DEAD-Box Protein Ddx46 Is Required for the Development of the Digestive Organs and Brain in Zebrafish

Shunya Hozumi, Ryō Hirabayashi, Akio Yoshizawa, Mitsuko Ogata, Tohru Ishitani, Makiko Tsutsumi, Atsushi Kuroiwa, Motoyuki Itoh, Yutaka Kikuchi

1 Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima, Japan, 2 Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya, Japan

Abstract
Spatially and temporally controlled gene expression, including transcription, several mRNA processing steps, and the export of mature mRNA to the cytoplasm, is essential for developmental processes. It is well known that RNA helicases of the DEAD/H-box protein family are involved in these gene expression processes, including transcription, pre-mRNA splicing, and rRNA biogenesis. Although one DEAD/H-box protein, Prp5, a homologous of vertebrate Ddx46, has been shown to play important roles in pre-mRNA splicing in yeast, the in vivo function of Ddx46 remains to be fully elucidated in metazoans. In this study, we isolated zebrafish Moreno (mor), a mutant that shows developmental defects in the digestive organs and brain, and found that it encodes Ddx46. The Ddx46 transcript is maternally supplied, and as development proceeds in mutant, suggesting that Ddx46 may be required for pre-mRNA splicing during zebrafish development. Therefore, our results suggest a model in which zebrafish Ddx46 is required for the development of the digestive organs and brain, possibly through the control of pre-mRNA splicing.

Introduction
Precursor mRNA (pre-mRNA) splicing is essential for gene expression in metazoan cells, and the splicing reaction proceeds via a coordinated series of RNA-RNA, RNA-protein, and protein-protein interactions, which lead to exon ligation and the release of the intron lariat [1]–[4]. Pre-mRNA splicing is catalyzed by the macromolecular machinery known as the spliceosome, which consists of five small nuclear ribonucleoprotein particles (snRNPs: U1, U2, U4, U5, and U6) and >150 proteins. Non-snRNP proteins, which belong to a group of DEAD/H-box RNA-dependent ATPases/helicases, are required for pre-mRNA splicing process in yeast [1]–[4].

The DEAD/H-box RNA helicase family is a large protein group characterized by the presence of a helicase domain that is highly conserved from bacteria to humans [5]–[8]. The DEAD/H-box helicases share nine conserved motifs; motifs Q, I, II, and VI are required for NTP/ATP binding and catalyze its hydrolysis [5]–[8]. These proteins have been shown to play important roles in all aspects of RNA metabolism, including the modulation of RNA structures and association/dissociation of RNA-protein complexes, such as pre-mRNA splicing, rRNA biogenesis, transcription, RNA stability and turnover, RNA export, and translation [5]–[8]. In the yeast Saccharomyces cerevisiae, eight DEAD/H-box proteins-Suh2, Prp5, Prp28, Brt2, Prp2, Prp16, Prp22, and Prp43-act in specific steps of the splicing cycles to catalyze RNA-RNA rearrangements and RNP remodeling [2]–[4]. Among them, Prp5 (a homolog of vertebrate Ddx46) is necessary, along with ATP hydrolysis, for stable association of U2 snRNP with pre-mRNA and prespliceosome formation in S. cerevisiae and Schizosaccharomyces pombe [9]–[11]. In addition, human DDX46 has been shown to play roles in pre-mRNA splicing in vitro before or during prespliceosome assembly [12]. The in vivo function of Ddx46 in metazoans remains to be elucidated, however.

The zebrafish has emerged as an important model system for the investigation of vertebrate development and other complex
biological processes, including human disease [13], [14]. Analyses of zebrafish mutants and knock-down embryos have provided significant insights into the in vivo function of the genes responsible for the mutants or the targeting genes [13], [14]. Here, we discuss the function of Ddx46 in the development of the digestive organs and brain using a newly identified zebrafish Ddx46 mutant, morha4 (mor). Ddx46 is expressed maternally and ubiquitously, and its expression gradually becomes restricted to the digestive organs and brain. Phenotypic analysis of the Ddx46 mutant and the examination of various molecular marker expressions using whole-mount in situ hybridization of the digestive organs and brain showed that zebrafish Ddx46 is required for the development of these organs. Based on RT-PCR analyses, we propose that Ddx46 plays a role in pre-mRNA splicing in the digestive organs and brain during zebrafish development.

Results

The morha4 mutant has defects in the development of the digestive organs and brain

To elucidate the mechanisms that underlie the formation of the intestinal epithelium during development, we took a forward genetic approach. One mutant that we identified, morha4, had defects in intestinal epithelium and retinal development, and showed a recessive larval lethal phenotype. Phenotypic analyses of the morha4 mutant revealed that the swim bladder failed to inflate (Figure 1A–D), the intestine lacked folds (Figure 1C, D, G, and H), and the retinae were smaller than normal (Figure 1E and F) at 5.5 days post fertilization (dpf). In addition, histochemical and immunohistochemical analyses exhibited that the exocrine pancreas and liver in the morha4 mutant were smaller than those in wild-type (WT) larvae (Figure 1I–L, Figure S1), whereas the size of the endocrine pancreas was normal in this mutant (Figure 1I and J). We also found that cell death was increased in the brain, retinae, and intestine in the morha4 mutant but not in the WT at 3 dpf (brain and retinae) or 5 dpf (intestine) (Figure 1M–P). Conversely, the formation of somite was apparently unaffected (Figure 1A and B), and increased cell death was not detected in the morha4 somite at 5 dpf (Figure 1O and P). These results suggest that the morha4 mutant has defects in digestive organ and brain development.

