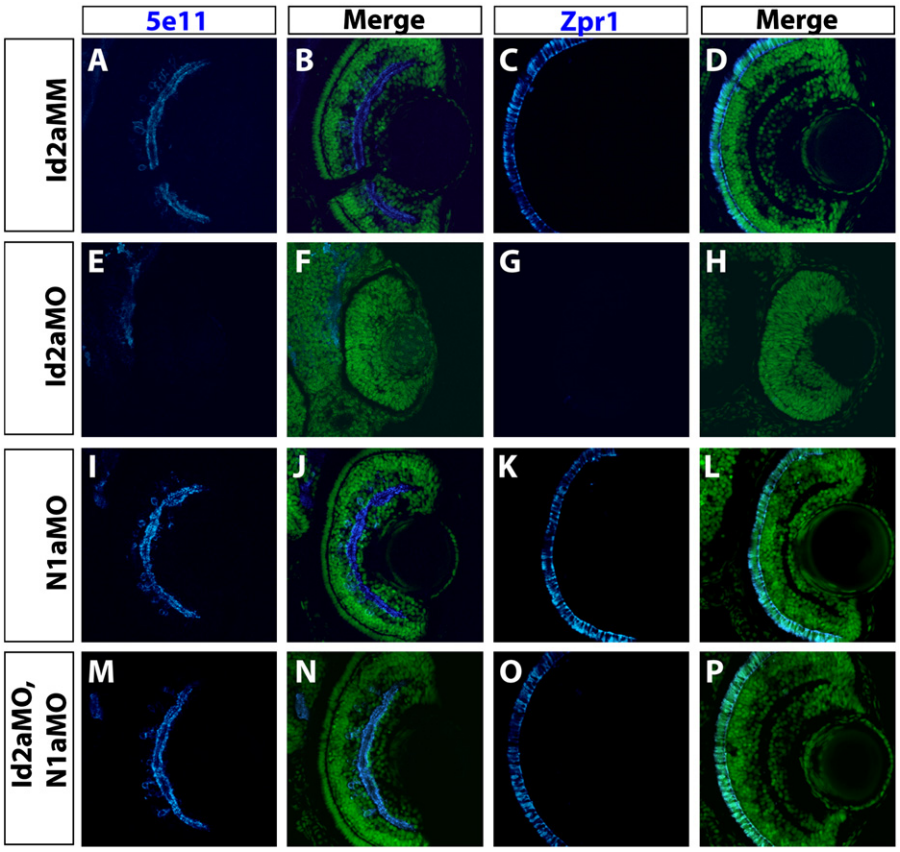

Fig. 5. Knockdown of Notch1a rescues terminal differentiation of retinal neurons in Id2a-deficient retinae. Transverse retinal sections from Id2aMM (A)–(D), Id2aMO (E)–(H), N1aMO (I)–(L) and (M)–(P) Id2aMO/N1aMO embryos at 72 hpf assayed for 5e11 expression (amacrine cells) or Zpr1 expression (red/green cones). Merged images show co-staining of retinal marker (blue) and nuclei (Sytox-green; green). Dorsal is up in all panels.

during retinal neurogenesis. Examining a subset of the Notch pathway genes upregulated in Id2a-deficient retinae, qRT-PCR demonstrated that the transcript levels of *n1a*, *ascl1a*, *dla* and *her4.1* were each significantly reduced in retinae isolated from *id2a*-overexpressing embryos at 33 hpf, when compared to levels in *gfp*-injected controls (Fig. 6I). Spatially, while there was no observable change in the distribution of *n1a* in *id2a*-injected retinae when compared to controls (Fig. 6A and E); however, the expression domains of *ascl1a*, *dla* and *her4.1* were all more restricted in *id2a*-injected retinae than in controls (Fig. 6C–H). *ascl1a* was enriched at the retinal margins in control embryos, and also observed throughout much of the central retina (Fig. 6B), while in *id2a*-overexpressing embryos, *ascl1a* was absent from much of the central retina (Fig. 6F). *dla* was expressed throughout the control retina (Fig. 6C), whereas in *id2a* retinae, *dla* expression was less pronounced within the central retina (Fig. 6G). Finally, in control retinae the distribution of *her4.1* was sharply confined to the ciliary marginal zones (Fig. 6D), while in *id2a*-overexpressing retinae, *her4.1* expression was barely detectable (Fig. 6H). These observations indicate that Id2a is sufficient to restrict the expression of Notch pathway genes during retinal development and when combined with the Notch pathway inhibition experiments, they support a model in which Id2a functions to limit Notch pathway activity during retinal development.

Id2 is a helix-loop-helix factor incapable of directly binding DNA; rather, it heterodimerizes with a variety of transcription factors and transcriptional regulators thereby influencing expression of downstream targets (Benezra, 2001; Jogi et al., 2002). Thus, its function in influencing Notch activity, if direct, must be mediated by some unidentified binding partners. It is also possible that Id2a-dependent regulation of the Notch pathway is indirect, whereby it regulates the expression of an intermediate factor and/or pathway that then regulates Notch pathway activity. In an attempt to gain an unbiased, global view of gene expression changes that occur as a result of Id2a deficiency in the retina, we next utilized a novel RNA-sequencing (RNA-seq) strategy to compare gene expression levels between Id2aMO and Id2aMM retinae and identify gene expression changes resulting from a deficiency in Id2a. Experimentally, RNA was isolated from retinae dissected from 48–50 hpf Id2aMM or Id2aMO injected embryos (lenses removed) and utilized for Illumina sequencing to quantify transcript abundance in either condition (Fig. 7A). Over 100 million 2×100 bp paired-end reads in two biological replicates of both Id2aMM control retinae and Id2aMO knockdown retinae were mapped to all known zebrafish cDNA sequences (Supplemental Table 1; see the Materials and methods section), and differentially expressed (DE) transcripts between Id2aMM and Id2aMO retinae were identified from these mapped reads. Setting a threshold for differential expression of a two fold change with significance of adjusted $p < 0.05$, and a mean of normalized RPK100M of either sample is greater than 500, 1877 DE transcripts were identified (corresponding to 1255 genes) (Fig. 7B and C; Supplemental Table 2).

