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ARS2 is required for retinal progenitor cell S-phase progression and Müller glial cell fate specification.

Connor O’Sullivan¹¶, Philip E.B. Nickerson²,#¶, Oliver Krupke², Jennifer Christie¹, Li-Li Chen², Monica Mesa-Peres¹, Minyan Zhu², Bridget Ryan², Robert L. Chow²*, Perry L. Howard¹*

¹Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada
²Department of Biology, University of Victoria, Victoria, BC, Canada

#* Current Address: Donald K Johnson Eye Institute, Krembil Research Institute, University Health Network, ON, Canada
* Corresponding authors

Email: phoward@uvic.ca (PLH); bchow@uvic.ca (RLC)

¶These authors contributed equally to this work

Short title: ARS2 requirements in the developing mouse retina
Abstract

During a developmental period that extends postnatally in the mouse, proliferating multipotent retinal progenitor cells produce one of seven major cell types (rod, cone, bipolar, horizontal, amacrine, ganglion, and Müller glial cells) as they exit the cell cycle in consecutive waves. Cell production in the retina is tightly regulated by intrinsic, extrinsic, spatial, and temporal cues and is coupled to the timing of cell cycle exit. ARS2 (also known as SRRT) is a component of the nuclear cap-binding complex involved in RNA Polymerase II transcription, and is required for cell cycle progression. We show that postnatal RPCs require ARS2 for proper progression through S phase, and ARS2 disruption leads to early exit from the cell cycle. Furthermore, we observe an increase in the proportion of cells expressing a rod photoreceptor marker, and a loss of Müller glia marker expression, indicating a role for ARS2 in regulating cell fate specification or differentiation. Knockdown of FLASH, which interacts with ARS2 and is required for cell cycle progression and 3’-end processing of replication-dependent histone transcripts, phenocopies ARS2 knockdown. These data implicate ARS2/FLASH-mediated histone mRNA processing in regulating RPC cell cycle kinetics and neuroglial cell fate specification during postnatal retinal development.

**Key words:** Ars2, retina, retinal progenitor cells, cell cycle, photoreceptor cells, Müller cells
Introduction

ARS2 (Arsenic-resistance protein 2), also known as SRRT (Serrate RNA effector molecule), is a stable component of the nuclear cap-binding complex and is required for the processing and transport of most RNA Polymerase II (RNAP II) transcripts (O’Sullivan 2016). Consistent with a central role in RNA processing, knockout of Ars2 is embryonic lethal in plants, fission yeast, fruit flies, zebrafish, and mice (Amsterdam et al. 2004; Golling et al. 2002; Kim et al. 2010; Lobbes et al. 2006; Oh et al. 2003; Wilson et al. 2008). Work from our lab identified that Ars2−/− mouse embryos die peri-implantation, and fail to progress past the blastocyst stage, indicating that ARS2 is essential for stem cell maintenance and/or differentiation in the early embryo (Wilson et al. 2008). A similar role for ARS2 has been demonstrated in adult mammals, as conditional knockout of Ars2 in subventricular zone neural stem cells decreases their self-renewal and neurogenic capacity (Andreu-Agullo et al. 2012), and conditional Ars2 knockout in hematopoietic tissues results in decreased cellularity in the bone marrow (Gruber et al. 2009). Although previous work has shown that ARS2 links the nuclear cap-binding complex to additional complexes necessary for RNAP II transcript termination, 3’-end processing, exosomal degradation, intranuclear transport, and export into the cytoplasm (O’Sullivan 2016), the mechanism of how ARS2 contributes to stem cell maintenance and differentiation has not been fully elucidated.

ARS2 has been implicated in cell cycle progression, maintenance, and differentiation of progenitor cells (Andreu-Agullo et al. 2012; Kiriyama et al. 2009; O’Sullivan et al. 2015). Cultured cells deficient in ARS2 display delayed cell cycle kinetics and accumulate in S-phase (Kiriyama et al. 2009; O’Sullivan et al. 2015), in part due to the role of ARS2 in replication-dependent histone (RDH) mRNA processing as part of the nuclear cap-binding complex (Gruber et al. 2012;
Kiriyama et al. 2009; O'Sullivan et al. 2015). The synthesis of replication-dependent histone proteins is tied closely to DNA synthesis to ensure sufficient histone levels are present to adequately package the newly synthesized DNA into chromatin. Critical to the timing of histone production is the 3'-end processing of RDH mRNA, which are not polyadenylated (Marzluff et al. 2008). Rather, they are regulated by stem-loop binding protein (SLBP), the U7 small nuclear ribonucleoprotein (snRNP), Flice Associated Huge protein (FLASH), and a complex of proteins that promote and regulate the temporal processing and export of RDH mRNA during S phase (Marzluff et al. 2008). ARS2 interacts with the 3'-end processing machinery through the protein FLASH, and is a necessary component for RDH mRNA processing and progression through S phase (Gruber et al. 2012; Kiriyama et al. 2009; O'Sullivan et al. 2015). The ARS2 zinc finger and RNA recognition motif (RRM) domains interact with the Flash ARS2 Binding motif (FARB) within FLASH, and the interaction is necessary for S-phase progression (Kiriyama et al. 2009; O'Sullivan et al. 2015).

