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Id2a is required for hepatic outgrowth during liver development in zebrafish

Mehwish Khaliq[a], **Tae-Young Choi**[a], **Juhoon So**[a], and **Donghun Shin**[a,*]

Mehwish Khaliq: mek118@pitt.edu; Tae-Young Choi: choity@pitt.edu; Juhoon So: juhoon@pitt.edu

[a]Department of Developmental Biology, McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA 15260, USA

Abstract

During development, inhibitor of DNA binding (Id) proteins, a subclass of the helix-loop-helix family of proteins, regulate cellular proliferation, differentiation, and apoptosis in various organs. However, a functional role of Id2a in liver development has not yet been reported. Here, using zebrafish as a model organism, we provide *in vivo* evidence that Id2a regulates hepatoblast proliferation and cell death during liver development. Initially, in the liver, *id2a* is expressed in hepatoblasts and after their differentiation, *id2a* expression is restricted to biliary epithelial cells. *id2a* knockdown in zebrafish embryos had no effect on hepatoblast specification or hepatocyte differentiation. However, liver size was greatly reduced in *id2a* morpholino-injected embryos, indicative of a hepatic outgrowth defect attributable to the significant decrease in proliferating hepatoblasts concomitant with the significant increase in hepatoblast cell death. Altogether, these data support the role of Id2a as an important regulator of hepatic outgrowth via modulation of hepatoblast proliferation and survival during liver development in zebrafish.

Keywords

Inhibitor of DNA binding; differentiation; biliary epithelial cell; hepatoblast; liver specification; helix-loop-helix

1. Introduction

Liver organogenesis is a multifaceted process involving hepatoblast specification from the ventral foregut endoderm, budding and outgrowth of the liver bud, and hepatoblast differentiation into either hepatocytes or biliary epithelial cells (BECs) (Zaret, 2002; Lemaigre, 2003). In both mice (Jung et al., 1999; Rossi et al., 2001) and zebrafish (Shin et al., 2007; Chung et al., 2008), inductive signals of Fibroblast Growth Factors (FGFs) and Bone Morphogenetic Proteins (BMPs) are essential for hepatoblast specification. In conjunction with the BMP and FGF signaling pathways, several homeobox transcription

*Corresponding author: 3501 5th Avenue BST3-5063, Pittsburgh, PA 15260; Tel: 1-412-624-2144; fax: 1-412-383-2211; donghuns@pitt.edu.

factors, including HHEX and PROX1, also regulate the initial stages of liver organogenesis (Si-Tayeb et al., 2010). HHEX regulates hepatoblast proliferation and delamination from the foregut endoderm as *Hhex*$^{-/-}$ mice lack a liver bud and the hepatoblasts fail to migrate into the surrounding septum transversum mesenchyme (Bort et al., 2004). PROX1 also regulates hepatoblast delamination from the liver diverticulum as hepatoblasts fail to migrate in *Prox1*$^{-/-}$ mice (Sosa-Pineda et al., 2000). Hepatocyte metabolic gene expression is altered in favor of biliary gene expression when *Prox1* is ablated in post-delaminated hepatoblasts (Seth et al., 2014). *hhex* (Wallace et al., 2001) and *prox1a* also regulate liver development in zebrafish. *prox1a*, specifically, marks the initiation of hepatoblast specification in zebrafish (Ober et al., 2006). Besides HHEX and PROX1, zebrafish and mammals share additional transcription factors critical for liver organogenesis, such as GATA6 and hepatic nuclear factors (HNFs) (Bossard and Zaret, 1998; Matthews et al., 2004; Holtzinger and Evans, 2005; Lokmane et al., 2008). However, a comprehensive understanding of the molecular mechanisms underlying transcriptional regulation during liver development still needs to be defined.

One family of transcriptional regulators essential in developmental processes, including cell lineage commitment, proliferation and differentiation, is the helix-loop-helix (HLH) family of transcription factors (Massari and Murre, 2000; Jones, 2004). The HLH domain, essential for dimerization, is important in the formation of homo- or hetero-dimers. While some HLH proteins are ubiquitously expressed (e.g., E proteins), other HLH proteins are tissue-specific (e.g., PTF1, HES1). HES1, in particular, downstream of Notch signaling, is essential for digestive system development, especially in extrahepatic bile duct development (Sumazaki et al., 2004). In *Hes1*$^{-/-}$ mice, no tubular structures form in the ductal plate during intrahepatic bile duct development (Kodama et al., 2004). In addition, the bHLH factor, heart and neural crest derivatives expressed 2 (Hand2), is expressed in tissues that surround the liver primordium, such as the lateral plate mesoderm in zebrafish, which later contributes to the hepatic stellate cells (Yin et al., 2012). Moreover, bHLH-PAS (Per-ARNT-Sim) factors, such as the hypoxia inducible factors (HIFs), participate in hepatic disease, regeneration, fibrosis, and hepatocellular carcinoma (Nath and Szabo, 2012). Hif2α (renamed as Epas1b) binds hypoxia response elements (HREs) and regulates hepatic outgrowth in zebrafish (Lin et al., 2014). The activity of bHLH factors can be regulated by the inhibitor of DNA binding (ID) family of proteins.

ID proteins lack the basic DNA binding domain and regulate HLH factors via heterodimerization and subsequent creation of nonfunctional, dominant negative complexes that lack DNA-binding capability (Norton, 2000). By heterodimerizing and sequestering ubiquitously expressed HLH factors, such as E-proteins (E47, E2-2, HEB, E12), or tissue-restricted HLH factors, ID proteins can thereby regulate cell proliferation, differentiation and apoptosis in a cell-context dependent manner (Sikder et al., 2003). In the pancreas, for instance, by binding and sequestering NeuroD, a bHLH factor implicated in pancreatic beta cell survival and differentiation, ID2 regulates pancreatic progenitor expansion (Hua et al., 2006). Non-bHLH factors can also bind and regulate ID protein function. For example, hypophosphorylated Retinoblastoma (Rb) tumor-suppressor protein interacts with ID2 during cell cycle arrest, preventing the latter from sequestering other transcription factors

and consequently allows differentiation to occur (Iavarone et al., 1994; Lasorella et al., 2002). Mice with a genetic deletion of *Id2* display a reduced number of natural killer cells, lack lymph nodes and experience 25% neonatal lethality (Yokota et al., 1999). To date, no study has examined the role of ID2 in hepatogenesis.

While the mammalian genome consists of four *Id* genes, *Id1–4* (Lasorella et al., 2014), five *id* genes are present in the zebrafish genome: *id1, id2a, id2b, id3* and *id4.* In the developing zebrafish liver, we observed that among the five *id* genes, only *id2a* is restrictively expressed in BECs. This unique expression pattern prompted us to investigate the function of *id2a* in liver development. Here, we show that initially, *id2a* is expressed in hepatoblasts and later, in BECs. In addition, using the morpholino knockdown approach, we show that Id2a regulates hepatic outgrowth, without affecting hepatoblast specification or differentiation, by modulating hepatoblast proliferation and survival.