The mor locus encodes Ddx46

The morha4 mutation was meiotically mapped to a region of chromosome 21 defined by two microsatellites, z10508 and z15212_1, in the Zv6 ensemble assembly of the zebrafish genome (Figure 2A). At this point, we learned that the Ddx46 mutation (Ddx46hi2137), which was isolated using a large insertional screening [15] and causes a similar phenotype in morha4 (http://web.mit.edu/hopkins/group11.html), was also positioned on the same region of chromosome 21 (see Figure 2A). Given the similarities between Ddx46hi2137 and morha4, we attempted to position the Ddx46 gene in relation to the mor locus. No recombination was observed between the morha4 phenotype and a Ddx46 intronic polymorphic marker, z12027_1 (see Figure 2A). Thus, both mapping and the phenotype of the Ddx46hi2137 mutant suggested that Ddx46 is a good candidate for the morha4 mutation. To see whether morha4 is a mutation of the Ddx46 gene, Ddx46 cDNA was cloned and sequenced from WT and mutant embryos. Sequencing of the morha4 mutant revealed a T-to-G transversion, which introduced a serine in place of an isoleucine at amino acid position 942 in the C-terminal region of the Ddx46 protein (Figures 2B and S2). The sequence alignment of the human, mouse, chicken, and zebrafish Ddx46 proteins showed a high level of conservation in.

Figure 1. Phenotype of the morha4 mutant. (A–F) Lateral (A–D) and dorsal (E, F) views of live WT and morha4 larvae at 5.5 dpf. The swim bladder failed to inflate (arrows in A, B), the intestine lacked folds (arrowheads in C, D), and the retinae were reduced in size (brackets in E, F) in the morha4 mutant. Conversely, somite formation in the morha4 mutant appeared normal (arrowheads in A, B). (G–L) Sagittal sections of 5.5-dpf larvae stained with hematoxylin and eosin. The intestine lacked folds and was thin walled (arrowheads in G, H), and the exocrine pancreas (blue dotted lines in I, J) and liver (blue dotted lines in K, L) were small in the morha4 mutant. In contrast, the endocrine pancreas (blue dotted lines in I, J) in WT larvae was indistinguishable from that in morha4 larvae. Scale bars, 50 μm. (M–P) Dorsal views, anterior to the top (M, N). Lateral views, anterior to the left (O, P). Apoptotic cells were detected using the TUNEL method. An increase in apoptotic cells was evident in the brain, retinae, and posterior intestine of the morha4 larvae (white arrowheads in O, P) compared to WT larvae, but not in the morha4 somite (white arrows in O, P). en, endocrine pancreas; ex, exocrine pancreas. doi:10.1371/journal.pone.0033675.g001
the C-terminal region among these vertebrates (see Figure S2). We confirmed that this lesion segregated with the mutant phenotypes in 200 meiotic events (data not shown).

Although the viral insertion site of the \( Ddx46^{hi2137} \) mutant was identified in intron 1 of the \( Ddx46 \) gene (Figure 2C; http://web.mit.edu/hopkins/group11.html), no \( Ddx46 \) transcript was detected in the \( Ddx46^{hi2137} \) mutants at 3.5 dpf (Figure 2D). These data indicated that the viral insertion strongly abrogates the transcription of \( Ddx46 \) or transcript stability, as observed previously [16]. To confirm that the loss of \( Ddx46 \) function accounted for the \( mor^{het} \) phenotype, we performed complementation analysis between the \( mor^{het} \) and \( Ddx46^{hi2137} \) alleles. In transheterozygote \( (mor^{het}/Ddx46^{hi2137}) \) larvae, the swim bladder failed to inflate, the intestine lacked folds, and the retinae were smaller than normal-the same phenotype of the \( mor^{het} \) mutant (see Figure S3).

We next performed rescue experiments using both alleles \( (mor^{het} \) and \( Ddx46^{hi2137}) \). As observed with histological section data, the size of the exocrine pancreas, which is detected through \( actb1 \) expression, was markedly reduced in \( egfp \) mRNA-injected \( mor^{het}/Ddx46^{hi2137} \) larvae (17 of 18 larvae rescued; Figure 3B–D). As in \( mor^{het} \) mutants, the size of the exocrine pancreas was also markedly reduced in \( egfp \) mRNA-injected \( Ddx46^{hi2137}/Ddx46^{hi2137} \) mutants (16 of 16 larvae rescued; Figure 3B–D). This result suggested that the function of \( Ddx46 \) is abolished by the \( mor^{het} \) point mutation. Moreover, to investigate the importance of the ATPase activity of \( Ddx46 \) to its function in zebrafish larvae, we introduced a mutation into motif I of the DEAD box (see Figure 3A; substitution from lysine to alanine at amino acid position 402), which is known to disrupt ATPase activity in \( S. pombe \) Prp5 [10]. Overexpression of \( Ddx46^{K402A} \) mRNA in the \( mor^{het} \) mutant larvae failed to rescue the size of the exocrine pancreas (0 of 26 larvae rescued; Figure 3E and F). Our results showed that the defects of the pancreas in both \( mor^{het} \) and \( Ddx46^{hi2137} \) larvae were rescued by the overexpression of \( Ddx46^{hie2137} \) mRNA. Together, genetic data, complementation analysis, and rescue experiments indicated that the \( mor \) gene corresponds to \( Ddx46 \).