The one glial and six neuronal cell types of the mouse retina are derived from multipotent retinal progenitor cells (RPCs) that are generated in an overlapping, sequential order starting embryonically and continuing postnatally (Young 1985a). Proliferation of RPCs is necessary to maintain the progenitor pool throughout retinogenesis (Young 1985b). While there are numerous intrinsic and extrinsic factors that contribute to cell fate decisions and terminal differentiation in the retina (Agathocleous and Harris 2009; Alexiades and Cepko 1996), cell cycle length and timing of exit play a key role. For example, deletion of cyclin D1 (Ccnd1−/−) in mice delays retinal cell cycle progression in G1, hastens cell cycle exit, and increases the proportion of ganglion cells and photoreceptors produced by RPCs (Das et al. 2009). Additionally, inducing premature cell cycle exit by misexpression of cyclin-dependent kinase inhibitors alters cell fate.
determination (Dyer and Cepko 2001; Ohnuma et al. 2002). In the mouse, retinal progenitor cells undergo several rounds of cell division postnatally, giving rise to the majority of rod photoreceptor, bipolar, and Müller glial cells (Cepko et al. 1996). This postnatal period of progenitor cell proliferation and differentiation, coupled with the ability to manipulate these cells in newborn pups, makes the neural retina an excellent model for studying the interplay between neural progenitor cell cycle progression and cell fate decisions.

In this report, we examine the phenotype of ARS2 knockdown in the postnatal mouse retina. We show that ARS2 is expressed in the neuroblastic layer during development, and in all cell types of the adult retina. ARS2 knockdown (KD) disrupts cell cycle progression of retinal progenitor cells and leads to premature cell cycle exit. Furthermore, ARS2 KD increases the proportion of cells expressing a rod photoreceptor-specific fate reporter in the outer nuclear layer, with a concomitant decrease in Müller glial cell reporters. The knockdown of FLASH phenocopies ARS2 disruption, providing evidence that ARS2/FLASH-mediated histone mRNA processing regulates cell cycle kinetics and proper cell fate specification during postnatal retinal development.

Materials and methods

Animals and ethics statement

This study was carried out in accordance with the guidelines of the Canadian Council on Animal Care. The protocol was approved by the University of Victoria Animal Care Committee (Permit Numbers: 2013-013(1), 2014-023). All mice were group-housed within HEPA-filtered, ventilated racks within the animal care unit. All cages included enrichment in the form of huts, crinkle paper, nestlets, and sunflower seeds. All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. CD-1 mice were obtained from Charles River.
**Plasmids**

All shRNA plasmids (pGFP-V-RS vector) were purchased from Origene, had a GFP cassette to assess electroporation efficiency, and had the following targeting sequences: Ars2 sh78 ATGCAGCTGTCATTAAGATGG; Ars2 sh79, CAGGCTGAGATGACAGTTCCAACGATGA; Ars2 sh80, GCAAGGATAAGTGGCTATGTCCCTCAGT; Ars2 sh81, CCAGGCTTTATGCAGTGCCACTGTCAGA; non-targeting GCACTACCAGAGCTACTAGTAGTACT; FLASH sh, AACATTGTGCCAATAATGTCTGGTCACGT; Dr osha, GTTCATTGAGCGGAAATACAGACAGAGT. pNrl-DsRed (Addgene plasmid # 13764) [23], pHes1-DsRed (Addgene plasmid #13767) [23], pCebp5-DsRed (Addgene plasmid # 11157) [24], and pCralbp-DsRed (Addgene plasmid # 11158) (Matsuda and Cepko 2007 [24]) were provided by Dr. Connie Cepko. pEGFP-C1 was used to express enhanced GFP (eGFP) as a control in Fig 3B. pcDNA3.1(+) (Thermo Fisher Scientific) was used as an empty vector control.

**In vivo electroporation**

Newborn mice were electroporated as described in (Matsuda and Cepko 2007). Briefly, once anesthetized using isoflurane, a small incision was made in the eyelid and sclera using a 30-gauge needle under a dissecting microscope. DNA solutions (~3-5 µg/µL) in Phosphate Buffered Saline (PBS, 0.127 M NaCl; 0.0027M KCl; 0.01 M Na₂HPO₄; 0.0018M KH₂PO₄) containing 0.1% fast green were injected (~0.3 µL) into the subretinal space using a Hamilton syringe with a 32-gauge blunt-end needle. Electrodes (BTX Harvard Apparatus) briefly soaked in PBS were gently placed on the heads of the pups, and five square pulses of 50-ms duration with 950-ms intervals at 80 V were applied using a pulse generator (BTX Harvard Appartus).
Pups were then placed on a warm bed and monitored continuously until recovered from the anesthesia. Once recovered, pups were placed back with the dam and monitored at least once daily for coloration, presence of milk spot, size and condition relative to siblings, and whether they grouped normally or were isolated. There were no adverse clinical signs during the course of these experiments.

**In vitro electroporation and retinal explant culture**

Retinas were dissected and transferred to a micro-electroporation chamber containing DNA solution (~1 µg/µL) in Hank’s Balanced Salt Solution (Thermo Fisher Scientific). Five square pulses of 50-ms duration with 950-ms intervals at 30 V were applied using a pulse generator (BTX Harvard Apparatus). Electroporated retinas were placed on Nucleopore track-etched membranes (Whatman, 1.0 µm pore) with Neurobasal medium (Thermo Fisher Scientific) supplemented with 1× Glutamax (Thermo Fisher Scientific), GS21 (Sigma-Aldrich), and Penicillin-Streptomycin solution (5000 U/ml; Thermo Fisher Scientific), and were incubated for the indicated time points at 37˚C.