Among the downregulated genes, factors involved in retinal neuron differentiation and function exhibited the most dramatic reduction in expression (Fig. 7D). These included genes that encode for retinal photoreceptor cell proteins such as Rhodopsin (*rho*), Opsin 1 medium-wave-sensitive (*opn1lw2*), Arrestin3a

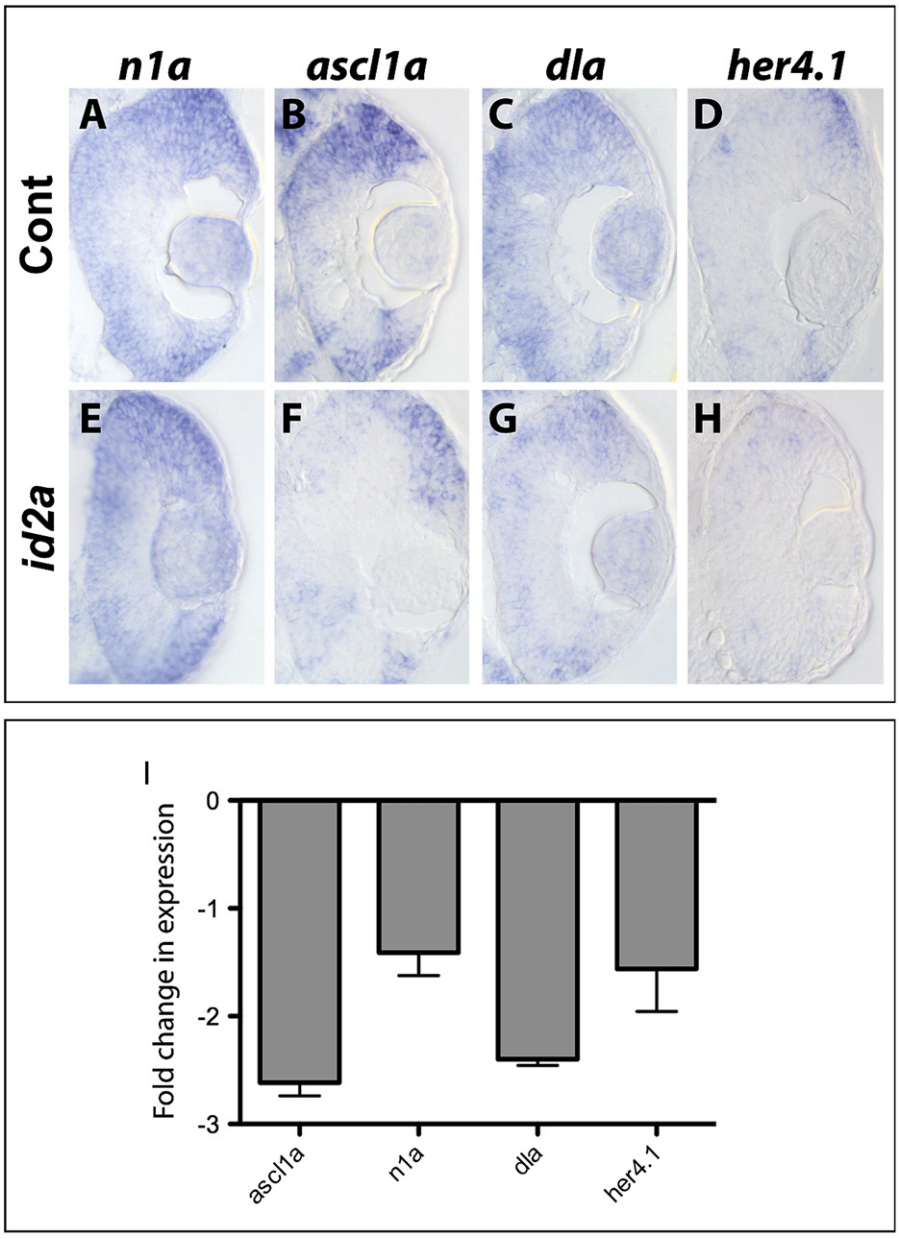

Fig. 6. *Id2a*-overexpression is sufficient to limit the retinal expression of Notch pathway component genes. Transverse sections reveal the expression domains for *n1a, ascl1a, dla* and *her4.1* at 33hpf in *gfp*-injected control (A)–(D) and *id2a*-overexpressing (E)–(H) embryos following in situ hybridization ($n=6$ sectioned embryos/condition). (I) qRT-PCR quantification of *ascl1a*, *n1a*, *dla* and *her4.1* levels in *id2a* retinas compared to *gfp* control retinas at 33 hpf. Transcript levels were normalized to *tubulin, alpha 1* and the fold-change in expression in *gfp*-injected vs. *id2a*-injected retinae presented. Error bars represent SEM, $^{**}p < .05$, $n=3$ biological replicates.

(*arr3a*) and S-antigen; retina and pineal gland b (*sagb*) (Fig. 7D). This is not surprising given the lack of terminally differentiated neurons in Id2aMO retinae and served as an excellent internal control for the RNA-Seq approach and DE analyses. Numerous other genes of interest were noted within the DE downregulated list; for example, several that encode proteins functioning in signaling pathways that feed into the cell cycle such as Cyclin dependent kinase inhibitor 1b (p27) (*cdkn1b*), Adenomatosis polyposis coli (*apc*) and Mitogen-activated kinase 10 (*mapk10*).