**Effect of the \( mor^{het} \) point mutation on \( Ddx46 \) function.**

To investigate the effect of the \( mor^{het} \) point mutation on \( Ddx46 \) function, we also performed rescue experiments using the \( mor^{het} \) mutant gene \( Ddx46^{I942S} \) (Figure 3A). The expression of \( try \) in the \( Ddx46^{I942S} \) mutant was not rescued by the overexpression of \( Ddx46^{I942S} \) mRNA (0 of 21 larvae rescued; Figure 3E and G). Our results showed that the defects of the pancreas in the \( mor^{het} \) and \( Ddx46^{I942S} \) larvae were rescued by the overexpression of \( Ddx46^{I942S} \) mRNA. Together, genetic data, complementation analysis, and rescue experiments indicated that the \( mor \) gene corresponds to \( Ddx46 \).
**Ddx46 for the Digestive Organ and Brain Formation**

**Figure 3. Defects of exocrine pancreas formation in both mor/ha4 and Ddx46 hi2137/hi2137 mutants are rescued by the overexpression of Ddx46 mRNA but not mutated Ddx46 mRNA.** (A) Scheme of the Ddx46 protein structure. The yellow, red, and orange boxes indicate the N-terminal, DEAD-box helicase, and C-terminal domain, respectively. Mutations were introduced into the Ddx46 protein; in Ddx46-I942S, an isoleucine in the C-terminal domain of Ddx46 was changed to serine, which is the same mutation as that in the mor/ha4 mutant; in Ddx46-K402A, KGT in motif I, which is important for ATPase activity in Ddx46 homologues, was changed to GAT. (B–I) All dorsal views, anterior to the top. The expression of try, a molecular marker for the exocrine pancreas, was examined using whole-mount in situ hybridization at 3.5 dpf. The try expression in the exocrine pancreas was markedly reduced in egfp mRNA-injected mor/ha4 (C) and Ddx46 hi2137/hi2137 mutants (F) compared to egfp mRNA-injected control larvae (B, E). The try expression was rescued in the Ddx46 mRNA-injected mor/ha4 (D) and Ddx46 hi2137/hi2137 mutants (G), whereas no rescue was achieved by the overexpression of Ddx46 hi2137+ (H) or Ddx46 hi2137- (I) mRNA into Ddx46 hi2137/hi2137 mutants. Control larvae-sibling WT or mor/ha4 larvae (B–D), sibling WT or Ddx46 hi2137+ larvae (E–I) had normal phenotypes.

doi:10.1371/journal.pone.0033675.g003

**Ddx46 expression is restricted to developing digestive organs and brain**

To define the spatiotemporal expression of Ddx46 in developing embryos and larvae, we performed whole-mount in situ hybridization. Ddx46 was found to be a maternally supplied transcript that was expressed ubiquitously during early somitogenesis (Figure 4A and B). Its expression became restricted to the head region by 24 hours post-fertilization (hpf) (Figure 4C). By 2 dpf, Ddx46 was expressed in the head, retina, digestive organs, and pectoral fin bud (Figure 4D–G), and at 4 dpf, its expression was even more confined to the retinae, telencephalon, midbrain, midbrain-hindbrain boundary, branchial arches, esophagus, liver, pancreas, and intestinal bulb (Figure 4H–K). Transverse section data revealed the presence of the Ddx46 transcript in pancreatic exocrine cells but not in pancreatic endocrine cells (Figure 4L and M). Further, we showed that Ddx46 transcripts were not present in the somite after 4 dpf (see Figures 4H). These Ddx46 expression patterns were consistent with nearly all aspects of the mor/ha4 mutant phenotype.

**Gene expression in the digestive organs and brain is down-regulated in the Ddx46 hi2137/hi2137 mutants**

We showed that the Ddx46 mutant displays defects in the development of the digestive organs and brain. To explore these defects during development, we examined the expression of various molecular markers using whole-mount in situ hybridization. At 2.5 dpf, the expression level and pattern of foxa3 [18] of the control and Ddx46 hi2137/hi2137 larvae were indistinguishable (Figure S4), indicating that the budding of the digestive organs was normal in the Ddx46 hi2137/hi2137 mutants. The expressions of deltaA (dio) [19], [20], and her6 [21] in the brain or retinae were markedly reduced in the Ddx46 hi2137/hi2137 larvae at 3 dpf, however (Figure 5A–D). In addition, we found that the expressions of intestinal epithelium marker fabp2 [22], liver marker fabp10a [23], and exocrine pancreatic marker pancreas specific transcription factor, 1a (ptf1a) [24] were also markedly reduced in the Ddx46 hi2137/hi2137 mutants at 3.5 dpf (Figures 5E–5J). In contrast, expressions of endocrine pancreatic marker preproinsulin (ins) [25] and a myogenesis marker of the somite, myogenic differentiation 1 (myod1) [26], did not change in the Ddx46 hi2137/hi2137 mutant (Figure 5K and L; Figure S5). Consistent with this result, Ddx46 was not expressed in pancreatic endocrine tissues (Figure 4M) or the somite (Figure 4H). We also examined the expression of various molecular markers in the mor/ha4 mutant. Downregulation of the expression levels of dla, fabp2, fabp10a, and ptf1a in the mor/ha4 mutant was less severe than that in the Ddx46 hi2137/hi2137 mutant (Figure 5, Figure S6), suggesting that mor/ha4 is a hypomorphic allele.

We next tested whether the down-regulation of these mRNAs is due to the loss of tissues in the liver and exocrine pancreas in the Ddx46 hi2137/hi2137 mutant. Transverse section data of the Ddx46 hi2137/hi2137 mutant showed that although the size of the
liver and exocrine pancreas is smaller than normal, the tissues of these organs are still present at 3.5 dpf (Figure 5M–P). These results suggested that the amount of mRNAs in these organs is reduced specifically in this mutant.

