

Developmental Roles of D-bifunctional Protein-A Zebrafish Model of Peroxisome Dysfunction

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The peroxisome is an intracellular organelle that responds dynamically to environmental changes. Various model organisms have been used to study the roles of peroxisomal proteins in maintaining cellular homeostasis. By taking advantage of the zebrafish model whose early stage of embryogenesis is dependent on yolk components, we examined the developmental roles of the D-bifunctional protein (Dbp), an essential enzyme in the peroxisomal β -oxidation. The knockdown of *dbp* in zebrafish phenocopied clinical manifestations of its deficiency in human, including defective craniofacial morphogenesis, growth retardation, and abnormal neuronal development. Overexpression of murine *Dbp* rescued the morphological phenotypes induced by *dbp* knockdown, indicative of conserved roles of Dbp during zebrafish and mammalian development. Knockdown of *dbp* impaired normal development of blood, blood vessels, and most strikingly, endoderm-derived organs including the liver and pancreas - a phenotype not reported elsewhere in connection with peroxisome dysfunction. Taken together, our results demonstrate for the first time that zebrafish might be a useful model animal to study the role of peroxisomes during vertebrate development.

INTRODUCTION

Peroxisomes perform crucial functions to maintain cellular homeostasis (Islinger et al., 2012; Van Veldhoven, 2010). Animal models of peroxisome dysfunction, at the level of its biogenesis

or separately at an individual enzyme level, were generated to elucidate the molecular mechanisms responsible for disease phenotypes in humans (Baes and Van Veldhoven, 2012). In particular, knockout mouse models provided convincing evidence showing that peroxisome is an active, multifunctional organelle communicating in response to environmental changes with other cellular compartments for maintaining normal physiological functions (Baes and Veldhoven, 2012; Faust et al., 2011; Huyghe et al., 2006). In mice, peroxisome dysfunction affects mitochondrion and Endoplasmic Reticulum (Thoms et al., 2009). However, mice develop *in utero* and receive nutrients from their mother through the umbilical cord during embryogenesis, making it difficult to gain adequate access to developing embryos. Additionally, such dependence makes it difficult to interpret developmental changes associated with metabolic alterations contributed solely by peroxisome dysfunction. The zebrafish is a well-established genetic and developmental model for studying human-diseases, particularly since its embryos are transparent and develop fast *ex utero* consuming only the yolk during the initial stages of development (Lieschke and Currie, 2007).

In this study, we examined whether zebrafish embryogenesis can be used as an effective *in vivo* model for studying peroxisome function, which might permit an organism-level analysis while providing mechanistic insights into development. Specifically, we analyzed the developmental roles of D-bifunctional protein (DBP, also known as 17- β -hydroxysteroid dehydrogenase 4), a peroxisomal enzyme involved in β -oxidation of fatty acids that generates acetyl-CoA and short-chain acyl CoA for intracellular energy and metabolic homeostasis (Moller et al., 2001). Mutations in humans may lead to DBP deficiency in which severe neonatal abnormalities, including growth retardation, neuropathy, craniofacial malformation, and hypotonia occur within an early period of life (Mehtälä et al., 2013; Moller et al., 2001; Suzuki et al., 1997; Van Grunsven et al., 1998; 1999). Mice deficient in *Dbp* exhibited a similar cohort of phenotypes as those found in humans that included severe growth retardation, male infertility and massive mortality within the first two postnatal weeks (Baes et al., 2000; Ferdinandusse et al., 2005; Huyghe et al., 2006; Verheijden et al., 2013). At a molecular level, DBP is the primary enzyme in the peroxisomal β -oxidation pathway that processes both saturated very long chain and branched chain fatty acids.

Here we report that *dbp* knockdown in zebrafish embryos

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Table 1. Primer list and its sequences used in real-time PCR, cDNA cloning of *dbp*, or efficacy test for *dbp* splicing-blocking morpholino