Upregulated in Id2a-deficient retinae were also a number of relevant genes and pathways. Indeed, several genes encoding proteins involved in cell cycle progression were upregulated in Id2a-deficient retinae, and these included Cyclin D1 (*ccnd1*), Cyclin G1 (*ccng1*), Cyclin dependent kinase 6 (*cdk6*), Checkpoint homologue 2 (*chek2*), S-phase kinase-associated protein 2 (*skp2*) and Cyclin dependent kinase inhibitor 1a (*cdkI1a*) (Fig. 7D). This increase in cell cycle pathway components was also not surprising, given Id2a's role in modulating retinoblast cell cycle progression (Uribe and Gross, 2010). In addition, several Wnt pathway components were also upregulated. The Wnt pathway is known to regulate the proliferative state of retinoblasts as well as their neurogenic potential (Kubo et al., 2005; Agathocleous et al., 2009). Transcripts encoding R-spondin homologue (*rspo1*), Frizzled 6 (*fzd6*) and Transcription factor 7-like 1b (*tcf7l1b*) were enriched in Id2a-deficient retinae (Fig. 7D). Rspo1 is a secreted Wnt pathway agonist (Carmon et al., 2011), while Fzd6 is a Wnt receptor (Katoh, 2008) and Tcf7l1b (formerly known as Tcf3b) is a downstream mediator of the Wnt pathway (Amoyel et al., 2005). qRT-PCR validation of the RNA-seq dataset for a subset of the DE genes (*ccnd1*, *cdk6*, *skp2*, *cdkI1a, fzd6, rspo1 and tcf7l1b*) indicated that

