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Zfyve9a regulates the proliferation of hepatic cells during zebrafish embryogenesis

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ABSTRACT Zfyve9 is a FYVE domain protein first identified as a binding partner for SMAD2/3. *In vitro* studies indicate that it can function either positively or negatively in the TGF-β signaling pathway depending on the cell lines used. However, the *in vivo* function of this protein remains to be investigated. We first analyzed the tissue distribution of zebrafish *zfyve9a* by *in situ* hybridization. To investigate the *in vivo* function of this gene, we performed morpholino mediated loss-of-function assays. We analyzed the expression patterns of liver (*cp* and *fabp10a*), pancreas (*trypsin* and *insulin*) or gut (*fabp2*) specific markers to determine whether the formation of these organs is affected by *zfyve9a* knockdown. We determined the specification of hepatoblast in the *zfyve9a* morphants (*prox1a*) and investigated the proliferation and survival of hepatic cells in the morphants by P-H3 staining and TUNEL assay respectively. We report here that *zfyve9a* is enriched in the zebrafish embryonic liver and required for hepatogenesis. Morpholino mediated knockdown of *zfyve9a* inhibits the formation of liver by day 4 while the other endoderm-derived organs appear unaffected. We demonstrated that the specification of hepatoblasts is normal in the *zfyve9a* morphants; however, the proliferation rate of these cells is reduced. Thus, our results reveal the liver-specific function of *zfyve9a* during early embryogenesis and indicate that the *zfyve9a* mediated signal is essential for the proliferation of hepatic cells during the expansion of liver bud.

KEY WORDS: *zfyve9a, liver development, proliferation, zebrafish*

Introduction

The liver is a visceral organ in vertebrates that performs essential roles in metabolism, secretion, detoxification and homeostasis. Structurally, the major components of liver are hepatocytes and bile duct cells and both are derived from the endoderm. Formation of liver during embryogenesis starts with the specification of hepatoblasts from the foregut endoderm which forms the 'liver bud'. These bi-potent hepatoblasts then undergo further growth and differentiate into either the hepatocytes or the bile duct cells (Si-Tayeb *et al.*, 2010). Studies from various models have revealed that the Bmp and Fgf signaling pathways play vital roles during liver specification (Chen *et al.*, 2003, Shin *et al.*, 2007, Zhang *et al.*, 2004) as well as maturation (Berg *et al.*, 2007, Calmont *et al.*, 2006, Jung *et al.*, 1999, Rossi *et al.*, 2001, Sekhon *et al.*, 2004, Shin *et al.*, 2007, Yanai *et al.*, 2008). On the other hand, inhibition of Wnt signaling is required for liver specification(McLin *et al.*, 2007) while the same pathway plays positive roles in later stage of liver development(Micsenyi *et al.*, 2004, Monga *et al.*, 2003, Suksaweang *et al.*, 2004, Tan *et al.*, 2008). Other signaling molecules and transcriptional factors required for liver formation have been identified as well (Chu and Sadler, 2009, Sanchez and Fabregat, 2010, Si-Tayeb *et al.*, 2010, Tao and Peng, 2009).

Zfyve9 (Zinc finger FYVE domain-containing protein 9), also known as SARA (Smad anchor for receptor activation), is a

Abbreviations used in this paper: cp, ceruloplasmin; ERBIN, erbb2 interacting protein; gata6, GATA-binding protein 6; hpf, hours post fertilization; fabp2, fatty acid binding protein 2, intestinal; fabp10a, fatty acid binding protein 10a, liver basic; myca, myelocytomatosis oncogene a; prox1a, prospero-related homeobox gene 1a; P-H3, phospho-histone H3; SMAD2/3, SMAD family member 2/3; TGF-β, transforming growth factor-beta; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Zfyve9, zinc finger FYVE domain-containing protein 9; Zfyve9a, zinc finger, FYVE domain containing 9a.

