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An error was published in *Development* **137**, 3763-3774.

On p. 3764, the Id2a morpholino sequences were incorrect. The correct sequences are:

Id2a-MO, 5'-GCCTTCATGTTGACAGCAGGATTTC3'; and Id2a-MM, 5'-GCGTTGATGTTACAGCACGAATTC-3'.

The nucleotides in bold differ from the published ones.

The authors apologise to readers for this mistake.

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Id2a influences neuron and glia formation in the zebrafish retina by modulating retinoblast cell cycle kinetics

Rosa A. Uribe¹ and Jeffrey M. Gross^{1,2,*}

SUMMARY

Inhibitor of differentiation (Id) family helix-loop-helix proteins regulate the proliferation, survival and differentiation of numerous cell types during development; however, their functions during retinal development have not been analyzed. Using loss-of-function and overexpression assays in zebrafish, we demonstrate that Id2a levels modulate retinoblast cell cycle kinetics and thereby influence neuron and glia formation in the retina. Id2a-deficient retinas possess increased numbers of cells occupying S phase, at the expense of mitotic cells, and kinetic analyses demonstrate that Id2a is required for S-phase progression and/or the transition from S to M phase. Id2a-dependent defects in retinoblast proliferation lead to microphthalmia and to an absence of nearly all differentiated inner and outer nuclear layer cell types. Overexpression of *id2a* has the opposite effect on retinoblast cell cycle kinetics: *id2a*-overexpressing retinoblasts progress from S to M phase more rapidly and they undergo mitosis more frequently, which results in macrophthalmia. Mosaic analyses reveal that Id2a function in facilitating both cell cycle progression and neuronal differentiation in the retina is non-cell-autonomous, suggesting that Id2a functions upstream of the extrinsic pathways that regulate retinogenesis.

KEY WORDS: Id2a, Neuronal differentiation, Retinoblast proliferation, Zebrafish

INTRODUCTION

During retinogenesis, retinal progenitor cells (retinoblasts) proliferate, and then in waves – starting with the ganglion cell layer (GCL) and emanating outward to the inner nuclear layer (INL) and outer nuclear layer (ONL) – they are specified into distinct cell fates, exit the cell cycle following a terminal mitosis, begin to express markers of terminal differentiation and, finally, undergo neuronal or glial morphogenesis (Cayouette et al., 2006; Zaghoul et al., 2005). The molecular mechanisms underlying the shift from retinoblast proliferation to neuronal/glial differentiation are not well understood, and both intrinsic and extrinsic factors have been identified that modulate this process (Agathocleous and Harris, 2009; Bilitou and Ohnuma, 2010). For example, intrinsically, a diverse network of transcription factors, including those of the basic helix-loop-helix (bHLH) family, is required for normal proliferation, specification and cell cycle exit events during retinogenesis (Hatakeyama and Kageyama, 2004; Vetter and Brown, 2001), as are cell cycle modulators, such as *cyclin D1* (*ccnd1*) and *p57kip2* (*cdkn1c* – Zebrafish Information Network) (Dyer and Cepko, 2001a; Dyer and Cepko, 2001b; Levine and Green, 2004). Cell cycle exit also involves additional intrinsic regulatory factors such as *histone deacetylase 1* (*hdac1*) (Stadler et al., 2005; Yamaguchi et al., 2005), and terminal differentiation and neuronal/glial morphogenesis requires the chromatin-remodeling factor *brahma-related gene1* (*brg1*; *smarca4* – Zebrafish Information Network) (Gregg et al., 2003). Extrinsically, the Notch, BMP, Shh, FGF and Wnt signaling pathways influence retinoblast proliferation, as well as the specification, commitment and

differentiation of these cells, although complicating this are species-specific differences in the ways that these pathways influence retinal development (Yang, 2004; Agathocleous and Harris, 2009).

The Inhibitor of differentiation (Id) family of HLH proteins is involved in the intrinsic control of proliferation and differentiation during development (Coppe et al., 2003; Desprez et al., 2003; Kowanez et al., 2004; Lasorella et al., 2002). The four Id family members (Id1–4) resemble bHLH transcription factors but lack the ‘basic’ DNA-binding domain and therefore cannot bind directly to DNA (Benezra, 2001). bHLH factors must heterodimerize to exert their transcriptional control and Ids antagonize this function by sequestering bHLH factors and preventing them from binding to DNA (Ghil et al., 2002; Lasorella et al., 2001; Norton, 2000; Ying et al., 2003). Several in vitro studies have highlighted Id family roles in maintaining a proliferative and pluripotent state in expressing cells (Jung et al., 2009; Ying et al., 2003; Yokota, 2001), and the Ids are also contributing factors in numerous types of cancer (Lasorella et al., 2001).

Id2 is expressed in the mouse (Yokota, 2001) and zebrafish (Chong et al., 2005; Thisse et al., 2004) retina, and *Id2*^{-/-} mice are microphthalmic (Yokota et al., 1999); however, its roles during retinal development have not been directly examined. Given the integral roles of bHLH proteins during retinal development, and the precise spatial and temporal controls that exist in the developing retina to facilitate proliferation, cell fate specification and neuronal differentiation, we focused on Id2 to determine whether it plays a role in any of these events in zebrafish. Using loss-of-function and overexpression strategies, our experiments reveal two novel and interrelated functions during retinogenesis for Id2a, one of the two zebrafish Id2 paralogs. First, Id2a regulates INL and ONL cell fate specification events by influencing the expression of factors that are essential for retinal neurogenesis. In Id2a-deficient retinas, these factors are not properly expressed, proliferative retinoblasts do not exit the cell cycle, and differentiated INL and ONL neurons are absent. Second, Id2a modulates the progression of retinoblasts

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through the cell cycle, specifically S-phase progression and/or the duration between the S and M phase. Alterations to *Id2a* levels impact growth of the eye, with *Id2a*-deficient embryos being microphthalmic and *id2a*-overexpressing embryos being macrophthalmic. Mosaic analyses reveal that *Id2a* function in facilitating both cell cycle exit and neuronal differentiation is non-cell-autonomous, suggesting that *Id2a* functions upstream of the extrinsic pathways that regulate retinogenesis.

MATERIALS AND METHODS

Zebrafish maintenance

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

Morpholino and mRNA injections

Id2b-MO (5'-ACCTCACCGACTGACTGCCTTCAT-3'), *Id2a*-MO (5'-GCCTTCATGTTGACAGCAGGATTC-3') and *Id2a* mismatch (*Id2a*-MM, 5'-GCGTTGATGTTACAGCACGAATC-3') morpholinos (MOs) were purchased from Gene Tools (Philomath, OR, USA) and injected at 1, 4 and 4 ng, respectively. For mRNA rescue injections, *id2a* was PCR amplified starting from the first ATG, thereby removing 16 bp of MO recognition sequence from the 5'UTR. Additionally, a silent G→A change was made in the second codon of *id2a*. cDNAs were subcloned into pCS2, linearized, and capped mRNAs were transcribed using the mMessage Kit (Ambion).

Riboprobes and in situ hybridization

Hybridizations were performed essentially as described (Jowett and Lettice, 1994). *id2a* was cloned from whole-embryo cDNA at 24 hours post-fertilization (hpf). cDNA clones encoding *id2b*, *pax6a*, *sox2*, *tfap2a*, *crx*, *pcna*, *ccnd1* and *neurod4* were purchased from ZIRC (Eugene, OR, USA), *mab2112* and *six3b* were provided by Mary Ellen Lane (Rice University) and *ath5* (*atoh7* – Zebrafish Information Network) was provided by Brian Link (Medical College of Wisconsin).

Histology

Histology was performed as described (Nuckels and Gross, 2007).

Immunohistochemistry

Immunohistochemistry was performed as described (Uribe and Gross, 2007). The following antibodies and dilutions were used: anti-*Id2* (1:100; sc-489, Santa Cruz Biotech); anti-phosphohistone H3 (pH3) (1:250; Upstate Signaling); the ZIRC antibodies *zpr1* (1:200), *zpr3* (1:200), *zn8* (1:100) and *zrf1* (1:200); 5e11 [1:100; gift of Dr James Fadool, Florida State University (Hyatt et al., 1996)]; anti-protein kinase C α (PKC; Prk α – Zebrafish Information Network) (1:100; sc-10800, Santa Cruz Biotech); and goat anti-mouse, anti-rabbit or anti-streptavidin secondary antibodies (1:200; Jackson ImmunoResearch). Nuclei were counterstained with SYTOX Green (1:10,000; Molecular Probes).

Eye measurements

Absolute eye size was measured at 48 hpf in living embryos ($n=6$) using an eye-piece reticle on a Leica 6SE microscope.