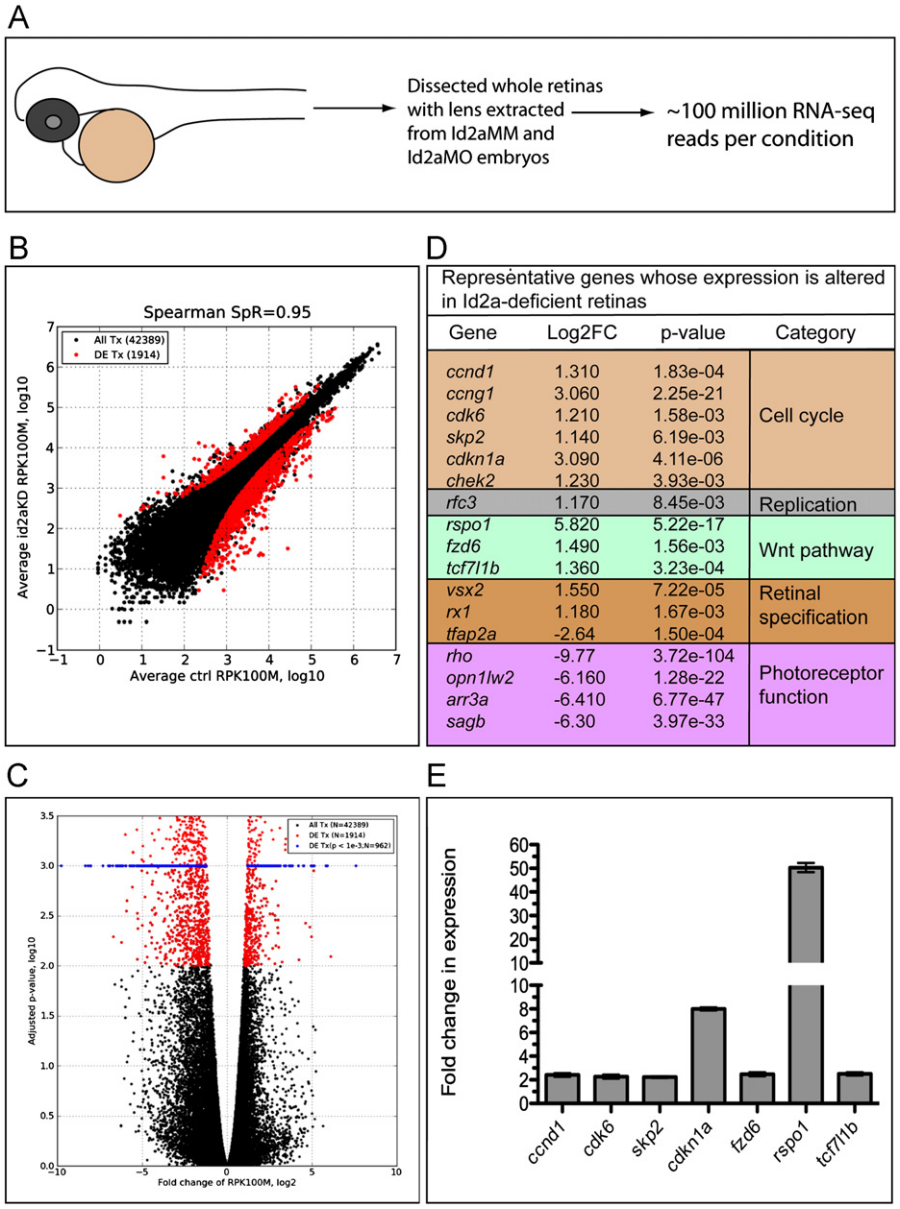

Fig. 7. RNA-Seq analysis of differential retinal gene expression resulting from Id2a-deficiency. (A) Schematic of RNA-Seq experiment. Whole retinas were dissected from Id2aMM and Id2aMO embryos, their lenses were removed and total retinal RNA was extracted and used for Illumina RNA sequencing. ~100 million Illumina reads were obtained for each condition, and the experiment was repeated in two biological replicates for each condition (see the Materials and methods section for details). (B) The number of reads per each transcript was normalized with total number of mapped reads and transcript length, converting all raw read counts to 'number of reads per 1000 bases per 100 million reads' (RPK100M). '100 million reads' were chosen to get positive values of $\log_{10}$-transformed normalized values. A scatter plot shows the correlation between Id2aMO and Id2aMM control normalized reads (Spearman = 0.95). Differentially expressed transcripts with fold changes greater than 2 and adjusted *p*-value less than 0.01 are indicated with red dots. (C) Volcano plot illustrating that out of 42,389 mapped transcripts, 1914 were significantly differentially expressed (DE), $p < 0.01$ (red dots), and, for visualization purposes, of those 962 were highly significant, with an adjusted *p*-value < 0.001 (blue dots). (D) A table depicting representative genes that exhibit significant changes in gene expression between Id2aMM and Id2aMO retinas; genes were categorized based upon known pathways and are color coded. (E) qRT-PCR validation of RNA-Seq data for a subset of DE genes. Transcript levels of *ccnd1*, *cdk6*, *skp2*, *cdkn1a*, *fzd6*, *rspo1* and *tcf7l1b* in Id2aMO retinae compared to Id2aMM retinae at 48hpf. Transcript levels were normalized to tubulin and the fold-change in expression in Id2aMO retinae compared to Id2aMM retinae presented. Error bars represent SEM, $^{**}p < 0.05$, $n = 3$ biological replicates.

Id2a-deficient retinae exhibited elevated transcript levels over those of Id2a-MM controls and supported the validity of the DE dataset and thus, the RNA-Seq and bioinformatic approach (Fig. 7E).

Pathway and GO term enrichment tests of the DE genes, via the DAVID program, identified various pathways and biological processes affected by Id2a-deficiency (Supplemental Tables 3 and 4). For those genes downregulated in Id2a-deficient retinae, enriched pathways included the biological processes of 'calcium ion binding' and 'cell adhesion' (Supplemental Table 3). Amongst the upregulated genes, enriched pathways included 'RNA processing', 'nitrogen compound biosynthesis', 'nucleotide biosynthesis', 'p53 signaling pathway', 'cell cycle' and 'DNA replication' (Supplemental Table 4). In all, pathway enrichment analyses revealed a number of potentially interesting pathways as being influenced downstream of Id2a function during retinogenesis, and as such, these identified pathways and genes should serve as excellent candidates for future studies to determine how Id2a mediates proliferation and differentiation during retinogenesis.

Discussion

The timing and distribution of Notch pathway activity is fundamental in dictating the choice between proliferation and neurogenesis during retinal development. Notch pathway activity maintains retinal progenitor cells in a proliferative state in order to ensure that there are sufficient numbers of progenitors available for successive waves of retinal neurogenesis (Henrique et al., 1997; Cepko, 1999; Jadhav et al., 2006a). In mice, sustained Notch activity within retinal progenitor cells leads to an upregulation of multiple "progenitor" genes, such as *Notch1*, *Fgf15* and *Ccnd1* (Jadhav et al., 2006a), and this correlates with the maintenance of a proliferative progenitor state. In contrast, when Notch signaling activity is ectopically induced in newly postmitotic retinal cells, cells ultimately adopt a glial cell fate, demonstrating that temporal control of Notch activity is also critical during retinal development (Jadhav et al., 2006a). In the zebrafish retina, ectopic expression of a constitutively active Notch1a variant impairs neuronal differentiation; retinal progenitor cells either differentiate into glia or remain undifferentiated and eventually undergo apoptosis (Scheer et al., 2001). Conversely, Notch inhibition causes retinoblasts to prematurely exit the cell cycle and differentiate into early born retinal cell types (Bernardos et al., 2005).

Our previous study demonstrated that Id2a levels modulated retinoblast cell cycle kinetics and that Id2a was required for the terminal differentiation of late born retinal cell types, i.e., amacrine and bipolar neurons, rod and cone photoreceptors, and Müller glia, phenotypes reminiscent of enhanced Notch activity. Indeed, mosaic analyses revealed that these roles were cell nonautonomous, indicating that Id2a functions upstream of extrinsic regulators of retinal development (Uribe and Gross, 2010). In the present study, we expanded on these observations and our data support a model in which Id2a function is required to limit Notch pathway activity during retinal development. The Notch ligand genes *dla* and *dlc*; the Notch receptor gene *n1a*; and the Notch pathway target genes *ascl1a, her4.1* and *hes6* were all significantly upregulated in retinae lacking Id2a. Conversely, the expression levels of *ascl1a*, *n1a*, *dla* and *her4.1* were downregulated in retinae overexpressing *id2a. dla*, *dlc* and *n1a* are expressed in a graded fashion along the apical-basal axis within the zebrafish retinal neuroepithelium and this graded localization is thought to dictate the level of Notch signaling a retinoblast is exposed to during interkinetic nuclear migration (IKNM) (Del Bene et al., 2008). *Dll1*, orthologous to zebrafish *dla*, is expressed in chicken retinoblasts (Nelson and Reh, 2008; Nelson et al., 2009), and its ectopic expression prevents retinal progenitor cells from differentiating (Henrique et al., 1997). Regulated expression of each of these factors is therefore critical to control the balance between proliferation and differentiation in retinoblasts, and this balance is disrupted in Id2a-deficient retinae.