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FYVE-type zinc finger containing protein. It was initially identified as a SMAD2/3-binding protein that recruits these SMADs to the TGF-β receptors and it is required for the SMAD2-dependent TGF-β signaling in a mink cell line (MvlLu) or human mesangial cells (Di Guglielmo *et al.*, 2003, Runyan *et al.*, 2005, Tsukazaki *et al.*, 1998). Recently, ERBIN was identified as a binding partner for both Zfyve9 and SMAD2/3. The binding of Zfyve9 to ERBIN releases the inhibitory effect of ERBIN on SMAD2/3 and induces the SMAD2/3-dependent signaling (Sflomos *et al.*, 2011). However, the exact role of Zfyve9 in TGF-β signaling appears to be context-dependent. For example, it is dispensable for TGF-β signaling in COS-7 cells, HeLa cells or B-cell lymphoma cell lines (Bakkebo *et al.*, 2012, Goto *et al.*, 2001). Furthermore, over-expression of Zfyve9 is able to reduce the TGF-β signaling in T cells (Kunzmann *et al.*, 2003). In cultured human kidney epithelial cells, Zfyve9 is required for the maintenance of epithelial cell phenotype through the down-regulation of SMAD2 and up-regulation of mesenchymal markers (Runyan *et al.*, 2009). In *Drosophila*, Zfyve9 negatively regulates Dpp signaling by recruiting the type 1 serine/threonine protein phosphatase to dephosphorylate the type I TGF-β receptor (Bennett and Alphey, 2002). In addition, the Zfyve9 endosomes are involved in the maintenance of Dpp signaling levels across mitosis in the developing wing epithelial cells (Bokel *et al.*, 2006). The *in vivo* function of Zfyve9 in vertebrates remains to be elucidated.

We identified zebrafish homologs of mammalian *zfyve9* gene and analyzed their embryonic expression patterns. We report here that the *zfyve9a* is a liver-enriched gene and it is required for the proliferation but not specification of hepatoblasts during embryonic liver development.

Results

The embryonic expression pattern of zfyve9a

We performed BLAST search and identified two zebrafish homologs of mammalian *zfyve9* gene: *zfyve9a* (Accession #: XM_001344468, Ensembl(Zv9): ENSDARG00000023701) and *zfyve9b* (Ensembl(Zv9): ENSDARG00000087295). We first determined the embryonic expression patterns of these genes by whole-mount *in situ* hybridization. We found that maternal mRNA of *zfyve9a* was clearly detectable at 2-cell stage (Fig. 1A). Unrestricted low level expression of *zfyve9a* was observed at gastrulation and somitogenesis stages (Fig. 1 B,C). The expression of *zfyve9a* then became more restricted and it was highly enriched in liver at day 2 and 3 (Fig. 1 G-J). It was also highly expressed in brain and neural tube at day 3 (Fig. 1 I-J). On the other hand, the staining of *zfyve9b* was very weak and unrestricted at early stages. Relatively high expression level of *zfyve9b* was detected at eyes at day 4 (Supp. Fig. S1). Based on these results, we focused on characterizing the *in vivo* function of *zfyve9a* in the development of endoderm-derived organs in this study.

Zfyve9a is required for embryonic liver development

The hepatic expression of *zfyve9a* implies that it could be involved in liver formation. We performed morpholino mediated loss-of-function study to test this possibility. We designed a translation blocking morpholino (MO-AUG) which was able to effectively block the translation of mRNA encoding a Zfyve9a-GFP fusion protein (Fig. 2B). We also synthesized a splicing blocking morpholino (MO-SP) targeting the intron2/exon2 junction of the