**Tissue fixation and sectioning**

Retinas were dissected, fixed in 4% paraformaldehyde (PFA) in PB (0.2 M sodium phosphate buffer, pH 7.2) for ~ 4 h at 4˚C, washed in PBS, and incubated in 30% sucrose in PB overnight at 4˚C. Retinas were then washed twice in Optimal Cutting Temperature (O.C.T.) compound (Sakura), placed in an O.C.T. block, and flash frozen on dry ice with 70% ethanol. Retinas were then sectioned between 14-18 µm using a cryostat (Leica), placed onto Superfrost slides (Thermo Fisher Scientific), and let dry in the dark overnight at room temperature.
Immunohistochemistry and microscopy

Slides with sectioned retinas were washed three times for 10 mins in PBS. Retinas were permeabilized in 1% Triton-X 100 for 30 mins, washed in PBS, and incubated in 1:1,000 rabbit anti-Recoverin (EMD Millipore) overnight at 4°C. Next, slides were washed in PBS and incubated in anti-mouse antibodies conjugated to either AlexaFluor 488 or 555 (Thermo Fisher Scientific) as appropriate for 1 hour at room temperature. Slides were then washed in PBS. Slides were then mounted with coverslips using Immuno-Mount (Thermo Fisher Scientific), pH adjusted to ~8.0. Images were taken on a confocal microscope (Nikon CS3 or Zeiss LSM710).

Knockdown quantification

Retinas electroporated with control or Ars2 sh79 plasmids at P1 and dissected at P5 were fixed, sectioned, mounted to slides, stained for ARS2, and imaged using confocal microscopy. Corrected total cell ARS2 fluorescence, measured as integrated density – (area of selected cell × mean fluorescence of background) using ImageJ software, was performed on green fluorescent protein (GFP) transfected cells individually, which were randomly sampled from sections taken from three independent electroporated replicates. Quantification was done so that the individual performing the quantification was blind to the sample identity.

Western blotting

Retinas were dissected at the indicated time points, flash frozen in liquid nitrogen, and homogenized in 2× laemmli sample buffer with 1× protease inhibitor cocktail (Sigma-Aldrich) and 0.1 mM Phenylmethanesulfonyl fluoride (PMSF,Sigma-Aldrich). Samples were incubated in boiling water for 20 minutes and centrifuged at 12,000 xg for 10 mins at 4°C. Lysates were resolved using SDS-PAGE (10%) and transferred to a Polyvinylidene difluoride (PVDF)
membrane (EMD Millipore). The membrane was blocked in 5% dehydrated milk in Tris-buffered saline–Tween 20 (0.5%) (TBST) for 1 h. Primary antibodies were diluted in TBST with 1% dehydrated milk at the following concentrations: 1:4,000 for mouse anti-Actin (Sigma-Aldrich), and 1:2,000 for rabbit anti-ARS2 (XL12.2), and were incubated on the membrane with shaking overnight at 4°C. Blots were washed 3× with TBST and incubated with 1:10,000 goat anti-mouse or goat anti-rabbit conjugated to horse radish peroxidase (HRP) (Bio-Rad) for 1 h, washed with TBST, incubated in enhanced chemilumiscence (Thermo Fisher Scientific) and exposed on X-ray film (Thermo Fisher Scientific).

**IdU/CldU pulse labeling experiments**

Retinas were electroporated *in vitro* as described with the indicated plasmids, and incubated with either 37 µM 5-Iodo-2’-deoxyuridine (IdU) (Sigma-Aldrich) or 37 µM 5-Chloro-2’-deoxyuridine (CldU) (Sigma-Aldrich) in Neurobasal supplemented with 1 × Glutamax, GS21 and Penicillin-Streptomycin solution (5000 U/ml; Thermo Fisher Scientific), for the indicated time points. Retinas were then fixed and sectioned as described above. Slides were then washed three times for 10 mins in PBS, permeabilized in 1% Triton-X 100 for 30 mins, and incubated in 1:2000 rabbit anti-turbo GFP (Origene) overnight at 4°C. Next, slides were washed in PBS and incubated in 1:500 donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific) for 1 h at room temperature. Slides were then washed in PBS, treated with 2N hydrochloric acid (HCl) for 45 mins, washed in PBS again, and incubated in 1:250 mouse anti-5’Bromo-2’-deoxyuridine (BrdU)/IdU (BD Biosciences) and 1:250 rat anti-BrdU/CldU (Abcam) overnight at 4°C. Slides were then washed in PBS, and incubated in 1:500 donkey anti-mouse Alexa Fluor 647 (Thermo Fisher Scientific) and 1:500 donkey anti-rat Cyanine 3 (Cy3) (EMD Millipore) for 1 h at room temperature. Slides were then washed, mounted and imaged using confocal microscopy.
Counting of IdU/CldU was done in a manner in which the individual performing the counting was blind to the experimental treatment.