2. Results

2.1. *id2a* expression in the developing liver

Using whole-mount *in situ* hybridization (WISH), we examined *id2a* expression during liver development in detail. We first detected *id2a* expression in the liver-forming region from 30 hours post fertilization (hpf) (Fig. 1A), when hepatoblast specification has already occurred. At this stage, the liver tissue consists of hepatoblasts, which are liver progenitor cells, capable of differentiating into either hepatocytes or BECs. Following hepatoblast differentiation, around 72 hpf, we noted that *id2a* expression displayed a branching pattern in the liver, indicative of the intrahepatic biliary network consisting of BECs. The BEC-specific expression was maintained even at 5 dpf (Fig. 1A). To confirm *id2a* expression in BECs, we conducted immunostaining in conjunction with WISH utilizing the *Tg(prox1a:YFP), Tg(Tp1:GFP)* and *Tg(kdrl:GFP)* lines, which express fluorescent proteins in hepatoblasts (Bussmann and Schulte-Merker, 2011), BECs (Parsons et al., 2009), and liver endothelial cells (Beis et al., 2005), respectively. As initially observed, *id2a* was specifically detected in *prox1a:*YFP-positive (Fig. 1B) and *Tp1:*GFP-positive cells (Fig. 1C), but not in the endothelial cells (Fig. 1D), indicating that in the liver, *id2a* is initially expressed in hepatoblasts and later restricted to BECs.

Since the zebrafish genome contains five *id* genes, we further investigated the expression patterns of the remaining four *id* genes, *id1, id2b, id3*, and *id4*, in the liver during embryonic development. At 30 hpf, *id2b, id3*, and *id4* are not expressed in the liver-forming region; however, it was not clear whether *id1* is expressed in the liver-forming region due to its broad expression (Fig. 2A). Double labeling of *id1* and *sox17:*GFP, which labels all endodermal cells (Chung and Stainier, 2008), showed *id1* expression in the liver-forming region (Fig. 2E; brackets). At 48 hpf, none of the four genes are expressed in the liver. At 72 hpf, *id2b* and *id3*, but not *id1* or *id4*, are expressed in the liver (Fig. 2B and 2C; arrows); however, their expression does not mimic the biliary branching pattern of *id2a* expression. Altogether, these expression data indicate that both *id1* and *id2a* are expressed in the liver-forming region at 30 hpf and that only *id2a* expression is restricted to BECs.

2.2. *id2a* knockdown causes an intrahepatic biliary network defect in the developing liver

Given the restricted expression pattern of *id2a* in BECs, we sought to determine whether *id2a* is important for intrahepatic biliary development. We conducted loss-of-function analyses using published *id2a* morpholino oligonucleotides (MO) (Uribe and Gross, 2010; Das and Crump, 2012; Uribe et al., 2012). Consistent with previous reports, *id2a* MO-injected embryos were microcephalic and microphthalmic (Uribe and Gross, 2010), a phenotype also observed in $Id2^{-/-}$ mice (Yokota et al., 1999). Importantly, the small liver phenotype observed in *id2a* MO-injected embryos was partially rescued by *id2a* mRNA injection (Fig. S1A and S1B), further validating the *id2a* MO. Since *id2a* is expressed specifically in BECs at later stages of liver development, we used the *Tg(Tp1:GFP)* line to examine BECs. Using epifluorescence microscopy, we detected very few GFP-positive cells in the livers of *id2a* MO-injected embryos (Fig. 3A; squares), suggesting BEC number was greatly reduced. To further analyze the intrahepatic biliary structure, whole-mount immunostaining combined with confocal microscopy was used. In *id2a* MO-injected embryos, not only was the liver size reduced, but the intrahepatic biliary network failed to branch, appearing aggregated (Fig. 3B). Taken together, these data imply that lack of *id2a* results in defective biliary structure and reduced BEC number, suggesting that *id2a* may regulate intrahepatic biliary development.

2.3. *id2a* knockdown reduces liver size but does not block hepatoblast specification or hepatocyte differentiation

The main steps of liver development include hepatic competence, hepatoblast specification, hepatocyte or BEC differentiation, and hepatic outgrowth (Zaret, 2002). Since *id2a* is expressed in the liver-forming region from 30 hpf (Fig. 1A) after hepatoblast specification, which occurs around 22 hpf in zebrafish (Ober et al., 2006), it is unlikely that *id2a* is implicated in hepatic competence. Thus, we examined the expression of the following markers in *id2a* MO-injected embryos: the early hepatoblast markers, *hhex* and *prox1a* (Field et al., 2003; Ober et al., 2006), for hepatoblast specification and maintenance; and the hepatocyte markers, *fabp10a, cp* and *sepp1b*, for hepatocyte differentiation and hepatic outgrowth. *hhex* and *prox1a* expression was detected in the livers of the MO-injected embryos at 36 hpf (Fig.3C and 3D, arrows), suggesting that *id2a* does not regulate hepatoblast specification or its maintenance (Fig.3C and 3D, arrows). Hepatic *fabp10a, cp* and *sepp1b* expression was also detected in the MO-injected embryos at 48 hpf (Fig. 3E–G, arrows), suggesting that *id2a* does not regulate hepatocyte differentiation. However, the liver size was reduced following *id2a* knockdown, implicating *id2a* in regulating hepatic outgrowth. Additionally, since *id2a* is expressed strongly in the gut and intestinal regions during development (Fig. 1A; bracket), we examined the expression of *cdx1b*, an intestinal bulb marker (Cheng et al., 2008), expecting a similar outgrowth phenotype as observed in the liver. As expected, lack of *id2a* had no effect on *cdx1b* induction; however, the intestinal bulb failed to grow at 48 hpf in *id2a* MO-injected embryos (Fig. 3H, brackets), indicative of an intestinal outgrowth defect. We further sought to determine whether *id2a* knockdown resulted in a general outgrowth defect of all endoderm-derived organs or specific organs in which *id2a* is expressed. Since *id2a* is not expressed in the dorsal pancreas (Fig. 1A and Fig. S2A), we performed WISH to examine the expression of insulin, which marks the pancreatic

beta cells of the dorsal pancreas (Argenton et al., 1999). We found no difference in the size of the dorsal pancreas between control and *id2a* MO-injected embryos (Fig. S2B).

Previous studies have implicated ID proteins in the maintenance of neural stem cells. *Id1* and *Id3* double-knockout mice exhibit precocious neuronal differentiation, whereas *ID2* overexpression in the chick hindbrain inhibits neuronal differentiation (Bai et al., 2007). Thus, it is still possible that *id2a* knockdown may result in precocious hepatocyte differentiation. To test this possibility, we examined *fabp10a* expression at 36 hpf, when *fabp10a* expression is not yet detected in the livers of wild-type embryos. However, *fabp10a* expression was not detected in *id2a* MO-injected embryos (data not shown), ruling out this possibility. Moreover, we examined whether Id2a overexpression could increase liver size. However, liver size was not further increased in *id2a* mRNA-injected embryos at 72 hpf compared with controls (Fig. S1E), indicating that Id2a is not sufficient for liver outgrowth.

Altogether, these data indicate that during liver development, *id2a* is not required for hepatoblast specification or hepatocyte differentiation, but rather for hepatic outgrowth. Moreover, the outgrowth defect observed in *id2a* MO-injected embryos may also apply to the development of other organ systems, such as the intestinal bulb.