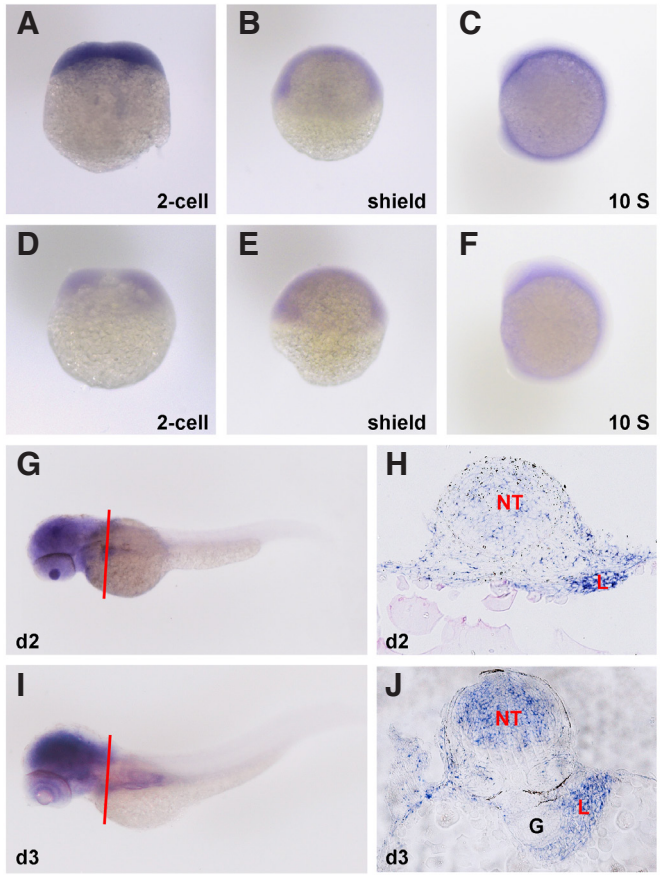

Fig. 1.The embryonic expression pattern of zebrafish *zfyve9a*. *Embryos were hybridized to an antisense probe to* zfyve9a *(A-C, G-J) or a sense control probe (D-F).* **(A)** *Maternal* zfyve9a *mRNA was detected at 2-cell stage.* **(B-C)** *Low level expression of* zfyve9a *at the shield and 10-somite stages.* **(G-I)** zfyve9a *was enriched in the liver at day 2 and 3. It was also highly expressed in the brain and neural tube at day 3.* **(H)** *is the cross-section of* **(G)** *at the position indicated by the red line.* **(J)** *is the cross-section of* **(I)** *at the indicated position. NT, neural tube; L, liver; G, gut.*

zfyve9a gene which effectively induced the alternative splicing of *zfyve9a* mRNA as determined by RT-PCR and DNA sequencing (Fig. 2C). We injected embryos with these morpholinos and found the morphants often had mild cardiac edema but otherwise appeared normal (Fig. 2D). We evaluated liver development in these morphants by *in situ* hybridization using the hepatocyte marker *fabp10a* as a probe. As shown in Fig. 2E, both morpholinos severely reduced the expression of *fabp10a* at day 3 (25/26 for MO-AUG and 30/32 for MO-SP). The liver defect in the morphants was not a result of general developmental delay since similar defect was obtained at day 4 (46/50 for MO-AUG and 40/43 for MO-SP, Fig. 2F). We further analyzed the expression pattern of *cp* (*ceruloplasmin*) which is another marker of hepatocytes and found that the level of *cp* was similarly down-regulated in the morphants (45/45 for MO-AUG and 42/43 for MO-SP, Fig. 2G). On the other hand, the development of other endoderm derived tissues such as the endocrine pancreatic β-cell (*insulin*) (16/16 for MO-AUG and 13/13 for MO-SP, Fig. 2H), the exocrine pancreas (*trypsin*) (23/25 for MO-AUG and 15/18 for MO-SP, Fig. 2I) or the intestine (*fabp2*) (24/24 for MO-AUG and 12/12 for MO-SP,

Fig. 2J) was not disrupted in the *zfyve9a* morphants. Together, these results suggested that *zfyve9a* is required for the liver but not pancreas or gut development in zebrafish.

