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 bms1lsq163 mutant which carries a T to A mutation in the gene bms1l. This mutation results in L152 to Q152 substitution in a GTPase motif in Bms1l. Surprisingly, bms1lsq163 mutation confers hypoplasia specifically in the liver, exocrine pancreas and intestine after 3 days post-fertilization (dpf). Consistent with the bms1lsq163 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 bms1lsq163 giving rise to an under-developed 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).

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) processome 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 processome. Bms1 is a G-domain-containing protein characterized first in yeast (Karbstein et al., 2005; Karbstein and Doudna, 2006). Nevertheless, studies on Bms1 motif that functions intra-molecularly (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, bms1lpq163, 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 L152 to Q152 substitution in the GTPase motif. Genetic evidences from co-segregation analysis, morpholino knockdown and mRNA rescue experiment unequivocally demonstrated that the bms1lpq163 mutation is responsible for the small liver phenotype we observed. Our study on bms1lpq163 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 bms1lpq163 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 bms1lpq163 mutant was identified as a small liver mutant from a total of 524 F2 ENU-mutagenized families. Map-based cloning method was used to clone the bms1lpq163 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 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’-ctgactgagagctgtgctgttc-3’; reverse primer: 5’-ctcagttctcgtgagct-3’) and cloned into the pcDNA3 vector. The plasmid harboring bms1l full length cDNA was used to synthesize bms1l 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’-ctactatgcttgctataata-3’) specifically targeting the 5’-UTR (5’-untranslated region) of bms1l mRNA (Bms11-5’-UTR MO) was designed and synthesized by Gene Tools (Philomath, USA). One nanolitre of Bms11-MO (0.75 mmol/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 described previously (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
each in PBST (140 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L Na$_2$HPO$_4$, 1.4 mmol/L KH$_2$PO$_4$, 0.1% Tween-20, pH 7.5). Samples were blocked in 4% bovine serum albumin for 30 min and incubated with primary antibody against P-H3 (sc-8656R, Santa Cruz, USA) at a dilution of 1:200 for 1 h at room temperature or overnight at 4°C. Five washes of 20 min each were followed to remove excess antibody. The fluorescence-conjugated second antibody, Alexa Fluor 568 goat anti-rabbit IgG (H + L) (SKU# A-11011, Molecular Probes, USA) was applied to sample at a dilution of 1:400 for 1 h at room temperature or overnight at 4°C. After the removal of excess antibody by similar washing conditions used to get rid of the first antibody, the samples were mounted for imaging.

2.7. TUNEL assay

Cryo-sections (8 microns) were fixed in 4% PFA for 20 min, washed 30 min with PBS, and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. TUNEL assay was carried out with In Situ Cell Death Detection Kit, TMR red (Roche) according to the instructions provided.

3. RESULTS

3.1. bms1lsq163 confers a small liver phenotype

Genetic screening for no and/or small liver mutants was carried out at 3 dpf via WISH when a liver proper has been well-defined. Being strongly expressed in the zebrafish embryonic liver from 1.5 dpf to 4 dpf (Ober et al., 2003), prox1 was selected as the probe to assess liver status in the F3 progenies. The bms1lsq163 mutant was isolated as a small liver mutant with weaker prox1 signals (Fig. 1A), with otherwise overall normal morphological development (Fig. 1B). Further characterization showed that mesoderm-derived organs such as the somite (examined with a troponin C probe) (Fig. 1C), pronephric duct and blood vessels of the mutant embryos (examined with AP staining) (Fig. 1D and E) also appeared normal at 3 dpf. The mutation does not appear to affect skeletal cartilage development up to 5 dpf (Fig. 1F). The small liver phenotype was confirmed by similar signal reduction with the liver specific gene fabp10a (Fig. 1G). By checking trypsin (exocrine pancreas) and insulin (islet) expression, we found that the mutant exocrine pancreas was affected significantly in size (Fig. 1H), while no visible defect in the islet was observed (Fig. 1I). The gut of the mutant was also noted to exhibit reduced signal when compared to that observed in the wild type using the intestinal marker fabp2 (Fig. 1J). These defects found on the three major digestive organs in the mutant were reflected more profoundly at 4 dpf. By 4 dpf, the left lobe of a normal liver should have accumulated in size comprehensively and extended over forming the right lobe, as observed in the wild type. However, the liver in bms1lsq163 experienced drastic growth arrest where the size remained the same as that in 3 dpf, with no sign of the second lobe (data not shown). The exocrine pancreas in the mutant also suffered obviously delayed expansion where the organ failed to broaden and lengthen posteriorly as that seen in wild type (data not shown). Apart from the absence of looping, the
mutant failed to form a fully expanded intestinal bulb (data not shown). Mutant embryos do not survive beyond 5 dpf.