2.4. *id2a* knockdown reduces liver size via decreased hepatoblast proliferation and increased cell death

To determine whether the small liver observed in *id2a* MO-injected embryos was caused by reduced proliferation and/or enhanced cell death, we conducted anti-phospho-Histone 3 (pH3) immunostaining and EdU labeling for proliferation and TUNEL labeling for cell death. Although the percentage of $pH3^+$ cells among *prox1a:*YFP^+ hepatic cells in *id2a* MO-injected embryos at 40 hpf was not significantly different from that in controls, there was a trend of reduced $pH3^+$ cell number in the MO-injected liver compared to the control liver (0–3 versus 3–5) (Fig.4A and 4B). EdU labeling revealed about a 40% decrease in the percentage of EdU^+ cells among *sox17:*GFP^+ hepatic cells in *id2a* MO-injected embryos at 40 hpf compared with controls (Fig.4C and 4D), indicating reduced proliferation. In addition, we observed TUNEL and Prox1 double-positive cells in *id2a* MO-injected embryos at 40 hpf, but not in controls (Fig.4E and 4F). MO-mediated knockdown can often induce apoptosis mediated via aberrant p53 activation; thus, concurrent knockdown of *tp53* can ameliorate apoptosis induced by MO off-targeting (Robu et al., 2007). Therefore, we performed simultaneous knockdown of *tp53* and *id2a*. We did not detect any differences in microphthalmic, microcephalic, or small liver phenotypes between single *id2a* and double *id2a/tp53* MO-injected embryos at 60 hpf (Fig. S1C and S1D), indicating that *id2a* knockdown phenotypes are independent of the p53 pathway. Altogether, these data indicate that *id2a* regulates hepatic outgrowth by promoting hepatoblast proliferation and repressing cell death.

3. Discussion

In this study, we sought to determine the role of Id2a in liver development. We report three important findings. First, by using WISH followed by immunostaining, we discovered that *id2a* is initially expressed in the liver-forming region from 30 hpf and following hepatoblast

differentiation at 48 hpf, *id2a* expression is restricted to BECs. Second, *id2a* knockdown did not affect hepatocyte differentiation or hepatoblast specification, which correlates with a lack of *id2a* liver expression during the hepatoblast specification stage (i.e., 22 hpf). Lastly, our data revealed that *id2a* knockdown inhibited hepatic outgrowth during development as supported by the reduced liver size in *id2a* MO-injected embryos.

Similar to the phenotype observed in *id2a* MO-injected embryos, in which hepatic outgrowth was compromised while hepatoblast specification and hepatocyte differentiation appeared unaffected, additional genes are also implicated in regulating hepatic outgrowth in zebrafish. Classified as a tumor suppressor gene that functions as a transcriptional activator, core promoter element binding protein (*copeb*; renamed as *klf6a*) is expressed in the zebrafish digestive organs, including the liver, the pancreas, and intestine. In *copeb* MO-injected embryos, the expansion of the liver is impaired (Zhao et al., 2010). Moreover, the failure of hepatic outgrowth in *copeb* MO-injected larvae is also attributed to a decrease in cell proliferation, as observed in *id2a* MO-injected embryos (Zhao et al., 2010). A similar phenotype is observed in the cell cycle modulator ubiquitin-like with PHD and ring finger domains 1 (*uhrf1*) mutants, which exhibit smaller livers as a result of a proliferation defect during the hepatic outgrowth phase (Sadler et al., 2007). In addition, knockdown of either *sfrp5* (Stuckenholz et al., 2013) or *nav3a* (Klein et al., 2011) also results in a defect in hepatoblast outgrowth and subsequent liver size in 40-hpf zebrafish embryos. These results correlate with the well-known role of ID proteins as master regulators of cell proliferation (Lasorella et al., 2014), especially evident during early development.

Early development is a process defined by rapid cell proliferation followed by cell differentiation, generating distinct, mature tissues. Generally, *Id* expression is usually upregulated during the cell proliferation phase of early tissue development, and subsequently downregulated in mature, differentiated cells (Biggs et al., 1992; Ellmeier et al., 1992). However, exceptions exist; therefore, ID protein-mediated proliferation is cell contextdependent. For example, overexpression of *Id1*, *Id2*, or *Id3* in neural stem cells derived from the mouse embryonic forebrain maintains the cortical neural stem cells in a proliferative, self-renewing state, and simultaneously inhibits neuronal differentiation (Jung et al., 2010). In contrast, upon differentiation of hematopoietic progenitor cells, *Id2* expression increases (Cooper et al., 1997). Previously, Uribe et al. reported on the role of *id2a* in zebrafish retinogenesis. Upon *id2a* knockdown, proliferative retinoblasts (in the S-phase) increased as mitotic retinoblasts (in the M-phase) decreased, which demonstrates a role of Id2a in regulating the S- to M-phase progression during the cell cycle (Uribe and Gross, 2010). In contrast, we noted a significant decrease in the number of proliferating hepatoblasts at 40 hpf in *id2a* MO-injected embryos. As aforementioned, this phenotypic difference observed in proliferative cells during retinogenesis and liver development alludes to the dependence on cellular contexts in which *id* genes were studied. Nonetheless, *id2a* appears to play an important role in regulating proliferation in diverse tissue contexts including the developing liver.

In addition to proliferation, ID proteins also regulate apoptosis in a cell-dependent manner (Florio et al., 1998; Norton and Atherton, 1998). While apoptosis is significantly increased in mammary epithelial cells of $Id2^{-/-}$ pregnant mice (Mori et al., 2000), overexpression of

Id1, *Id2*, or *Id3* induces apoptosis in serum-deprived rat embryonic fibroblasts (Deed et al., 1997). In *id2a* MO-injected embryos, we observed a significant increase in the number of TUNEL-positive hepatoblasts at 40 hpf, highlighting the role of Id2a in regulating apoptosis in the developing liver.

Although intrahepatic biliary defects were also observed in *id2a* MO-injected embryos, it is unclear if the defects are either attributed to (1) a primary phenotype due to *id2a* knockdown or (2) a secondary phenotype due to compromised hepatic outgrowth. BEC-specific knockdown or knockout of *id2a* should conclusively establish a direct or indirect role of *id2a* in intrahepatic biliary morphogenesis. Currently, however, it is a challenge to create such a tool in zebrafish. As *id2a* expression is restricted in BECs during liver development, it will be interesting to explore the role of *id2a* in biliary-driven liver regeneration. Previously, we reported on a novel hepatocyte-specific genetic ablation model in zebrafish: following severe hepatocyte loss, BECs contribute to the repopulation of the liver (Choi et al., 2014). Few reports have explored the role of ID2 in liver regeneration. In two different models of liver injury in rats, partial hepatectomy and bile duct ligation, *Id2* is immediately upregulated during the hepatocyte priming phase and ID2 expression is detected in the proliferating hepatocytes, respectively (Rodriguez et al., 2006). However, the role of *Id2* in liver regeneration has not been reported yet. In addition, although ID2 has been shown to interact with various factors, including MyoD during myogenesis (Langlands et al., 1997), future analysis should consider the currently unknown binding factor of Id2a in the developing zebrafish liver. Since *id2a* knockdown reduced the liver size, we speculate the binding partner of Id2a to function as a suppressor of hepatic outgrowth and a negative regulator of hepatoblast proliferation.

Our findings validate the role of *id2a* in promoting hepatic outgrowth and development. Future studies should explore the mechanism of action of Id2a, including its binding partner, in liver development. Discerning the molecular mechanisms regulating liver development will improve our comprehension of the biological relevance of hepatic diseases and methods to enhance innate liver regeneration.

4. Experimental Procedures

4.1. Zebrafish Maintenance

Embryos and adult zebrafish (*Danio rerio*) were raised and maintained under standard laboratory conditions (Westerfield, 2000) with protocols approved by the University of Pittsburgh IACUC.

4.2. Zebrafish Strains

We used the following transgenic lines: *TgBAC(prox1a:Citrine)*^{hu338} (Bussmann and Schulte-Merker, 2011) [referred to as *Tg(prox1a:YFP)*], *Tg(EPV.Tp1-Mmu.Hbb:EGFP)*^{um14} (Parsons et al., 2009) [referred to as *Tg(Tp1:GFP)*], *Tg(kdrl:EGFP)*^{s843} (Beis et al., 2005) [referred to as *Tg(kdrl:GFP)*], *Tg(ptf1a:EGFP)*^{jh1} (Godinho et al., 2005) [referred to as *Tg(ptf1a:GFP)*], *Tg(sox17:GFP)*^{s870} (Chung and Stainier, 2008), and *Tg(fabp10a:dsRed,ela31:GFP)*^{gz12} (Korzh et al., 2008) [referred to as *Tg(fabp10a:dsRed)*].