Zfyve9a is dispensable for the specification of hepatoblasts

Liver development in zebrafish begins with the specification of hepatoblasts which form a liver bud at about 30 hours post fertilization (hpf), then these progenitor cells are expanded and differentiated into either hepatocytes or bile duct cells (Chu and Sadler, 2009, Tao and Peng, 2009). The liver defect observed in the *zfyve9a* morphants could be a result of the failure of either process. We first tested whether the specification of hepatoblasts was affected by the inhibition of *zfyve9a*. As shown in Fig. 3A, the expression of a pan-endodermal marker *foxa3* appeared normal in *zfyve9a* morphants at 30 hpf (9/9). *prox1a* is one of the earliest liver specific markers and we found that the hepatic expression of this gene was not disrupted in *zfyve9a* morphants at 30 hpf (19/19, Fig. 3B). We noticed that the brain was underdeveloped and the neuronal expression of *prox1a* was reduced at this stage in the morphants. These results suggest that the specification of hepatoblasts was normal in the morphants.

We further found that the *prox1* expressing area was slightly reduced in *zfvye9a* morphants at day 2 (9/9, Fig. 3C). Similar result was observed with *gata6*, which is another pan-endodermal marker (9/9, Fig. 3D). We examined the expression of additional hepatic markers (*myca* and *cp*) and both of them were expressed with slightly reduced level at day 2 (9/9 and 18/18 respectively, Fig. 3 E,F). Taken together, these results indicated that the specification of hepatoblasts was normal in the morphants; however, the expansion of liver bud could be affected by the inhibition of *zfyve9a*.