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>mDbp</i>	ACCGGTATGGCTTCGCCGCTGAGGTTTCG	ACCGGTTTCAGAGCTTGGCATAGTCTTTAAG
<i>dbp</i>	CGGTCTATCCTGGTCAGTCT	TTTCCCCATCACCACCTCCA
<i>gnpatl</i>	CGTCTTCCTCAGAGCCATTC	CACTCTGAAGTCTGTTCTGGTC
<i>agps</i>	GTCCTCCATATTCACCTCATTG	CACTCTCTTATTATCCTCTCCT
<i>pex5</i>	GGGAGAGAGAGAAAGAGAGT	ACAGAGAGTGCGGCAGAGAAA
<i>pgcla</i>	CCTCCTTCTCTCAGCTCT	CCCTCTCACATTCCTGGTTTC
<i>pparab</i>	GGAGAAGCTAAGGTTGAAGG	GGGCACAGATTTGGGGAATTC
<i>esrra</i>	GTTCTGGATGAGGAGATGTC	TGAGGAGAGGCAGGGTGAG
<i>β-actin</i>	ACCTCATGAAGATCCTGACC	TGCTAATCCACATCTGCTGG
<i>dbp-C</i>	TCTCCAATCCTCCACGGTTTGTGTT	CGTTCCTCAAATATGAGCGTTAGACAGA
<i>dbp-E1</i>	CATCTCATCATGTCTGTGCC	
<i>dbp-E4</i>		CTTCTCTCCATCCTCCACT

leads to morphological malformations, defective yolk consumption, abnormal neuronal development, and growth retardation phenotypes similar to those observed in humans with *DBP* mutations. A detailed analysis also revealed phenotypes not found earlier in humans or mice, including defects in the formation of blood, blood vessels, and cartilage. Strikingly, *dbp* knock-down nearly blocked digestive organ development. This is probably a result of reduced transcriptional activities critical for functional peroxisomes as well as mitochondrial biogenesis. These morphological phenotypes were rescued by the expression of murine *Dbp*, indicating its functional conservation between zebrafish and mouse. Taken together, our results demonstrate that zebrafish is a promising animal model that may be used to uncover new roles of peroxisomes during vertebrate development. Our study is the first to demonstrate the developmental consequences of peroxisome dysfunctions by using the zebrafish model.

MATERIALS AND METHODS

Zebrafish care, transgenic animals, and microinjections

The zebrafish and their embryos were handled and staged according to standard protocols (Kimmel et al., 1995). Transgenic zebrafish lines were kindly provided by Drs. C-H Kim, H-C Park, Y-K Bae, Y Kee and S-Y Choi [*islet1:GFP*, *gfap:EGFP*, *mbp:EGFP*, *gata1:RFP*, MLS (mitochondria localization sequence)-EGFP], and by the Zebrafish Organogenesis and Mutant Bank [*lfabp:DSRED* and *elastase:GFP*]. Control (5'-AACATACATCAGTTTAATATATGTA-3'), splicing-blocking (5'-TCGGTGATGAAGAAGTACCTCCGC-3') and translation-blocking (5'-CGTCTCGAATCTCAAAGGCACAGACAT-3') morpholinos (MOs) were purchased from Gene Tools. Approximately 8 ng MO was microinjected at the 1-cell stage of wild type or transgenic zebrafish embryos as previously described (Choe et al., 2011). For rescue experiment, 50 μ g *in vitro* synthesized murine *Dbp* mRNA (Ambion) was co-injected with *dbp* MO and resulting phenotypes were documented.

Constructs

Murine *Dbp* was amplified using a standard PCR protocol and cloned into Agel site in modified pcDNA3.1+myc vector. For the rescue experiments, murine *Dbp* was subcloned into pCS2+ vector between BamHI and SnaBI sites. A 3' partial zebrafish *dbp* was PCR amplified. The amplicon was cloned into pCRII-TOPO vector (Invitrogen) and was used to generate antisense probe for *in situ* hybridization. All the primer sequences used

are listed in Table 1.

Whole-mount Oil Red-O staining

Embryos at various stages of development were fixed with 4% paraformaldehyde at 4°C overnight, washed 3 times with PBT, and preincubated in 60% isopropanol for 30 minutes. Embryos were then incubated with freshly filtered 0.3% solution of Oil Red-O (Sigma) in 60% isopropanol for 3 hours, rinsed 3 times with PBT and photographed.

In situ hybridization, immunostaining and microscopy

In situ hybridization and immunostaining were performed as described previously (Choe et al., 2009). The *dbp* antisense probe was synthesized using a standard protocol. For immunostaining, antibodies to Znp-1 (1:1,000, DSHB) and acetylated tubulin (1:1,000, Sigma) followed by secondary Alexa Fluor-conjugated anti-Mouse antibodies (1:400, Invitrogen) were used. Embryos were imaged with a Leica M165FC microscope equipped with Leica DFC500.

