Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

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

Stem-loop binding protein is required for retinal cell proliferation, neurogenesis, and intraretinal axon pathfinding in zebrafish

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ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form
5 July 2014
Accepted 23 July 2014

Keywords:
Zebrafish
Retina
Proliferation
Neurogenesis
Stem-loop binding protein
Notch

ABSTRACT

In the developing retina, neurogenesis and cell differentiation are coupled with cell proliferation. However, molecular mechanisms that coordinate cell proliferation and differentiation are not fully understood. In this study, we found that retinal neurogenesis is severely delayed in the zebrafish stem-loop binding protein (slbp) mutant. SLBP binds to a stem-loop structure at the 3′-end of histone mRNAs, and regulates a replication-dependent synthesis and degradation of histone proteins. Retinal cell proliferation becomes slower in the *slbp1* mutant, resulting in cessation of retinal stem cell proliferation. Although retinal stem cells cease proliferation by 2 days postfertilization (dpf) in the slbp mutant, retinal progenitor cells in the central retina continue to proliferate and generate neurons until at least 5 dpf. We found that this progenitor proliferation depends on Notch signaling, suggesting that Notch signaling maintains retinal progenitor proliferation when faced with reduced SLBP activity. Thus, SLBP is required for retinal stem cell maintenance. SLBP and Notch signaling are required for retinal progenitor cell proliferation and subsequent neurogenesis. We also show that SLBP1 is required for intraretinal axon pathfinding, probably through morphogenesis of the optic stalk, which expresses attractant cues. Taken together, these data indicate important roles of SLBP in retinal development.

Introduction

The vertebrate retina contains six major classes of neurons and one class of glial cells, which are organized into retinal layers (Dowling, 1987). Throughout vertebrates, retinal cell differentiation proceeds in a histogenetic sequence, with retinal ganglion cells (RGCs) born first and Müller cells born last, suggesting that birth date influences retinal cell fate decision (Holt et al., 1988; Hu and Easter, 1999). Early cell lineage studies using frog and mouse retinas demonstrated apparent randomness of clonal size and cell fate distribution (Holt et al., 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988). These observations suggest the model that retinal progenitor cells are multipotent, giving rise to all retinal cell types in response to environmental cues (Agathocleous and Harris, 2009; Livesey and Cepko, 2001). However, transcription factors that modulate competence of retinal progenitor cells have been reported (Elliott et al., 2008), suggesting that an intrinsic cell mechanism also regulates retinal cell fate determination (Agathocleous and Harris, 2009). Furthermore, variable retinal cell-type combinations are generated from equipotent progenitor cells, suggesting that some stochastic mechanisms contribute to the formation of retinal neural circuit, where the right number and proportions of neuronal cell types are generated (Gomes et al., 2011; He et al., 2012). Thus, it is important to elucidate how cell-cycle exit and subsequent neurogenesis are regulated in retinal progenitor cells, and how this neurogenic regulation cooperates with cell extrinsic and intrinsic mechanisms that determine retinal cell fate.

In the developing zebrafish retina, neurogenesis is initiated in the cell adjacent to the optic stalk and progresses into the entire neural retina (Hu and Easter, 1999; Masai et al., 2000), suggesting that zebrafish retina provides a good model for studying spatio-temporal regulation of neurogenesis. We and other groups found that fibroblast growth factor and Hedgehog (Hh) signaling pathways regulate the initiation and progression of retinal neurogenesis, respectively (Martinez-Morales et al., 2005; Masai et al., 2005; Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003). We also found that Wnt and Notch signaling pathways promote cell proliferation and inhibit neurogenesis in the zebrafish retina, and that histone deacetylase 1 (Hdac1) antagonizes both Wnt and Notch signaling pathways to promote neurogenesis (Yamaguchi et al., 2005). Furthermore, cell polarity regulators, N-cadherin and a zebrafish Stardust homolog, Nagie oko, influence the timing of retinal neurogenesis through the modulation of

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http://dx.doi.org/10.1016/j.ydbio.2014.07.020
0012-1606/

Notch signaling in zebrafish (Yamaguchi et al., 2010). However, the molecular mechanism that regulates the spatiotemporal pattern of retinal neurogenesis in zebrafish still remains to be elucidated.

The synthesis of histone protein is tightly coupled to DNA replication to ensure that histone protein production is sufficient to assemble newly replicated DNA into chromatin. In metazoans, histone protein production is temporally controlled through a replication-dependent regulation of histone mRNA levels (Marzluff, 2005; Marzluff and Duronio, 2002; Marzluff et al., 2008). The regulation of histone mRNA occurs at multiple steps, including an increase in the rate of histone gene transcription at the beginning of S phase, as well as a decrease in the half-life of histone mRNA at the end of S phase. Most importantly, the most critical step that drives the levels of histone mRNA, to an S phase level 35-fold higher than in G1 phase, is an increase in the efficiency of histone pre-mRNA processing during the S phase. Replication-dependent histone mRNAs are not polyadenylated, but end in a highly conserved stem-loop. This stem-loop structure is a critical element for histone metabolism, including translation and degradation. Stem-loop binding protein (SLBP) (Martin et al., 1997) binds to this element and cooperates with other factors such as U7 snRNP to promote histone pre-mRNA processing, and its translation into protein (Marzluff et al., 2008). Analyses of genetic mutations or knockdown of SLBP in *Caenorhabditis elegans* (Kodama et al., 2002; Pettitt et al., 2002), *Drosophila melanogaster* (Sullivan et al., 2001), mouse (Arnold et al., 2008), and human cultured cells (Zhao et al., 2004) suggest that SLBP is required for cell cycle-coupled histone mRNA production. Nevertheless, although vertebrate retinal neurogenesis is coupled with cell cycle progression, the role of SLBP and SLBP-mediated histone metabolism in retinal neurogenesis has not been studied.