BrdU assays

BrdU incorporation assays were performed as described (Baye and Link, 2007). Anti-BrdU antibody (1:250; Abcam) was used to detect BrdU⁺ nuclei on central retinal cryosections. Four to five embryos per condition were utilized, and counts were performed on sections from one retina per embryo for quantification. SYTOX Green was used for total cell counts and BrdU⁺ cells were calculated as a percentage of total cells. Statistical significance and s.e.m. were determined using a two-parametric unpaired *t*-test (GraphPad Prism).

TUNEL assays

TUNEL assays were performed on cryosections using the In Situ Cell Death Detection Kit, TMR-Red (Roche).

Flow cytometry

FACS DNA-content analysis was performed as described (Bessa et al., 2008). DNA content was quantified on a FACsCalibur flow cytometer (BD Biosciences) in three separate biological replicates using 50 eyes per condition (more than 25,000 cells per condition). Cell cycle analyses were performed using FlowJo software and statistical analysis was performed as above.

Hsp70l constructs and overexpression

Gateway cloning (Invitrogen) and Tol2 (Kwan et al., 2007) were utilized to generate hsp70l-nlsmCherry-IRES-eGFPcAAxP and hsp70l-*Id2a*-IRES-eGFPcAAxP. DNA (50 pg) and transposase mRNA (25 pg) were injected into 1-cell stage embryos. Embryos were heat shocked at 38°C and embryos exhibiting GFP⁺ retinal clones were fixed for cryosectioning and immunohistochemistry. GFP⁺ clones were detected using an anti-GFP antibody (Santa Cruz Biotech), and mitotic cells were detected using anti-pH3 (Upstate Signaling). A minimum of 20 clones from ten eyes per condition were analyzed, and the proportion of pH3⁺ cells to GFP⁺ cells within each clone was calculated. Statistical significance and s.e.m. were determined as above.

Mosaic analyses

Shield-stage transplants were performed as described (Carmany-Rampey and Moens, 2006). Donor embryos were injected with Alexa Fluor 568 and lysine-fixable biotin-dextran (MW 10,000; Molecular Probes) in 0.2 M KCl.

Cell cycle analyses

BrdU (10 mM) was injected into the yolk of 48 hpf embryos, which were then fixed 30 minutes later for BrdU immunohistochemistry. Cryosectioned retinas containing clones on the ventral side of the central retina were analyzed by confocal microscopy ($n=4-5$ clones per condition). Statistical significance and s.e.m. were determined as above.

Neuronal differentiation

Embryos were fixed at 61 hpf for immunohistochemistry ($n=3$ clones per condition).

In vivo imaging

Embryos were co-injected at the 1-cell stage with 150 pg *nls-kaede* mRNA along with either *Id2a*-MM, *Id2a*-MO or 100 pg *id2a* mRNA, and at 30 hpf they were mounted in 0.9% low-melting-point agarose dissolved in fish water. Small clones of cells were photoswitched in the dorsal-anterior retina using a Zeiss Pascal confocal microscope equipped with a UV laser. The retina was immediately imaged to identify photoconverted cells, and imaged again at 39 hpf to identify progeny ($n=3$ embryos per condition). Photoconverted cells were counted at each time point from confocal sections or projections and the fold increase per clone was calculated and averaged for each condition. Statistical significance and s.e.m. were determined as above.

RESULTS

Id2a expression during retinogenesis

Zebrafish possess two *Id2* paralogs, *id2a* and *id2b*. *id2a* transcripts are detected throughout the retina and lens at 24 hpf (Chong et al., 2005; Thisse et al., 2004) (data not shown). In the retina, *id2a* expression is restricted to the developing INL at 48 hpf and is maintained there at 72 hpf, as well as being expressed in the ciliary marginal zones (CMZs). *id2b* is not detected in the retina at 24 hpf (see Fig. S1A in the supplementary material), and at 48 hpf small groups of cells near the retinal ganglion cell (RGC) layer begin to express *id2b* (see Fig. S1B in the supplementary material).

Utilizing a polyclonal antiserum generated against a C-terminal epitope of mouse *Id2*, western blot analysis of 48 hpf lysates derived from embryos injected with a 5 bp-mismatch control MO (*Id2a*-MM) indicated that the antiserum detects a ~15 kDa band, a similar size to *Id2* (see Fig. S1G in the supplementary material).

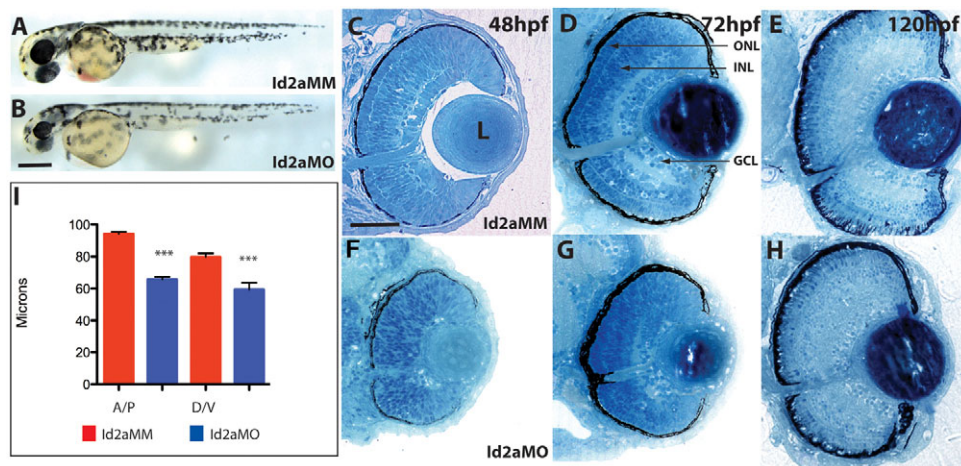


Fig. 1. Id2a knockdown results in microphthalmia and defects in retinal lamination. (A,B) Id2a-MM-injected (control) (A) and Id2a-MO-injected (B) zebrafish embryos at 48 hpf display a reduction in eye and brain size. (C-H) Transverse histological sections of Id2a-MM and Id2a-MO retinas at 48 (C,F), 72 (D,G) and 120 (E,H) hpf. Id2a-MO eyes are smaller than Id2a-MM eyes, and the inner nuclear layer (INL) and outer nuclear layer (ONL) are not morphologically identifiable in Id2a-MO retinas at 72 hpf (G). (I) Measurements of absolute eye size along the anteroposterior (A/P) and dorsoventral (D/V) axes at 48 hpf. $n=6$ embryos; ***, $P<0.001$. Error bars indicate \pm s.e.m. Dorsal is up in all images. GCL, ganglion cell layer; L, lens. Scale bars: 150 μ m in A,B; 80 μ m in C-H.

This band was reduced in lysates from embryos injected with a translation-blocking MO targeting *id2a* (Id2a-MO) (see Fig. S1G in the supplementary material). MO knockdown of Id2b (Id2b-MO) did not affect the levels of the 15 kDa band, suggesting that the antiserum might be specific to Id2a. In some extracts, two additional bands were detected at \sim 25 and 35 kDa, the identities of which are unknown. The Id2 antiserum labeled cells throughout the retina and lens at 24 hpf (see Fig. S1C in the supplementary material) and in the dorsal CMZ at 48 hpf (see Fig. S1D in the supplementary material). Id2 protein was not detected in Id2a-MO retinas at either time point (see Fig. S1E,F in the supplementary material).

Id2a influences growth of the eye and is required for neuronal and glial differentiation

Id2a morphants were microphthalmic when compared with Id2a-MM-injected embryos (Fig. 1A,B,I). Co-injection of 85 pg of *id2a* mRNA that could not be targeted by Id2a-MO rescued eye size in Id2a morphants (see Fig. S2A-D in the supplementary material). Id2a morphants also exhibited decreases in brain size; however, other aspects of embryonic development, such as body length, pigmentation and yolk consumption, appeared largely normal, indicating that the Id2a-MO effects are unlikely to result from a non-specific MO-induced developmental delay.

Retinal histology from Id2a-MM-injected embryos at 48 hpf revealed a well-laminated retina that, by 72 hpf, possessed a morphologically distinct GCL, INL and ONL (Fig. 1C,D). By contrast, Id2a-MO retinas were poorly laminated at 48 hpf, and at 72 hpf, although they appeared to possess a GCL, the INL and ONL were not morphologically discernable, with cells in these layers remaining progenitor-like in appearance (Fig. 1F,G). At 120 hpf, although Id2a-MO retinas recovered lamination and had grown substantially, as a likely reflection of MO dilution, the eyes remained smaller than those of Id2a-MM controls (Fig. 1E,H).