Quantitative RT-PCR

Total RNAs were prepared from 10 embryos at desired developmental stages using Trizol (Ambion) reagent following manufacturer's instructions. First strand cDNA was synthesized (Roche) and quantitative PCR was performed. All the primer sequences used are listed in Table 1. Gene expression levels relative to that of *β -actin*, is presented as an average of values from three independent experiments. Statistical significance was determined in Microsoft Excel, and $p < 0.05$ was considered to be significant.

RESULTS

dbp is maternally deposited during zebrafish development

We first compared protein sequence of zebrafish *Dbp* with that of human or mouse, and found that *DBP* is well conserved in vertebrates, especially in the conserved, catalytic domains (Fig. 1A). We next examined *dbp* expression during zebrafish embryogenesis by performing quantitative RT-PCR. As detected at 2-4 cell-stage embryos, Zebrafish *dbp* mRNA was found to be maternally delivered (Fig. 2A). Expression level of maternal *dbp* mRNAs decreased at the onset of zygotic transcription. As embryogenesis proceeded, expression of *dbp* mRNAs showed recovery and began to increase gradually. We also performed *in situ* hybridization to examine whether *dbp* expression is restricted to a specific cell type during zebrafish embryogenesis.

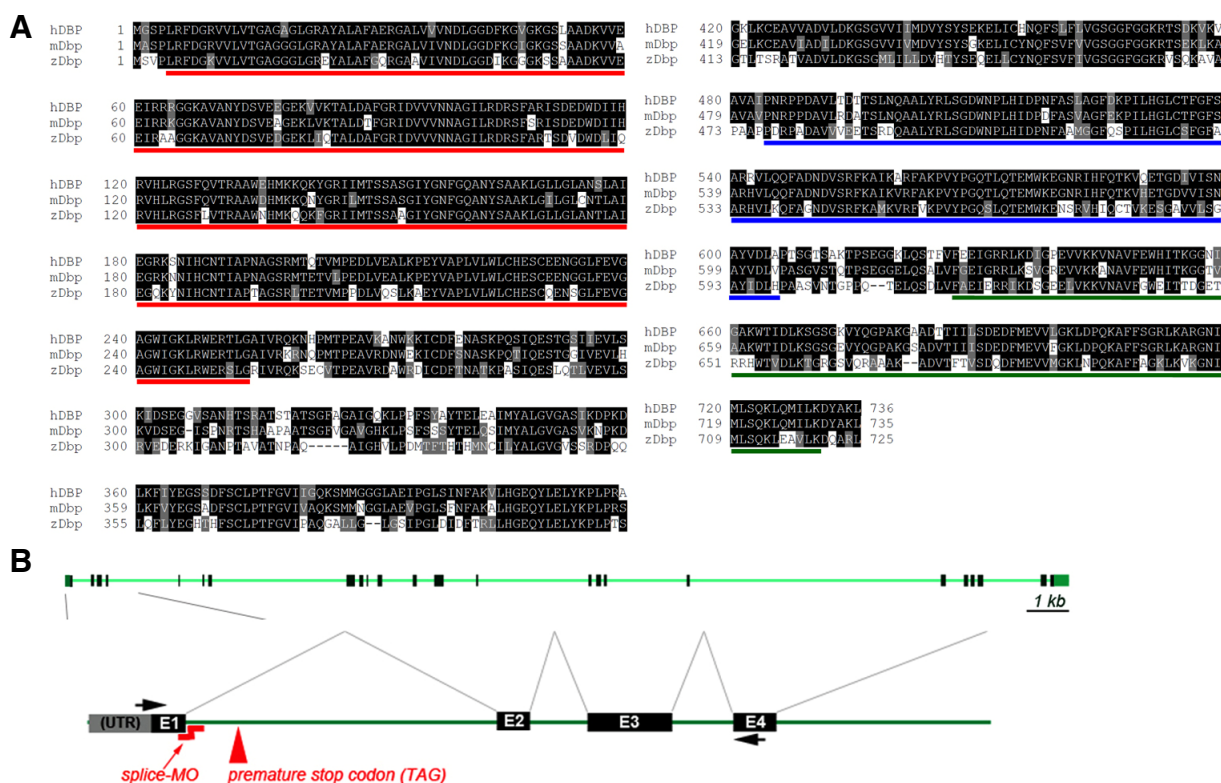


Fig. 1. Amino acid sequence alignment of human, mouse, and zebrafish DBP homologs and genomic structure of zebrafish *dbp*. (A) Amino acid sequence alignment of human, mouse, and zebrafish homologs of DBP using ClustaW. Amino acids conserved in at least two species are highlighted in black and similar amino acids in gray. Conserved domains are underlined in red (NADB_Rossmann fold), blue (Hotdog fold) and green (SCP2). (B) Diagram depicting genomic structure of zebrafish *dbp* and the target site of *dbp* splicing-blocking morpholino. *dbp* splicing-blocking MO interferes with the splicing event by blocking the splice donor region of the exon-intron boundary, which may result in translation of aberrant, truncated 27 amino acids from the intron sequences. A primer set (black arrows) was used to test the efficacy of *dbp* splicing-blocking MO.

In situ hybridization with *dbp* antisense probe showed *dbp* was ubiquitously expressed at least from 2-cell stage of embryos (not shown), consistent with maternal deposition of *dbp* mRNA determined by quantitative RT-PCR.

dbp knockdown impairs embryonic development