3.2. **bms1** does not affect liver initiation

The above results indicate that the mutation in **bms1** does not exclusively affect the development of the liver but impinge on the growth of other digestive organs as well. Meanwhile, despite displaying much reduced expression, positive *fabp10a*, *trypsin* and *fabp2* signals were detected in **bms1** mutant embryos. This observation suggests that the differentiation of hepatocytes, exocrine pancreatic cells and intestinal epithelial cells from their corresponding precursors did take place in the mutant although the reduced expression of these markers could be due to the compromised differentiation of these cell types. To determine the onset of observable defects, earlier markers were used to assess the status of the earlier events of liver formation. One of the earliest events of liver development in zebrafish is competency acquisition where factors such as *foxA1*, *foxA3*, *gata4* and *gata6* are known to confer endodermal cells with competency proper to develop as hepatic cells and these genes continue to express in the endodermal lineage during early development of digestive organs (Lee et al., 2005; Zhao et al., 2005). Upon comparing the expression of these four early markers between wild type and **bms1** at 2 dpf, it was found that though subtle, the liver bud in the mutant is noticeably smaller than that in its wild type siblings. All data at this stage suggests that liver bud expansion but not liver bud initiation is affected in **bms1**. Interestingly, the pancreas appeared to be most drastically affected where there was little or no detectable expression of *foxA1*, *gata4* and *gata6* in the pancreatic bud whereas the liver was only slightly smaller (Fig. 2). Intriguingly, unlike the drastic defects as revealed by *foxA1*, *gata4* and *gata6* probes, WISH using a *foxA3* probe showed no evidence of any abnormal phenotype (Fig. 2). One explanation for this observation is that, though present, the mutant pancreatic cells somehow failed to be stained by *foxA1*, *gata4* and *gata6* due to certain yet to be defined alterations caused by the mutation. In addition, we observed that, at as early as 2 dpf, the thickening and left-looping of the mutant gut tube were also affected (Fig. 2).

3.3. **bms1** does not block hepatoblast specification

The stage following competency acquisition is the specification of hepatoblast from competent hepatic endoderm cells. *prox1* and *hhex* are the two of the earliest markers of definitive hepatoblasts (Ober et al., 2006). To check the hepatoblasts in **bms1** we examined the expression of these two genes at 2 dpf. Consistently, *prox1* revealed a smaller liver bud in **bms1** compared to wild type (Fig. 2), implying that the mutant suffers deficiency in sustaining definitive hepatoblast. Interestingly, no noticeable difference was detected in the liver bud between **bms1** and wild type when another hepatoblast marker, *hhex* was used (Fig. 2). Comparison of the staining patterns of *prox1* and *hhex* suggests that these two markers appear to mark different sub-type of cells in the liver bud at 2 dpf (Fig. 2).

3.4. Positional cloning of **bms1**

After backcrossing four rounds with wild type AB fish to purify the genetic background, **bms1** heterozygous mutants were outcrossed with the WIK line to generate mapping families for positional cloning. To identify markers closely linked to **bms1** by BSA, DNA from 26 homozygous mutant embryos and 26 siblings from the same mapping families were pooled, respectively. DNA from the variant and siblings pools was used as PCR templates for each of the 266 SSLP markers. Two SSLP markers Z4830 (north marker) and Z4397 (south marker) were found to be linked to each side of **bms1** in linkage group 12 (Fig. 3A). Further genetic mapping revealed Z35706 as a new north marker closer to **bms1** than Z4830. Screening with Z4397 identified
The conversion of the conserved L152 to Q152 (indicated by green asterisk) in software (http://danio.mgh.harvard.edu/markers/ssr.html) and new SSLP markers were designed with the help of a free (Fig. 3A). Based on the BAC sequences in these contigs, full sequence available. Marker fc06f12-9 was located on BAC which is composed of 219 overlapping BAC clones, most with sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish) locked both and Genome Fingerprinting Project database (http://www.ensembl.org/Danio_rerio/index.html) database (http://www.ensembl.org/Danio_rerio/index.html) to become the closest informative marker that lies just north of the mutation (Fig. 3A). A search in the Ensembl database showed that this 155 kb genomic fragment contains 5 open reading frames (ORFs) encoding for G-protein coupled receptor Gpr123 (gi125831439), zinc finger CTCF (gi125831441), Mxtx2 (gi24371281), Bms1l (gi125831426) and SH2-like protein (gi125831443) (data not shown). Systematic exons sequencing was embarked on these genes in hunt for the mutation. Consisting of 23 exons, exon 5 of the bms1l gene was found to harbor a nucleotide change from T to A (Fig. 3B), resulting in an amino acid substitution from Leu to Gin on the 152nd amino acid (Fig. 3B) in the conserved GTPase domain of Bms1l (Figs. 3C and 4). Sequencing the remainder of the bms1l coding region revealed no other mutations.