Ids are involved in sustaining proliferation while preventing differentiation (Norton, 2000). Therefore, it was possible that the lack of lamination in the Id2a-MO retina actually reflected a

precocious differentiation event, and that the entire retina was composed of RGCs, which are the first retinal neurons to differentiate in zebrafish (Schmitt and Dowling, 1996). To test this hypothesis and determine whether abnormalities in neuronal differentiation contributed to the lack of lamination in Id2a morphants, the expression of immunohistochemical markers for differentiated neurons and glia was compared between Id2a-MO and Id2a-MM retinas (Fig. 2). Although Id2a-MO retinas contained differentiated RGCs, these cells were limited to the inner retina in a similar pattern to Id2a-MM controls, indicating that precocious neuronal differentiation had not occurred (Fig. 2A,B). Surprisingly, however, other than an occasional Müller glia cell (Fig. 2G,H), rod (Fig. 2I,J) or red/green cone (Fig. 2K,L; data not shown), Id2a-MO retinas did not express markers for any differentiated INL or ONL cell type (Fig. 2C-F). Co-injection of *id2a* mRNA was able to rescue neuronal differentiation in Id2a morphants (see Fig. S2A-D in the supplementary material; data not shown).

Id overexpression is known to induce proliferation (Benezra, 2001; Kowantetz et al., 2004), and Ids are often upregulated in multiple types of cancers (Lasorella et al., 2001). Therefore, we were curious whether overexpression of *id2a* mRNA could lead to increased eye growth. Whereas 85 pg of *id2a* mRNA did not induce any obvious phenotypes (see Fig. S2 in the supplementary material), overexpression of 100 pg increased eye size in 58.3% of injected embryos (see Table S1 in the supplementary material), leading to a \sim 13% increase in size over *mgfp*-injected controls (Fig. 3A-C). *id2a* mRNA-overexpressing embryos also appeared to be slightly more developed than their *mgfp*-injected controls, exhibiting larger heads and more highly pigmented bodies (Fig. 3A,B). Of those *id2a* mRNA-overexpressing embryos that did not possess enlarged eyes, a subset exhibited other ocular phenotypes, including microphthalmia and cyclopia (see Table S1 in the supplementary material). Embryos were exquisitely sensitive to *id2a* levels, as injection of more than 100 pg *id2a* mRNA was toxic to most embryos, and therefore these additional phenotypic groups might simply reflect this sensitivity. Although we limited the studies reported herein to analysis of Id2a function during the later

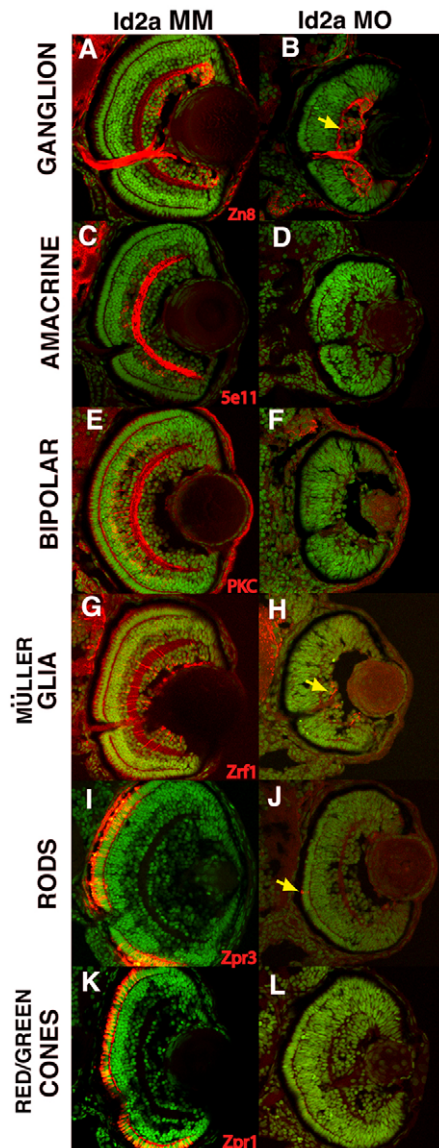


Fig. 2. Id2a is required for the differentiation of late-born retinal neurons and Müller glia. Immunohistochemical analysis of markers (red) of neuronal and glial differentiation in Id2a-MM and Id2a-MO zebrafish embryos at 72 hpf. (A,B) Ganglion cells (zn8), (C,D) amacrine cells (5e11), (E,F) bipolar cells (PKC), (G,H) Müller glia (zrf1), (I,J) rods (zpr3) and (K,L) red/green cones (zpr1). Although Id2a-MO retinas contain ganglion cells (B) and an occasional rod cell (J, arrow) or Müller glia cell (H, arrow), all late differentiated cell types are absent. DNA, green.

phases of eye development, given that *id2a* is expressed in the early eye field (Chong et al., 2005), it is possible that these phenotypes might indicate a biologically relevant sensitivity to increases in *id2a* levels during early development.

Histological examination of *id2a*-overexpressing embryos at 48 and 72 hpf revealed no obvious defects in retinal lamination or patterning (Fig. 3D-G). Analysis of differentiation markers revealed no differences in the cell type composition in *id2a*-overexpressing retinas compared with *mgfp* mRNA-injected controls at 72 hpf (data not shown); this is a surprising result given that overexpression of *Id2* in other contexts, e.g. neural stem cells (Ying et al., 2003), neural progenitor cells (Zhang et al., 2010) or

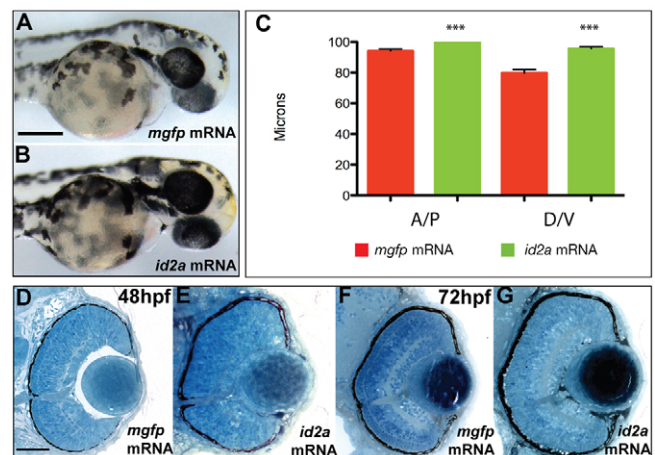


Fig. 3. Overexpression of Id2a leads to macrophthalmia but does not affect retinal lamination. (A,B) *mgfp* mRNA-injected (control) (A) and *id2a* mRNA-injected (B) zebrafish embryos at 48 hpf. Approximately 58% of embryos injected with *id2a* mRNA exhibit enlarged eyes compared with embryos injected with *mgfp* mRNA. (C) Quantification of eye size in *mgfp* mRNA-injected and *id2a* mRNA-injected embryos at 48 hpf. $n=5$ embryos; **, $P=0.015$. (D-G) Transverse histological sections of *mgfp* mRNA and *id2a* mRNA-injected embryos at 48 (D,E) and 72 (F,G) hpf. The *id2a* mRNA retina at 48 hpf (E) is noticeably larger than the *mgfp* mRNA-injected control (D). Retinal lamination is normal at both time points. Error bars indicate \pm s.e.m. Scale bars: 150 μ m in A,B; 80 μ m in D-G.

in neuroectodermal tissues (Lyden et al., 1999), leads to an inhibition of differentiation and to maintenance of a proliferative/progenitor state.

Id2a is essential for specification of late-born retinal cell types

Retinogenesis can be divided into five general steps: (1) designation of retinal progenitor identity during the optic vesicle and optic cup stages (McCollum et al., 2007; Zaghoul and Moody, 2007); (2) expression of transcription factors involved in retinal cell type specification (Inoue et al., 2002; Kageyama et al., 2005); (3) cell cycle exit following a terminal mitosis (Dyer and Cepko, 2000; Dyer and Cepko, 2001a; Dyer and Cepko, 2001c; Park et al., 2005; Shkumatava and Neumann, 2005); (4) expression of terminal differentiation factors (Sernagor, 2006; Harada et al., 2007); and (5) neuronal or glial morphogenesis.