We used a morpholino (MO)-based knockdown approach to determine the role of Dbp in zebrafish embryogenesis. A splicing-blocking MO effectively reduced the amount of mature *dbp* mRNA without generating any mis-spliced transcripts that probably underwent nonsense-mediated decay, a well-known process in response to a blockage of a splicing event (Figs. 1B and 2B). Both translation-blocking and splicing-blocking MOs generated morphologically abnormal embryos starting from 1 day post-fertilization (dpf) (Figs. 2C and 2D). Embryos occasionally exhibited curved body axis (not shown). At 2 dpf and later during development, edema was clearly visible in the pericardium and around the yolk. As compared to wild type embryos, the size of the yolk was noticeably larger in *dbp* knockdown embryos (Figs. 2E-2H). Therefore, defective β -oxidation in the peroxisome resulting from the *dbp* knockdown likely impaired normal embryogenesis in zebrafish.

dbp knockdown suppresses yolk consumption

Unlike mammals where embryos *in utero* receive nutrients from

mother through the umbilical cord, zebrafish embryos are limited in their energy source to the yolk components for the initial 5 days of development (Flynn et al., 2009). Zebrafish embryos consume the yolk, eventually depleting it by the time they are ready to be fed at about 6-7 dpf. We used Oil Red-O staining to detect neutral lipids stored in developing zebrafish embryos, and found that consistent with a previous report (Schlegel and Stainier, 2006), neutral lipids were largely located in the yolk and tissues such as brain, heart, and muscles for the first 3 days of normal embryonic development (Fig. 3A). Normally developing embryos consumed most yolk lipids by 4 dpf and almost completely depleted them at 5 dpf (Figs. 3C and 3E). In contrast, *dbp* knockdown embryos failed to use much of the yolk lipids, as was evident starting at 3 dpf (Figs. 3B, 3D, and 3F). Therefore, peroxisomal β -oxidation may constitute a key component for yolk consumption that supports embryonic growth during development.

dbp knockdown alters neuronal development

DBP deficiency in humans has been shown to result in neuropathy including demyelination (Moller et al., 2001; van Grunsven et al., 1999). We explored whether *dbp* knockdown generates neuronal abnormality with characteristics similar to that of DBP deficiency in human. Analysis of transgenic zebrafish expressing green fluorescent protein (GFP) in primary motor