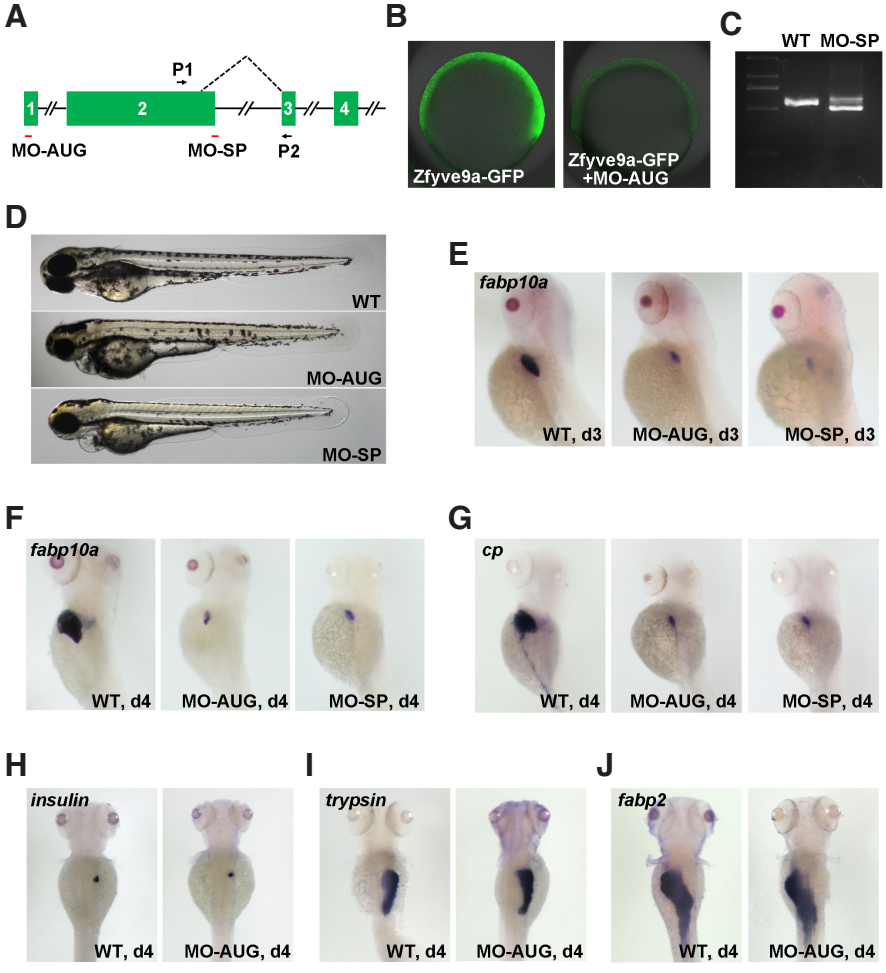

Fig. 2. Zfyve9a is required for liver development. (A) *A cartoon of the* zfyve9a *gene structure shown the positions of MO-AUG, MO-SP and primers used in RT-PCR reaction in (C).* **(B)** *MO-AUG (4 ng) effectively inhibited the translation of* Zfyve9a-GFP *mRNA (100 pg) as determined at about 70% epiboly stage.* **(C)** *RT-PCR analysis for the effect of MO-SP. PCR products from wild type and* zfyve9a *morphants were cloned and sequenced. The lower band in MO-SP treated sample corresponds to a deletion of the C-end of exon 2.* **(D)** *Representative live images of wild type embryo and* zfyve9a *morphants at day 3.* **(E-F)** *Expression of* fabp10a *in WT, MO-AUG or MO-SP injected embryos at day 3 (E) and day 4 (F).* **(G)** *Expression of another liver marker,* cp, *in WT embryos and* zfyve9a *morphants at day 4.* **(H-J)** *Knockdown of* zfyve9a *did not affect the development of pancreas or gut at day 4.* insulin *labels the pancreatic beta-cells,* trypsin *indicates the exocrine pancreas and* fabp2 *marks the intestine.*

Zfyve9a regulates the proliferation but not survival of hepatic cells

We then investigated whether the proliferation/survival of hepatic cells was affected upon the knockdown of *zfyve9a*. The gutGFP^{s854} is a transgenic zebrafish line where the whole endoderm cells are labeled by GFP (Field *et al.*, 2003). The liver cells are clearly distinguishable from other endoderm tissues due to its distinct locations after day 2. We counted the number of GFP-positive hepatic cells and found that it was reduced from 187±22 in the control morpholino (MO-CTL) treated embryos (N=19) to 134±26 in the MO-AUG injected embryos (N=17) at day 2, which was consistent with our previous *in situ* hybridization results. We investigated whether the proliferation of hepatic cells was reduced in the morphants by performing whole mount anti-phosphorylated histone 3 (P-H3) staining. As shown in Fig. 4 A,B, the percentage of P-H3 positive hepatic cells was 7.16% in the MO-CTL treated embryos at 48 hpf (N=3546). However, this rate was reduced to 2.85% in *zfyve9a* morphants (N=2144, P=2.029E-06). Similar results were observed at 60 hpf (reduced from 6.86% (N=3355) to 3.90% (N=2156), P=2.7329E-07). We also performed the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to determine whether the survival of hepatic cells was affected by *zfyve9a* knockdown. As shown in Fig. 4 C,D, the ratio of apoptosis in hepatic cells were comparable between the control embryos and *zfyve9a* morphants at all stages examined (P>0.25 in all cases). These results revealed that the activity of Zfyve9a is required for the proliferation but not survival of hepatic cells.

Discussion

Zfyve9 is a SMAD2/3 binding protein which has been implicated in the TGF-β signaling. Previous