**Flow cytometry**

Retinas were dissected at the indicated time points and dissociated to a single-cell suspension using the neural tissue dissociation kit for postnatal neurons (MACS Miltenyi Biotec) according to the manufacturer’s instructions. For cell fate analysis using discosoma red fluorescent protein (DsRed) reporters, samples were immediately run on a BD FACSCalibur flow cytometer following dissociation, and ~1.0 × 10⁶ events were acquired per sample.

**Results**

**ARS2 knockdown affects cell cycle progression and exit**

As shown in Fig.1A, transient expression of 4 independent shRNA against ARS2 reduced the expression of ~130 kDa band in mouse C2C12 myogenic progenitor cells, confirming that polyclonal antibody (XL12.2) recognizes ARS2 and our ability to knockdown its expression in mouse cells. To determine ARS2 expression in the retina, we performed western blotting on mouse retinal extracts using this antibody and detected a single band for ARS2 in the retina during embryogenesis (E14.5), postnatal differentiation (up to P14), and in the mature retina (8 weeks), confirming the ARS2 expression in the developing and adult retina (Fig 1B). While expression was most abundant in the embryo and early postnatal, ARS2 expression continues in adult post-mitotic tissue. We next electroporated retinal explants with a plasmid containing a GFP cassette and either control shRNA or shRNA-79 targeting Ars2 which we have also validated previously in myoblast cells (O'Sullivan et al. 2015). Electroporation targets mitotic retinal progenitor cells. Given the relatively low electroporation efficiency, we quantified the
level of ARS2 knockdown by measuring ARS2 fluorescence in GFP-positive transfected cells from micrographs using Image J at 4 days post-electroporation. ARS2 levels from 3 independent electroporations were reduced ~3-fold in Ars2 shRNA treated cells relative to control shRNA (Fig 1C,D), confirming the ability of the Ars2 shRNA to suppress Ars2 expression in the retina. Our previous work showed that myogenic progenitor cells in which Ars2 expression is knocked down, are delayed in progression through S-phase (Fig.1SA) (O'Sullivan et al. 2015). In addition, disruption of ARS2 function by overexpression in retinal explants resulted in defective interkinetic nuclear migration (INM) with cells accumulating in basal position associated with S-phase (Fig.1SB) (Nickerson et al. 2013). To assess whether ARS2 is required for S-phase progression in retinal progenitors, we first analyzed the cell cycle profile by propidium iodide incorporation using flow cytometry. Relative to control, Ars2 shRNA-treated cells accumulated in S-phase (48.8% vs 33.6%) 24 hours after electroporation (Fig 1D), consistent with previous work (Kiriyama et al. 2009; O'Sullivan et al. 2015). To confirm this result, we performed thymidine analogue labeling to label cells in S-phase. Retinal explants were electroporated with control or Ars2 shRNA at P0 and were given a 30 minute IdU pulse at 21 hours post-electroporation to label S-phase cells. ARS2 KD increased the proportion of IdU+ve cells ~2-fold relative to control shRNA (Fig 1E), indicating an increased proportion of cells in S-phase at this time point. Although this result could indicate either an increase in the number of cycling cells or a delay in S-phase progression, previous work in other cell types have shown an S-phase delay (Kiriyama et al. 2009; O'Sullivan et al. 2015). This, coupled with our previous data showing a defect in INM and accumulation of knockdown nuclei at basal positions in the retina (Nickerson et al. 2013), led us to suspect that ARS2-deficient cells are delayed in cell cycle progression through S-phase as was seen with myogenic progenitors (O'Sullivan et al. 2015).
To test this hypothesis, we performed a chase-chase experiment in which we labeled the electroporated retinal explants with IdU from 0 to 48 hours and CldU from 48 to 192 hours post-electroporation to determine whether actively cycling cells at the time of electroporation (IdU\(^{+ve}\)) continue to cycle (IdU\(^{+ve}/\)CldU\(^{+ve}\)) or exit the cell cycle (IdU\(^{+ve}/\)CldU\(^{-ve}\)) (Fig. 2A-C). Although the proportion of IdU\(^{+ve}/\)CldU\(^{+ve}\) cells trended lower in the ARS2 KD relative to control, the difference was not significant (Fig 2B). However, the proportion of cells IdU\(^{+ve}/\)CldU\(^{-ve}\) increased ~3-fold in the ARS2 knockdowns (Fig 2C). This data indicates that actively cycling progenitor cells deficient in ARS2 preferentially exit the cell cycle. Taken together these data strongly suggest that retinal progenitor cells deficient in ARS2 are delayed in S-phase progression and as a result preferentially exit the cell cycle. Of course, we cannot rule out that these cells are deficient in other phases of the cell cycle, or are completely arrested. However, premature cell cycle exit would be expected to impact neurogenesis and result in a reduction in cellularity. To test this, we examined overall retinal thickness. ARS2-deficient retinas were reduced by approximately 25 % in size relative to control knockdown retinas 96 hours post-electroporation (Fig 2D). Therefore, we conclude that ARS2 KD cells are compromised in S-phase progression and that dividing ARS2-deficient progenitors most likely prematurely exit the cell cycle.