While the inverse relationship between Id2a levels and Notch pathway components was not surprising, upregulation of *hes6* in Id2a-deficient retinae was, given that Hes6 is typically associated with proneural activity (Bae et al., 2000; Koyano-Nakagawa et al., 2000). For example, in mouse retinal explants, ectopic expression of *Hes6* during late phases of retinogenesis causes retinal cells to predominately differentiate into rod photoreceptors, by overcoming Hes1-mediated repression of neurogenesis and biasing retinoblasts towards neuronal fates (Bae et al., 2000). While we observed increased *hes6* expression in Id2a-deficient retinae, Id2a-deficient retinae lack nearly all neurons and Müller glia, indicating that this upregulation did not result in a proneural bias. Given the significant upregulation of other Notch pathway components, and antagonistic interactions between these factors, it could be that the increase in *hes6* expression was not sufficient to overcome the overall net increase in Notch pathway activity within Id2a-deficient retinae, and thus, retinoblasts remained proliferative. Hes6 itself has also recently been identified as a regulator of cell proliferation in glioma cells (Haapa-Paananen et al., 2012), and it could also feasibly function as a contributor to the overall enhanced proliferative state of Id2a-deficient retinoblasts.

Pharmacological inhibition of Notch pathway activity rescued both proliferation and differentiation defects in Id2a-deficient retinae, as did knockdown of Notch1a expression, suggesting that upregulation of Notch pathway activity contributes to the retinoblast proliferation and differentiation defects in Id2a-deficient embryos. In Id2a-deficient retinae, we previously reported that *crx* expression was absent and cone photoreceptors failed to differentiate (Uribe and Gross, 2010). Notch1 functions to suppress cone photoreceptor fates, at least in part, by suppressing the expression of key transcription factors required for their genesis, such as *crx* (Yaron et al., 2006). It could be that elevated Notch1a levels within Id2a-defcient retinae inhibit the expression of genes required for photoreceptor cell specification and differentiation, therefore abolishing photoreceptor production. In support of this model, we found that knockdown of Notch1a in Id2a-deficient retinae was sufficient to fully restore cone photoreceptor production. Although our data do not rule out the possibility that Id2a and Notch function within parallel pathways regulating retinoblast proliferation and differentiation, the transcriptional changes in Notch pathway gene expression occurring as a result of modulating Id2a levels lead us to favor a model in which Id2a acts upstream of the Notch pathway in a common gene regulatory network governing retinogenesis, where Id2a serves to limit Notch pathway activity (Fig. 8).

How would Id2a regulate Notch pathway gene expression in such a network? If Id2a directly regulates the pathway, given that Ids do not directly bind to DNA, Id2a's effect would have to be achieved by antagonizing a regulatory factor that ultimately functions to positively drive the expression of Notch pathway genes. We do not know to what factors Id2a binds in the zebrafish retina; however, Id3, an Id family member, is known to regulate the expression of *Notch1* in developing T cells and it is thought that this occurs by Id3 binding to members of the bHLH complex E2A, a heterocomplex of E12 and E47, thereby antagonizing E2A formation and its ability to activate *notch1* expression (Yashiro-Ohtani et al., 2009). Id2 can bind to and sequester E12 and E47 proteins in fibroblasts, thereby modulating the expression of cell cycle regulators, indicating that Id2 has the capacity to inhibit E2A heterocomplex formation (Trabosh et al., 2009). Id2a could also sequester the bHLH protein Ascl1a, a Notch pathway target gene itself, which functions to activate the expression of *delta-like* and other Notch pathway targets in the retina (Nelson et al., 2009). Indeed, numerous factors have been shown to regulate

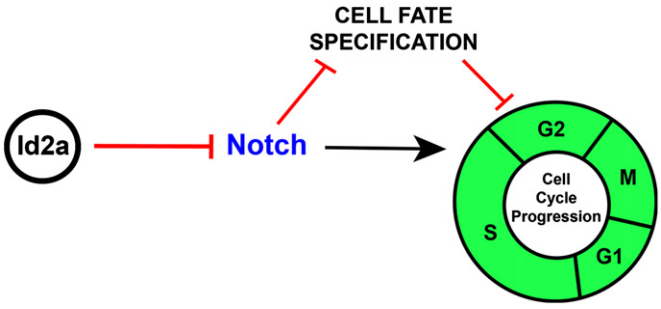

Fig. 8. Model for Id2a/Notch regulation of retinoblast proliferation and differentiation. (A) Id2a negatively regulates Notch pathway component expression and pathway activity. When active, the Notch pathway maintains retinoblasts in a proliferative, progenitor state.

Notch pathway gene expression, both transcriptionally and post-transcriptionally, and may of these also represent possible candidates for Id2a antagonism. For example, in mouse and chicken neuroepithelia, *Notch1* and *Dll1* transcripts are expressed in a cell cycle dependent manner (Cisneros et al., 2008), and in mouse neuroepithelial cell lines and knockouts, they are stabilized during M-phase via interaction with Elavl1/HuR (Garcia-Dominguez et al., 2011). In any of these scenarios, Id2a would serve to antagonize the function of an immediate upstream regulator of Notch pathway gene expression and thereby exert its influence on Notch pathway gene expression and activity. Importantly however, direct Id-Notch regulatory interactions are also likely to be tissue specific as, for example, in olfactory bulb development Id2 is required for dopaminergic neuron specification and Id2 knockouts possess decreased levels of *Hes1* and *Ascl1*in their olfactory bulbs (Havrda et al., 2008). Thus, Id-dependent antagonism is likely context dependent and determined by the interacting factors present in the Id-expressing tissues (i.e., whether Ids are antagonizing positively acting or negatively acting factors that then regulate Notch pathway gene expression in this context dependent manner).