We also examined the expressions of molecular markers such as dla, fabp10a, ptf1a, and ins in transheterozygote (morha4\textbackslash Ddx46hi2137) larvae at 3 or 3.5 dpf, and found that, with the exception of ins, they were markedly reduced (Figure S7), as observed in the Ddx46hi2137\textbackslash hi2137 mutants. These results further supported the conclusion that the mor gene corresponds to Ddx46.

Furthermore, we found that the expressions of other molecular markers for the digestive organs and brain- her4 (brain and retina) [27], [28], neurogenin 1 (neurog1: brain) [29], neurod (brain and retina) [29], homeo box HB9 like a (hbx9la: exocrine pancreas) [30], carboxypeptidase A5 (cpa5: exocrine pancreas) [31], gata6 (intestine, liver, and exocrine pancreas) [32], and dehydrogenase/reductase member 9 (dhrs9: intestine and liver) [33]-were markedly reduced in the Ddx46hi2137\textbackslash hi2137 larvae from 3 to 3.5 dpf (Figure S8). These results suggested that Ddx46 is required for gene expression in the digestive organs and brain.

Ddx46hi2137\textbackslash hi2137 mutant has defects in pre-mRNA splicing in the digestive organs and brain

Because yeast Prp5 and human DDx46 are known to be involved in pre-mRNA splicing, we tested whether the Ddx46 mutant had defects in this process. For the analyses of pre-mRNA splicing in the Ddx46hi2137\textbackslash hi2137 mutants, we examined the splicing status of four genes (dla and her6 in the brain, and fabp10a and ptf1a in the digestive organs) by performing an RT-PCR analysis that is often used to detect unspliced forms of mRNAs [34]–[36]. The analysis showed that the unspliced mRNAs were retained in the Ddx46hi2137\textbackslash hi2137 mutants at 3 or 4 dpf (Figure 6), suggesting that the pre-mRNA splicing process is defective in this mutant, as observed in yeast.

To test whether the effect on pre-mRNA splicing is restricted to a subset of genes or general, we further examined the pre-mRNA splicing of various genes, including housekeeping genes. Unspliced mRNAs of a housekeeping gene, beta-2-microglobulin (\textit{b2m}) [37], were retained in the heads of Ddx46hi2137\textbackslash hi2137 mutants (Figure S9). In contrast, we found that the splicing of actb1 in the heads of Ddx46hi2137\textbackslash hi2137 mutants was normal compared to that in the heads of control larvae (Figure S9). These results suggest that the effect of pre-mRNA splicing may be specific to a certain set of genes in the Ddx46hi2137\textbackslash hi2137 mutants.

Discussion

Functional significance of the ATPase domain and C-terminal region in Ddx46

All DExD/H-box proteins have nine conserved motifs, which are required for ATP binding and hydrolysis, RNA binding, and helicase activity [5]–[8]. It has been clearly shown that the ATP
hydrolysis of Prp5 is necessary for the stable association of U2 snRNP with pre-mRNA and pre-spliceosome formation in **S. cerevisiae** and **S. pombe** [6], [7]. In this study, our rescue experiments showed that the introduction of a point mutation into the ATPase domain of Ddx46, which disrupts the ATPase activity of **S. pombe** Prp5 (SpPrp5), leads to the loss of the rescue capability of Ddx46 for the Ddx46hi2137/hi2137 mutant phenotype (Figure 3). Therefore, the ATP hydrolysis by the ATPase domain in Ddx46 may be required for the Ddx46 to function during zebrafish development.

In addition to the involvement of the ATPase domain, the role of the N-terminal region in Ddx46 has been reported in SpPrp5 and human DDX46 [10]. Both proteins physically associate with the U1 and U2 snRNPs through their N-terminal regions [10], when they function in pre-mRNA splicing. SpPrp5 contains distinct U1- and U2-interacting domains in its N-terminal region that are required for pre-spliceosome assembly [10]. In contrast to N-terminal region functioning, the function of the C-terminal region of Ddx46 proteins has not yet been analyzed. The

---

**Figure 5. Expression of molecular markers for digestive organs and brain is reduced in the Ddx46hi2137/hi2137 mutant.** (A–D) The expression of dla and her6 was examined using whole-mount in situ hybridization at 3 dpf. All lateral views, anterior to the left. (E–L) The expression of fabp2, fabp10a, ptf1a, and ins was examined using whole-mount in situ hybridization at 3.5 dpf. All dorsal views, anterior to the top. In the Ddx46hi2137/hi2137 mutants, the intensity and area of dla, her6, fabp2, fabp10a, and ptf1a expression were markedly reduced at 3 or 3.5 dpf (A–J; arrowheads in H, J). In contrast, the ins expression in the Ddx46hi2137/hi2137 mutant did not change at these developmental stages (K, L). (M–P) Transverse sections of 3.5-dpf Ddx46hi2137/hi2137 mutant larvae stained with hematoxylin and eosin. The transverse sections were cut at the levels indicated by black dotted lines in E–L. The tissues in the intestinal bulb, liver, and exocrine pancreas were still present in the Ddx46hi2137/hi2137 mutant larvae at 3.5 dpf. Scale bars, 50 μm. en, endocrine pancreas; ex, exocrine pancreas; ib, intestinal bulb; l, liver. Control larvae were sibling WT or Ddx46hi2137/+ larvae and had normal phenotypes. doi:10.1371/journal.pone.0033675.g005
alignment of the Ddx46 proteins of various vertebrates reveals high homology in the C-terminal region (see Figure S2), but to date, no specific motif has been reported in this region. Although the phenotype of the morha4 mutant and our rescue experiments using a morha4 mutant form of Ddx46 (Ddx46-I942S) indicated that the C-terminal region of Ddx46 is critical for its function in zebrafish development, further studies are needed to uncover the role of the Ddx46 C-terminal region and the influence of the morha4 point mutation on Ddx46 function.