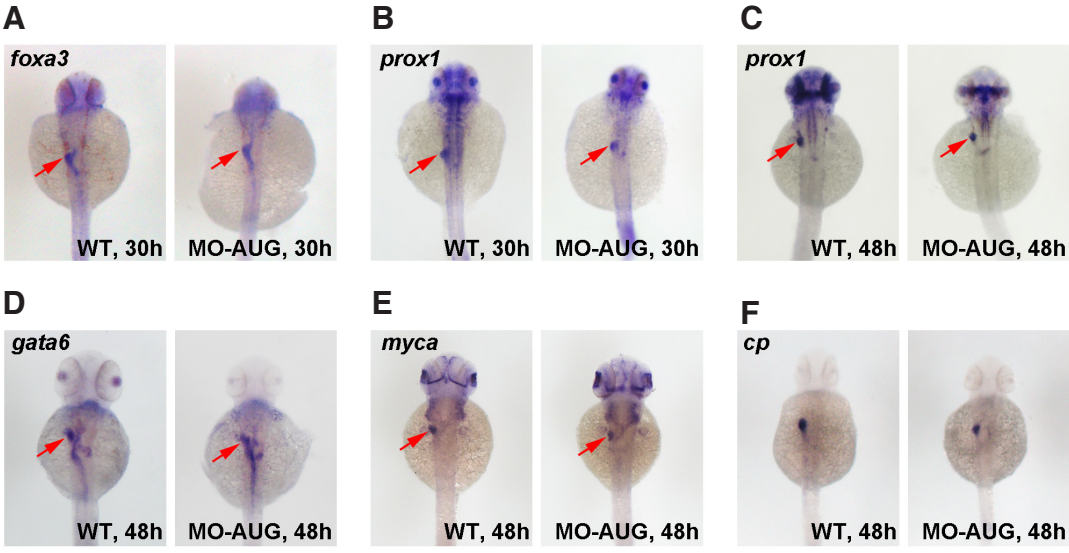

Fig. 3. The specification of hepatoblasts is normal in *zfyve9a* morphants. (A) *The expression of pan-endoderm marker* foxa3 *in WT embryos and* zfyve9a *morphants at 30 hpf.* **(B,C)** *The expression of* prox1a *in liver bud was clearly detected in* zfyve9a *morphants at 30 and 48 hpf. The neuronal expression of* prox1a *was severely reduced in morphants at these stages.* **(D)** *Expression of* gata6 *at day 2 in the endoderm of WT embryos and* zfyve9a *morphants.* **(E,F)** *Hepatic expression of* myca *and* cp *were initiated normally in* zfyve9a *morphants at day 2. Arrows in all panels indicate the liver bud.*

studies indicate that it functions either as a positive or a negative regulator of the TGF-β pathway *in vitro* depending on the cell lines used. During the *Drosophila* wing disc development, it is able to regulate the level of Dpp signaling. However, the *in vivo* function of this gene in vertebrates remains to be characterized. We identified two homologs of mammalian *zfyve9* gene from the zebrafish genome and found that one of them (*zfyve9a*) is enriched in the embryonic liver and neuronal tissues. Loss-of-function analysis revealed that the *zfyve9a* gene is required for the formation of liver but not other endoderm-derived organs such as pancreas and gut. We further found that knockdown of *zfyve9a* does not affect the specification of hepatoblasts but it is required for the proliferation of these cells during subsequent liver development. A recent study reported that down-regulation of *zfyve9* decreases the level of SMAD2 as well as the SMAD2 mediated signaling in a renal epithelial cell line (Runyan *et al.*, 2009). It is interesting to note that haploinsufficiency of both *smad2* and *smad3* in the *smad2+/-*; *smad3+/-* mice reduces the proliferation of hepatocytes and results in liver defect similar to that in the *zfyve9a* morphants (Weinstein *et al.*, 2001). It is possible that a defect in the SMAD2/3 signaling is responsible for the liver defect in *zfyve9a* morphants. In fact, we found that knockdown of *zfyve9a* in zebrafish embryos reduced the level of phosphorylated-SMAD2 (P-SMAD2, Supp. Fig. S2 A). Furthermore, direct inhibition of the TGF-β receptor by small chemical inhibitor Repsox reduced the growth of liver bud (Supp. Fig. S2 B,C). These observations suggest that the Zfyve9a might regulate liver development through the TGF-β/SMAD pathway. However, the TGF-β/SMAD pathway is essential for early developmental events such as the gastrulation. Injection of an active form of SMAD2 at one-cell stage disrupts the proper gastrulation of the embryo. We were not able to specifically activate this pathway during the expansion of liver bud to rescue the MO-AUG induced liver defects. It remains a challenging task to dissect the late-stage and tissue-specific functions of the TGF-β/SMAD signaling during embryogenesis. Further investigations are required to elucidate whether or not *zfyve9a* can function as a tissue-specific regulator of the TGF-β/SMAD pathway during liver development.

