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Yap1/Taz are essential for the liver development in zebrafish

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ABSTRACT

Hippo pathway regulates cell proliferation and differentiation. Yes-associated protein (Yap) and transcriptional coactivator with PDZ-binding motif (Taz) are effectors of Hippo pathway. The function of Yap/Taz in embryonic liver development has yet to be reported. Here *yap1* and *taz* were found expressed in liver and other digestive organs in zebrafish embryos, and knockout of *yap1* or *taz* did not lead to visible defects during embryogenesis. Interestingly, Taz was significantly increased in *yap1* mutants, which may account for their normal development, albeit losing Yap1. However, $yap1^{-/-}$; $taz^{+/-}$ embryos exhibited smaller digestive organs, and more than half of them showed bilateral livers and pancreas and non-looped intestines. Further analysis revealed that the disrupted gene function in $yap1^{-/-}$; $taz^{+/-}$ embryos did not disturb liver bud formation, but instead impaired cell proliferation in liver and movement of the neighboring lateral plate mesoderm (LPM). Overexpression of wild type *yap1* or *taz* could rescue the defective liver phenotypes in $yap1^{-/-}$; $taz^{+/-}$ embryos, indicating that Yap1 cooperate with Taz to regulate the liver development. In addition, Yap1 was found to function in a Tead-dependent manner in the liver development. Our results suggest that Yap1/Taz regulate LPM movement and promote cell proliferation to ensure proper liver development in zebrafish.

1. Introduction

Liver is derived from the endoderm, where the developmental process and molecular mechanism are conserved among vertebrates [1,2]. In zebrafish, liver development includes two stages: budding and growth. At 24 h post fertilization (hpf), endodermal cells aggregate and form a rod underneath the middle line, and a subset of cells undergo specification into hepatoblasts. Liver primordium buds from the endodermal rod at 28 hpf and develops into a distinct organ by 34 hpf. The liver budding process is accompanied by a leftward looping of the intestinal rod, termed "gut looping", to achieve a final location at the left side of the body [3]. Gut looping is controlled by Nodal signaling, and driven by the asymmetric migration of the left and right lateral plate mesoderm (LPM), which is neighbor to the intestinal rod [4]. Starting from 50 hpf, the liver bud increases its size quickly, and hepatoblasts differentiate into functional hepatocytes and cholangiocyte [3].

The framework of molecular mechanism regulating hepatogenesis has been drawn, which mainly includes two parts: mesodermal induction and action of tissue specific factors. Mesodermal signaling factors, including FGFs, BMPs and WNTs, are required for hepatic specification and proliferation in both spatial and temporal manners [5–9]. Pan-endodermal transcription factors Foxas and Gatas are necessary to enable the presumptive hepatic endoderm to respond to mesodermal signals [10,11], the liver specific factors Hhex, Prox1 and Hnf4α are responsible for cell proliferation, differentiation, migration and organ morphogenesis [12–14]. Although the detailed molecular mechanisms have been reviewed by Gordillo et al. and Tao et al. [1,2], many fields remain to be explored.

Hippo signaling pathway plays a diversity of roles in growth, tumorigenesis, regeneration and development. It mainly consists of a kinase cascade and core effectors YAP and TAZ. Upon the activation of Hippo pathway, large tumor suppressor 1/2 (LATS1/2) phosphorylate YAP/TAZ and lead to cytoplasmic retention and degradation. When the pathway is switched off, YAP/TAZ can translocate into nucleus and bind to transcription factors, such as TEADs, to activate the transcription of downstream genes [15]. The most well studied function of Yap1/Taz is to promote cell proliferation and inhibit cell death [16], and they have been demonstrated to play important roles in embryonic development and

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organogenesis. While the deletion of *Yap* in mice results in embryonic lethality at embryonic day 8.5 (E8.5) [17], some *Taz* knockout mice can be given birth with smaller body size and develop polycystic kidney [18,19]. Using conditional knockout mice, tremendous work have shown the essential roles of Hippo pathway in various organs in size control, cell differentiation and organ regeneration [15]. Liver specific inactivation at perinatal stage in mice results in increased hepatocyte turnover and disrupted biliary system [20], and over-activated Yap in the liver leads to tissue overgrowth (hepatomegaly) and eventually hepatocellular carcinoma [16,21]. In zebrafish, knockout of *yap1* leads to the partial loss of the retinal pigment epithelium (RPE), and the phenotype is more severe in $yap1^{-/-}$; $taz^{+/-}$ embryos [22]. Double mutant of *yap1* and *taz* displays a defective posterior body during somitegenesis due to mis-assembly of Fibronectin [23]. Interestingly, the medaka *hirame*/*yap1* mutant shows a collapsed and flattened body shape, mirroring an exacerbated phenotype of zebrafish *yap1* and *taz* double mutant [24]. Until now, there is no report about the roles in liver development at embryonic stage.

Here, we report that *yap1* and *taz* are expressed in the digestive organs in zebrafish embryos, and knockout of *yap1* or *taz* does not affect embryogenesis. However, $yap1^{-/-}$; $taz^{+/-}$ embryos are defective in digestive organs development, with smaller and bilateral livers and pancreas and non-looped intestines. Further investigation reveals that the disruption of genes function in $yap1^{-/-}$; $taz^{+/-}$ embryos leads to impaired proliferation of hepatocyte and movement of LPM which may account for the phenotypes, and overexpression of *taz* can rescue the liver phenotypes in $yap1^{-/-}$; $taz^{+/-}$ embryos as *yap1* does. Our data reveal a redundant function of Taz and Yap1 in liver development in zebrafish.