2 recombinants out of 3500 mutants. At the same time, screening with Z35706 identified 30 recombinants out of 3538 mutant embryos (Fig. 3A). Subsequently, fc06f12-9 took over Z35706 to become the closest informative marker that lies just north of the mutation (Fig. 3A). A search in the Ensembl database (http://www.ensembl.org/Danio_rerio/index.html) and Genome Fingerprinting Project database (http://www.sanger.ac.uk/Projects/D_rerio/WebFPC/zebrafish) located both fc06f12-9 and Z4397 markers onto one BAC contig 1189, which is composed of 219 overlapping BAC clones, most with full sequence available. Marker fc06f12-9 was located on BAC clone DKEY-145P15 while Z4397 on DKEY-288I10 (Fig. 3A). Based on the BAC sequences in these contigs, new SSLP markers were designed with the help of a free software (http://danio.mgh.harvard.edu/markers/ssr.html) and were tested on recombinants (data not shown). The final 2 (south) and 1 (north) recombinants could not be reduced further upon assessment with finer north SSLP CH211-214E3-11 and south SSLP CH211-214E3-1 markers, respectively (on CH211-214E3) (Fig. 3A). These two final SSLPs define a critical region with an estimated physical distance of 155 kb (Fig. 3A).

3.5. bms1l<sup>p1463</sup> alters a conserved domain in Bms1l

To unequivocally prove that the T to A substitution in bms1l gene was found to harbor a nucleotide change from T to A (Fig. 3B), resulting in an amino acid substitution from Leu to Gin on the 152nd amino acid (Fig. 3B) in the conserved GTPase domain of Bms1l (Figs. 3C and 4). Sequencing the remainder of the bms1l coding region revealed no other mutations.

To confirm whether the gene identified is indeed the mutated gene responsible for the mutant phenotypes observed, segregation analysis was carried out. Male and female bms1l<sup>p1463</sup> heterozygotes were mated and the progenies were subjected to WISH analysis using fabp10 probe to group the embryos based on liver size. One hundred and five embryos and 26 embryos showing normal and small liver, respectively, were identified after WISH. Genomic DNA from these siblings was isolated and DNA fragment containing the mutation was amplified via PCR and sequenced. Embryos showing normal sized liver were expected to show either T/T (wild type) or A/T (heterozygote) whilst embryos showing small liver would be A/A (homozygote) at the nucleotide of query. Results showed that embryos with normal sized liver exhibited T/T (33/105) and A/T (72/105) at the anticipated ratio of 1:2 while A/A genotype was strictly restricted to the mutant homozygotes.

To unequivocally prove that the T to A substitution in bms1l is responsible for the bms1l<sup>p1463</sup> phenotype, complementation test was carried out. The mRNA encoding wild type and mutant bms1l was injected into one-cell stage embryos independently and the embryos are genotyped later after assessing the liver status via in situ hybridization. At 5 dpf, ~57% of the mutants injected with wild type bms1l mRNA (34 out of 60 injected mutant embryos examined) had restored the expression of the liver specific gene fabp10 fully or partially (Fig. 5A). In contrast, both the T to A mutant bms1l mRNA and a mRNA harboring a premature stop codon failed to rescue the mutant phenotype (0/6 and 0/18 mutant embryos examined, respectively) (Fig. 5A). These results definitively prove that the L<sup>152</sup> to Q<sup>152</sup> substitution in Bms1l in bms1l<sup>p1463</sup> brought about the small liver phenotype we observed.