In vivo function of zebrafish Ddx46 during development

In this study, we showed that the unspliced mRNAs of dla, her6, ptf1a, and fabp10a were retained in the Ddx46hi2137/hi2137 mutant (Figure S6). We further showed that the splicing of the housekeeping gene actb1, but not b2m, was normal in the heads of Ddx46hi2137/hi2137 mutants (Figure S9). These results, combined with functional analyses of yeast Prp3 and human DDX46, suggest that zebrafish Ddx46 may be required for pre-mRNA splicing during development, and that the effect of splicing may be specific to a certain set of genes in the affected organs. Since four genes (dla, her6, fabp10a, and ptf1a) were selected as simple markers for organ development, it is possible that the defects in the pre-mRNA splicing of genes other than these four lead to the phenotypes of the Ddx46 mutant.

Assessment of pre-mRNA status by RT-PCR, which is not a quantitative analysis, showed that the spliced mRNAs of the five genes (dla, her6, fabp10a, ptf1a, and b2m) we tested were still present in the Ddx46hi2137/hi2137 mutants (Figures 6 and S9). There are two possible explanations for this finding. One is that Ddx46 protein from the maternally inherited transcript may rescue pre-mRNA...
splicing. A previous study revealed that the maternally derived minichromosome maintenance protein 5 persists beyond 3 dpf in zebrafish larvae [38]. This indicates that the maternally derived protein is very stable during early development. Alternatively, it is possible that the five genes (dla, her6, foalp10a, pfla, and k2m) require Ddx46 for their splicing in a subset of tissues, but not in other tissues where splicing may occur in a Ddx46-independent manner. Detailed biochemical analyses will be needed to elucidate the in vivo function of Ddx46 during vertebrate development.

Organ-specific requirement of Ddx46 in zebrafish development

Recent microarray profiling and expression cloning analyses have revealed that some housekeeping genes are expressed in specific tissues or organs, but others have shown ubiquitous expression during development [39], [40]. In zebrafish, microarray profiles and in situ analyses have shown that nucleolar genes, which are generally thought to be ubiquitously expressed, are preferentially expressed in the developing gastrointestinal tract [39]. Consistent with these results, zebrafish mutations in RNA polymerase III [41] and nucleolar protein RBM19 [42] showed specific defects in digestive organ formation during development because these genes are expressed in the digestive organs [41], [42].

Expression cloning screens in Xenopus laevis have revealed that some pre-mRNA splicing genes demonstrate the tissue- or organ-specific expression and function analyzed using whole-mount in situ hybridization and overexpression experiments during development [40]. Moreover, analyses of zebrafish mutants and knockdown experiments have revealed the tissue-specific function of some splicing or splicing-related factors during vertebrate development: subtelomal knockdown of the pre-mRNA processing factor 31 (Prp31) predominantly affects retinal transcripts [43]; the splicing factor proline/glutamine rich (spgr) gene, which is strongly expressed in the developing brain, is required for cell survival and neuronal development [44]; the ubiquitin specific peptide 39 (usp39) gene is involved in embryonic pituitary homeostasis by regulating the retinoblastoma 1 pre-mRNA splicing and E2F transcription factor 4 expression [35]; and a mutation of a p110 protein, which functions in recycling of the U4/U6 snRNPs, leads to organ-specific defects during development [45].

These reports, combined with our results, suggest that some splicing genes may be specific to digestive organ and brain development in X. laevis and zebrafish. It is possible that another redundant DExD/H-box helicase functions in the pre-mRNA splicing in other tissues or organs, where Ddx46 is not expressed.

An alternative possibility is that some transcriptional/post-transcriptional genes, including splicing factors, are not specific to digestive organ and brain development. As observed with Ddx46 knockdown in digestive organ and brain development, high expression levels of some transcriptional/post-transcriptional genes are needed to maintain a high number of cell cycles, because these organs grow particularly fast during larval stages. Further study will be necessary to elucidate the organ-specific requirement of Ddx46 in zebrafish development.

In summary, we demonstrated that a mutation in Ddx46 is responsible for defects in the digestive organs and brain of the zebrafish mutant morha4. Consistent with the phenotype of morha4 or Ddx46 mutant larvae, the expression of Ddx46 was gradually restricted to these organs as development proceeded after 2 dpf. Our rescue experiments revealed that both ATPase and the C-terminal domains of Ddx46 are necessary for its function. Based on our findings, we propose a model in which Ddx46 is specifically expressed in the digestive organs and brain and is required for pre-mRNA splicing in these organs. Future investigations of the function of Ddx46 should lead to a better understanding of the splicing processes during vertebrate development.

Materials and Methods

Ethics statement

At present no approval needs to be given for research on zebrafish because in accordance with Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71 (June 1, 2006) there is no rule on fish use at Hiroshima University.