2. Materials and methods

2.1. Fish lines and maintenance

The zebrafish (*Danio rerio*) AB strain was used as wild type. The *yap1* mutant was generated by TALEN technology [25], and left and right arms were designed to target the sequence (5′-CCGAACCAGCACAAC-3′) (5′-CCCCGAACATGGACGAT-3′) in the exon 1 of *yap1* gene. The *taz* mutant generated by CRISPR/Cas9 technology was described in our submitted manuscript to other journal. Fish was raised and maintained in the fish facility in Southwest University according to standard procedures.

2.2. Zebrafish Yap1 rabbit polyclonal antibody generation and purification

Zebrafish *yap1* cDNA full-length sequence was amplified using primers *yap1*-fw (5′-CCGCTCGAGACATGGATCCGAACCAGCACAACCCT-3′) and *yap1*-rv (5′-CCGCTCGAGTAGCCAGGTTAGAAAGTTCTCCTT-3′), and cloned into pET-28a (+) (Clontech) at XhoI sites. Yap1 protein was expressed in *E. coli* BL21, purified and used as antigen for rabbit immunization. After one primary and three booster injections, serum were collected and antibody was affinity purified using Yap1 protein.

2.3. Western blot

Western blot was performed as described previously using the following antibodies: Taz (Cell Signaling Technology), Gapdh (California Bioscience) and Yap1 (generated by ourselves) [26].

2.4. Quantitative PCR

Total RNAs were extracted from 36 hpf embryos using Tripure reagent (Roche), and cDNAs were synthesized by SuperScript™ II (Invitrogen). QPCRs were performed for three biological replicates using SYBR Green Master (Roche). Primers used were listed as following: *lats2*-fw: 5′-TCCGATGGACTCACAACTCA-3′, *lats2*-rv: 5′-AGCATCTCAAACAGGATCACT-3’. *nf2a*-fw: 5′-TTGTCTGCCAACTCTCTTCCT-3′, *nf2a*-rv: 5′-TAGTCCAGTGTGTCTGAAGGGT-3’. *nf2b*-fw: 5′-ACATATGCCTGGCTGAAGCCT-3′, *nf2b*-rv: 5′-GGCAAGAGTTCATCTTGAGCT-3’. *ef1a*-fw: 5′-CTTCGCCCCTGCCAATGTAAC-3′, *ef1a*-rv: 5′-GCAGGCGATGTGAGCAGTGTG-3’. *elongation factor 1a* (*ef1a*) was used for normalization.

2.5. Immunofluorescence staining and TUNEL assay

Embryos were cryo-processed into 16 μm sections for immunofluorescence staining of PH3 (Santa Cruz), aPKC (Santa Cruz) and β-catenin (Upstate), Detection was achieved by fluorescence labeled secondary antibody (Invitrogen). The same processed samples were used for TUNEL assay with the In Situ Cell Death Detection Kit TMR red (Roche) as previously described [26]. Whole mount embryos at 7 somite stage were used for anti-acetyl-tubulin (Sigma) immuno-staining to label cilia in the Kupffer's vesicle (KV). Cell nuclei were stained by DAPI (Roche). Samples were visualized under a confocal microscope (Zeiss LSM700).

2.6. Whole mount in situ hybridization (WISH)

WISH was carried out as described previously using the following anti-sense RNA probes: *yap1*, *taz*, *foxa1*, *prox1*, *lfabp*, *ifabp*, *trypsin* and *spaw* [8].

2.7. Generation of plasmids and mRNA rescue experiments

Full-length *yap1* and *taz* cDNA were amplified and cloned into pCS2+ vector. The mutant constructs $yap1^{S54A}$ and $yap1^{\Delta 11}$ were generated using PfuTurbo® DNA Polymerase (Stratagene) site-directed mutagenesis. The mRNAs were synthesized using the mMESSAGE mMACHINE kit (Ambion) and injected into one-cell stage embryos obtained from the cross between $yap1^{-/-}$; $taz^{+/-}$ and $yap1^{-/-}$. The genotypes of embryos were determined after imaging.

3. Results

3.1. yap1 and taz transcripts were enriched in digestive organs in zebrafish embryos

To investigate the possible roles of Yap1 and Taz in the liver development in zebrafish, the expression pattern of *yap1* and *taz* were examined during embryogenesis. WISH results showed that *yap1* was maternally deposited at one cell stage (data not shown), ubiquitously expressed at 25 hpf, and enriched in the head, branch arches and digestive organs, which include liver, exocrine pancreas and intestine at 72 hpf (Fig. 1A, C). Similar to *yap1*, its paralogous gene *taz* was also expressed in the digestive organs (Fig. 1B, D).

The enriched expressions of *yap1*/*taz* suggested possible roles in digestive organs development. To test this speculation, *yap1* and *taz* zebrafish mutants were generated by TALEN and CRISPR/Cas9 technology, respectively. An 11-nucleotide deletion was targeted at exon 1 of *yap1*, which resulted in open reading frame shift and an early stop codon (Fig. 1E), which might result in the loss of function of the gene.

3.2. $yap1^{-/-}$; $taz^{+/-}$ embryos displayed developmental defects

To assess the effectiveness of gene knockouts at protein levels,

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