3.7. Knockdown of bms1l gene phenocopies the small liver phenotype in bms1l<sup>p1463</sup>

In zebrafish, morpholino-mediated gene knockdown is commonly used to study gene function. A 5'-UTR morpholino
was designed to block the 5'-UTR region of bms1l preventing efficient translation of the gene. The small liver phenotype observed in bms1lsq163 was phenocopied in morphants injected with bms1l-5'-UTR where 100% of the 93 morphants exhibited small liver, a specific phenotype that was not detected in any of the 89 embryos injected with a control morpholino.

Fig. 4. Alignment of Bms1l proteins from different species.

Alignment of Bms1l proteins from zebrafish (NM_001111150), human (GI:40788900), mouse (GI:39930555) and baker's yeast (GI:6325039). The conserved GTPase domain is underlined. The mutated amino acid Leu152 is marked with a green asterisk.

with bms1l-5'-UTR where 100% of the 93 morphants exhibited small liver, a specific phenotype that was not detected in any of the 89 embryos injected with a control morpholino.
The specificity of this observation was further verified by the rescue of these morphants by co-injected bms1l mRNA, where more than 80% was fully or partially rescued (Fig. 5B). This provides additional genetic evidence that the mutated bms1l is responsible for the small liver phenotype seen in bms1lsq163.

3.8. Bms1l is highly conserved in different species

Blast search revealed that proteins showing strong sequence similarity to Bms1l are encoded in a diverse range of genomes, including that of Homo sapiens, Drosophila melanogaster, Caenorhabditis elegans, Schizosaccharomyces pombe and even Arabidopsis thaliana, suggesting that it is highly evolutionarily conserved. The approximately 400 amino acids long N- and C-terminal domains (domain N and domain C, respectively) are the most conserved regions of the proteins (Fig. 4), implying functional significance of these two regions.

3.9. Expression patterns of bms1l

To explore the possible roles of Bms1l in the development of the liver and other digestive organs, we first analyzed bms1l expression patterns in zebrafish. WISH using a fragment of bms1l containing the GTPase domain as a probe on wild type zebrafish embryos revealed strong specific signals in the various endoderm-derived organs including liver, pancreas and gut (Fig. 6A). Probes generated from the middle and C-terminal of the gene showed similar enriched signals in these organs (data not shown). Northern blot of bms1l in wild type embryos showed that bms1l transcripts were expressed at similar levels from unfertilized eggs (maternal expression) and
1–5 dpf, indicating that it is required throughout embryogenesis (Fig. 6B).

3.10. \textit{bms1lsq163} affects the processing of pre-rRNA

All 28S, 18S and 5.8S rRNAs are derived from the same pre-rRNA precursor (Fig. 6C). In yeast, depletion of Bms1 caused aberrant processing of 35S pre-rRNA (Karbstein et al., 2005; Karbstein and Doudna, 2006). To find out whether Bms1l plays similar function in zebrafish, we performed Northern blot to analyze the status of mature 18S and 28S rRNA and their intermediate precursors using corresponding probes (Azuma et al., 2006). Our result showed that \textit{bms1l}-5'UTR morphants accumulated pre-18S precursors although the final amount of 18S rRNA remained stable (Fig. 6D).

3.11. \textit{bms1l}^\textit{pq163} small liver phenotype is due to impaired hepatoblast proliferation rather than elevated apoptosis

Growth or otherwise, is an output after balancing proliferation and apoptosis. A direct supposition for the small liver phenotype in \textit{bms1l}^\textit{pq163} therefore is reduced proliferation of the hepatoblasts or increased apoptosis or both. To find out which is the case, P-H3 staining and TUNEL assay were performed to compare the proliferation and cell death status, respectively, in wild type and mutants. P-H3 assay uses an antibody against the phospho-histone 3 protein (P-H3) as a marker of proliferating cells. To better distinguish the hepatocytes from the surrounding cells, we performed this experiment in the background of \textit{Tg(fabp10a:RFP)} transgenic fish, in which the reporter RFP is specifically expressed in the liver. By examining sectioned embryos at 3 dpf, we found that
the mutant had approximately 4-fold less P-H3-positive cells (6 positive cells out of 645 cells counted) than the wild type (67 positive cells out of 1744 cells counted) in the liver region, respectively (Fig. 7A–E). Meanwhile, we noticed that the P-H3-positive cells in the mutant intestine were reduced approximately 1.5 folds at 3 dpf (Fig. 7A–E). The \( bms1l^{sq163} \) mutation does not seem to affect proliferation of the cells in the neural tube (Fig. 7E). This result suggests that one of the contributing factors for a reduced size liver observed is the impairment of hepatoblast proliferation. Concurrently, increased apoptosis could add onto the phenotype observed. Apoptotic activities in both the wild type and mutant embryos were examined via TUNEL method and no apoptotic cells were found in wild type or mutant in the liver region examined at 2 dpf (data not shown) and 4 dpf (Fig. 7F and G).