That Id2a-MO retinas do not contain morphologically identifiable neurons and glia (Fig. 1), and that these cells do not express terminal differentiation markers (Fig. 2) other than those of RGCs, indicate that Id2a functions during retinogenesis upstream of steps 4 and 5. These data, however, do not indicate at which point Id2a function is required, and so we examined each of these upstream steps to begin to determine how Id2a regulates retinal neuron and glia formation. To assess the first phase of retinogenesis, the acquisition of retinal progenitor identity, we analyzed the expression of the retinal progenitor identity markers *pax6a*, *six3b*, *mab2112* and *sox2* at the optic vesicle stage (14-16 hpf) and *six3b* and *sox2* also at the optic cup stage (24 hpf) (see Fig. S3 in the supplementary material). Although the eye field as a whole was slightly smaller in Id2a morphants, progenitor markers were otherwise expressed normally, indicating that Id2a is unlikely to be required for the specification of retinal progenitor cell identity.

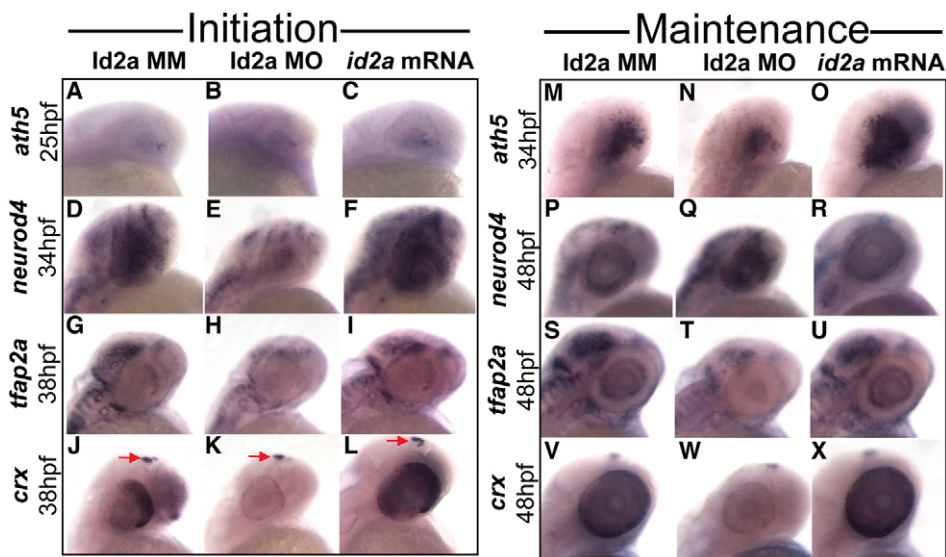


Fig. 4. Changes in Id2a levels alter the expression of neurogenic factors involved in retinal cell fate specification. (A–X) Lateral views of whole-mount in situ hybridizations detecting the initiation (A–L) and maintenance (M–X) of the indicated genes at the indicated time points in Id2a-MM, Id2a-MO and *id2a*-overexpressing zebrafish embryos. Pineal body expression of *crx* is indicated by arrows in J–L.

To determine whether Id2a is required for the second phase of retinogenesis, expression of the retinal cell type specification factors *ath5*, *neurod4*, *tfap2a* and *crx* was examined by in situ hybridization. In Id2a-MM retinas, *ath5* expression initiated at 25 hpf in a small patch of cells in the ventro-nasal retina (Fig. 4A) coinciding with the onset of retinal neurogenesis (Masai et al., 2000; Kay et al., 2001), and by 34 hpf *ath5* had swept around the retina to the ventro-temporal region (Fig. 4M). Although *ath5* expression initiated normally in Id2a-MO retinas, it was restricted to a much smaller domain (Fig. 4B). By 34 hpf, although *ath5* had swept around the entire retina in Id2a morphants, it was restricted to a smaller vitreal domain than in control embryos (Fig. 4N). *id2a* mRNA-overexpressing embryos initiated and maintained *ath5* normally (Fig. 4C,O), although expression extended further along the scleral axis at 34 hpf (Fig. 4O). Despite these differences, RGC specification and morphogenesis were unaffected in both Id2a morphants and *id2a*-overexpressing embryos (Figs 2 and 3).

neurod4 expression initiated in control retinas at 34 hpf (Fig. 4D), whereas *tfap2a* and *crx* initiated at 38 hpf (Fig. 4G,J). In Id2a-MO retinas, no expression of these factors was detected (Fig. 4E,H,K). Expression in the brain (*neurod4* and *tfap2a*) and pineal body (*crx*) appeared relatively unaffected, however. Later retinal expression of these markers was also disrupted in Id2a morphants. At 48 hpf, *neurod4* refined to a ring of expression in the INL in Id2a-MM controls (Fig. 4P), whereas in Id2a morphants expression had initiated but transcripts were distributed throughout the entire retina (Fig. 4Q). *tfap2a* expression was maintained in the INL at 48 hpf in control embryos (Fig. 4S), whereas *crx* was expressed in the presumptive ONL (Fig. 4V). Id2a morphants lacked detectable expression of either of these markers at 48 hpf (Fig. 4T,W). Overexpression of *id2a* did not impact the initiation or maintenance of *neurod4* or *tfap2a* (Fig. 4F,I,R,U); however, the initial expression domain of *crx* was expanded at 38 hpf (Fig. 4L).

Id2a modulates retinoblast cell cycle kinetics

Step 3 of retinogenesis, cell cycle exit following a terminal mitosis, is a prerequisite for neuron and glia differentiation and has been linked to the expression and function of bHLH and homeobox factors in a number of contexts (e.g. Farah et al., 2000; Kay et al., 2001; Le et al., 2006; Kanekar et al., 1997; Pujic et al., 2006; Jomary and Jones, 2008). To determine whether Id2a function is

required for retinoblast cell cycle exit, a series of BrdU incorporation assays was performed. Embryos were exposed to BrdU from 28–30, 48–50 and 72–74 hpf, and then immediately sacrificed and processed for BrdU immunohistochemistry to determine the percentage of S-phase cells (BrdU⁺) out of total retinal cells (stained by SYTOX Green). At all time points analyzed, an increase in the percentage of BrdU⁺ cells was detected in Id2a-MO retinas when compared with Id2a-MM controls (Fig. 5A). At 30 hpf, Id2a-MM retinas possessed 42.8% BrdU⁺ cells, whereas Id2a-MO retinas contained 51% ($P=0.073$; Fig. 5A). At 50 hpf, concomitant with differentiation within the GCL and INL, control retinas contained 29.3% BrdU⁺ cells, whereas Id2a-MO retinas contained 43% ($P=0.0279$). BrdU⁺ cells were limited to the peripheral retina and CMZs of Id2a-MM eyes, whereas in Id2a-MO retinas BrdU⁺ cells were located ectopically throughout the central retina (Fig. 5A). Id2a morphants also continued to express the proliferative markers *pna* and *ccnd1* in the central retina at 34 and 48 hpf, respectively, a region devoid of expression in Id2a-MM embryos (see Fig. S4 in the supplementary material). At 74 hpf, only 4.6% of Id2a-MM retinal cells were BrdU⁺, whereas 41.3% of Id2a-MO retinal cells remained in S phase ($P=0.0067$). As with eye size and neuronal differentiation, cell cycle exit could be rescued in a dose-dependent fashion in Id2a morphants when *id2a* mRNA was co-injected (see Fig. S2E–I in the supplementary material).

BrdU analyses indicated that retinoblasts in Id2a-MO retinas remain in the cell cycle, which raised the question of why Id2a morphants were microphthalmic when these cells remained proliferative. Two possible models might explain these observations. First, increases in the number of proliferative retinoblasts are balanced out by increases in cell death, similar to what has been observed in *p27Kip1* and *p57Kip2* (*Cdkn1b* and *Cdkn1c*, respectively – Mouse Genome Informatics) knockout mice (Dyer and Cepko, 2000; Dyer and Cepko, 2001a) and *caf-1b* (*chaf1b* – Zebrafish Information Network) mutants (Fischer et al., 2007). Second, cell cycle kinetics are perturbed, such that retinoblasts are delayed in cell cycle progression and unable to exit the cell cycle on time.