Zebrafish husbandry and N-ethyl-N-nitrosourea mutagenesis

Zebrafish were obtained from the Zebrafish International Resource Center (Oregon, USA). Adult zebrafish and zebrafish embryos were maintained under a 14-h day/10-h night cycle at 28.5°C. Embryos were incubated in 1/3 Ringer’s solution [39] NaCl, 0.97 mM KCl, 1.8 mM CaCl2, 1.7 mM HEPES, pH 7.2] at 28.5°C and staged according to Kimmel et al. [46]. The Ddx46 allele hi2137 was isolated during an insertional mutagenesis screening [15] (http://web.mit.edu/hopkins/group11.html), and the Ddx46hi2137/hi2137 fish was obtained from the Zebrafish International Resource Center.

A morha4 mutant was isolated during a mutagenesis screen performed in our laboratory. G0 males (AB strain) were mutagenized with N-ethyl-N-nitrosourea as described previously [47]. F1 progeny were grown from G0 males crossed to AB strain females. The F2 family was established by crossing F1 male and female fish. F3 larvae obtained by crossing pairs of F2 fish were fixed at 60 hpf and screened by whole-mount in situ hybridization for the expression of foax3 [18] (number of mutated genotypes screened, 269). Families of larvae that showed abnormal expression of foax3 were subjected to further analyses.

Positional cloning

The mor gene was mapped on a hybrid genetic background, AB/India, via bulked segregant analysis between microsatellite markers Z10508 and Z13212_1 on LG 21 [48]. Based on the Zv6 zebrafish genome database (http://wwwensembl.org/Danio_rerio/Info/Index), the closest marker, Z10207_1, was in the intron of the Ddx46 gene. The cDNA was prepared from pools of mutant or WT sibling larvae using RT-PCR with the following primers: 5’-AAGCTTAGCAGAGAGCCAGAGGAGCG-3’ and 5’-AGACGTGACCTTCCACCTTG-3’. The amplified DNA fragments were amplified with the following PCR primers: 5’-GGATCCGGCGAAGAATTGGGCGAGAGACG-3’ and 5’-AGCTTACAGGAGGCAACCACGAGG-3’, and was sequenced to find the mutaion. To confirm that Ddx46 was tightly linked to the mor mutant, DNA fragments were amplified with the following PCR primers: 5’-TGTGTTGGGCTGAACGCTTG-3’ and 5’-AGACGTGACCTCCACCTTG-3’. The amplified products were digested with MboI and resolved on 1% agarose gels. The mor mutant abolished an MboI site.

Whole-mount in situ hybridization, histology, genotyping, immunohistochemistry, and detection of cell death

Whole-mount in situ hybridizations and histological analyses were performed as described previously [49], [50], and riboprobes were prepared according to published instructions. For histological analysis, embryos were embedded in JB4 (Polysciences), and 7-μm sections were cut with a microtome and stained with hematoxylin and eosin. After whole-mount in situ hybridization and histological analyses, the mor mutant larvae were genotyped as described above. Ddx46hi2137 mutants were confirmed with genotyping using two pairs of primers: one pair derived from the LacZ gene (5’-CAGAGAGCCAGAGGAG-CAGCAGAGGAGCG-3’ and 5’-AGACGTGACCTCCACCTTG-3’).
mRNA injections
To introduce point mutations, we performed site-directed mutagenesis using a QuickChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s instructions. The cDNA fragments for Ddx46 were prepared from control and mutant larvae at 2.5 dpf. Both larvae were processed for carboxypeptidase A immunohistochemistry. The size of the exocrine pancreas was markedly reduced at 3 or 3.5 dpf in the mor^hat^ mutant compared to the WT larva. Scale bars, 50 μm.

Figure S2 The C-terminus region of Ddx46 is highly conserved among vertebrates. Amino acid sequence alignment of the Ddx46 proteins of different vertebrates. The yellow, red, and orange boxes represent the N-terminal, DEAD-box helicase, and C-terminal domains, respectively. The C-terminal region of zebrafish Ddx46 was compared with those of human, mouse, and chicken Ddx46 proteins. Conserved amino acids in at least two species and similar amino acids are highlighted in black and gray, respectively. The red arrowhead and box indicate the mutated amino acid isoleucine found in the mor^hat^ mutant.

Figure S3 Transheterozygote (mor^hat^/Ddx46^{hi2137}) of mor^hat^ and Ddx46^{hi2137} shows the phenocopy of the mor^hat^ mutant. (A-F) Lateral (A–D) and dorsal (E, F) views of live control and mor^hat^/Ddx46^{hi2137} larvae at 5 dpf. The swim bladder failed to inflate (arrows in A, B), the intestine lacked folds (arrowheads in C, D), and the retinæ were reduced in size (brackets in E, F) in the mor^hat^/Ddx46^{hi2137} mutant. Conversely, somite formation in the mor^hat^/Ddx46^{hi2137} mutant appeared normal (arrowheads in A, B). Control larvae were sibling WT, mor^hat^+/+ or Ddx46^{hi2137+/+} larvae and had normal phenotypes.

Figure S4 Expression of foxa3 is unaffected in the Ddx46^{hi2137/hi2137} mutant at 2.5 dpf. (A, B) Expression of foxa3 was examined using whole-mount in situ hybridization. Dorsal views, anterior to the top. The foxa3 expression in control larva (A) was indistinguishable from that in the Ddx46^{hi2137/hi2137} mutant (B) at 2.5 dpf. Control larvae were sibling WT or Ddx46^{hi2137/hi2137} larvae and had normal phenotypes.

Figure S5 Expression of myod1 is normal in the Ddx46^{hi2137/hi2137} mutant. (A, B) Expression of myod1 was examined using whole-mount in situ hybridization. Lateral views, anterior to the left. The myod1 expression in control larva (A) was indistinguishable from that in the Ddx46^{hi2137/hi2137} mutant (B) at 3.5 dpf. Control larvae were sibling WT or Ddx46^{hi2137/hi2137} larvae and had normal phenotypes.