**ARS2 knockdown increases expression of a rod photoreceptor marker**

The P0-P8 postnatal period is dominated by the birth of rod photoreceptors, bipolar cells, and Müller glial cells (Bassett and Wallace 2012; Young 1985a), providing an opportunity to determine the impact of ARS2 KD on the specification and differentiation of these late-born cell
types. To determine the requirement of ARS2 for late-born rod photoreceptor production, we co-electroporated newborn pup retinas \textit{in vivo} with either control or Ars2 shRNA along with a reporter containing the neural retina leucine zipper (NRL) promoter coupled to DsRed (pNrl-DsRed) (Matsuda and Cepko 2007). NRL is both necessary and sufficient for rod fate in postmitotic photoreceptor cells (Mears et al. 2001; Oh et al. 2007), and pNrl-DsRed is restricted to rod photoreceptors (Matsuda and Cepko 2007). Analysis of retinas electroporated with pNrl-DsRed showed DsRed positive cells were localized as expected to the Outer Nuclear Layer (ONL) (Fig 3B). Electroporation targets the mitotic cells at the time of electroporation and these cells will take up GFP and DsRed expression plasmids equally (Matsuda and Cepko 2007). Strikingly, ARS2 KD increased the proportion of Nrl-DsRed/GFP double positive cells approximately 3-fold when analyzed by immunofluorescence of retinal sections or by flow cytometry at P8 \textit{in vivo} (Fig 3B, C). To confirm that the Nrl reporter was indeed identifying photoreceptor cells, we also stained for recoverin, a marker for photoreceptors (Dizhoor et al. 1991). As shown in Fig. 3D, most Nrl+ve cells were also positive for recoverin, confirming that there is an increase in photoreceptor cells in the Ars2 knockdowns.

**ARS2 knockdown decreases Müller glial cells**

Since disrupting ARS2 impacts NRL expression, a rod photoreceptor marker, we next tested whether ARS2 KD affects Müller glial and bipolar cell production, as they are also late-born cell types (Bassett and Wallace 2012). To assess Müller glial generation, a DsRed reporter containing the promoter for Hairy and enhancer of split 1 (\textit{Hes1}) was initially used. \textit{Hes1} is expressed in progenitor and Müller glial cells (Matsuda and Cepko 2007), and is required for Müller glial cell production in the mouse retina (Furukawa et al. 2000). Mice were co-
electroporated with control or Ars2 shRNA and pHes1-DsRed, incubated until P8 in vitro, and analyzed using flow cytometry. ARS2 KD resulted in a ~2.5-fold reduction in Hes1-DsRed positive cells relative to control (Fig 4A). We did not observe a difference in vivo for bipolar cell production using a DsRed reporter under the control of the calcium binding protein 5 (CABP5) promoter, which is restricted to bipolar cells (data not shown)(Haeseleer et al. 2000; Matsuda and Cepko 2004).

Since HES1 is potentially expressed in a small pool of remaining progenitor cells at this time point and to corroborate this result with an additional Müller glial marker, a DsRed reporter containing the promoter for cellular retinaldehyde-binding protein (CRALBP) was also used; the Cralbp promoter is restricted to Müller glial cells (Bunt-Milam and Saari 1983; Matsuda and Cepko 2004). Mice were co-electroporated at P0 in vivo with control or Ars2 shRNA and pCralbp-DsRed and analyzed at P14 (Fig 4B, C). We noticed that the cytomegalovirus (CMV)-GFP expression from the shRNA plasmid was largely limited to the ONL in all cases, potentially indicating a limited ability to electroporate non-photoreceptor destined neurogenic progenitors (Figs 3B and 4B). However, the Cralbp-DsRed reporter was expressed in the inner nuclear layer (INL) and in cells displaying Müller glial morphology as expected (Fig 4C)(Matsuda and Cepko 2007), indicating that Müller glial-destined progenitors were successfully targeted by electroporation. Based on this, we reasoned that the restricted GFP expression is due to CMV promoter silencing in the other layers of the retina as has been reported (Klein et al. 1998; Miyoshi et al. 1997). Consistent with this interpretation, ARS2 KD, where the shRNA construct was driven by a U6 promoter and therefore not expected to be silenced, abolished Cralbp-DsRed activity as observed using flow cytometry or micrograph analysis (Fig 4B,C). No differences in
the number of GFP fluorescent cells were observed between ARS2 KD and control conditions (data not shown), consistent with previous work showing that ARS2 KD in neural progenitors does not affect cell survival (Andreu-Agullo et al. 2012). Taken together, these results indicate that ARS2 KD results in an increased proportion of Nrl-DsRed positive rod photoreceptor cells at the expense of Müller glia.