To test the first model, TUNEL immunohistochemistry was performed and apoptotic nuclei quantified in Id2a-MM and Id2a-MO retinas at 28 and 48 hpf. No increases in the number of

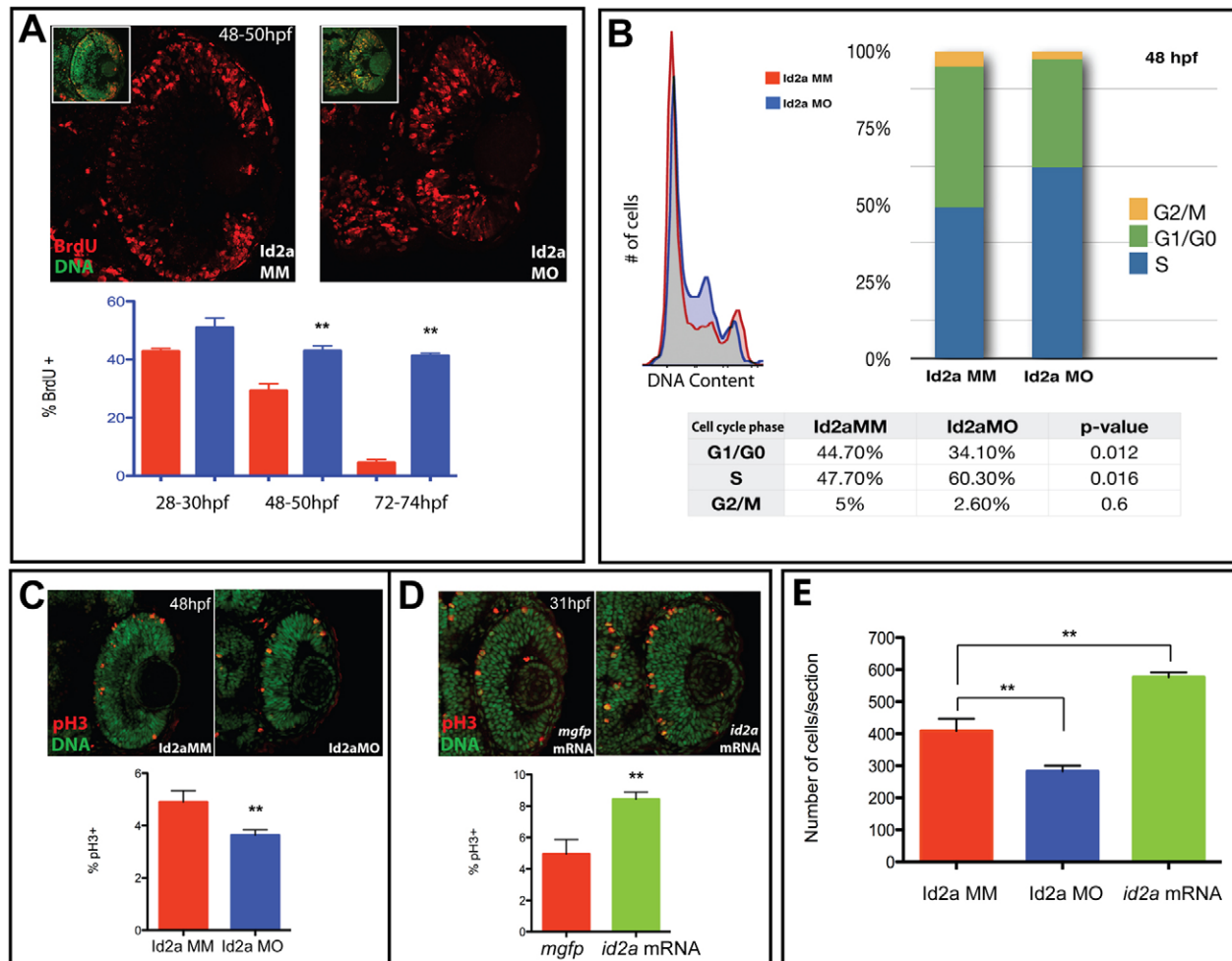


Fig. 5. Id2a is required for S-phase progression and is sufficient to enhance retinoblast mitotic activity. (A) BrdU exposures from 28–30, 48–50 and 72–74 hpf reveal an increased proportion and mislocalization of S-phase cells in Id2a-MO retinas compared with Id2a-MM retinas. $n=4$ retinas; **, $P<0.05$. Insets are examples of SYTOX Green/BrdU overlays used for cell counts. (B) FACS DNA-content analysis of Id2a-MM and Id2a-MO retinas indicates that Id2a-MO retinal cells contain an increased percentage of retinoblasts in S phase, concomitant with decreases in G0/G1 and G2/M (data averaged from three independent experiments). (C) pH3 localization in Id2a-MM and Id2a-MO retinas at 48 hpf. $n=5$ retinas; **, $P<0.05$. (D) pH3 in *mgfp* mRNA-injected and *id2a*-overexpressing retinas at 31 hpf. Id2a-MO retinas contain a lower percentage of mitotic cells and *id2a*-overexpressing retinas contain a higher percentage than controls. $n=5$ retinas; **, $P<0.05$. (E) Average cell numbers in Id2a-MM, Id2a-MO and *id2a*-overexpressing retinas. $n=4$ retinas; **, $P<0.05$. Error bars indicate \pm s.e.m.

TUNEL⁺ cells were detected at either time point, indicating that there were no apparent increases in cell death (see Fig. S5 in the supplementary material).

To test the second model, FACS DNA-content analysis was utilized to quantify the percentage of cells that occupy each phase of the cell cycle (Bessa et al., 2008). Analyses at 48 hpf revealed that 47.7% of cells in Id2a-MM retinas were in S phase (Fig. 5B) compared with 60.3% of cells in Id2a-MO retinas ($P=0.016$). Increased S-phase occupancy was accompanied by a decrease in the percentage of cells in G0/G1; Id2a-MM control retinas contained 44.5% in G0/G1, whereas Id2a-MO retinas contained only 34.1% ($P=0.012$). Although Id2a-MO retinas also contained fewer cells in G2/M (2.6% versus 4.9% in Id2a-MM retinas), this decrease was not statistically significant ($P=0.61$). Quantification of the percentage of pH3⁺ cells at 48 hpf, however, revealed a significant decrease in Id2a-MO retinas ($P=0.029$; Fig. 5C). Given the reciprocal phenotypes between Id2a morphant and *id2a* mRNA-overexpression embryos, it was logical to expect that increased Id2a levels might also enhance retinoblast mitotic

activity. To address this possibility, the mitotic state of retinoblasts in *id2a*-overexpressing and *mgfp* mRNA control retinas was determined by quantifying the percentage of pH3⁺ cells at 31 hpf, a time point at which most of the retina is actively proliferating. Whereas *mgfp*-injected retinas contained 4.9% M-phase retinoblasts, *id2a*-overexpressing retinas contained 8.4% ($P=0.015$; Fig. 5D).

These observations support a model in which Id2a function is required for S-phase progression or exit in proliferating retinoblasts. Id2a knockdown leads to an increased number of S-phase retinoblasts, a decreased number of M-phase retinoblasts and microphthalmia. Conversely, increases in Id2a levels lead to an increase in the number of mitotic retinoblasts and to enlarged eyes. In support of this model, quantification of the average number of cells per section in Id2a-MM control, Id2a-MO and *id2a*-overexpressing embryos at 48 hpf revealed that Id2a-MO retinas contained, on average, 126 fewer cells than control retinas ($P=0.0426$), whereas *id2a*-overexpressing retinas contained 168 more cells ($P=0.0155$; Fig. 5E).

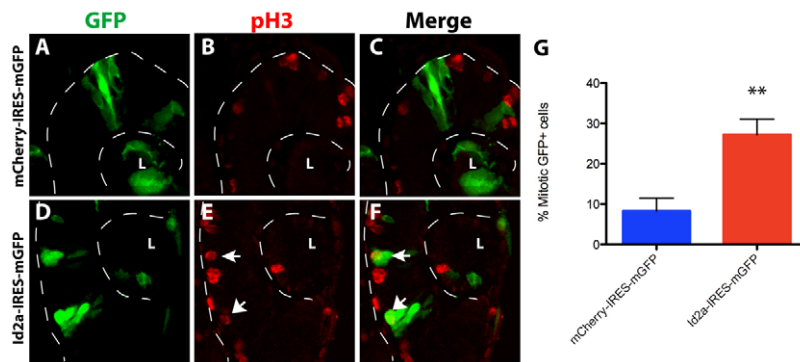


Fig. 6. Clonal overexpression of *id2a* is sufficient to enhance retinoblast mitoses. (A-F) Confocal micrographs of transverse immunosections from heat shock-induced mCherry-IRES-mGFP (A-C) and Id2a-IRES-mGFP (D-F) zebrafish embryos at 34 hpf. GFP⁺ cells that were pH3⁺ (arrows in E,F) were counted. The lens (L) and posterior boundary of the optic cup are outlined (dashed lines). Dorsal is up in all images. (G) The percentage of mitotic GFP⁺ cells from control mCherry-expressing cells and *id2a*-expressing cells. **, $P=0.0012$. Error bars indicate \pm s.e.m.