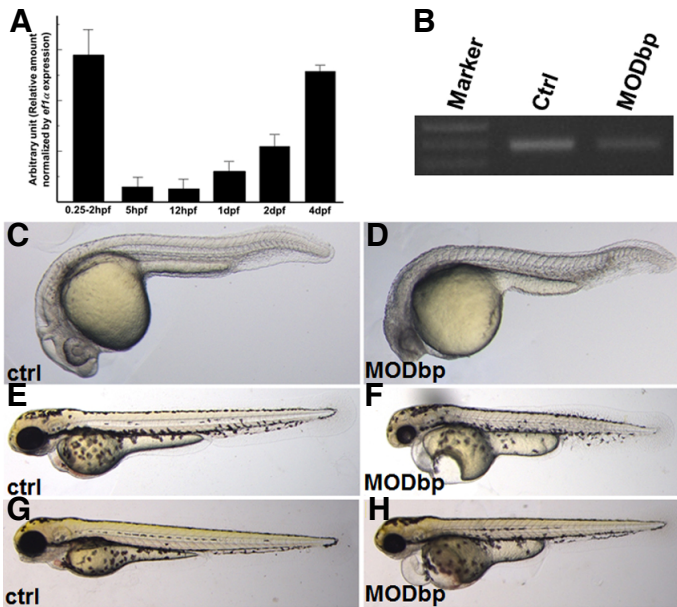


Fig. 2. *dbp* expression is essential for zebrafish embryogenesis. (A) *dbp* expression during development. Quantitative RT-PCR was used to assay *dbp* expression during development. The highest expression of *dbp* is at 0-2 hpf (maternally deposited) after which it is significantly decreased but increased gradually at 1dpf onwards. The graph shows relative amount of *dbp* mRNA normalized by *ef1α* expression. (B) *dbp* splicing-blocking morpholino (MO) efficiently reduced synthesis of mature mRNA. Total RNA extracted from control and MO-injected embryos at 1 dpf were used for cDNA synthesis followed by RT-PCR. A ~390 bp band in the control lane was significantly reduced in the morpholino-injected lane (MODbp), indicating high efficiency of MO. (C-H) *dbp* knockdown generates morphologically distinct phenotypes. MO-injected embryos displayed phenotypes, including small head, pericardial edema, and voluminous yolk compared to control embryos [compare (C, E, and G) with (D, F, and H)]. As embryogenesis continued, phenotypes became more severe [(C) and (D) (1dpf); (E) and (F) (2 dpf); (G) and (H) (3 dpf)]. Embryos are shown in lateral view with anterior to the left.

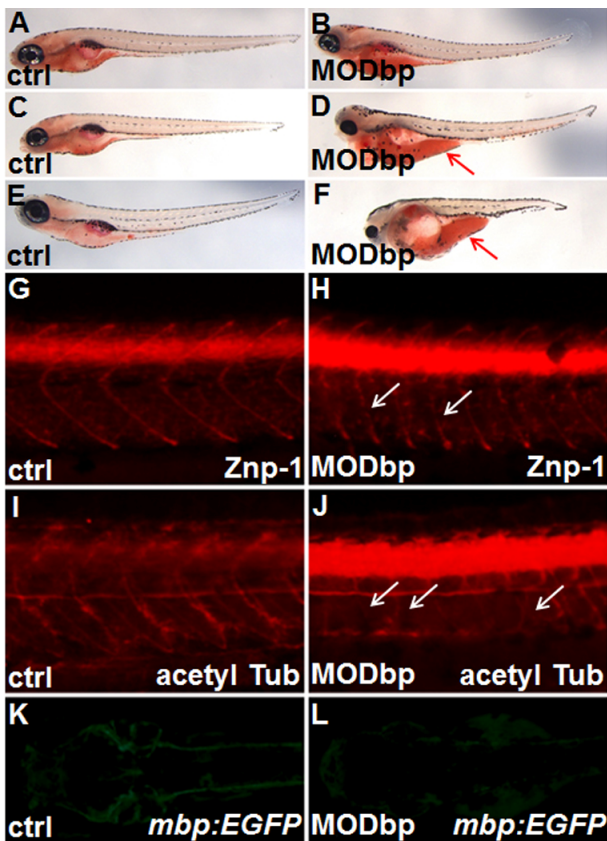


Fig. 3. *dbp* knockdown affects yolk consumption and neuronal development. (A-F) Embryos with *dbp* knockdown showed accumulated neutral lipids in the yolk. Oil Red-O staining revealed a significant delay or defect in yolk lipid consumption in *dbp* knockdown embryos on or after 3 dpf, (A) and (B) (3 dpf); (C) and (D) (4 dpf); (E) and (F) (5 dpf). As compared to control embryo, greater quantities of yolk lipids (visualized in red) were retained in a *dbp* knockdown embryo. Red arrows in (D) and (F) indicate accumulated lipids in the yolk. (G-L) *dbp* knockdown impaired neuronal development. Immunostaining using anti-Znp-1 antibody (G, H) or anti-acetylated tubulin (I, J) revealed defective neuronal development, such as discontinued axonal projections [indicated by white arrows in (H)] or suppression of differentiating motor axons [indicated by white arrows in (I)] upon *dbp* knockdown. (K, L) GFP expression driven by the *myelin basic protein (mbp)* promoter (indicative of myelinating cells) in an *mbp:EGFP* transgenic line was significantly reduced following *dbp* knockdown. Embryos are shown in lateral view with anterior to the left, except in (K) and (L) where embryos are in dorsal view. Partial trunk regions of embryos are shown in (G-J).