Figure S6 Expression of molecular markers for digestive organs and brain is reduced in the mor^hat^/hat^ mutant. (A-B) The expression of dla was examined using whole-mount in situ hybridization at 3 dpf. All lateral views, anterior to the left. (C-J) The expression of fabp2, fabp10a, ptf1a, and ins was examined using whole-mount in situ hybridization at 3.5 dpf. All dorsal views, anterior to the top. Although the expression of dla, fabp2, and fabp10a was slightly reduced, the ptf1a expression was markedly reduced at 3 or 3.5 dpf in the mor^hat^/hat^ mutants (A–H). In contrast, the ins expression in the mor^hat^/hat^ mutant did not change at these developmental stages (I, J). Control larvae were sibling WT or mor^hat^+/+ larvae and had normal phenotypes.

Figure S7 Expression of molecular markers for digestive organs and brain is also reduced in the transheterozygote mor^hat^/Ddx46^{hi2137} mutant. (A, B) The expression of dla was examined using whole-mount in situ hybridization at 5 dpf. All lateral views, anterior to the left. (C–H) The expression...
of fabp10a, pfg1a, and ins was examined by whole-mount in situ hybridization at 3.5 dpf. All dorsal views, anterior to the top. The intensity and area of dtx, fabp10a, and pfg1a expression were markedly reduced at 3 or 3.5 dpf in the hoxb4Ddx46hi2137 mutants. In contrast, ins expression in this transheterozygote was unchanged at these developmental stages. These phenotypes are the same as those of the Ddx46hi2137/kd2137 mutant. Control larvae were sibling WT, hoxb4+/−/+, or Ddx46hi2137/+ larva and had normal phenotypes.

Figure S8 Expression of various molecular markers for digestive organs and brain is reduced in the Ddx46hi2137/kd2137 mutant. (A–F) The expression of her1, neurog1, and neurod for brain was examined using whole-mount in situ hybridization at 3 dpf. All lateral views, anterior to the left. (G–N) The expression of ghb6/2137, gata6, and ddx9 for digestive organs was examined using whole-mount in situ hybridization at 3.5 dpf. All dorsal views, anterior to the top. In the Ddx46hi2137/kd2137 mutants, the intensity and area of all of these gene expressions were markedly reduced at 3 or 3.5 dpf. Control larvae were sibling WT or Ddx46hi2137/+ larva and had normal phenotypes. (TIF)

Figure S9 Pre-mRNA splicing of the housekeeping gene actb1, but not b2m, is unaffected in the Ddx46hi2137/kd2137 mutant. (A–D) Scheme of the b2m and actb1 pre-mRNA regions analyzed for splicing (boxes, exons; lines, introns; arrows, primers) (A, C). The splicing status of b2m and actb1 pre-mRNA was monitored using RT-PCR with the primers indicated in scheme A and C, respectively. Total RNA was isolated from the heads of Ddx46hi2137/kd2137 mutants (mut) and control (con) larvae. Unspliced b2m mRNAs were retained in the Ddx46hi2137/kd2137 mutants compared to the control larvae (arrowheads in B), whereas the splicing of actb1 was unaffected in the Ddx46hi2137/kd2137 mutants (arrowheads in D). Unspliced and spliced PCR products were verified by sequencing. +RT refers to the validation reaction itself, −RT represents the respective control reaction without reverse transcriptase. 18S RNA was used as a loading control. M, DNA size markers (sizes in bp). Control larvae were sibling WT or Ddx46hi2137/+ larva and had normal phenotypes. (TIF)

Table S1 The list and sequence of primers used for RT-PCR analysis. (XLS)

Table S2 PCR thermal cycler program for RT-PCR. (XLS)

Acknowledgments

We thank Drs. Masahiko Hibi and Masataka Nikaido for providing DNA templates; and Dr. Nobuyoshi Shimoda for helpful advice on positional cloning. During the revision of our paper, we have observed the weak expression of ddx46 in caudal hematopoietic tissue at 4 dpf. Moreover, we have also found that the expression pattern of ddx46 overlaps with that of a molecular marker for hematopoietic stem cells. We intend to report these results elsewhere in the near future.

Author Contributions

Conceived and designed the experiments: SH YK. Performed the experiments: SH RH MO TI MT AK MI YK. Analyzed the data: SH RH YK TI AK MI YK. Wrote the paper: SH YK. Read and edited the manuscript: SH RH MO TI MT AK MI YK.