**ARS2 knockdown cell fate defect phenocopies FLASH**

Intrinsic delaying of cell cycle progression or exiting the cell cycle prematurely can itself increase the proportion of rod photoreceptors, especially shortly after birth, which corresponds to the peak of rod photoreceptor production (Das et al. 2009; Dyer and Cepko 2001). Given the role of ARS2 in RDH mRNA processing and the importance of RDH during S-phase, we hypothesized that the S-phase progression defect following ARS2 KD was directly contributing to the altered cell fate decisions. If this hypothesis is correct, one would expect that disrupting the expression of another component of the RDH processing machinery would produce a similar phenotype. Therefore, we compared ARS2 and FLASH KD using the Nrl-DsRed reporters. Strikingly, ARS2 or FLASH KD increased the proportion of Nrl-DsRed/GFP double positive cells approximately 2-fold relative to control shRNA (Fig 5A). Since ARS2 is also required for microRNA (miRNA) production (Gruber et al. 2009; O'Sullivan et al. 2015), as a control we also analyzed Nrl-DsRed following DROSHA KD, the enzyme required for processing pri-miRNA (Nguyen et al. 2015). Notably, DROSHA KD decreased the proportion of electroporated cells expressing Nrl-DsRed relative to control shRNA, implying a miRNA biogenesis defect is not contributing to the cell fate phenotype following ARS2 KD (Fig 5A).
To confirm that disruption of ARS2 function in neuronal progenitor cells results in a deficit in RDH mRNA processing, we expressed a RDH reporter (O'Sullivan et al. 2015) in neurogenic N2a cells. The reporter consists of partial cDNA from histone H2A and the H2A 3’UTR, including the stem-loop and histone downstream element, fused in-frame to the cDNA of a DsRed reporter construct (O'Sullivan et al. 2015; Wagner et al. 2007). Under normal conditions, the recruitment of the U7snRNP and processing machinery results in cleavage of the transcript between the stem loop and histone downstream element (HDE) and results in no expression of DsRed. However, under conditions in which RDH mRNA processing is impaired, cleavage does not occur and the transcript is polyadenylated, exported, and translated to produce a Histone 2A (H2A)-DsRed fusion protein. As shown in Figure 5B, knockdown of ARS2, FLASH, or LSM11, a component of the U7 snRNP, all resulted in increased DsRed expression as expected. In contrast, the control shRNA or DROSHA shRNA did not affect the expression of the reporter (Fig 5B). These results show that knockdown of ARS2 or other core components of the RDH mRNA processing machinery impairs RDH mRNA processing in neuronal lineages. The RDH processing and cell cycle progression defects, combined with the phenocopy observed with the Nrl-DsRed reporter in the ARS2 or FLASH knockdowns, suggest that delayed S-phase progression due to histone deficiency results in early cell cycle exit. Both the timing of cell cycle exit during the first few days after birth and likely alterations in competency, bias these RPCs towards producing photoreceptors (Das et al. 2009; Degterev et al. 2001; Dyer and Cepko 2001).

Discussion

We have demonstrated that ARS2 is expressed in the developing and adult retina. Although previous work has suggested ARS2 expression is limited to cells actively undergoing cell division in vitro (Gruber et al. 2009), the expression we detected in post-mitotic adult retinal cells (8 weeks)
is consistent with the known functions of ARS2 in the nuclear cap-binding complex, and regulation of RNAP II transcription (Andersen et al. 2013; Chi et al. 2014; Gruber et al. 2012; Gruber et al. 2009; Hallais et al. 2013; Kiriyama et al. 2009), processes not restricted to dividing cells. Indeed, ARS2 expression in the adult mouse brain does not correlate with markers of proliferation and is instead expressed in quiescent neural stem cells within the subventricular zone where it maintains neural stem cell number and their neurogenic capacity (Andreu-Agullo et al. 2012).

We have shown that ARS2-deficient RPCs accumulate in S-phase and prematurely exit the cell cycle. This requirement of ARS2 for efficient S-phase progression agrees with our previous work showing ARS2-deficient RPC nuclei are blocked in INM and accumulate in basal positions (Nickerson et al. 2013). INM is intertwined with cell cycle progression in the retina, as nuclei of proliferating cells oscillate between the apical and basal positions of the neuroblastic layer according to the phase of the cell cycle, with S-phase cells occupying the most basal and M-phase cells occupying the most apical positions (Taverna and Huttner 2010). Additionally, cells with nuclei that travel greater distances basally during the preceding mitotic cycle are more likely to produce neurogenic daughter cells (Baye and Link 2007). Although we cannot rule out additional defects in other phases of the cell cycle, the accumulation of RPCs in S-phase, basal positioning, and INM defect following ARS2 disruption collectively support the importance of ARS2 for S-phase progression in retinal progenitors and open the possibility that cell fate alterations that we observed may be linked to cell cycle progression.