4. DISCUSSION

Phenotypic characterization of the \( bms1l^{sq163} \) mutant using specific digestive organ markers suggests that liver bud growth but not bud initiation was affected in the mutant. The lesion continued to impinge on the expansion of the liver, as well as other digestive organs such as the intestine and pancreas, resulting in their retardation after 3 dpf. The fact that three of the major organs were formed suggests that the differentiation of these organs is compromised but not totally abolished. This is in contrast to liverless mutants like \( mypt1^{sq181} \) and \( prometheus \) (\( prt \)) (Ober et al., 2006; Huang et al., 2008). By exploiting the polymorphisms exhibited in 226 pairs of SSLP markers and polymorphic mapping families, the BSA protocol mapped \( bms1l^{sq163} \) to linkage group 12. From ~7000 meiotic events, subsequent intermediate and fine mapping in combination with candidate gene approach identified a T to A mutation at \( bms1l \), which results in L152 to Q152 substitution in a GTPase motif in Bms1l. Three lines of evidence from co-segregation analysis, morpholino knockdown and wild type transcript rescue clearly proved that \( bms1l^{sq163} \) is responsible for the small liver phenotype. Consistent with the \( bms1l^{sq163} \) phenotypes, WISH on wild type embryos showed that \( bms1l \) was enriched in the entire digestive tract and its accessory organs. Therefore, the mutation in the GTPase domain of Bms1l in \( bms1l^{sq163} \) might lead to the inability or reduced efficiency of the GTPase in either the conversion of GTP into GDP, or the recruitment of proper partners to exert its function in organogenesis. Rcl1 is an interacting partner of Bms1l in ribosomal synthesis and processing (Wegierski et al., 2001). One working hypothesis is that in \( bms1l^{sq163} \), the interaction (either physical or functional) between Bms1l and Rcl1 has been compromised resulting in the small liver phenotype. Future work is required to address this proposition.

Ribosome, responsible for protein manufacturing in all living cells, is a large ribonucleoprotein complex made up of 65% rRNA and 35% ribosomal proteins. Eukaryotes have 80S ribosomes, each consisting of a small (40S) and large (60S) subunit. The large subunit is composed of a 5S RNA (120 nucleotides), a 28S RNA (4700 nucleotides), a 5.8S subunit (160 nucleotides) and ~49 proteins. The 40S subunit has a 1900 nucleotide (18S) RNA and ~33 proteins (Ben-Shem et al., 2011). Synthesis and processing of rRNAs and the assembly of ribosomes in eukaryotic cells occur in the nucleolus following a complex pathway where rRNA is transcribed, processed and assembled with ribosomal proteins producing ribosomal subunits. The machinery responsible for rRNA processing is a large ribonucleoprotein complex containing multiple ribosomal proteins and accessory nucleolar trans-acting factors that associate with the nascent pre-rRNA (Kressler et al., 1999; Fromont-Racine et al., 2003). rRNA processing has been most extensively studied in the yeast \( Saccharomyces cerevisiae \) and many trans-acting factors, both proteins and ribonucleoproteins, required for the process have been characterized in the organism as well. Rcl1p is a putative endonuclease essential for pre-rRNA processing at specific sites of the ribosomes (Billy et al., 2000). Bms1lp was discovered via a yeast two-hybrid screen using Rcl1p as the bait (Wegierski et al., 2001). Like Rcl1p, Bms1lp is an evolutionarily conserved nucleolar protein required for pre-rRNA processing at specific sites. Bms1lp possesses structural features common to the regulatory GTP/GDP binding protein (G-proteins) at the N-termini domain and later was shown to indeed, function as a GTPase for the biogenesis of 40S ribosomal subunits (Karbstein et al., 2005). Almost the entirety of Bms1l functional study to date is focused on its conserved role of GTPase in ribosomal assembly, where a large proportion of the investigation is biochemical in nature, exploring the ordering and kinetics of complex formation and disassociation by components reconstitution assays.