neurons (*islet1:GFP*) of the central nervous system (CNS) indicated normal GFP expression in *dbp* knockdown embryos (not shown). This suggested that Dbp might not be required for motor neuron specification in the CNS. However, Znp1, an axonal marker, was affected, featuring discontinued CaP pro-

jections occasionally in the trunk (Figs. 3G and 3H). Additionally, severe suppression of motor axon differentiation in the trunk was also detected in *dbp* knockdown embryos (Figs. 3I and 3J). Further, GFP expression driven by the promoter of *myelin basic protein* in Schwann cells of the control *mbp:EGFP* transgenic

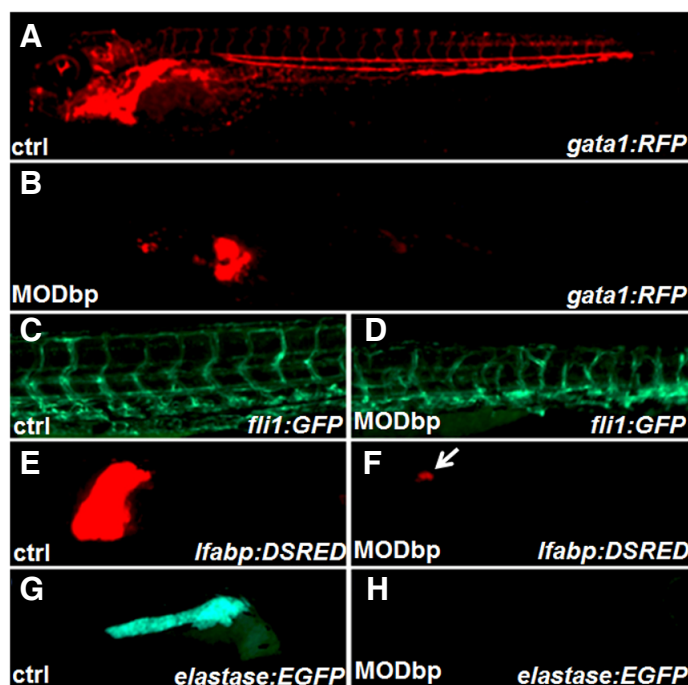


Fig. 4. *dbp* knockdown affects development of blood, blood vessels and digestive organs. Embryos carrying different transgenes were used to determine tissue-specific effects upon *dbp* knockdown. (A-D) *dbp* knockdown affects red blood cell development and blood vessel patterning. Upon *dbp* knockdown, blood cells were severely reduced [shown by *gata1:RFP* transgenic line, (A) and (B)] and blood vessels were formed abnormally [shown by *fli1:EGFP*, (C) and (D)]. (E-H) *dbp* knockdown on doubly transgenic fish line (*lfabp:DSRED* in liver and *elastase:EGFP* in pancreas) shows significantly reduced liver size (F) and absence of pancreas (H). Representative embryos at 3 dpf are shown in lateral view with anterior to the left, except (G) and (H) where presented in lateral view with anterior to the right.