4.3. Morpholino and mRNA injection

id2a MO (5'-`GCCTTCATGTTGACAGCAGGATTTC-3') (Uribe and Gross, 2010) and *tp53* MO (5'-GCGCCATTGCTTTGCAAGAATTG-3') (Langheinrich et al., 2002) were purchased from GeneTools (Philomath, OR, USA). 3–4 ng of *id2a* MO or 2 ng of *tp53* MO was injected into one-cell stage embryos. For rescue experiments, 3 ng of the *id2a* MO and 150 pg of *id2a* mRNA, which is resistant to the *id2a* MO, was sequentially injected into one-cell stage embryos. The *id2a* mRNA was generated using the mMessage mMachine SP6 kit (Life Technologies, Grand Island, NY, USA).

4.4. Whole-mount *in situ* hybridization and immunohistochemistry

Whole-mount *in situ* hybridization was performed as previously described (Alexander et al., 1998). cDNA from 24-hpf embryos was used as a template for PCR to amplify *id1*, *id2a*, *id2b*, *id3*, and *id4* genes. We also used the following probes: *hhex* (Ho et al., 1999), *prox1* (Glasgow and Tomarev, 1998), *fabp10a* (Her et al., 2003), *sepp1b* (Kudoh et al., 2001), *cp* (Korzh et al., 2001), and *cdx1b* (Flores et al., 2008). Whole-mount immunostaining was performed as previously described (Dong et al., 2007), using the following antibodies: chicken polyclonal anti- GFP (1:100; Aves Labs, Tigard, OR, USA), rabbit polyclonal anti-Prox1 (1:1000; Millipore, Billerica, MA, USA), mouse monoclonal anti-Anxa4 (also named as 2F11; 1:100; Abcam, Cambridge, MA USA), rabbit polyclonal anti-dsRed (1:200; Clontech, Mountain View, CA, USA), mouse monoclonal anti-phospho-Histone H3 (1:100; Cell Signaling, Danvers, MA, USA) and conjugated secondary antibodies, including Alexa Fluor 405-, 488-, 568-, and 647 (1:300; Life Technologies, Grand Island, NY, USA). Hoechst 33342 (2.5 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) was used for DNA staining. Zeiss LSM700 was used for confocal microscopy.

4.5. TUNEL and EdU assays

Apoptotic cell death was analyzed according to the protocol of the In Situ Cell Death Detection Kit, TMR Red (Roche, Switzerland). Following whole-mount immunostaining, TUNEL labeling was applied. Cell Proliferation was performed using the protocol outlined in the Click-iT EdU Alexa Fluor 647 Imaging Kit (Life Technologies, Grand Island, NY, USA). Larvae were incubated with EdU solution at 39 hpf for one hour, and at 40 hpf, they were harvested for EdU staining. Unpaired two-tailed Student's t-tests were used for statistical analysis; $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Alexander J, Stainier DY, Yelon D. Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. Dev Genet. 1998; 22:288–299. [PubMed: 9621435]

Argenton F, Zecchin E, Bortolussi M. Early appearance of pancreatic hormone-expressing cells in the zebrafish embryo. Mech Dev. 1999; 87:217–221. [PubMed: 10495291]

Bai G, Sheng N, Xie Z, Bian W, Yokota Y, Benezra R, Kageyama R, Guillemot F, Jing N. Id sustains Hes1 expression to inhibit precocious neurogenesis by releasing negative autoregulation of Hes1. Dev Cell. 2007; 13:283–297. [PubMed: 17681138]

Beis D, Bartman T, Jin SW, Scott IC, D'Amico LA, Ober EA, Verkade H, Frantsve J, Field HA, Wehman A, Baier H, Tallafuss A, Bally-Cuif L, Chen JN, Stainier DY, Jungblut B. Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. Development. 2005; 132:4193–4204. [PubMed: 16107477]

Biggs J, Murphy EV, Israel MA. A human Id-like helix-loop-helix protein expressed during early development. Proc Natl Acad Sci U S A. 1992; 89:1512–1516. [PubMed: 1741406]

Bort R, Martinez-Barbera JP, Beddington RS, Zaret KS. Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. Development. 2004; 131:797–806. [PubMed: 14736744]

Bossard P, Zaret KS. GATA transcription factors as potentiators of gut endoderm differentiation. Development. 1998; 125:4909–4917. [PubMed: 9811575]

Bussmann J, Schulte-Merker S. Rapid BAC selection for tol2-mediated transgenesis in zebrafish. Development. 2011; 138:4327–4332. [PubMed: 21865323]

Cheng PY, Lin CC, Wu CS, Lu YF, Lin CY, Chung CC, Chu CY, Huang CJ, Tsai CY, Korzh S, Wu JL, Hwang SP. Zebrafish cdx1b regulates expression of downstream factors of Nodal signaling during early endoderm formation. Development. 2008; 135:941–952. [PubMed: 18234726]

Choi TY, Ninov N, Stainier DYR, Shin D. Extensive Conversion of Hepatic Biliary Epithelial Cells to Hepatocytes After Near Total Loss of Hepatocytes in Zebrafish. Gastroenterology. 2014; 146:776–788. [PubMed: 24148620]

Chung WS, Shin CH, Stainier DYR. Bmp2 Signaling Regulates the Hepatic versus Pancreatic Fate Decision. Developmental Cell. 2008; 15:738–748. [PubMed: 19000838]

Chung WS, Stainier DYR. Intra-endodermal interactions are required for pancreatic beta cell induction. Developmental Cell. 2008; 14:582–593. [PubMed: 18410733]

Cooper CL, Brady G, Bilia F, Iscove NN, Quesenberry PJ. Expression of the Id family helix-loop-helix regulators during growth and development in the hematopoietic system. Blood. 1997; 89:3155–3165. [PubMed: 9129018]

Das A, Crump JG. Bmps and Id2a Act Upstream of Twist1 To Restrict Ectomesenchyme Potential of the Cranial Neural Crest. PLoS Genet. 2012; 8

Deed RW, Hara E, Atherton GT, Peters G, Norton JD. Regulation of Id3 cell cycle function by Cdk-2-dependent phosphorylation. Mol Cell Biol. 1997; 17:6815–6821. [PubMed: 9372912]

Dong PD, Munson CA, Norton W, Crosnier C, Pan X, Gong Z, Neumann CJ, Stainier DY. Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. Nat Genet. 2007; 39:397–402. [PubMed: 17259985]

Ellmeier W, Aguzzi A, Kleiner E, Kurzbauer R, Weith A. Mutually exclusive expression of a helix-loop-helix gene and N-myc in human neuroblastomas and in normal development. EMBO. J. 1992; 11:2563–2571. [PubMed: 1628620]

Field HA, Ober EA, Roeser T, Stainier DY. Formation of the digestive system in zebrafish. I. Liver morphogenesis. Dev Biol. 2003; 253:279–290. [PubMed: 12645931]

Flores MVC, Hall CJ, Davidson AJ, Singh PP, Mahagaonkar AA, Zon LI, Crosier KE, Crosier PS. Intestinal Differentiation in Zebrafish Requires Cdx1b, a Functional Equivalent of Mammalian Cdx2. Gastroenterology. 2008; 135:1665–1675. [PubMed: 18804112]