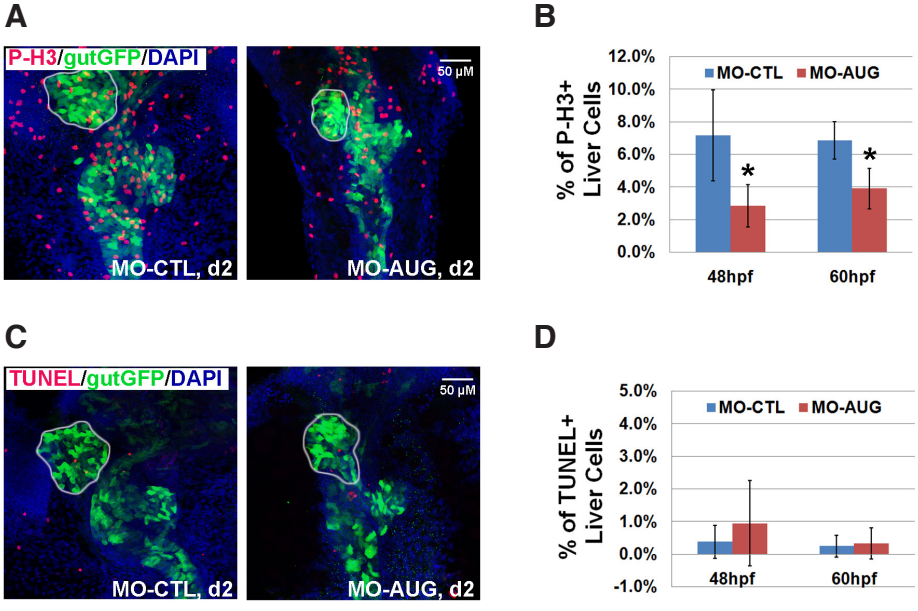

Fig. 4. Zfyve9a regulates the proliferation but not survival of hepatic cells. (A) *Representative images of P-H3 staining of embryos at day 2. Green indicates the GFP-labeled endoderm cells. The outlined area is the liver.* **(B)** *Statistical results of the percentage of P-H3 positive hepatic cells in MO-CTL or MO-AUG injected embryos at 48 and 60 hpf. Stars indicates P<0.05 as analyzed by the t-test method.* **(C)** *Representative images of TUNEL assay at day 2.* **(D)** *The percentage of apoptotic hepatic cells in MO-CTL or MO-AUG treated embryos at 48 and 60 hpf. Error bars in (B) and (D) present the SD.*

Materials and Methods

Zebrafish manipulation

Zebrafish adults were bred and embryos were staged using standard protocols as previously described (Westerfield, 1995). Tü and gutGFP^{s854} lines were used in this study. Zebrafish experiments were approved by the GIBH Institutional Animal Care and Use Committee. Morpholino antisense oligonucleotides to *zfyve9a* were purchased from Gene-Tools (Corvallis, OR): MO-AUG (CCTCAGCCTGGAAGTAATTCTCCAT, 4 ng/embryo), MO-SP (AATGAACTAGAGACTTTACCTTGCC, 10-12 ng/embryo). The standard control morpholino (CCTCTTACCTCAGTTACAATTTATA, 4 ng/embryo) was used as the injection control. Morpholinos were injected at 1-cell stage. For testing the efficiency of MO-AUG, embryos were first injected with the mRNA encoding the Zfyve9a-GFP (100 pg) then injected again with the MO-AUG (4 ng/embryo). The expression level of Zfyve9a-GFP was evaluated at the 60-70% epiboly stage. For validation of the splice-blocking morpholino, the WT or the MO-SP injected embryos were dechorionated at 36 hpf and total RNAs were extracted using the RNAqueous®-4PCR Kit (Ambion, CA). Reverse-transcription was performed using the ReverTra Ace (TOYOBO, Japan). A fragment of *zfyve9a* was amplified by PCR using the following primers: TGACAGAGGAAAAAGAAATAGAGG (P1) and ACACAAACCCTCGCTTCTTT (P2) and the sequences of PCR products determined by DNA sequencing.