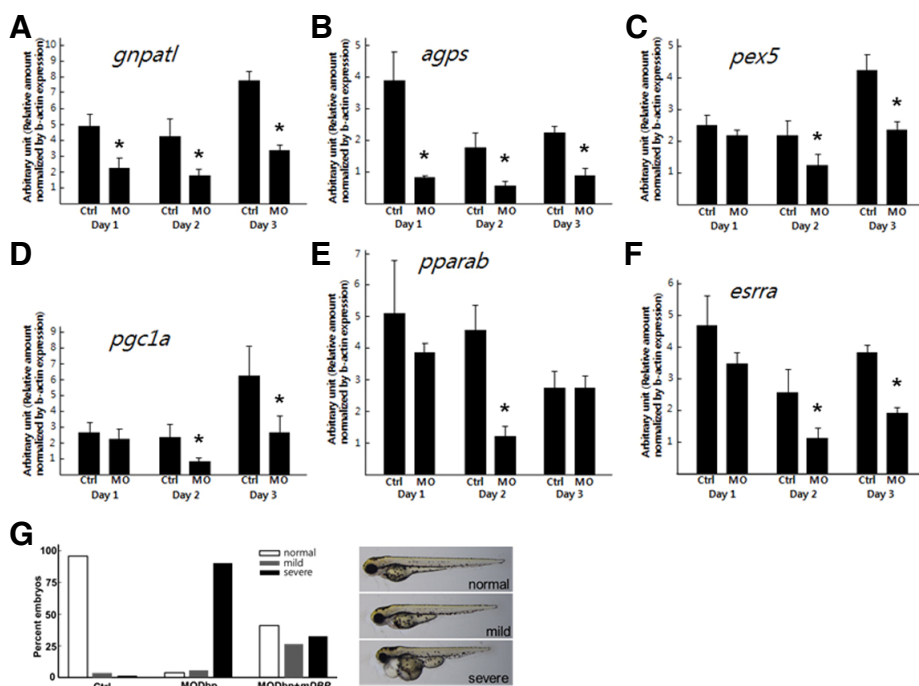


Fig. 5. Knockdown of *dbp* affects expression of genes involved in peroxisome functions and mitochondrial biogenesis. (A-F) Quantitative RT-PCR was used to assay expression of genes involved in ether phospholipid synthesis: *gnpat1* (A) and *agps* (B), a gene in peroxisomal protein import: *pex5* (C), and genes in mitochondrial biogenesis: *pgc1a* (D), *pparab* (E) and *esrra* (F). *dbp* knockdown altered the expression of genes involved in ether phospholipid synthesis starting at 1 dpf (A-B) and that of genes in peroxisomal protein transport or mitochondrial biogenesis at 2 dpf. * indicates statistical significance ($p < 0.05$) between control and *dbp* MO samples. (G) DBP is functionally conserved between mice and zebrafish. Rescue experiment was performed by co-injecting murine *Dbp* mRNA together with *dbp* MO. Embryos belonging to each category of phenotypic severity shown

in the right were counted from three independent experiments and presented as percentage of total injected embryos.

zebrafish was almost abrogated in *dbp* knockdown embryos (Figs. 3K and 3L). These results strongly suggest that the zebrafish *Dbp* may act similarly to that in mice and humans to promote neuronal development and integrity (Moller et al., 2001; van Grunsven et al., 1999; Verheijden et al., 2013).

***dbp* knockdown impairs organogenesis and tissue development**

While using various transgenic lines to detect tissue-specific abnormalities upon *dbp* knockdown, we discovered several developmental programs being impaired in addition to observ-

ing the phenotypes expected based on that found in mice and human (Baes and Van Veldhoven, 2012; Huyghe et al., 2006; Van Veldhoven et al., 2010). For examples, the numbers of red blood cells were reduced (detected by the *gata1:RFP* line, Figs. 4A and 4B) and blood vessels were abnormally patterned (by the *fli1:EGFP* line, Figs. 4C and 4D) in *dbp* knockdown embryos. Additionally, abnormal cartilage formation was also observed (not shown). Starting from 3 dpf, both liver and pancreas (by the *lfabp:DSRED* and *elastase:GFP* lines, respectively) developed poorly in *dbp* knockdown embryos (Figs. 4E-4H). *In situ* hybridization showed that expression of *foxa3*, a marker of early endodermal organs, was severely reduced or absent in liver and pancreas of *dbp* knockdown embryos (not shown). These results demonstrated that *dbp* knockdown in zebrafish impaired a number of developmental programs including those that have not been previously reported in connection with peroxisome dysfunction in humans or mice.