Florio M, Hernandez MC, Yang H, Shu HK, Cleveland JL, Israel MA. Id2 promotes apoptosis by a novel mechanism independent of dimerization to basic helixloop- helix factors. Mol Cell Biol. 1998; 18:5435–5444. [PubMed: 9710627]

Glasgow E, Tomarev SI. Restricted expression of the homeobox gene prox 1 in developing zebrafish. Mech Dev. 1998; 76:175–178. [PubMed: 9767161]

Godinho L, Mumm JS, Williams PR, Schroeter EH, Koerber A, Park SW, Leach SD, Wong RO. Targeting of amacrine cell neurites to appropriate synaptic laminae in the developing zebrafish retina. Development. 2005; 132:5069–5079. [PubMed: 16258076]

Her GM, Yeh YH, Wu JL. 435-bp liver regulatory sequence in the liver fatty acid binding protein (L-FABP) gene is sufficient to modulate liver regional expression in transgenic zebrafish. Dev Dyn. 2003; 227:347–356. [PubMed: 12815620]

Ho CY, Houart C, Wilson SW, Stainier DY. A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. Curr Biol. 1999; 9:1131–1134. [PubMed: 10531010]

Holtzinger A, Evans T. Gata4 regulates the formation of multiple organs. Development. 2005; 132:4005–4014. [PubMed: 16079152]

Hua H, Zhang YQ, Dabernat S, Kritzik M, Dietz D, Sterling L, Sarvetnick N. BMP4 regulates pancreatic progenitor cell expansion through Id2. J Biol Chem. 2006; 281:13574–13580. [PubMed: 16547003]

Iavarone A, Garg P, Lasorella A, Hsu J, Israel MA. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. 1994; 8:1270–1284. [PubMed: 7926730]

Jones S. An overview of the basic helix-loop-helix proteins. Genome Biol. 2004; 5:226. [PubMed: 15186484]

Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science. 1999; 284:1998–2003. [PubMed: 10373120]

Jung S, Park RH, Kim S, Jeon YJ, Ham DS, Jung MY, Kim SS, Lee YD, Park CH, Suh-Kim H. Id proteins facilitate self-renewal and proliferation of neural stem cells. Stem Cells Dev. 2010; 19:831–841. [PubMed: 19757990]

Klein C, Mikutta J, Krueger J, Scholz K, Brinkmann J, Liu D, Veerkamp J, Siegel D, Abdelilah-Seyfried S, le Noble F. Neuron navigator 3a regulates liver organogenesis during zebrafish embryogenesis. Development. 2011; 138:1935–1945. [PubMed: 21471154]

Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology. 2004; 127:1775–1786. [PubMed: 15578515]

Korzh S, Emelyanov A, Korzh V. Developmental analysis of ceruloplasmin gene and liver formation in zebrafish. Mech Dev. 2001; 103:137–139. [PubMed: 11335121]

Korzh S, Pan X, Garcia-Lecea M, Winata CL, Pan X, Wohland T, Korzh V, Gong Z. Requirement of vasculogenesis and blood circulation in late stages of liver growth in zebrafish. BMC Dev Biol. 2008; 8:84. [PubMed: 18796162]

Kudoh T, Tsang M, Hukriede NA, Chen X, Dedekian M, Clarke CJ, Kiang A, Schultz S, Epstein JA, Toyama R, Dawid IB. A gene expression screen in zebrafish embryogenesis. Genome Res. 2001; 11:1979–1887. [PubMed: 11731487]

Langheinrich U, Hennen E, Stott G, Vacun G. Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. Curr Biol. 2002; 12:2023–2028. [PubMed: 12477391]

Langlands K, Yin X, Anand G, Prochownik EV. Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. J Biol Chem. 1997; 272:19785–19793. [PubMed: 9242638]

Lasorella A, Benezra R, Iavarone A. The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. Nat Rev Cancer. 2014; 14:77–91. [PubMed: 24442143]

Lasorella A, Boldrini R, Dominici C, Donfrancesco A, Yokota Y, Inserra A, Iavarone A. Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. Cancer Res. 2002; 62:301–306. [PubMed: 11782392]

Lemaigre FP. Development of the biliary tract. Mech Dev. 2003; 120:81–87. [PubMed: 12490298]

Lin TY, Chou CF, Chung HY, Chiang CY, Li CH, Wu JL, Lin HJ, Pai TW, Hu CH, Tzou WS. Hypoxia-inducible factor 2 alpha is essential for hepatic outgrowth and functions via the regulation of leg1 transcription in the zebrafish embryo. PLoS One. 2014; 9:e101980. [PubMed: 25000307]

Lokmane L, Haumaitre C, Garcia-Villalba P, Anselme I, Schneider-Maunoury S, Cereghini S. Crucial role of vHNF1 in vertebrate hepatic specification. Development. 2008; 135:2777–2786. [PubMed: 18635606]

Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol. 2000; 20:429–440. [PubMed: 10611221]

Matthews RP, Lorent K, Russo P, Pack M. The zebrafish onecut gene hnf-6 functions in an evolutionarily conserved genetic pathway that regulates vertebrate biliary development. Dev Biol. 2004; 274:245–259. [PubMed: 15385156]

Mori S, Nishikawa SI, Yokota Y. Lactation defect in mice lacking the helix-loop-helix inhibitor Id2. EMBO. J. 2000; 19:5772–5781. [PubMed: 11060028]

Nath B, Szabo G. Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. Hepatology. 2012; 55:622–633. [PubMed: 22120903]

Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci. 2000; 113(Pt 22):3897–3905. [PubMed: 11058077]

Norton JD, Atherton GT. Coupling of cell growth control and apoptosis functions of Id proteins. Mol Cell Biol. 1998; 18:2371–2381. [PubMed: 9528806]

Ober EA, Verkade H, Field HA, Stainier DY. Mesodermal Wnt2b signalling positively regulates liver specification. Nature. 2006; 442:688–691. [PubMed: 16799568]

Parsons MJ, Pisharath H, Yusuff S, Moore JC, Siekmann AF, Lawson N, Leach SD. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mech Dev. 2009; 126:898–912. [PubMed: 19595765]

Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC. p53 activation by knockdown technologies. PLoS Genet. 2007; 3:e78. [PubMed: 17530925]

Rodriguez JL, Sandoval J, Serviddio G, Sastre J, Morante M, Perrelli MG, Martinez-Chantar ML, Vina J, Vina JR, Mato JM, Avila MA, Franco L, Lopez-Rodas G, Torres L. Id2 leaves the chromatin of the E2F4-p130-controlled c-myc promoter during hepatocyte priming for liver regeneration. Biochem. J. 2006; 398:431–437. [PubMed: 16776654]

Rossi JM, Dunn NR, Hogan BL, Zaret KS. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev. 2001; 15:1998–2009. [PubMed: 11485993]

Sadler KC, Krahn KN, Gaur NA, Ukomadu C. Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, uhrf1. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:1570–1575. [PubMed: 17242348]

Seth A, Ye J, Yu N, Guez F, Bedford DC, Neale GA, Cordi S, Brindle PK, Lemaigre FP, Kaestner KH, Sosa-Pineda B. Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment. Development. 2014; 141:538–547. [PubMed: 24449835]

Shin D, Shin CH, Tucker J, Ober EA, Rentzsch F, Poss KD, Hammerschmidt M, Mullins MC, Stainier DY. Bmp and Fgf signaling are essential for liver specification in zebrafish. Development. 2007; 134:2041–2050. [PubMed: 17507405]

Si-Tayeb K, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. Dev Cell. 2010; 18:175–189. [PubMed: 20159590]

Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. Id proteins in cell growth and tumorigenesis. Cancer Cell. 2003; 3:525–530. [PubMed: 12842081]

Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. Nat Genet. 2000; 25:254–255. [PubMed: 10888866]

Stuckenholz C, Lu L, Thakur PC, Choi TY, Shin D, Bahary N. Sfrp5 modulates both Wnt and BMP signaling and regulates gastrointestinal organogenesis [corrected] in the zebrafish, Danio rerio. PLoS One. 2013; 8:e62470. [PubMed: 23638093]

Sumazaki R, Shiojiri N, Isoyama S, Masu M, Keino-Masu K, Osawa M, Nakauchi H, Kageyama R, Matsui A. Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. Nat Genet. 2004; 36:83–87. [PubMed: 14702043]

Uribe RA, Gross JM. Id2a influences neuron and glia formation in the zebrafish retina by modulating retinoblast cell cycle kinetics. Development. 2010; 137:3763–3774. [PubMed: 20943708]

Uribe RA, Kwon T, Marcotte EM, Gross JM. Id2a functions to limit Notch pathway activity and thereby influence the transition from proliferation to differentiation of retinoblasts during zebrafish retinogenesis. Dev Biol. 2012; 371:280–292. [PubMed: 22981606]

Wallace KN, Yusuff S, Sonntag JM, Chin AJ, Pack M. Zebrafish hhex regulates liver development and digestive organ chirality. Genesis. 2001; 30:141–143. [PubMed: 11477693]

Westerfield, M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio). University of Oregon Press; 2000.

Yin CY, Evason KJ, Maher JJ, Stainier DY. The basic helix-loop-helix transcription factor, heart and neural crest derivatives expressed transcript 2, marks hepatic stellate cells in zebrafish: Analysis of stellate cell entry into the developing liver. Hepatology. 2012; 56:1958–1970. [PubMed: 22488653]

Yokota Y, Mansouri A, Mori S, Sugawara S, Adachi S, Nishikawa S, Gruss P. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. Nature. 1999; 397:702–706. [PubMed: 10067894]

Zaret KS. Regulatory phases of early liver development: paradigms of organogenesis. Nat Rev Genet. 2002; 3:499–512. [PubMed: 12094228]

Zhao X, Monson C, Gao C, Gouon-Evans V, Matsumoto N, Sadler KC, Friedman SL. Klf6/copeb is required for hepatic outgrowth in zebrafish and for hepatocyte specification in mouse ES cells. Dev Biol. 2010; 344:79–93. [PubMed: 20430021]

Highlights

- *id2a* is initially expressed in hepatoblasts of the zebrafish liver.
- After hepatoblast differentiation, *id2a* is expressed in biliary epithelial cells.
- Hepatic outgrowth is compromised upon *id2a* loss.
- *id2a* is required for hepatoblast proliferation and survival.

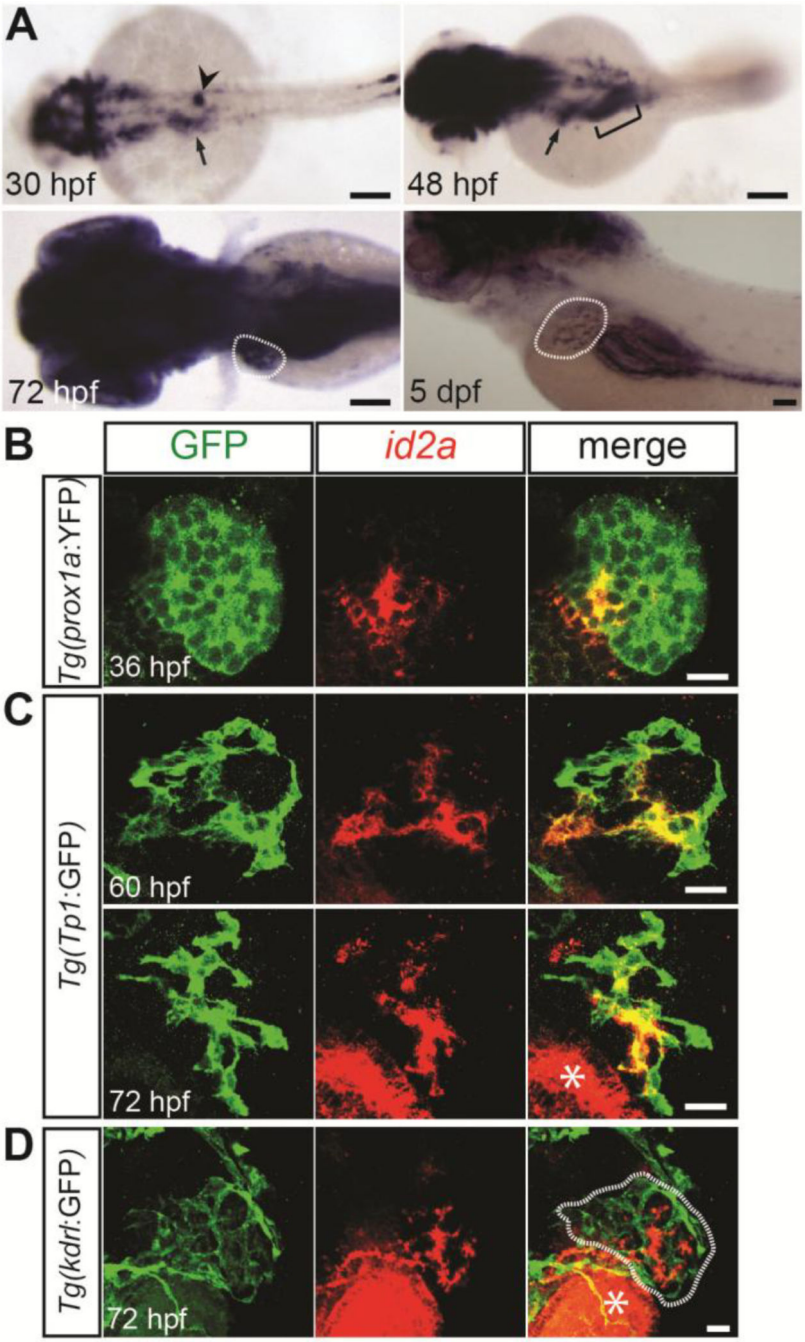

Figure 1. *id2a* expression in the developing liver

(A) WISH reveals *id2a* expression in the liver-forming region at 30 hpf (arrow) and in the liver at 48 hpf (arrow), 72 hpf and 5 dpf (dotted lines). From 72 hpf to, at least, 5 dpf, *id2a* expression in the liver appears to be restricted to BECs. Arrowhead points to the interrenal primordium; bracket denotes the intestinal bulb. Dorsal (30–72 hpf) or lateral (5 dpf) views, anterior to the left. **(B–D)** *id2a* in situ hybridization (red) combined with anti-GFP immunostaining (green) in *Tg(prox1a:YFP)* (B), *Tg(Tp1:GFP)* (C), or *Tg(kdrl:GFP)* (D) embryos reveals *id2a* expression in hepatoblasts at 36 hpf and BECs at 60 and 72 hpf, but

not in the endothelial cells of the liver (dotted line), respectively. Asterisks mark *id2a* expression in the intestinal bulb. Single confocal section (B) or projections of z-stack confocal sections (C, D). Scale bars: 100 (A), 20 (B– D) μm.