It is possible that the increases in retinoblast proliferation in *id2a*-overexpressing embryos resulted from an Id2a-dependent signal derived from a non-retinal source, and therefore that increases in Id2a levels do not directly influence retinoblast proliferation. To test this possibility, we generated Tol2 hsp70l-inducible constructs that express either *id2a* or mCherry and, through an IRES sequence, also a membrane-tethered GFP to identify overexpressing clones (Kwan et al., 2007). Injection of these constructs led to mosaic expression when heat-shocked at 27–28.5 hpf, and this resulted in small clones expressing either mCherry or Id2a plus mGFP (Fig. 6). Quantification of the percentage of GFP⁺ retinal cells that were also pH3⁺ at 34 hpf revealed a significant increase in mitoses in *id2a*-expressing cells when compared with mCherry-expressing cells (Fig. 6G, $P=0.0012$). Thus, overexpression of *id2a* within retinoblasts was sufficient to increase their proliferation, supporting a direct role for Id2a in the retina.

BrdU incorporation, pH3 immunohistochemistry and FACS analyses provide static windows into the effects that alterations in Id2a levels have on the cell cycle, but they do not enable an assessment of how these alterations affect its overall kinetics. Therefore, two additional cell cycle experiments were performed. The first utilized a ‘percent labeled mitoses’ (PLM) paradigm (Locker et al., 2006) to determine the time required to progress from S to M phase in Id2a-MM, Id2a-MO and *id2a*-overexpressing retinoblasts. Embryos were exposed to 15-minute BrdU pulses at 31 hpf and then fixed at 15- and 30-minute increments thereafter, until 2.5 hours post-exposure (hpe). Immunohistochemistry was then utilized to identify M-phase cells at the time of fixation (pH3⁺) and retinoblasts that were in S phase during the BrdU pulse (BrdU⁺). Cells that were pH3⁺ BrdU⁺ had progressed from S to M phase during the time between exposure and fixation (Fig. 7A,B), whereas those that were only pH3⁺ were not in S phase during the time of the pulse. The proportion of pH3⁺ cells that were also BrdU⁺ represents the PLM, and graphing this percentage over time provides an approximate readout of the duration between the S and M phases.

In Id2a-MM control retinas, almost 100% of retinoblasts progressed from S to M phase within ~1.5–2 hpe (Fig. 7C). By contrast, Id2a-MO retinoblasts progressed from S to M phase much more slowly: by 1.5 hpe, ~50% had reached M phase ($P=0.0002$), in agreement with the decrease in total pH3⁺ cells in the morphant retina (Fig. 5C). Conversely, *id2a*-overexpressing retinoblasts exhibited an accelerated rate of S-to-M phase progression, such that more retinoblasts had progressed to M phase as early as 15 minutes post-exposure ($P=0.0002$), and this increase was maintained at 1 hpe ($P=0.0205$; Fig. 7C).

These results suggested that Id2a levels modulate the overall cell cycle kinetics of proliferative retinoblasts during retinogenesis. To further test this possibility, we performed a second assay that utilized a nuclear-localized Kaede protein (nls-Kaede) to ‘label’ a small clone of cells and calculate their rate of proliferation in vivo. *nls-kaede* was co-injected with either Id2a-MM, Id2a-MO or *id2a* mRNA, and at

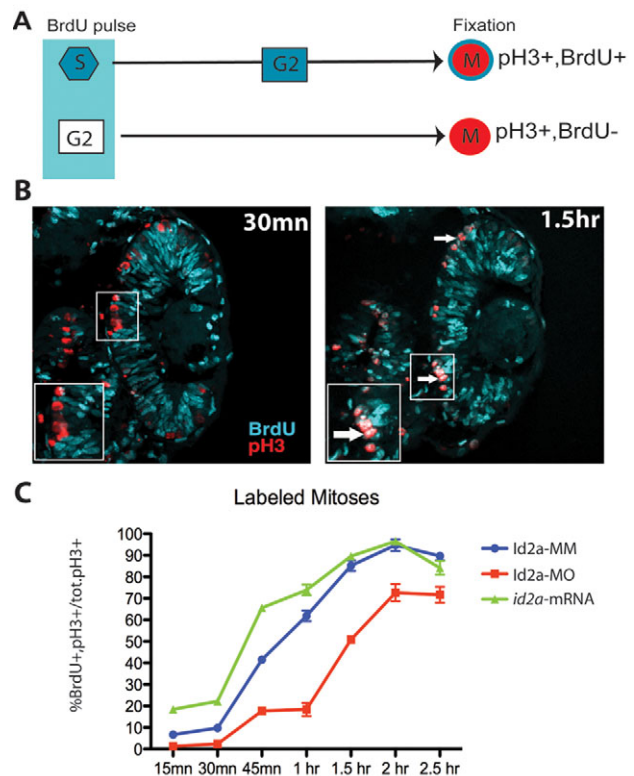


Fig. 7. Id2a modulates the progression between S and M phase in proliferating retinoblasts. (A) Schematic of the percent labeled mitoses (PLM) paradigm. A 15-minute BrdU pulse marks S-phase retinoblasts, which then progress to M phase over time. Detection of M-phase cells with pH3 identifies those that were in S phase at the time of BrdU exposure. (B) Control retinas showing BrdU incorporation (cyan) and pH3 staining (red) at 30 minutes and 1.5 hours post-exposure. Many S-phase cells have progressed to M phase within 1.5 hours and are detected by BrdU/pH3 co-labeling (inset, arrow). (C) Graphical representation of PLM from 15 minutes to 2.5 hours post-exposure. Id2a-MO cells are delayed in progressing from S to M phase at all time points examined ($n=4$ retinas; **, $P<0.003$), whereas at 15 minutes, 30 minutes and 1 hours post-exposure, *id2a*-overexpressing retinoblasts have progressed from S to M phase more rapidly ($n=4$ retinas; **, $P<0.025$).

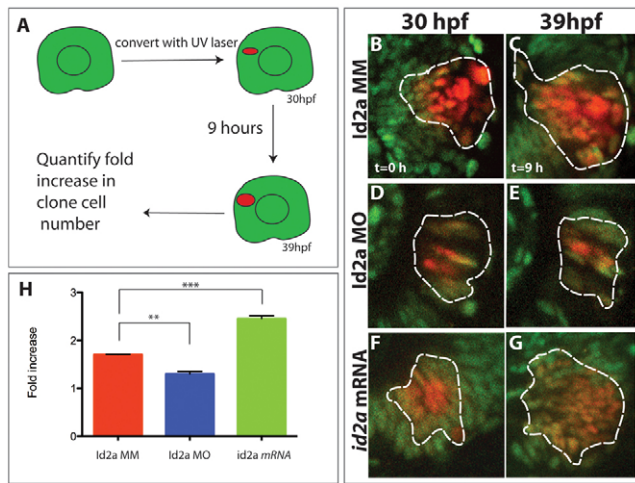


Fig. 8. Id2a levels modulate retinoblast proliferation in vivo. (A) Kaede clonal analysis paradigm. (B–G) Clonal expansion between 30 and 39 hpf in Id2a-MM (B,E), Id2a-MO (C,F) and *id2a* mRNA retinas (D,G). Projections from confocal z-stacks with the approximate position of each clone outlined. (H) Quantification of fold increase in cell number per clone ($n=3$ retinas). Id2a-MM clones exhibit a 1.7-fold increase in cell number, Id2a-MO clones exhibit a 1.29-fold increase (**, $P=0.0019$), and *id2a*-overexpressing clones exhibit a 2.45-fold increase (***, $P=0.002$) over time. Error bars indicate \pm s.e.m.

30 hpf nls-Kaede was photoconverted in small clones (Fig. 8A; see Fig. S6 in the supplementary material). Clones were imaged upon photoconversion, and then again at 39 hpf to determine the rate of proliferation. In agreement with our PLM data, whereas control clones exhibited a 1.7-fold increase in cell number over this time (Fig. 8B,C,H), Id2a-MO clones only exhibited a 1.29-fold increase ($P=0.0019$; Fig. 8D,E,H), and *id2a*-overexpressing clones exhibited a 2.45-fold increase ($P=0.0002$; Fig. 8F,G,H).

Id2a function is non-cell-autonomous in the retina

Intrinsic factors have been shown to regulate the proliferative state of retinal progenitors in both a cell-autonomous manner, such as with N-myc (Martins et al., 2008), and a non-

autonomous manner, such as with Brg1 (Gregg et al., 2003). Given the role of Id2a in modulating retinoblast cell cycle progression and its subsequent requirement for the differentiation of late-born cell types, we investigated whether Id2a function was required cell-autonomously or non-autonomously with respect to cell cycle progression and neuronal differentiation. Shield-stage transplants (Carmany-Rampey and Moens, 2006) were utilized to create mosaic eyes between Id2a-MO and wild-type (WT) embryos, and at 48 hpf the embryos were pulsed with BrdU for 30 minutes and immediately processed for BrdU immunohistochemistry to assay cell cycle exit (Fig. 9; see Fig. S7 in the supplementary material), or they were fixed at 61 hpf to assay for the presence of red/green cone photoreceptors at the onset of terminal differentiation (Fig. 10).