Downstream factors or pathways whose expression or activity depends on Id2a function could also mediate the regulatory network interactions between Id2a and the Notch pathway in an indirect fashion. With this in mind, and to gain insight into gene expression changes resulting from loss of Id2a function, we utilized a novel RNA-Seq approach to identify differentially expressed genes between Id2aMO and Id2aMM retinae. Upregulated genes belonging to the 'cell cycle' and 'Wnt pathway' were identified (Fig. 7, Supplemental Table 4), and downregulated genes involved in the processes of 'calcium ion binding', 'ion transport', 'cell adhesion' and 'ion channel activity' were identified, among others (Supplemental Table 3).

The identification of the cell cycle pathway as being influenced downstream of Id2a correlates well with the proliferative defects in Id2a-deficient retinoblasts (Uribe and Gross, 2010) and with the Id2a-dependent upregulated Notch pathway activity described here. *ccnd1*, *ccng1*, *cdk6*, *skp2*, *cdkn1a* and *chek2* were all significantly enriched within Id2a-deficient retinae, while *cdkn1b* was downregulated. Many of these factors have been shown to influence, or be influenced by, Notch pathway activity (e.g., Sarmento et al., 2005; Bienvenu et al., 2010; Ronchini and Capobianco, 2001). Of particular interest in this group is CyclinD1; in addition to its well-characterized role in regulating cell cycle progression, it also possesses a transcriptional activator role and positively regulates Notch1 expression in the retina (Bienvenu et al., 2010). The Notch pathway feeds into positive regulation of CyclinD1 expression, which then maintains a proliferative and undifferentiated state (Ronchini and Capobianco, 2001).

Id2a-dependent regulation of cell cycle progression itself could also contribute to the elevated Notch pathway activity in Id2a-deficient retinae. Skp2, S-phase kinase-associated protein 2, is an important regulator of cell cycle maintenance because it promotes the degradation of p21, p27 and p57 proteins (Chan et al., 2010). Skp2 expression can be activated directly by Notch signaling (Sarmento et al., 2005) and by E2F transcription factors (Chan et al., 2010), and thus elevated Skp2 levels would maintain Id2a-deficient retinoblasts in a proliferative state. Moreover, in the zebrafish retinal neuroepithelium there is an apical (high) to basal (low) gradient of Notch activity, and during IKNM, progenitor nuclei are exposed to Notch-dependent neurogenic versus proliferative signals based on their location within each proliferating retinoblast (Del Bene et al., 2008). Id2a-deficient retinoblasts progress through the cell cycle more slowly, with S to M phase progression significantly delayed (Uribe and Gross, 2010). While we have not quantified IKNM in Id2a-deficient retinae, it is possible that the delay in S to M phase progression manifests as a prolonged IKNM of Id2a-deficient neuroblast nuclei, and in this scenario, Id2a-deficient retinoblast nuclei would spend longer periods of time apically and thus be exposed to a higher duration of Notch signaling, resulting in the maintenance of a progenitor state.

Wnt pathway gene expression was upregulated in Id2a-deficient retinae; *rspo1*, *fzd6* and *tcf7l1b* were all expressed at higher levels than in control retinae. The Wnt pathway is a key regulator of retinogenesis (Kubo et al., 2005), and in Xenopus, ectopic activation of the Wnt pathway inhibits retinal neuron differentiation and maintains the retinal progenitor pool by activating Notch signaling, indicating that there is crosstalk between the Wnt and Notch pathways to regulate proliferation and differentiation events in the retina (Agathocleous et al., 2009). Rspo1 is a secreted positive regulator of Wnt signaling events, but has not yet been implicated in retinal development (MacDonald et al., 2009; Carmon et al., 2011).

In summary, our data support a model in which Id2a, an intrinsic regulator of retinogenesis, acts upstream of the Notch pathway to regulate the balance between proliferation and differentiation during retinogenesis (Fig. 8). Alterations in Id2a levels result in prolonged S to M phase progression during the retinoblast cell cycle and a lack of terminal differentiation of retinal neurons and glia. Notch pathway gene expression is inversely correlated with Id2a levels, and blocking Notch pathway activity pharmacologically or by targeting the Notch 1a receptor rescues both proliferation and differentiation defects in Id2a-deficient retinae. Analysis of global gene expression changes resulting from Id2a deficiency revealed that a number of regulatory pathways involved in modulating retinoblast proliferation and differentiation were affected by loss of Id2a, and several of these have known interactions with the Notch pathway. Future studies analyzing these pathways, their dependence on Id2a and their downstream effects on Notch activity will continue to shed light on how the balance between proliferation and differentiation is maintained in the retina in order to form a retina of proper size and cell type composition.

Acknowledgements

This work was supported by N.I.H. grants RO1-EY18005 to J.M.G. and F31-EY019239 to R.A.U., grants from the N.I.H., N.S.F., Cancer Prevention Research Institute of Texas, U.S. Army Research (58343-MA) and Welch Foundation (F1515) to E.M.M. and the University of Texas at Austin Graduate School Continuing Fellowship to T.K. We are grateful to Declan MacManus for technical support and to members of the Gross lab for helpful suggestions throughout the course of this work. cDNAs and antisera were obtained from ZIRC, supported by NIH-NCRR grant P40-RR012546.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.08.032.

References

Agathocleous, M., Harris, W.A., 2009. From progenitors to differentiated cells in the vertebrate retina. Annu. Rev. Cell. Dev. Biol. 25, 45–69.

Agathocleous, M., Iordanova, I., Willardsen, M.I., Xue, X.Y., Vetter, M.L., Harris, W.A., Moore, K.B., 2009. A directional Wnt/beta-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the Xenopus retina. Development 136, 3289–3299.