In this report, we identified a zebrafish mutant, namely *rw440*, in which retinal neurogenesis is severely delayed. We found that the *rw440* mutant gene encodes SLBP1. Next, we examined cell proliferation in the *rw440* mutant retina. Cell proliferation was slow in the *rw440* mutant retina, and retinal stem cells stopped proliferation at 2 days postfertilization (dpf). Interestingly, although retinal progenitor cell proliferation was slow, cells continued to proliferate and generated retinal neurons in the central retina of the *rw440* mutant, at least until 5 dpf. We found that proliferation of central retinal cells depended on Notch signaling, suggesting that Notch signaling maintains retinal progenitor proliferation in the reduction of SLBP1 activity. Thus, SLBP1 is required for retinal stem cell maintenance. SLBP1 and Notch signaling are required for retinal progenitor proliferation and subsequent neurogenesis. We also found that retinal axons failed to exit from the optic cup, and were rather extended astray inside the neural retina in the *rw440* mutant, suggesting a role of SLBP1 in intraretinal axon pathfinding. In zebrafish, chemokine receptor and ligand, *cxcr4b* and *cxcl12a*, are expressed in RGCs and the optic stalk, respectively, and promote retinal axons to find the exit point of the optic cup. In the *rw440* mutant, *cxcr4b* expression was normally observed in RGCs; however, *cxcl12a* expression was observed in the forebrain but failed to extend inside the retina. Furthermore, expression of an optic stalk marker, *pax2.1*, showed that morphogenesis of the optic cup, including choroid fissure closure, was affected in the *rw440* mutant. These observations suggest that SLBP1 is required for intraretinal pathfinding through regulation of optic stalk morphogenesis, and taken together, these data indicate important roles of SLBP in retinal development.

Material and methods

Fish

Zebrafish (*Danio rerio*) were maintained according to standard procedures (Westerfiled, 2000). RIKEN-wako (RW) and WIK strains were used as wild type for mutagenesis and mapping, respectively. Mutagenesis was performed as previously described (Masai et al., 2003). The *rw440* mutant and a *mib* mutant allele, *mib*^{ta52b} (Jiang et al., 1996), were used. Tg[ath5:GFP]^{rw021} was used to monitor *ath5* gene expression (Masai et al., 2005).

Mutagenesis, mapping, and cloning of the rw440 mutant gene

Mutagenesis, mapping, and cloning were performed as previously described (Masai et al., 2003). The polymorphic markers zk169H1 and z34510 were used for restricting of the genomic region covering the *rw440* mutation.

Rescue experiments

To make the DNA construct Tol2[*EF1α*: SLBP1], a full-length cDNA of the *slbp1* gene was amplified with specific primers using the polymerase chain reaction, and subcloned into a Tol2 transposon-based vector, pT2AL200R150G (Urasaki et al., 2006), which drives *egfp* mRNA transcription under the control of the *EF1α* promoter, by replacing the *egfp* coding region with *slbp1* cDNA. To make the DNA construct Tol2[*EF1α*: GFP-SLBP1], *slbp1* cDNA was fused to the 3′-end of the GFP coding sequence in frame, and the fusion DNA fragment was subcloned into pT2AL200R150G by replacing the *egfp* coding region with *gfp-slbp1* cDNA. Five to 25 ng/μL of DNA plasmid encoding Tol2[*EF1α*: SLBP1] or Tol2[*EF1α*: GFP-SLBP1] was injected with 250 ng/μL RNA encoding transposase into zebrafish fertilized eggs produced by *rw440* mutant heterozygous fish pairs. Injected embryos were maintained at 28.5 °C and fixed with 4% paraformaldehyde (PFA) at the stage when phenotypes were examined. Embryos were separated into heads and bodies, which were used for histological analyses and genotyping, respectively.

Histology

Immunostaining of cryosections and plastic sectioning were performed as described previously (Imai et al., 2010). Zn5 antibody (Zebrafish international resource center (ZIRC), Eugene, Oregon), zpr1 antibody (ZIRC, Eugene, Oregon), anti-acetylated α-tubulin antibody (Clone 6-11B-1; Sigma), anti-phosphorylated histone H3 (anti-pH3) antibody (SC-8656; Santa Cruz Biotechnology), and anti-Pax6 antibody (Covance; PRB-278P) were used at dilutions of 1:100, 1:100, 1:1000, 1:1000, and 1:100, respectively. Nuclear staining was performed using 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) or 50 nM SYTOX Green (Molecular Probes). Filamentous actin (F-actin) was stained using 0.1 μM rhodamine-conjugated phalloidin (Molecular Probes). Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) was performed using an In Situ Cell Death Detection Kit (Roche). Images were scanned using a LSM510 (Carl Zeiss) or FV1000 (Olympus) confocal laser scanning microscope.

In situ hybridization

In situ hybridization was performed as described previously (Imai et al., 2010). Full-length versions of the *slbp1*, *slbp2*, *c-myc2* (Yamaguchi et al., 2005), *rx1* (Chuang et al., 1999), *ath5* (Masai et al., 2000), *delta-C* (Haddon et al., 1998), *her4* (Pasini et al., 2004), *cxcr4b* (Li et al., 2005), *cxcl12a* (Li et al., 2005), and *pax2.1* (Krauss et al., 1991) genes were used.

Western blot

Two and three dpf embryos were used for western blotting as described previously (Imai et al., 2010). Anti-histone H3 (Abcam;

Please cite this article as: Imai, F., et al., Stem-loop binding protein is required for retinal cell proliferation, neurogenesis, and intraretinal axon pathfinding in zebrafish. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.07.020