We predict that RPCs depleted of ARS2 do not re-enter the cell cycle and instead prematurely exit cycling, which influences their neurogenic capacity. This is based on the observed increase in IdU+ve/CldU-ve and increase in Nrl+ve cells. While we cannot rule out that cells are arrested in S-phase and do not complete the cell cycle, this would not be expected to alter cell fate
in favour of photoreceptor (Nrl+ve) cells. The simplest interpretation is that the IdU+ve cycling cells at the time of the first pulse exit the cell cycle and do not return. Furthermore, since we did not observe difference in the number of GFP +ve cells between the knockdown and control electroporated cells, it is unlikely that difference in survival are contributing to the phenotype. This interpretation is consistent with loss of cyclin D1 in the retina, which regulates the transition from G1 into S-phase. Cyclin D1 deletion results in a delayed cell cycle progression, early cell cycle exit, and increase in rod photoreceptor cells (Das et al. 2009). Furthermore, overexpression of the cell cycle inhibitor p27Kip1, which inhibits cyclin E/CDK2, also leads to early cell cycle exit and an increase in rod photoreceptors and decrease in Müller glia (Dyer and Cepko 2001). Therefore, disrupting cell cycle progression and the timing of cell cycle exit has consequences on cell fate. One reason for the cell cycle delay may be histone deficiency during S-phase. In support of this, we found that ARS2-deficient RPCs phenocopy FLASH-deficient RPCs with respect to the increase in NRL-reporter expression. The finding of early cell cycle exit following replication-dependent histone mRNA misprocessing is consistent with work in the zebrafish retina, where loss of stem-loop binding protein (SLBP), a critical component of RDH mRNA 3’-end processing, resulted in delayed cell cycle progression, early cell cycle exit, and delayed neurogenesis (Imai et al. 2014). Of course, we cannot rule out that changes in the processing, trafficking, or stability of other RNAP II transcripts are contributing to this phenotype. Further work is also required to understand how these cells progress through the rest of the cell cycle and whether this results in DNA damage. However, our results showing that Ars2 and Flash deficiency phenocopy one another suggest that disruption of cell cycle-coupled histone metabolism is a major contributing factor to the alteration in cell fate seen with the NRL reporter.
Previous work has shown that ARS2 is required for multipotency and self-renewal of neural stem cells (NSCs) in the subventricular zone of the adult mouse brain, and ARS2 KD in these cells results in the loss of mature neuronal populations and an accompanying increase in astroglial cells (Andreu-Agullo et al. 2012). Intriguingly, ARS2 deficiency in these NSCs could be rescued by ectopic expression of SOX2, a transcription factor required for NSC maintenance (Andreu-Agullo et al. 2012). In the developing retina, SOX2 is required for maintaining proliferating RPCs in the neuroblastic layer, and its expression is maintained in Müller glia, a cell type that retains neurogenic capacity in the adult retina (Lin et al. 2009; Taranova et al. 2006). The effects of Sox2 on both RPC and Müller glia maintenance are in part mediated through NOTCH1 in the retina (Taranova et al. 2006), which is expressed in an apical (high) to basal (low) gradient (Del Bene et al. 2008; Furukawa et al. 2000). The Notch pathway is necessary for maintaining RPCs, is a determinant of Müller glial cell fate, and suppresses photoreceptor cell fate (Furukawa et al. 2000; Jadhav et al. 2009; Luo et al. 2012; Surzenko et al. 2013). Recent studies suggest that Notch signaling stabilizes the expression of the downstream Sonic Hedgehog (Shh) effector gene, Gli2, thereby priming retinal progenitor cells to respond to Shh signaling (Ringuette et al. 2016). Intriguingly, we found that ARS2 KD cells exhibit a decreased expression of a Hes1 reporter, a direct downstream target of the Notch-Shh axis in the retina (Wall et al. 2009). Therefore, we propose a mechanism whereby ARS2-deficient nuclei are postponed in S-phase progression and accumulate basally, which may limit their exposure to Notch signaling. Cell cycle delay and premature cell cycle exit coupled with low NOTCH1 exposure may cause these cells to adopt a photoreceptor cell fate (Fig 6). We did attempt to rescue the cell fate phenotype by ectopically expressing Notch Intracellular Domain (NICD), however, for technical reasons we were unable to obtain sufficient expression with three plasmid
electroporation to draw a conclusion. Therefore, whether SOX2 and NOTCH1 contributes to the ARS2 KD phenotype in the retina will require further investigation.

In summary, ARS2 is required for progression through S-phase in late-born RPCs and for these cells to become Müller glia. Depletion of ARS2 in late-born RPCs leads to premature cell cycle exit and the adoption of a photoreceptor cell fate. We speculate that delayed S-phase progression and basal accumulation leads to a deficit in SOX2/NOTCH1 signaling, which biases ARS2-deficient cells to adopt a photoreceptor cell fate.

Acknowledgements

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References


Protein (CABP) Subfamily with Similarity to Calmodulin. Journal of Biological Chemistry 275, 1247-1260.


**Figure Legends**

**Fig 1 – ARS2 is expressed in the developing and adult mouse retina.** A) Western blots show knockdown of ARS2 expression in C2C12 myoblasts using 4 shRNAs (shRNAs-78, 79, 80, and 81) targeting different regions of the Ars2 mRNA. B) Western blot show endogenous ARS2 expression in the developing and adult retina at embryonic day 14.5 (E14.5), postnatal day 0 (P0), P4, P7 and 8 weeks (8w). C) Representative retinal explants electroporated with either scrambled shRNA or Ars2 shRNA 4 and immunostained with anti-ARS2 antibodies D) Retinal explants from 4 independent electroporations were analyzed 4 days post-electroporation with either control shRNA (sh) or Ars2 sh79 using immunofluorescence and quantified using Image J. The corrected total cell ARS2 fluorescence of GFP transfected cells was calculated, and a two-tailed unpaired t test was performed ($t$(65) = 5.83; $P<$0.001). E) Retinal explants from 4
Independent experiments were electroporated at P0, fixed, and DNA content was labeled using propidium iodide 24 hours post-electroporation. Retinas were dissociated, pooled and analyzed using flow cytometry to determine the proportion of cells in each cell cycle phase. F) Retinal explants were electroporated with either control or Ars2 shRNA-79 at P0, and 21 hours post-electroporation were given a 30 minute IdU pulse. The percent of electroporated (GFP+ve) IdU+ve cells is shown; a two-tailed unpaired t test was performed (t(10) = 3.67, P<0.01).