Based on close sequence homology, the structure of the G-domain of Bms1lp has been inferred from the closely related eubacterial protein elongation factors EF-Tu (Sanchez and Sali, 1998), containing five conserved polypeptide loops designated as G1 through G5, which form contact sites with guanine ring or coordinate Mg\(^{2+}\) ion. Mutational studies have shown that certain residue substitution at the loops of Bms1l affected its biological activity (Wegierski et al., 2001), hence defining specific amino acids crucial for the process. G-domain is known to be present in many regulatory GTPases, acting as molecular switches in diverse cellular processes such as translation, protein trafficking and signal transduction (Bourne et al., 1991). Therefore, it is of no surprise when a mutated Bms1lp resulted in unviable spores that failed to germinate, revealing reduced 40S subunits levels in the yeast mutant as demonstrated by polysome profiling (Wegierski et al., 2001). Similarly, \( bms1l^{sq163} \) zebrafish could only live up to 6 dpf. However, no significant decrease of mature rRNA was observed on Northern blots (data not shown). It is possible that the fluctuation of 40S level in the mutant is not sufficient enough to be detected by Northern blot. Alternatively, the effect of the \( bms1l \) mutation was not manifested through the reduction of 40S level in zebrafish. A more sensitive detection method will be needed to address this aspect, such as to monitor the status of newly synthesized rRNA instead of total rRNA.
Fig. 7. *bms1lP163* impairs hepatoblasts proliferation.

A–D: immunostaining using a P-H3 antibody (green) on 3 dpf wild type (wt) (A,C) and mutant embryo (mt) (B,D) sections. DAPI (blue) is used to mark cell nucleus. Hepatoblasts are labeled in red by staining RFP which is specifically expressed in the liver under driven by the *fabp10a* promoter. E: quantitative analysis of the proliferating cells in the liver, intestinal bulb and neural tube. Sections from three wt and three mt embryos across the liver and the intestinal bulb were used in counting the PH-3-positive cells and total DAPI positive cells. F and G: TUNEL analysis of apoptotic cells in wild type (wt) and mutant (mt) at 5 dpf. No apoptotic cells were found in both wt and mu in the liver region. Forty-six sections from three wild type embryos and 51 sections from three mutant embryos were examined. Ib: intestinal bulb; L: liver; Nt: neural tube; Nc: notochord; Pa: pancreas; Pd: pronephric duct; Ph: pharyngeal.
One puzzling question raised with the identification of bms1l as the gene responsible for the specific small liver phenotype in bms1l<sup>Pq163</sup> is the regional defects instead of the more expected general developmental defects with widespread phenotypes like those observed in the Minutes fly mutants, which were reported to be contributed by quantitative deficiencies of ribosomal protein (RP) genes (Lambertsson, 1998). One likely explanation is due to maternally expressed bms1l that compensates for bms1l<sup>Pq163</sup> at the early stage of embryogenesis. Another possible rationalization of the specific liver phenotype is that ribosomal protein genes could conceivably display some novel biological function independent of their role in the ribosome where inhibition of this function leads to specific consequence such as defective liver development. Such speculation is not groundless since individual ribosomal proteins have been implicated in a wide variety of biological functions, including cell cycle and progression, apoptosis and DNA damage responses (Volarivic et al., 2000; Lohrum et al., 2003), and it has also been suggested that their roles in these generic processes may arise independently of their role in the ribosome itself (Wool, 1996; Soulet et al., 2001). Therefore, a possible novel function of Bms1l in liver organogenesis cannot be ruled out. Alternatively, the selective manifestation of endodermal phenotype by an apparently housekeeping gene is due to different gene product dosage in different tissue types, which is similar to the reason for explaining why one-third of the mutated rp did not give rise to tumors (MacInnes et al., 2008). This is probably not surprising after all since it has been suggested that different tissues have different requirements for specific rp dosages hence expressing variable amounts of the same transcript (Bortoluzzi et al., 2001).

While the characterization work on bms1l<sup>Pq163</sup> reported so far is still at its infancy and is limited to phenotypic examination via molecular markers, a glimpse into possible reasons behind a small liver phenotype revealed impairment of hepatoblast proliferation. This is in contrast to the previous findings in the conditional knockout mouse for S6 (a ribosomal protein for the 40S subunit) where although the S6-deficient liver cells failed to enter S phase and proliferate, the livers from fasted mice were capable of increasing cell size in response to the re-addition of food despite a deficiency in 40S ribosomes. These results place a cell proliferation checkpoint but not one on growth as a consequence of deficiency in ribosomal biogenesis (Opferman and Zambetti, 2006). A different mechanism might be governed by the other ribosomal biogenesis proteins such as Bms1l.

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