Large clones (of more than 30 cells) of transplanted cells always behaved as their donor ‘genotype’, probably demonstrating a community effect (see Fig. S8 in the supplementary material), and therefore all experiments analyzed clones of fewer than 30 cells (Fig. 9; see Fig. S7 in the supplementary material). Analysis of cell cycle exit revealed that WT clones in a WT environment exhibited an average of 9.3% BrdU⁺ cells per clone ($n=4$) (Fig. 9B,C). By contrast, WT clones in an Id2a-MO environment exhibited a significantly higher proportion of BrdU⁺ cells per clone, at 31.6% ($n=5$; $P=0.0014$) (Fig. 9B,C). Conversely, whereas Id2a-MO clones in Id2a-MO retinas contained a high percentage of BrdU⁺ cells per clone at 34.7% ($n=5$), Id2a-MO clones placed in a WT environment exhibited a significantly lower percentage of BrdU⁺ cells, at 13.2% ($n=5$; $P=0.0003$).

Analysis of neuronal differentiation revealed that Id2a also functions non-cell-autonomously with respect to red/green cone cell differentiation (Fig. 10). As expected, WT clones in a WT environment successfully differentiated into red/green cones (Fig. 10A), and Id2a-MO clones in an Id2a-MO environment did not differentiate (Fig. 10D). Surprisingly, Id2a-MO cells in a WT environment were able to differentiate into red/green cones (Fig. 10B). Conversely, WT cells in an Id2a-MO environment failed to differentiate, behaving identically to their ‘morphant’ environment (Fig. 10C). These data indicate that Id2a functions non-cell-autonomously with respect to both cell cycle exit and neuronal differentiation during retinal development.

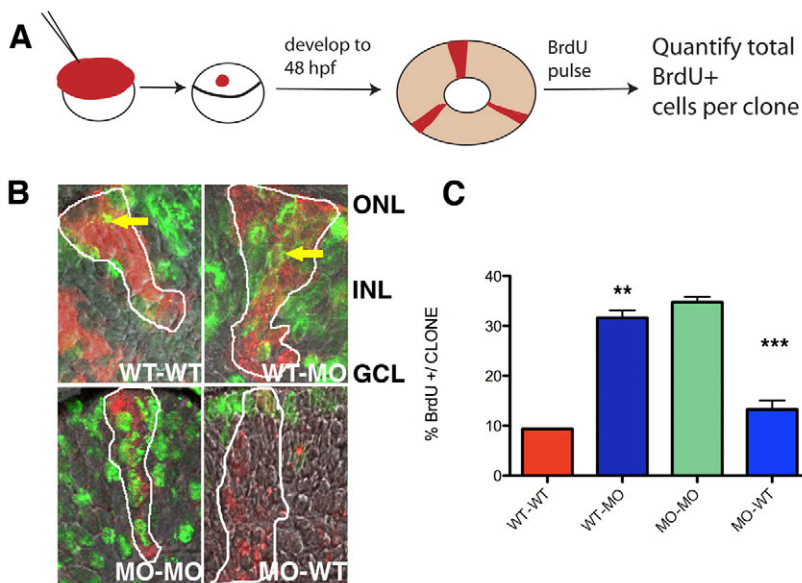


Fig. 9. Id2a downstream function in mediating retinoblast cell cycle progression is non-cell-autonomous. (A) Schematic of experiment. Biotin-dextran lineage-labeled donor cells were transplanted into shield-stage host zebrafish embryos, then mosaic embryos were pulsed with BrdU at 48 hpf to assay cell cycle exit. Clones on the ventral side of the central retina were analyzed. (B) Transplanted cells (red) and BrdU⁺ cells (green). Top left, wild-type (WT) cells in a WT host; top right, WT cells in an Id2a-MO host; bottom left, Id2a-MO cells in an Id2a-MO host; bottom right, Id2a-MO cells in a WT host. Arrows indicate an example of a BrdU⁺ cell within a clone. (C) Quantification of the percentage of BrdU⁺ cells per clone. $n=4-5$ clones per condition; **, $P<0.001$; ***, $P<0.0003$.

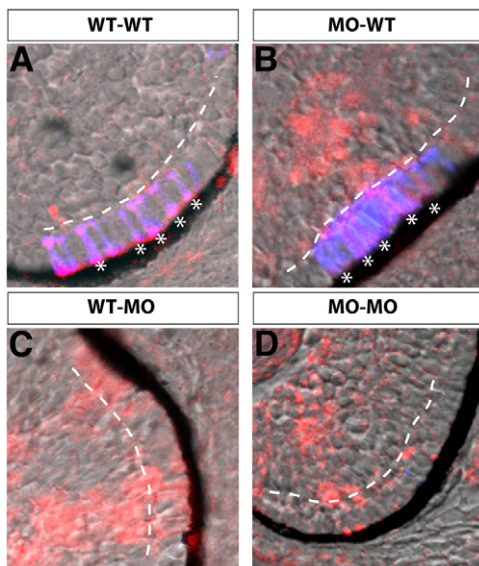


Fig. 10. Id2a downstream function in mediating neuronal differentiation is non-cell-autonomous. Ventral retinal clones (red) at 61 hpf stained for *zpr1* (blue) immunoreactivity in (A) WT-WT, (B) MO-WT, (C) WT-MO and (D) MO-MO mosaics. Dashed line, outer plexiform layer; asterisks, transplanted cells differentiated as red/green cones.

DISCUSSION

Id2a plays two interrelated roles during retinogenesis. First, Id2a influences eye size by modulating retinoblast cell cycle progression. Loss of Id2a function delays S-phase progression and/or the transition from S to M phase, resulting in decreased numbers of mitotic retinoblasts and, ultimately, microphthalmia. Overexpression of *id2a* shortens the cell cycle, which is manifest by a decrease in the duration between the S and M phases and in an increased number of mitotic cells, resulting in

macrophthalmia (Fig. 11A). Second, Id2a influences the specification of INL and ONL retinal cell subtypes. In Id2a-deficient retinas, late-born INL and ONL retinal cell types are not properly specified and differentiated INL and ONL neurons are absent from the retina (Fig. 11B).

Mosaic analyses revealed that Id2a functions non-cell-autonomously in the retina with respect to cell cycle exit and neuronal differentiation, suggesting that Id2a is required upstream of the extrinsic cues that regulate these processes. Extrinsic pathways, such as Notch, FGF, BMP, Wnt and Shh, have all been implicated in regulating retinoblast proliferation and differentiation (Levine and Green, 2004; Yang, 2004), and several of these are attractive candidates for analysis in Id2a-deficient retinas. For example, in the zebrafish retina, Shh pathway activity is required for cell cycle exit of proliferative retinoblasts (Shkumatava and Neumann, 2005) and for the differentiation of INL and ONL cell types (Shkumatava et al., 2004; Stenkamp and Frey, 2003; Stenkamp et al., 2000). Alterations in Shh pathway activity modulate the cell cycle kinetics of retinoblasts: inactivation of Shh signaling leads to an extended cell cycle and to reduced neurogenesis, whereas increased Shh signaling accelerates cell cycle kinetics, decreases cell cycle length and promotes neurogenesis (Locker et al., 2006), phenotypes that are reminiscent of Id2a-deficient and Id2a-overexpressing retinoblasts, respectively. Shh is expressed in two independent waves in the zebrafish retina: the first by differentiated RGCs (Neumann and Nusslein-Volhard, 2000) and the second by amacrine cells (Shkumatava et al., 2004). That Id2a-deficient retinas lack differentiated amacrine cells indicates that amacrine cell-dependent Hh expression might be compromised in Id2a morphants, which could contribute to the cell cycle exit and neuron/glia differentiation defects in these embryos. Notch pathway activity also maintains a proliferative and undifferentiated state in retinoblasts (Perron and Harris, 2000; Nelson et al., 2007), and the Wnt pathway has been shown to regulate the transition from proliferation to differentiation in retinoblasts (Agathocleous et al., 2009), making these interesting pathways to examine.