***dbp* knockdown alters expression of various genes involved in peroxisomal pathways and mitochondrial biogenesis**

Since *dbp* knockdown may also affect other peroxisomal functions that confer abnormality on developing embryos, we next examined expression of various genes important in major peroxisomal pathways. We found no differences in the expression of other enzymes in the β -oxidation pathway including *acox1*, *scp2a*, and *scp2b* between wild type and DBP knockdown embryos (not shown). In contrast, mRNA expression of the enzymes involved in the synthesis of ether phospholipids, such as *gnpat1* and *agps*, was significantly down-regulated in *dbp* knockdown embryos at 1 dpf (Figs. 5A and 5B). We also found that expression of *pex5*, the major carrier of peroxisome-targeted proteins, was significantly down-regulated starting from 2 dpf during development (Fig. 5C). Similarly, *dbp* knockdown also down-regulated expression of transcription factors known as upstream regulators of mitochondrial biogenesis (Figs. 5D-5F). These results suggested the presence of cross-talk between different functions of peroxisome. Additionally, our results suggest that dysfunctional peroxisome may affect other metabolic organelles such as mitochondria, potentially inducing phenotypic exacerbation as observed at later stages of development.

Exogenous murine *Dbp* can rescue embryonic phenotypes resulting from *dbp* knockdown in zebrafish

During the course of experiments, we noticed that morphological phenotypes were closely related to other abnormalities described. Since we found a significant homology between mice and zebrafish DBP protein sequences (Fig. 1), we used murine *Dbp* to rescue morphological defects observed in *dbp* knockdown embryos. As expected, murine *Dbp* over-expression efficiently rescued developmental abnormalities caused by *dbp* knockdown in zebrafish embryos (Fig. 5G). These results demonstrated that the observed phenotypes were specifically induced by *dbp* knockdown. Our results clearly indicated conserved functions of DBP in zebrafish and mammals.

DISCUSSION

In the study reported here, we assessed whether zebrafish can be used as an *in vivo* model for examining peroxisome functions by examining developmental roles of *Dbp*. We found that *dbp* knockdown in zebrafish gave rise to a phenotypic spectrum similar to that found in human patients. In particular, clinical

symptoms such as growth retardation, neuropathy, and craniofacial disfiguration in human patients were recapitulated in *dbp*-knockdown zebrafish embryos that displayed reduced yolk consumption, abnormal neuronal development, and cartilage malformation. Interestingly, phenotypes not found in previous mammalian models were also observed. In addition to defective blood and vessel formation, we found that *dbp* knockdown nearly blocked digestive system development. Although cases of hepatomegaly associated with peroxisome dysfunction in humans have been reported (Roels et al., 1993), severe defects in the development of digestive organs have not been observed in other organisms. Our results illustrate the usefulness of the zebrafish system in analyzing the role of metabolic pathways in development and diseases.

While DBP deficiency in mammals leads to compensatory activation of several peroxisomal enzymes, *dbp* knockdown in zebrafish did not affect expression of enzymes in the peroxisomal β -oxidation pathway. Instead, we found decreased expression of genes involved in other peroxisomal pathways, such as the import of peroxisomal matrix proteins and synthesis of ether phospholipids. This phenotypic difference between zebrafish and mammals may at least in part be due to the differences in stages of development analyzed. We found *dbp* knockdown-mediated transcriptional changes as early as 1 dpf when most organs were yet to be formed, while DBP-deficient mice or human patients were examined at single organ/tissue levels at several weeks of postnatal age (Baes et al., 2000; Ferdinandusse et al., 2005; Huyghe et al., 2006; Moller et al., 2001; Suzuki et al., 1997; van Grunsven et al., 1998; 1999). Accordingly, we found that phenotypes of developing zebrafish embryos with *dbp* knockdown described in this study were far more severe than those reported in *DBP*-deficient mammals.

We also analyzed the possibility that impaired β -oxidation in the peroxisome due to *dbp* knockdown may interfere with mitochondrial biogenesis, analogous to previous reports of abnormal mitochondrial morphologies observed in instances of peroxisome dysfunctions (Baes et al., 1997; Baumgart et al., 2001; Dirx et al., 2005; Muller-Hocker et al., 1984), *dbp* knockdown decreased expression of *pgc1 α* , *ppara* and *esrra* starting at 2 dpf. Given the importance of these genes regulating a number of pathways in mitochondria and peroxisomes, this result suggests that metabolic disturbance induced by peroxisomal β -oxidation may trigger to transcriptional suppression of upstream factors by an unknown feedback mechanism. Nonetheless, our results suggest that a functional link between the peroxisome and other metabolic organelles including mitochondria may be a critical factor for normal animal development.

In conclusion, the zebrafish system with metabolism confined within itself during early development may be an attractive animal model to analyze the molecular basis and physiological consequences of peroxisomal dysfunctions.

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