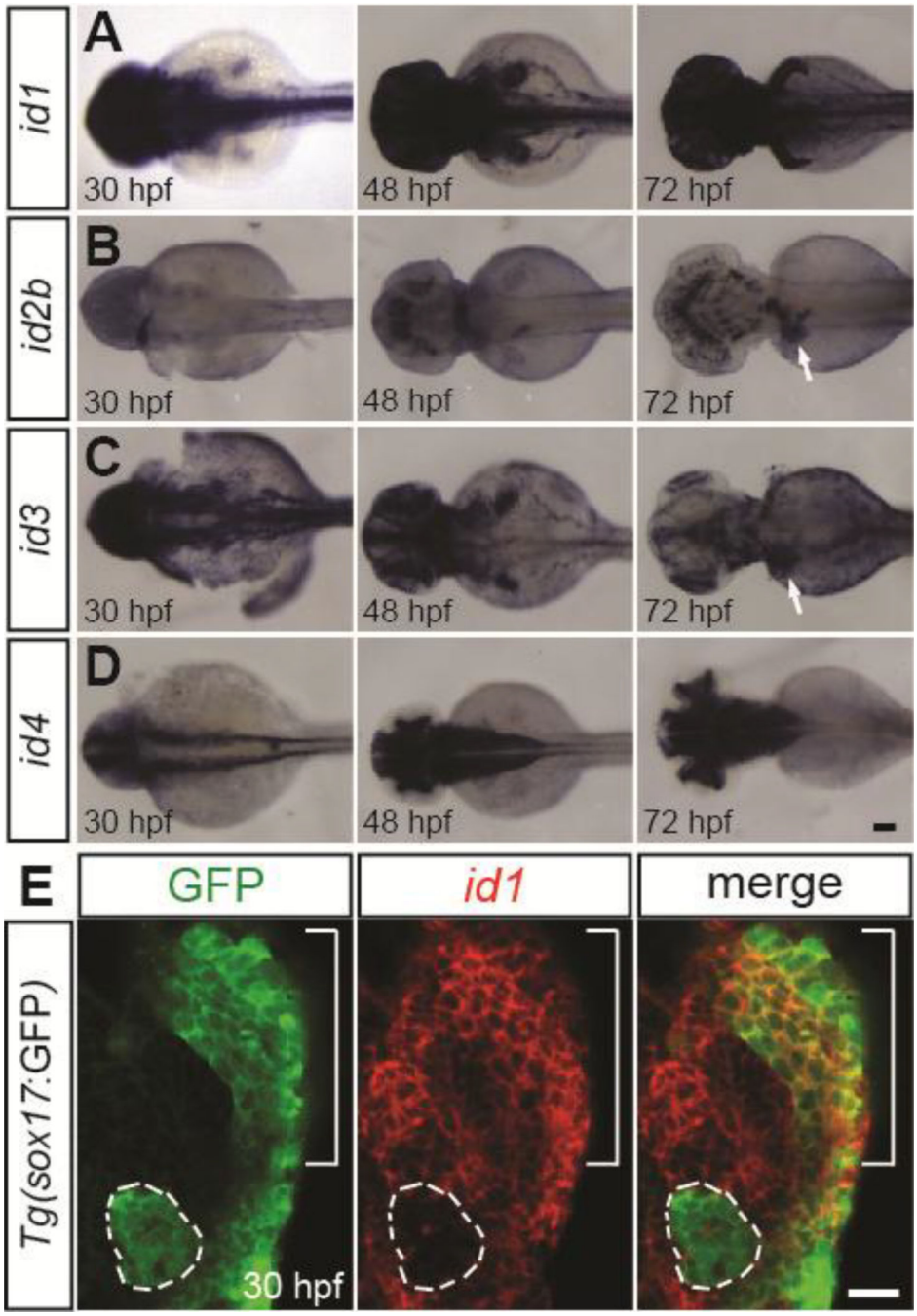

Figure 2. The expression patterns of *id1, id2b, id3,* and *id4* during liver development in zebrafish

(A–D) Wild-type embryos were processed for WISH analysis with *id1* (A), *id2b* (B), *id3* (C), and *id4* (D) probes. *id1* appears to be ubiquitously expressed at 30 hpf, but its expression is absent in the liver at 48 and 72 hpf. *id2b* and *id3* expression in the liver was detected at 72 hpf (arrows), but not at 30 or 48 hpf. *id4* is not expressed in the liver. Dorsal views, anterior to the left. **(E)** *id1 in situ* hybridization (red), combined with anti-GFP immunostaining (green), in *Tg(sox17:GFP)* embryos reveals *id1* expression in the liver-

forming region (brackets), but not in the dorsal pancreas (dotted lines) at 30 hpf. Single confocal optical section, ventral view, anterior up. Scale bars: 100 (A–D), 20 (E) μm.

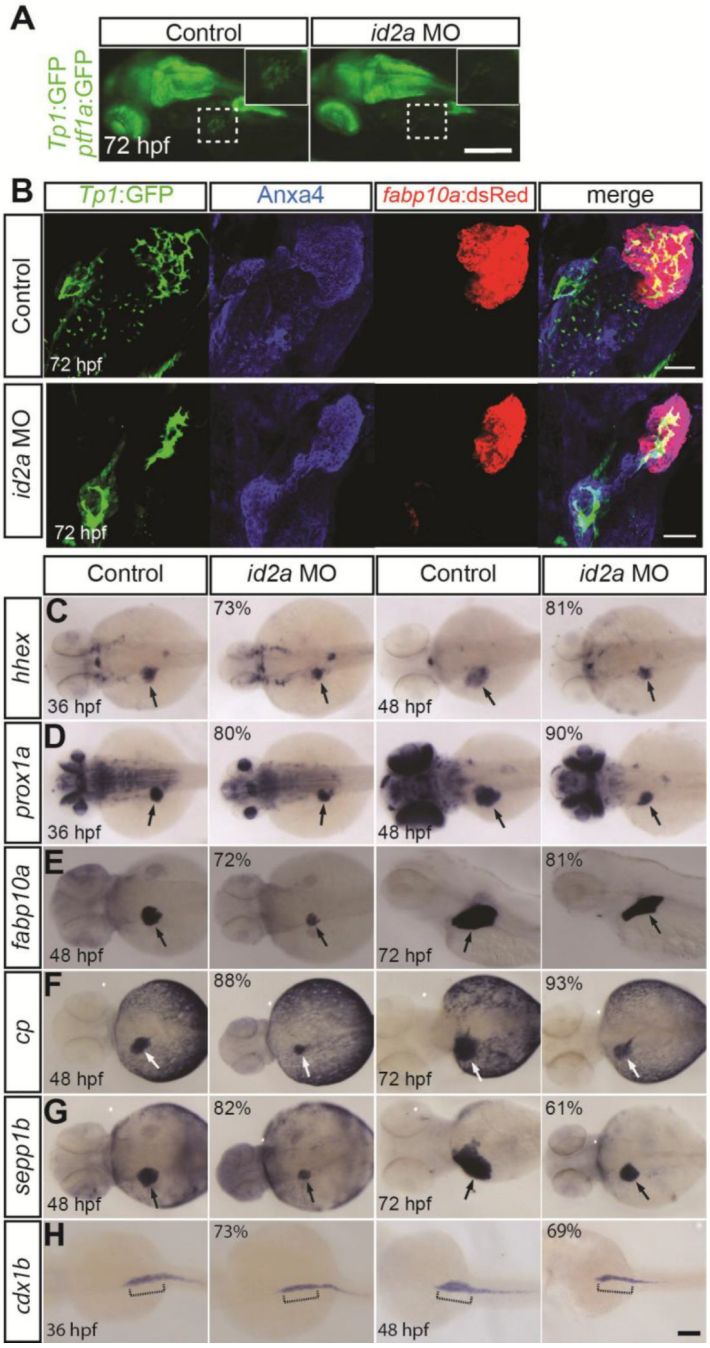

Figure 3. *id2a* knockdown reduces liver size but does not block hepatoblast specification or hepatocyte differentiation