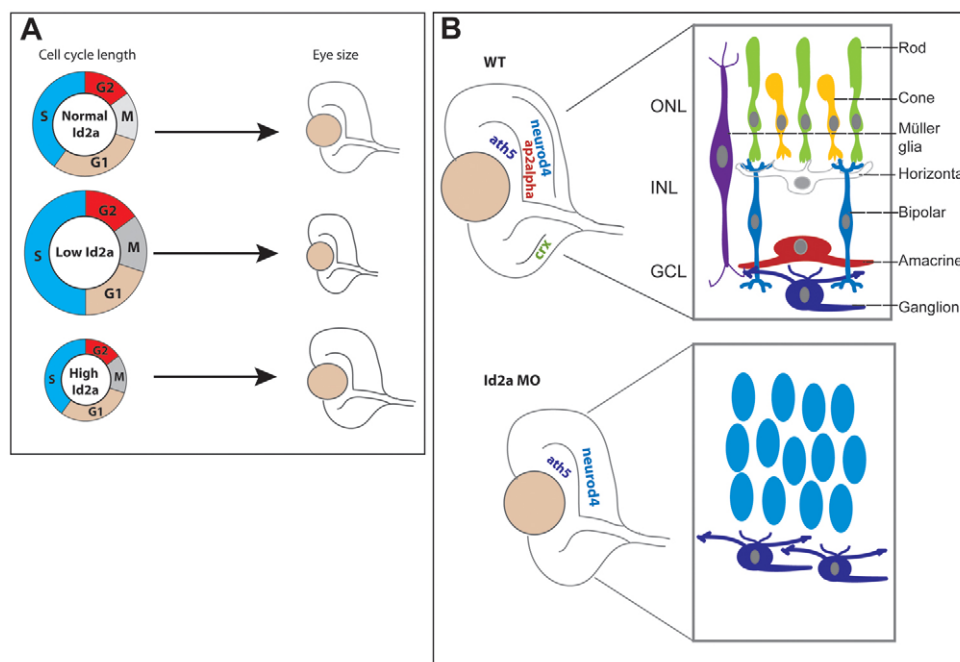


Fig. 11. Id2a function during retinogenesis. A schematic of interrelated Id2a functions during zebrafish retinogenesis. (A) Id2a modulates cell cycle kinetics in proliferative retinoblasts by effecting the progression through S phase and/or the transition from S to M phase. Id2a levels modulate S-phase progression and the duration between S and M phase, as indicated schematically by the size of the circles. Id2a-dependent changes in cell cycle kinetics correlate with changes in the overall rate of retinoblast proliferation and thereby influence growth of the eye. (B) Subsequently, Id2a is required for the expression of neurogenic transcription factors and the formation of INL and ONL neurons and glia.

The observation that an intrinsic factor, such as Id2a, may influence development of the retina by modulating extrinsic events is not unique. Mutations in *brg1*, which encodes a component of the Brahma chromatin-remodeling complex, also lead to non-cell-autonomous defects in neuronal and glial differentiation in the retina (Gregg et al., 2003). Leung et al. identified intrinsic and extrinsic regulators of retinal development, the expression of which depends on Brg1, and their analyses revealed that *id2a* and *id2b* are downstream of *brg1* during retinogenesis (Leung et al., 2008). Our mosaic analyses place Id2a in this network upstream of the extrinsic pathways and the specification factors that are required for the formation of INL and ONL neurons and glia.

Id2a in the specification of INL and ONL cell types

A requirement for Id2a in the specification of INL and ONL retinal cell subtypes was surprising because Ids are classically thought to inhibit differentiation (Norton, 2000). The predicted Id2a-deficient retinal phenotype was one in which retinoblasts exited the cell cycle precociously, resulting in a small retina, composed largely of RGCs. In Id2a morphants, however, despite a delay in the progression of *ath5* expression, RGCs differentiated normally and they were located in the appropriate retinal layer (Fig. 2B). Therefore, Id2a-deficient retinoblasts were neither precociously fated nor did they ectopically differentiate into early-born cell types. Rather, cells in the outer regions of the Id2a-morphant retina failed to exit the cell cycle and did not differentiate. Similarly, overexpression of Ids typically leads to the maintenance of a proliferative state and to an inhibition of differentiation (Lasorella et al., 2001). Although overexpression of Id2a did speed up retinoblast cell cycle kinetics, retinal differentiation was overtly normal; all cell types were present in *id2a*-overexpressing retinas and they were located in their proper laminar positions. Given that the expression of some cell fate markers was precocious (i.e. *ath5* and *crx*) and that the overall rate of development appeared to be mildly enhanced in *id2a*-overexpressing retinas, it is possible that the timing of neuronal differentiation was altered by *id2a* overexpression; however, the net result on cell fates was minimal as the *id2a*-overexpressing retina appeared normal with respect to cell type composition at 72 hpf.

Id2 has been shown to directly bind and antagonize both ubiquitous and tissue-specific bHLH proteins, acting as a dominant-negative inhibitor of their function (Norton, 2000). Id2 can also bind and antagonize members of the Pax and Ets families of transcriptional regulators (Roberts et al., 2001; Ji et al., 2008), factors that control multiple aspects of retinal development, including proliferation and differentiation (Harada et al., 2007). Thus, the role of Id2a in retinal neurogenesis could be direct, such that Id2a-dependent regulation of one or more of these factors might be required for the proper expression of neurogenic genes, and, in the absence of Id2a, their expression is unchecked and neurogenic gene expression is compromised. Conversely, the role of Id2a in regulating INL and ONL cell fates could be indirect, whereby it is required upstream of extrinsic regulatory pathways that feed into the activation (*ath5*, *tfap2a*, *crx*) or inhibition (*neurod4*) of expression of these factors. In the absence of Id2a function, one or more of these extrinsic pathways might be affected and, as a consequence, neurogenic factors in the INL and ONL are not properly expressed and retinoblasts remain proliferative.

Id2a in modulating cell cycle progression

Cell cycle exit is a prerequisite for retinal neuron and glia formation (e.g. Baye and Link, 2007; Bessa et al., 2008; Dyer and Cepko, 2001b; Fischer et al., 2007; Stadler et al., 2005), and our

cell cycle data demonstrate that Id2a function is necessary and sufficient for driving cell cycle progression. Kinetic analyses indicate that Id2a influences cell cycle progression by altering S-phase progression and/or the duration between S and M phase, and this directly impacts proliferative rates in vivo. bHLH and homeobox factors are known to drive proliferative progenitor cells out of the cell cycle and stimulate their differentiation (Farah et al., 2000; Kay et al., 2001; Le et al., 2006; Kanekar et al., 1997). Thus, the inability to properly specify INL and ONL fates could underlie the inability of Id2a-deficient retinoblasts to exit the cell cycle, and, moreover, given the cell non-autonomy of Id2a function in cueing cell cycle exit, this might also suggest a model in which Id2a is required upstream of one or more of these extrinsic regulatory pathways, and these, in turn, regulate the expression of neurogenic factors required for INL and ONL cell fate specification and retinoblast cell cycle exit prior to differentiation. Conversely, Ids are also directly linked to cell cycle progression through their functional and gene regulatory network interactions with core cell cycle regulatory proteins such as retinoblastoma (Rb), Cyclin D1 and cyclin-dependent kinase inhibitors (CKIs) (Lasorella et al., 2001; Zebedee and Hara, 2001). Through its HLH domain, Id2 interacts with pRb, abolishing its growth-suppressive capabilities during the G1/S phase transition and thereby allowing S-phase entry (Zebedee and Hara, 2001; Lasorella et al., 2001). Id2 expression peaks during the G1/S phase transition and its levels are maintained throughout S phase, during which it is subject to post-translational modifications by the core cell cycle machinery, including Cyclin E-Cdk2 and Cyclin A-Cdk2, and this serves to regulate Id2 function and stability (Hara et al., 1997; Zebedee and Hara, 2001; Lasorella et al., 2001). Given that Id2a is both necessary and sufficient for S-phase progression in retinoblasts, regulation of Id2a levels or activity through its interactions with the cell cycle machinery might play a direct role in the decision to exit the cell cycle and initiate the neurogenic program. Thus, it is also possible that Id2a operates intrinsically in a 'developmental timing mechanism', akin to that observed in oligodendrocytes (Durand and Raff, 2000), acting upstream of extrinsic signaling pathways that then feed back onto cell cycle progression and cell fate specification events in proliferating retinoblasts.

Finally, although our analyses focused on the role of Id2a in the embryonic retina, Id2a is also expressed in the retinal CMZs, which are regions of continual proliferation in fish and amphibians (Johns, 1977). Ids maintain progenitor-like states in numerous cell types in vitro (Ying et al., 2003; Jung et al., 2009). Therefore, it is feasible that in the CMZ, Id2a could play a more stereotypical 'Id' role in maintaining proliferation and preventing premature differentiation to ensure continued growth of the retina.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050484/-/DC1>

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