References

1. Staley JP, Guthrie C (1998) Mechanical devices of the spliceosome: motors, clocks, springs, and things. Cell 92: 315–326.
2. Brow DA (2002) Allosteric cascade of spliceosome activation. Annu Rev Genet 36: 373–390.
3. Smith DJ, Query CC, Konarska MM (2008) "Nought may endure but mutability": spliceosome dynamics and the regulation of splicing. Mol Cell 30: 151–164.
4. Odenthal J, Nüsslein-Volhard C (1998) fork head domain genes in zebrafish. Dev Genes 20: 245–258.
5. Biemar F, Argenton F, Schmidtke R, Epperlein S, Peers B, et al. (2001) Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. Dev Biol 230: 189–203.
6. Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, et al. (1996) Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. Development 122: 271–280.
7. Zecchin E, Mavropoulos A, Devos N, Filippi A, Tiso N, et al. (2004) Evolutive conserved role of ptaf in the specification of exocrine pancreatic fates. Dev Biol 268: 174–184.
8. Milewski W, Dugasay S, Chan S, Steinert D (1998) Conservation of PDX-1 structure, function, and expression in zebrafish. Endocrinology 139: 1440–1449.
9. Biemar F, Argenton F, Schmidtke R, Epperlein S, Peers B, et al. (2000) Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. Dev Biol 230: 189–203.
10. Silverman E, Edwalds-Gilbert G, Lin RJ (2003) DExD/H-box proteins and their metabolism. Nat Rev Mol Cell Biol 5: 232–241.
11. Smith DJ, Query CC, Konarska MM (2008) "Nought may endure but mutability": spliceosome dynamics and the regulation of splicing. Mol Cell 30: 151–164.
12. Biemar F, Argenton F, Schmidtke R, Epperlein S, Peers B, et al. (2001) Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. Dev Biol 230: 189–203.
13. Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, et al. (1996) Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. Development 122: 271–280.
14. Zecchin E, Mavropoulos A, Devos N, Filippi A, Tiso N, et al. (2004) Evolutive conserved role of ptaf in the specification of exocrine pancreatic fates. Dev Biol 268: 174–184.
15. Milewski W, Dugasay S, Chan S, Steinert D (1998) Conservation of PDX-1 structure, function, and expression in zebrafish. Endocrinology 139: 1440–1449.
16. Biemar F, Argenton F, Schmidtke R, Epperlein S, Peers B, et al. (2000) Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. Dev Biol 230: 189–203.
31. dilorio PJ, Moss JB, Shroga JL, Karlstrom RO, Moss LG (2002) Sonic hedgehog is required early in pancreatic islet development. Dev Biol 244: 75–84.
32. Yee NS, Lorret K, Pack M (2005) Exocrine pancreas development in zebrafish. Dev Biol 284: 84–101.
33. Nadalid L, Sanoval IT, Chidester S, Yost HJ, Jones DA (2004) Adenomatous polyposis coli control of retinoic acid biosynthesis is critical for zebrafish intestinal development and differentiation. J Biol Chem 279: 51561–51569.
34. König H, Matter N, Bader R, Thiele W, Müller F (2007) Splicing segregation: the minor spliceosome acts outside the nucleus and controls cell proliferation. Cell 131: 718–729.
35. Rios Y, Melmed S, Lin S, Liu NA (2011) Zebrafish spm39 mutation leads to rb1 mRNA splicing defect and pituitary lineage expansion. PLoS Genet 7: e1001271.
36. Rosel TD, Hung LH, Medenbach J, Donde K, Starke S, et al. (2011) RNA-Seq analysis in mutant zebrafish reveals role of U1C protein in alternative splicing regulation. EMBO J 30: 1965–1976.
37. Ono H, Figueroa F, O’Hugain C, Klein J (1993) Cloning of the beta 2-microglobulin gene in the zebrafish. Immunogenetics 38: 1–10.
38. Röösli JD, Erhardt S, Etzl AK, Driever W (2005) Depletion of minichromosome maintenance protein 5 in the zebrafish retina causes cell-cycle defect and apoptosis. Proc Natl Acad Sci U S A 102: 18467–18472.
39. Stuckenholz C, Lu L, Thaker P, Kamiyuki N, Balassy N (2009) FACS-assisted microarray profiling implicates novel genes and pathways in zebrafish gastrointestinal tract development. Gastroenterology 137: 1321–1332.
40. Dichmann DS, Fletcher RB, Harland RM (2008) Expression cloning in Xenopus identifies RNA-binding proteins as regulators of embryogenesis and Ddx46 for the Digestive Organ and Brain Formation
41. Yee NS, Gong W, Huang Y, Lorent K, Dolan AC, et al. (2007) Mutation of RNA Pol III subunit rpc2/polr3b Leads to Deficiency of Subunit Rrpc11 and disrupts zebrafish digestive development. PLoS Biol 5: e312.
42. Mayer AN, Fishman MC (2003) Nil per os encodes a conserved RNA recognition motif protein required for morphogenesis and cytodifferentiation of digestive organs in zebrafish. Development 130: 3917–3928.
43. Linder B, Dill H, Hirmer A, Brocher J, Lee GP, et al. (2011) Systemic splicing factor deficiency causes tissue-specific defects: a zebrafish model for retinitis pigmentosa. Hum Mol Genet 20: 368–377.
44. Lowery LA, Rubin J, Sive H (2007) Whitesnake/sfpiq is required for cell survival and neuronal development in the zebrafish. Dev Dyn 236: 1347–1357.
45. Trede NS, Medenbach J, Damianov A, Hung LH, Weber GJ, et al. (2007) Network of coregulated spliceosome components revealed by zebrafish mutant in recycling factor p110. Proc Natl Acad Sci U S A 104: 6608–6613.
46. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310.
47. Solnica-Krezel L, Schier A, Driever W (1994) Efficient recovery of ENU-induced mutations from the zebrafish germline. Genetics 136: 1401–1420.
48. Shimoda N, Knapp E, Zinni J, Sim C, Yanada E, et al. (1999) Zebrafish genetic map with 2000 microsatellite markers. Genomics 58: 219–232.
49. Westerfield M (1995) The zebrafish book. Eugene, OR: University of Oregon Press.
50. Mizoguchi T, Verkade H, Heath JK, Kuroiwa A, Kikuchi Y (2008) Sdf1/Cxcr4 signaling controls the dorsal migration of endodermal cells during zebrafish gastrulation. Development 135: 2521–2529.