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Ribosome Biogenesis Factor Bms1-like Is Essential for Liver Development in Zebrafish

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ABSTRACT

Ribosome biogenesis in the nucleolus requires numerous nucleolar proteins and small non-coding RNAs. Among them is ribosome biogenesis factor Bms1, which is highly conserved from yeast to human. In yeast, Bms1 initiates ribosome biogenesis through recruiting Rcl1 to pre-ribosomes. However, little is known about the biological function of Bms1 in vertebrates. Here we report that Bms1 plays an essential role in zebrafish liver development. We identified a zebrafish *bms1l*^{sq163} mutant which carries a T to A mutation in the gene *bms1-like* (*bms1l*). This mutation results in L^{152} to Q^{152} substitution in a GTPase motif in Bms1l. Surprisingly, *bms1l*^{sq163} mutation confers hypoplasia specifically in the liver, exocrine pancreas and intestine after 3 days post-fertilization (dpf). Consistent with the *bms1l*^{sq163} mutant phenotypes, whole-mount *in situ* hybridization (WISH) on wild type embryos showed that *bms1l* transcripts are abundant in the entire digestive tract and its accessory organs. Immunostaining for phospho-Histone 3 (P-H3) and TUNEL assay revealed that impairment of hepatoblast proliferation rather than cell apoptosis is one of the consequences of *bms1l*^{sq163} giving rise to an underdeveloped liver. Therefore, our findings demonstrate that Bms1l is necessary for zebrafish liver development.

KEYWORDS: Liver development; Digestive organ development; Ribosome biogenesis; Bms1-like; Zebrafish

1. INTRODUCTION

The liver is an essential organ that carries out many important functions. Most studies in liver development are carried out in mice and chick using reverse genetics and/or explants culture method (Zaret, 2002; Duncan, 2003). However, there are still a lot of missing gaps in the whole picture of liver organogenesis due to limitations of such approaches and early lethality of liver defects. Zebrafish, a recent model for vertebrate development, is particularly suitable for studying liver organogenesis through forward genetics (Tao and Peng, 2009). In zebrafish, liver organogenesis begins with the establishment of a population of cells gaining hepatic competency within the ventral foregut endoderm, instructed by Foxa and Gata factors; thereafter, mesodermal signals, including Fgfs, Bmps, Wnt2b and retinoic acid (RA), induce the specification of hepatoblasts; hepatoblasts then migrate and proliferate to form a discrete liver bud and finally hepatoblasts in the liver bud undergo rapid proliferation and differentiation, giving rise to bile duct cells and functional hepatocytes (Allende et al., 1996; Stafford and Prince, 2002; Zaret, 2002; Duncan, 2003; Field et al., 2003; Mayer and Fishman, 2003; Chen et al., 2005; Holtzinger and Evans, 2005; Ober et al., 2006; Sadler et al., 2007; Shin et al., 2007).

Nucleolus, a sub-cellular organelle in the nucleus, is best known as the location for the biosynthesis and processing of

Abbreviations: BSA, bulked-segregant analysis; dpf, days post-fertilization; *fabp10*, *liver fatty acid binding protein*; *fabp2*, *intestinal fatty acid binding protein*; hpf, hours post-fertilization; SSLP, simple sequence length polymorphism; WISH, whole-mount *in situ* hybridization.

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rRNA precursors and the assembly of two subunits of the ribosome. The small subunit (SSU) processosome is a complex responsible for the assembly of the 40S ribosome small subunit (Fromont-Racine et al., 2003). Bms1 and Rcl1 are two components of the SSU processosome. Bms1 is a G-domain-containing protein characterized first in yeast (Wegierski et al., 2001). By exerting GTPase activity on the conversion of recruited GTP into GDP, the N-terminal domain has been shown to promote 35S pre-rRNA processing at sites A_0, A_1 and A_2 during the 40S ribosomal subunit biogenesis events (Karbstein et al., 2005; Karbstein and Doudna, 2006). It is believed that Rcl1, a putative endonuclease, activates Bms1 by acting as a guanine-nucleotide exchange factor (GEF) to promote GDP/GTP exchange, and that activated (GTP-bound) Bms1 delivers Rcl1 to the pre-ribosomes. Apart from enzymatic functions, the GTPase domain has also been reported to recruit and to enhance binding affinity of interacting partners like Rcl1 *via* conformational changes. The C-terminal domain, on the other hand, contains a GTPase-activating protein (GAP) motif that functions intra-molecularly (Karbstein et al., 2005; Karbstein and Doudna, 2006). Nevertheless, studies on Bms1 thus far are limited to the role of this protein in ribosome biogenesis, with focus on the GTPase domain, using yeast as the model system.