Amoyel, M., Cheng, Y.C., Jiang, Y.J., Wilkinson, D.G., 2005. Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain. Development 132, 775–785.
Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S., Hopkins, N., 2004. Identification of 315 genes essential for early zebrafish development. Proc. Nat. Acad. Sci. U.S.A. 101, 12792–12797.
Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11, R106.
Bae, S., Bessho, Y., Hojo, M., Kageyama, R., 2000. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. Development 127, 2933–2943.
Bassett, E.A., Korol, A., Deschamps, P.A., Buettner, R., Wallace, V.A., Williams, T., West-Mays, J.A., 2012. Overlapping expression patterns and redundant roles for AP-2 transcription factors in the developing mammalian retina. Dev. Dyn. 241, 814–829.
Benezra, R., 2001. Role of Id proteins in embryonic and tumor angiogenesis. Trends Cardiovasc. Med. 11, 237–241.
Bernardos, R.L., Lentz, S.I., Wolfe, M.S., Raymond, P.A., 2005. Notch–Delta signaling is required for spatial patterning and Muller glia differentiation in the zebrafish retina. Dev. Biol. 278, 381–395.
Bienvenu, F., Jirawatnotai, S., Elias, J.E., Meyer, C.A., Mizeracka, K., Marson, A., Frampton, G.M., Cole, M.F., Odom, D.T., Odajima, J., Geng, Y., Zagozdzon, A., Jecrois, M., Young, R.A., Liu, X.S., Cepko, C.L., Gygi, S.P., Sicinski, P., 2010. Transcriptional role of cyclin D1 in development revealed by a genetic–proteomic screen. Nature 463, 374–378.
Bill, B.R., Balciunas, D., McCarra, J.A., Young, E.D., Xiong, T., Spahn, A.M., Garcia-Lecea, M., Korzh, V., Ekker, S.C., Schimmenti, L.A., 2008. Development and notch signaling requirements of the zebrafish choroid plexus. PLoS One 3 (9), e3114.
Carmon, K.S., Gong, X., Lin, Q., Thomas, A., Liu, Q., 2011. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc. Nat. Acad. Sci. U.S.A. 108, 11452–11457.
Cepko, C.L., 1999. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr. Opin. Neurobiol. 9, 37–46.
Chan, C.H., Lee, S.W., Wang, J., Lin, H.K., 2010. Regulation of Skp2 expression and activity and its role in cancer progression. Sci. World J. 10, 1001–1015.
Cisneros, E., Latasa, M.J., Garcia-Flores, M., Frade, J.M., 2008. Instability of Notch1 and Delta1 mRNAs and reduced Notch activity in vertebrate neuroepithelial cells undergoing S-phase. Mol. Cell. Neurosci. 37, 820–831.
Del Bene, F., Wehman, A.M., Link, B.A., Baier, H., 2008. Regulation of neurogenesis by interkinetic nuclear migration through an apical–basal notch gradient. Cell 134, 1055–1065.
Dowling, J.E., 2012. The Retina: An Approachable Part of the Brain. (Revised edition). The Belknap Press of Harvard Univeristy Press.
Fadool, J.M., Fadool, D.A., Moore, J.C., Linser, P.J., 1999. Characterization of monoclonal antibodies against zebrafish retina. Invest. Opth. Vis. Sci. Suppl. 40, 1251.
Garcia-Dominguez, D.J., Morello, D., Cisneros, E., Kontoyiannis, D.L., Frade, J.M., 2011. Stabilization of Dll1 mRNA by Elavl1/HuR in neuroepithelial cells undergoing mitosis. Mol. Biol. Cell 22, 1227–1239.
Geling, A., Steiner, H., Willem, M., Bally-Cuif, L., Haass, C., 2002. A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. EMBO Rep. 3, 688–694.
Gregg, R.G., Willer, G.B., Fadool, J.M., Dowling, J.E., Link, B.A., 2003. Positional cloning of the young mutation identifies an essential role for the Brahma chromatin remodeling complex in mediating retinal cell differentiation. Proc. Nat. Acad. Sci. U.S.A. 100, 6535–6540.
Gross, J.M., Perkins, B.D., Amsterdam, A., Egana, A., Darland, T., Matsui, J.I., Sciascia, S., Hopkins, N., Dowling, J.E., 2005. Identification of zebrafish insertional mutants with defects in visual system development and function. Genetics 170, 245–261.
Haapa-Paananen, S., Kiviluoto, S., Waltari, M., Puputti, M., Mpindi, J.P., Kohonen, P., Tynninen, O., Haapasalo, H., Joensuu, H., Perala, M., Kallioniemi, O., 2012. HES6 gene is selectively overexpressed in glioma and represents an important transcriptional regulator of glioma proliferation. Oncogene 31, 1299–1310.
Harada, T., Harada, C., Parada, L.F., 2007. Molecular regulation of visual system development: more than meets the eye. Genes Dev. 21, 367–378.
Havrda, M.C., Harris, B.T., Mantani, A., Ward, N.M., Paolella, B.R., Cuzon, V.C., Yeh, H.H., Israel, M.A., 2008. Id2 is required for specification of dopaminergic neurons during adult olfactory neurogenesis. J. Neurosci. 28, 14074–14086.
Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., Lewis, J., 1997. Maintenance of neuroepithelial progenitor cells by Delta–Notch signalling in the embryonic chick retina. Curr. Biol. 7, 661–670.
Hu, M., Easter, S.S., 1999. Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. Dev. Biol. 207, 309–321.
Huang da, W., Sherman, B.T., Lempicki, R.A., 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13.
Huang da, W., Sherman, B.T., Lempicki, R.A., 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.
Hyatt, G.A., Schmitt, E.A., Fadool, J.M., Dowling, J.E., 1996. Retinoic acid alters photoreceptor development in vivo. Proc. Nat. Acad. Sci. U.S.A. 93, 13298–13303.