(A) Epifluorescence images revealing a decreased number of *Tp1:*GFP^{+} BECs in the liver of *id2a* MO-injected embryos at 72 hpf compared with controls (squares). Higher magnification images of the square regions are shown in insets. Lateral view, anterior to the left. **(B)** Confocal projection images revealing *fabp10a:*dsRed (hepatocytes; red), *Tp1:*GFP (BECs; green) and Anxa4 (the hepatopancreatic ductal system; blue) expression (Zhang et al., 2014). In *id2a* MO-injected embryos, liver size was greatly reduced and intrahepatic

BECs appeared aggregated, displaying a branching defect. **(C–H)** *id2a* MO-injected and uninjected control embryos were processed for WISH with *hhex* (C), *prox1a* (D), *fabp10a* (E), *cp* (F), *sepp1b* (G), and *cdx1b* (H) probes. Overall liver size was greatly reduced in *id2a* MO-injected embryos as revealed by the hepatoblast markers (*hhex* and *prox1a*) and the hepatocyte markers (*fabp10a*, *cp*, and *sepp1b*). However, the expression of these genes was clearly detected in the MO-injected embryos (C–G, arrows), indicating normal hepatoblast specification and hepatocyte differentiation upon *id2a* knockdown. The induction of the intestinal bulb as assessed by *cdx1b* expression appeared normal in *id2a* MO-injected embryos at 36 hpf; however, the intestinal bulb failed to grow at 48 hpf (H, brackets). The percentage of *id2a* MO-injected embryos exhibiting the representative phenotype shown is indicated in the upper left corner (n=10–20). The remaining percentage of embryos exhibited an intermediate liver/intestinal bulb phenotype: their liver/intestinal bulb size was still smaller than that of the control embryos. Arrows point to the liver. Scale bar: 250 (A), 20 (B) and 100 (C–H) μm.

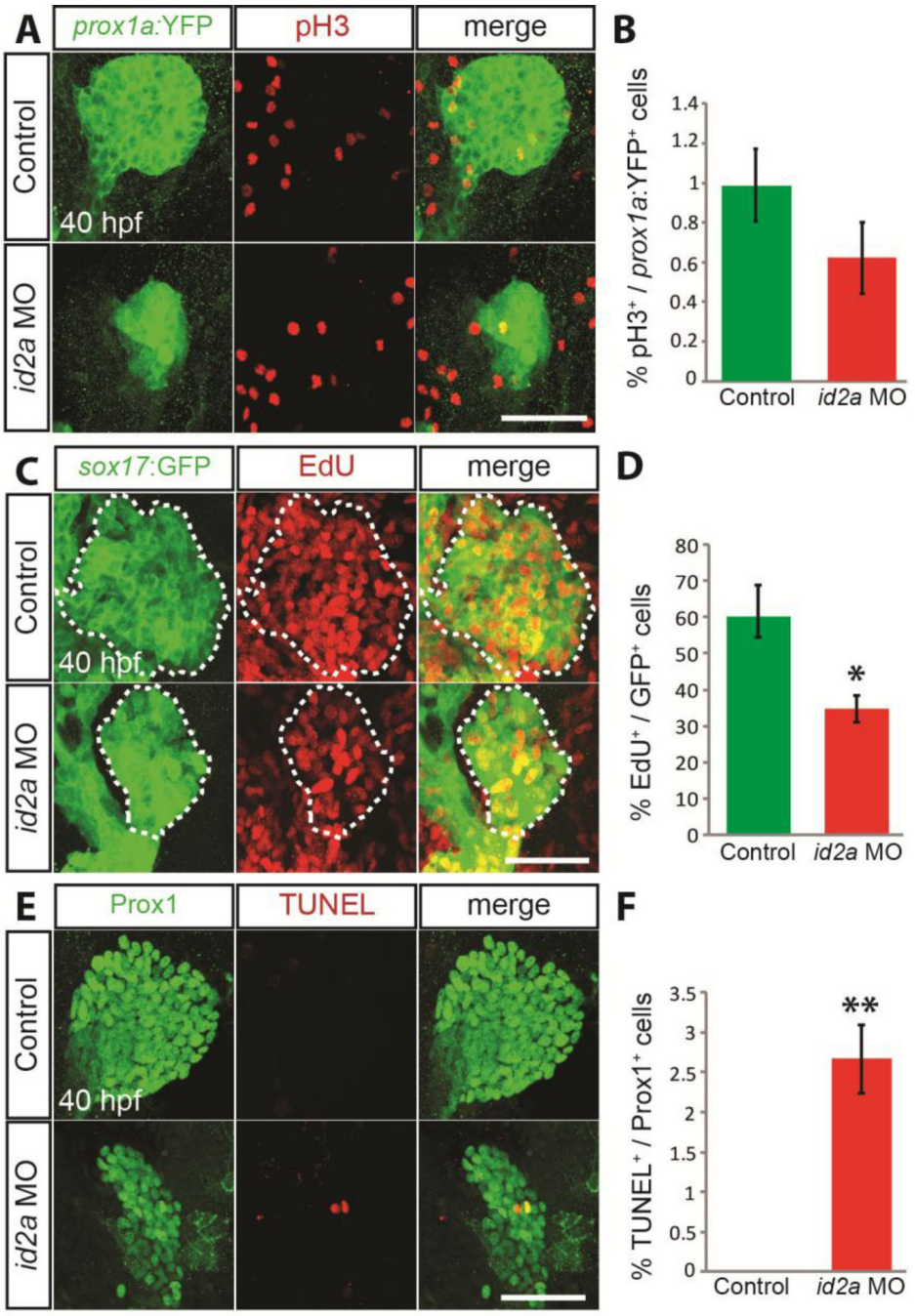

Figure 4. *id2a* knockdown decreases hepatoblast proliferation and increases cell death in the developing liver

(A) Whole-mount immunostaining with anti-pH3 (red) anti-GFP (green) antibodies in *Tg(prox1a:YFP)* embryos. The total number of *prox1a:*YFP$^+$ hepatic cells per liver is 316 ± 8.5 in controls and 156 ± 5.6 in *id2a* MO-injected embryos. **(B)** A graph showing the percentage of pH3$^+$ cells among *prox1a:*YFP$^+$ hepatic cells (n=10). **(C)** EdU labeling (red), combined with anti-GFP immunostaining (green), in *Tg(sox17:GFP)* embryos reveals a significant reduction of proliferation in the liver of *id2a* MO-injected embryos at 40 hpf

compared with controls. Dotted lines outline the liver. The total number of *sox17:*GFP$^+$ cells per liver is 220 ± 16.6 in controls and 127 ± 12.7 in *id2a* MO-injected embryos. **(D)** A graph showing the percentage of EdU$^+$ cells among GFP$^+$ hepatoblasts (n=10). **(E)** TUNEL labeling (red) combined with anti-Prox1 immunostaining (green) reveals apoptosis in the liver of *id2a* MO-injected embryos at 40 hpf, but not in controls. The total number of Prox1$^+$ cells per liver is 276 ± 20.5 in controls and 140 ± 8.6 in *id2a* MO-injected embryos. **(F)** A graph showing the percentage of TUNEL$^+$ cells among Prox1$^+$ hepatoblasts (n=10). *$p < 0.05$, **$p < 0.005$; error bars, ± s.e.m. Scale bars: 50 μm.