Molecular cloning

Molecular cloning was performed according to standard protocols. The full length zebrafish *zfyve9a* gene was cloned by RT-PCR method with the following primers: ATGGAGAATTACTTCCAGGCTGA and TTAAGAGATGATCTCCAGAATGTAGAA. The PCR product was cloned into the pCS2+ (for making mRNA) or the pGM-TEASY (for making probe) vectors. A chimera containing the 1-393 bp of *zfyve9a* fused to GFP was constructed in the pCS2+ vector (for testing the efficiency of MO-AUG). Partial zebrafish *zfyve9b* gene was cloned by RT-PCR method with the following primers: GAAGGTTTGGTTTGCAGATAATGTC and CCAAAGAGTGACTGAGGTGATTCAG. The PCR product was cloned into the pGM-TEASY (for making probe) vectors. All constructs were confirmed by DNA sequencing. Detailed information about these constructs is available upon request. mRNAs were synthesized using the mMESSAGE mMACHINE Kit (Ambion, CA).

Whole mount in situ hybridization

Embryos were fixed at the indicated stages and whole-mount *in situ* hybridization was performed as described (Westerfield, 1995). The sense and anti-sense probes were prepared from the *zfyve9a* and *zfyve9b* genes cloned in the pGM-TEASY vector by *in vitro* transcription as described (Westerfield, 1995). Other probes used in this study included *fabp10a, cp, myca, gata6, prox1a, fabp2, trypsin* and *insulin*.

Proliferation assays

Whole-mount fluorescence immunostaining was performed as described (Xu *et al*., 2012). Briefly, embryos were fixed with 3% formaldehyde in 0.1 M Pipes/1.0 mM $MgSO_4$/2 mM EGTA overnight at 4°C. The yolk was manually removed. Embryos were incubated in acetone for 7 min at -20°C, and then washed with PBST (1X PBS with 0.1% Tween-20) twice. Embryos were blocked in PBS/4% BSA/0.3% Triton X-100 for 1 h at RT and incubated with a primary antibody (Phospho-Histone H3 (Ser10) (6G3) Mouse mAb (Cell Signaling, 1:100 dilution) or anti-GFP antibody (ab290) (Abcam, 1:1000 dilution)) at 4°C overnight. After several washes with PBT (0.1% triton X-100 in PBS), embryos were incubated with a secondary antibody (Alexa Fluor®488 conjugated donkey anti-rabbit IgG or Alexa Fluor®568 conjugated donkey anti-mouse IgG, Molecular Probes, 1:200) for 2 h at RT. Finally, embryos were washed and counterstained with DAPI (1 μg/ml in PBS, 5 min) and images were obtained with the Zeiss LSM 710 NLO confocal microscope.

TUNEL assays

TUNEL assay was performed as described (Curado *et al*., 2007). Briefly, embryos were fixed as described above. Embryos then embedded in 4% low melting agarose/1% gelatin and sectioned with the Leica VT1000S vibratome into 100 μm thick slices. Sections were first incubated with the anti-GFP antibody (ab290) (Abcam, 1:1000 dilution), then stained with the *In situ* Cell Death Detection Kit (TMR red, Roche Diagnostics) according to the manufacturer's protocol. After several washes with PBT (0.1% triton X-100 in PBS), embryos were incubated with a secondary antibody (Alexa Fluor®488 conjugated donkey anti-rabbit IgG, Molecular Probes, 1:200) for 2 h at RT. Finally, embryos were washed and counterstained with DAPI (1 μg/ml in PBS, 5 min) and images were obtained as described above.

Acknowledgements

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