Here we report the characterization of a small liver mutant, *bms1l*^{sq163} obtained from our genetic screening in zebrafish and the subsequent cloning of the mutant gene through positional cloning. The mutation was identified to be a T to A conversion in the gene *bms1l* encoding ribosomal biogenesis protein Bms1l, which results in L^{152} to Q^{152} substitution in the GTPase motif. Genetic evidences from co-segregation analysis, morpholino knockdown and mRNA rescue experiment unequivocally demonstrated that the *bms1l*^{sq163} mutation is responsible for the small liver phenotype we observed. Our study on *bms1l*^{sq163} provides the first genetic evidence demonstrating that Bms1l possibly plays a specific function in vertebrate liver development. Since Bms1l is a key component in the 40S ribosomal biogenesis pathway that recruits many other ribosomal proteins onto the pre-ribosome−rRNA complex, our work provides the first evidence for the involvement of a seemingly housekeeping gene in a specific developmental process such as liver formation. This finding is instrumental in filling up some of the current gaps in our understanding of liver organogenesis.

2. MATERIALS AND METHODS

2.1. Generation of the *bms1l*^{sq163} mutant and genetic mapping of the mutant gene

Zebrafish were raised and maintained according to standard procedures. Ethylnitrosourea (ENU, 3 mmol/L) was used as the mutagen to mutagenize male fish (AB wild type strain). The mutagenized progenies were screened for small or no liver mutants using *prox1* as a probe in a high throughput whole-mount *in situ* hybridization (WISH) approach (Huang et al., 2008). The *bms1l*^{sq163} mutant was identified as a small liver mutant from a total of 524 F_2 ENU-mutagenized families. Map-based cloning method was used to clone the *bms1l*^{sq163} mutant gene. In order to create mapping families, two heterozygous pairs (163-5 and 163-10) were used to cross with two wild type WIK pairs (WIK-A2 and WIK-C5). The obtained homozygous mutant embryos were subjected to bulked-segregant analysis (BSA) (Shimoda et al., 1999) using 226 simple sequence length polymorphism (SSLP) markers generated by the Fishman (Shimoda et al., 1999) and Zon (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/Maps.htm) groups.

2.2. WISH

For WISH probe labeling, plasmids harboring *prox1*, *fabp10* (*liver fatty acid binding protein 10*), *trypsin*, *insulin*, *fabp2 (intestinal fatty acid binding protein 2)*, *hhex*, *foxA1*, *foxA3*, *gata4* and *gata6* sequences (Chen et al., 2005; Huang et al., 2008) were used to synthesize their corresponding mRNAs *via in vitro* transcription using appropriate RNA polymerases. All probes were labeled with digoxigenin (DIG, Roche Diagnostics, USA). WISH was performed as described previously (Chen et al., 2005).

2.3. Mutant phenotype rescue

Full length *bms1l* cDNA was obtained *via* RT-PCR (forward primer: 5′-ctcgagtctagaacagtggagagagtcacgttg-3′; reverse primer: 5′-ctcgagtacgtatctcgtgcagcgtcatcat-3′) and cloned into the pCS2+ vector. The plasmid harboring *bms1l* full length cDNA was used to synthesize *bsm1l* mRNA. For mutant phenotype rescue, 1 ng of *in vitro* transcribed *bms1l* mRNA was injected into fertilized eggs at the one-cell stage. Injected embryos at 3 days post-fertilization (dpf) were analyzed by WISH using the liver specific probe *fabp10*.

2.4. Morpholino (MO) injection

Morpholino (5′-ctcttttgcggttacatttattta-3′) specifically targeting the 5′-UTR (5′-untranslated region) of *bms1l* mRNA (Bms1l-5′-UTR MO) was designed and synthesized by Gene Tools (Philomath, USA). One nanolitre of Bms1l-MO (0.75 nmol/μL) was injected into one-cell stage embryos.

2.5. RNA analysis

Total RNA from different samples was extracted using TRIzol (Gibco BRL, USA) as instructed by the supplier. Probes were DIG-labeled *via* polymerase chain reaction (PCR) using plasmids harboring the target probes as templates. RNA gel blot hybridization was performed as previously described (Wen et al., 2005; Cheng et al., 2006).

2.6. Phospho-histone 3 (P-H3) immunostaining

Sectioned samples were fixed in 2% PFA (paraformaldehyde) for 20 min and washed three times of 20 min