Ishitani, T., Hirao, T., Suzuki, M., Isoda, M., Ishitani, S., Harigaya, K., Kitagawa, M., Matsumoto, K., Itoh, M., 2010. Nemo-like kinase suppresses Notch signalling by interfering with formation of the Notch active transcriptional complex. Nat. Cell Biol. 12 (3), 278–285.
Jadhav, A.P., Cho, S.H., Cepko, C.L., 2006a. Notch activity permits retinal cells to progress through multiple progenitor states and acquire a stem cell property. Proc. Nat. Acad. Sci. U.S.A. 103, 18998–19003.
Jogi, A., Persson, P., Grynfeld, A., Pahlman, S., Axelson, H., 2002. Modulation of basic helix-loop-helix transcription complex formation by Id proteins during neuronal differentiation. J. Biol. Chem. 277, 9118–9126.
Jowett, T., Lettice, L., 1994. Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. Trends Genet. 10, 73–74.
Katoh, M., 2008. WNT signaling in stem cell biology and regenerative medicine. Curr. Drug Targets 9, 565–570.
Koyano-Nakagawa, N., Kim, J., Anderson, D., Kintner, C., 2000. Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. Development 127, 4203–4216.
Kubo, F., Takeichi, M., Nakagawa, S., 2005. Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes. Development 132, 2759–2770.
Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25.
Larison, K.D., Bremiller, R., 1990. Development 109(3), 567–576.
Lorent, K., Yeo, S.Y., Oda, T., Chandrasekharappa, S., Chitnis, A., Matthews, R.P., Pack, M., 2004. Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. Development 131, 5753–5766.
Luo, H., Jin, K., Xie, Z., Qiu, F., Li, S., Zou, M., Cai, L., Hozumi, K., Shima, D.T., Xiang, M., 2012. Forkhead box N4 (Foxn4) activates Dll4-Notch signaling to suppress photoreceptor cell fates of early retinal progenitors. Proc. Nat. Acad. Sci. U.S.A. 109, E553–562.
MacDonald, B.T., Tamai, K., He, X., 2009. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev. Cell 17, 9–26.
Martinez-Morales, J.R., Del Bene, F., Nica, G., Hammerschmidt, M., Bovolenta, P., Wittbrodt, J., 2005. Differentiation of the vertebrate retina is coordinated by an FGF signaling center. Dev. Cell 8, 565–574.
Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628.
Murali, D., Yoshikawa, S., Corrigan, R.R., Plas, D.J., Crair, M.C., Oliver, G., Lyons, K.M., Mishina, Y., Furuta, Y., 2005. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. Development 132, 913–923.
Nelson, B.R., Hartman, B.H., Ray, C.A., Hayashi, T., Bermingham-McDonogh, O., Reh, T.A., 2009. Acheate-scute like 1 (Ascl1) is required for normal delta-like (Dll) gene expression and notch signaling during retinal development. Dev. Dyn. 238, 2163–2178.
Nelson, B.R., Reh, T.A., 2008. Relationship between Delta-like and proneural bHLH genes during chick retinal development. Dev. Dyn. 237, 1565–1580.
Perron, M., Harris, W.A., 2000. Determination of vertebrate retinal progenitor cell fate by the Notch pathway and basic helix-loop-helix transcription factors. Cell. Mol. Life Sci. 57, 215–223.
Ronchini, C., Capobianco, A.J., 2001. Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). Mol. Cell. Biol. 21, 5925–5934.
Sarmento, L.M., Huang, H., Limon, A., Gordon, W., Fernandes, J., Tavares, M.J., Miele, L., Cardoso, A.A., Classon, M., Carlesso, N., 2005. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. J. Exp. Med. 202, 157–168.
Scheer, N., Groth, A., Hans, S., Campos-Ortega, J.A., 2001. An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development 128, 1099–1107.
Schmitt, E.A., Dowling, J.E., 1994. Early eye morphogenesis in the zebrafish, Brachydanio rerio. J. Comp. Neurol. 344, 532–542.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.
Trabosh, V.A., Divito, K.A., B, D.A., Simbulan-Rosenthal, C.M., Rosenthal, D.S., 2009. Sequestration of E12/E47 and suppression of p27KIP1 play a role in Id2-induced proliferation and tumorigenesis. Carcinogenesis 30, 1252–1259.
Uribe, R.A., Gross, J.M., 2007. Immunohistochemistry on cryosections from embryonic and adult zebrafish eyes. Cold Spring Harbor Protoc., http://dx.doi.org/10.1101/pdb.prot4779.
Uribe, R.A., Gross, J.M., 2010. Id2a influences neuron and glia formation in the zebrafish retina by modulating retinoblast cell cycle kinetics. Development 137, 3763–3774.
van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S., Nusslein-Volhard, C., 1996. Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. Development 123, 153–164.
Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Maeda, R., Nojima, Y., Li, H., Okamoto, H., Masai, I., 2005. Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. Development 132, 3027–3043.
Yaron, O., Farhy, C., Marquardt, T., Applebury, M., Ashery-Padan, R., 2006. Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development 133, 1367–1378.

Yashiro-Ohtani, Y., He, Y., Ohtani, T., Jones, M.E., Shestova, O., Xu, L., Fang, T.C., Chiang, M.Y., Intlekofer, A.M., Blacklow, S.C., Zhuang, Y., Pear, W.S., 2009. Pre-TCR signaling inactivates Notch1 transcription by antagonizing E2A. Genes Dev. 23, 1665–1676.

Zaghloul, N.A., Yan, B., Moody, S.A., 2005. Step-wise specification of retinal stem cells during normal embryogenesis. Biol. Cell 97, 321–337.

Zhang, X.M., Yang, X.J., 2001. Regulation of retinal ganglion cell production by Sonic hedgehog. Development 128, 943–957.