Fig 2 – ARS2 knockdown affects cell cycle progression and exit A) Representative micrographs of electroporated retinas were given IdU from 0-48 hours post-electroporation, and CldU from 48-192 hours. B) The average proportion of cells that were IdU+ve/CldU+ve (B) or IdU+ve/CldU-ve (C) are shown for each condition. A two-tailed unpaired t test was performed (t(3) = 5.81, P<0.05 for IdU+ve/CldU-ve), n.s. indicates not significant. D) Retinas electroporated as in A) were fixed 96 hours post-electroporation and retinal thickness was measured from the apical to basal outer edges and using confocal microscopy, and a two-tailed unpaired t test was performed (t(18) = 4.74, P<0.001). Mean retina thickness was 95.1 ± 10.8 µm for control condition, and 74.2 ± 8.9 µm following ARS2 knockdown. Error bars represent SD.

Fig 3 - ARS2 knockdown increases rod photoreceptors. A) Schematic of the pNrl-DsRed and shRNA/GFP plasmids used to assess rod photoreceptor production. Nrl – rod photoreceptor-specific promoter, ATG – DsRed start codon, pA – polyA tail. New born mice (P0) electroporated in vivo with a plasmid containing Ars2 or control shRNA and a Nrl-DsRed reporter construct which is specifically expressed in photoreceptor cells B) Retinas co-electroporated as in A) were fixed and sectioned at P8 and assessed for Nrl-DsRed expression. DRAQ5 staining was used to identify nuclei using confocal microscopy. C) Retinas from 5 biological replicates were in vivo co-electroporated as in A), dissociated and analyzed at P8 using flow cytometry, and % Nrl-
DsRed$^{+ve}$ is shown relative to total transfected cells. A two-tailed unpaired $t$ test was performed ($t(9) = 3.16; P < 0.05$). Error bars represent standard deviation (SD). D) Retinas co-electroporated in triplicate as in A) with control shRNA or Ars2 shRNA-79 (green channel) and pNrl-DsRed (Red channel) were sectioned at P8 and stained for Recoverin (blue channel). ONL- outer nuclear layer, INL- inner nuclear layer.

**Fig 4 - ARS2 knockdown decreases Müller glia cells.** A) Retinas co-electroporated *in vitro* with either control shRNA or Ars2 shRNA and pHes1-DsRed were analyzed at P8 using flow cytometry. The GFP fluorescence compensation control (GFP Comp.) is shown in the upper left plot, and was used to draw the gate (pink box) representing Hes1-DsRed$^{+ve}$ events. 4 retinas were pooled per experimental condition, and the proportion of Hes1-DsRed$^{+ve}$ events relative to total transfected cells is shown within the gates. B) Retinas co-electroporated *in vivo* with either control shRNA, Ars2 shRNA or eGFP and pCralbp-DsRed were sectioned on P14 and analyzed using confocal microscopy. Nuclei were stained using DRAQ5 and are shown in blue. C) Retinas co-electroporated *in vivo* with control shRNA or Ars2 shRNA and pCralbp-DsRed were analyzed at P14 using flow cytometry, and percent Cralbp-DsRed$^{+ve}$ is shown relative to total transfected cells. A two-tailed unpaired $t$ test was performed ($t(8) = 4.21; P < 0.001$), and error bars represent SD.

**Fig 5 – ARS2 knockdown cell fate defect phenocopies FLASH KD.** A) Retinas were co-electroporated with either control, Ars2, FLASH or Drosha shRNA and the pNrl-DsRed reporter. Electroporated retinal explants were cultured *in vitro* to P8, at which point 4 retinas per condition were pooled and dissociated into a single cell suspension and analyzed using flow cytometry. The proportion of Nrl-DsRed$^{+ve}$ cells (pink gate) is shown relative to total transfected cells for each condition. B) Neurogenic N2a cells were transfected with either control, Ars2, FLASH,
Lsm11 or Drosha shRNA and the H2A-DsRed RDH processing reporter, and DsRed expression was analyzed by flow cytometry. A one-way analysis of variance (ANOVA) was performed followed by Tukey's multiple-comparison post hoc test ($F[4,20] = 84.99; P < 0.001$). ***, $P < 0.001$. n.s., not significant. Error bars represent SD.

**Fig 6 - Working model of ARS2 depletion phenotype in the mouse retina.** In control retinas (left panel) nuclei of proliferating progenitors undergo interkinetic nuclear migration and exit the cell cycle to produce the correct proportions of the major cell types. Following ARS2 disruption (right panel), cell cycle progression is delayed, nuclei spend more time at a basal position, and exit the cell cycle prematurely (extra green and black nuclei, respectively). Whether mitosis occurs at the apical margin or at an ectopic position is unknown and is indicated with a question mark. Additionally, ARS2 depletion increases rod photoreceptor reporter expression, and decreases Müller glia reporter expression. We hypothesize that the delayed cell cycle progression and early cell cycle exit is at least partially due to a defect in histone processing, and the increase in rod photoreceptors is a consequence of the timing of cell cycle exit.
ARS2 is expressed in the developing and adult mouse retina

130x155mm (300 x 300 DPI)
ARS2 knockdown affects cell cycle progression and exit
ARS2 knockdown increases rod photoreceptors.

184x244mm (300 x 300 DPI)
ARS2 knockdown decreases Müller glia cells.

190x175mm (300 x 300 DPI)
ARS2 knockdown cell fate defect phenocopies FLASH KD

140x97mm (300 x 300 DPI)