Mindbomb 2 is dispensable for embryonic development and Notch signalling in zebrafish
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ABSTRACT
The Mindbomb E3 ubiquitin protein ligase (Mib) family of proteins, Mib1 and Mib2, are RING finger ubiquitin ligases that share specific substrates. Mib1 is known to play essential roles in Notch signalling by ubiquitinating Notch ligands in vivo. Conversely, the functions of Mib2 in vivo are not fully understood, although Mib2 ubiquitinates multiple substrates, including Notch ligands, in vitro. To determine the Notch-dependent and Notch-independent functions of Mib2 in vivo, we generated mutant alleles of zebrafish mib2 using transcription activator-like effector nucleases (TALENs). We found that mib2 homozygous mutants were viable and fertile. Notch-mediated functions, such as early neurogenesis, somitogenesis, and pigment cell development, were not affected in mib2 mutant embryos. The lack of Notch-deficient phenotypes in mib2 mutants was not due to compensation by a mib2 maternal gene product because mib2 maternal-zygotic mutants also did not exhibit a distinct phenotype. We also showed that Mib2 does not redundantly act with Mib1 because the genetic ablation of mib2 neither enhanced mib1<sup>−/−</sup>-null nor did it alleviate antimorphic mib1<sup>hs52b</sup>-null phenotypes. Furthermore, the postulated Notch-independent roles of Mib2 in maintaining muscular integrity and N-methyl-D-aspartate receptor (NMDAR) activity were not evident: mib2 mutants did not show phenotypes different from those of the control embryos. These observations suggest that Mib2 is dispensable for embryonic development and does not have redundant functions with Mib1 in Notch signalling at least during early development stages in zebrafish.

KEY WORDS: Mib2, Notch signalling, Neuronal development, Zebrafish, Ubiquitin ligase

INTRODUCTION
Ubiquitination is a posttranslational modification that regulates protein functions and is associated with diverse cellular functions and human diseases (Komander and Rape, 2012; Popovic et al., 2014). E3 ubiquitin ligases play important roles in facilitating ubiquitin transfer from the ubiquitin-conjugating enzyme E2 to the substrate and determining substrate specificity (Pickart, 2001).

Mib2, which is also known as skeletrophin, is a RING finger ubiquitin ligase that was originally cloned from aggregated neuroblastoma cells and shown to interact with alpha-actin (Takeuchi et al., 2003). The domain organization and sequence of Mib2 are similar to those of Mib1, which plays essential roles in activating Notch signalling in metazoans (Itoh et al., 2003; Koo et al., 2005; Nguyen et al., 2007; Zhang et al., 2007a; Yamamoto et al., 2010). Notch signalling is a well-conserved signalling pathway that is required for several processes during embryonic development, such as the generation of neurons and somites, and adult tissue function (Guruharsha et al., 2012). Previous studies showed that Mib2 ubiquitimates Notch ligands in vitro because mouse or Drosophila Mib2 mutants do not show strong Notch loss-of-function phenotypes. Because maternal gene products deposited into eggs are involved in early embryonic development in metazoan, the lack of a strong phenotype of mib2 mutants may be due to the masking of phenotypes in homozygous mib2 mutants by a Mib2 maternal gene product during development. However, the maternal/zygotic function of Mib2 has not been explored.

Moreover, because Mib2 is homologous to Mib1, redundant functions between Mib1 and Mib2 have been examined using double knockout/knockdown animals. In mice, Mib1/Mib2 double knockout does not cause a more severe phenotype than Mib1 single knockout. In contrast Zhang et al. showed the roles of Mib2 and Mib1 are redundant in zebrafish, as evidenced by the fact that mib2 knockdown enhances the phenotypes of mib1-null mutant embryos. These conflicting reports may indicate species differences in Mib2 functions. Alternatively, the morpholino (MO)-mediated knockdown of Mib2 in zebrafish might cause non-specific effects, as evidenced by a recent report demonstrating that MO-mediated knockdown is associated with a high false-positive rate (Kok et al., 2015). Thus, zebrafish mib2 mutant analysis should help resolve this issue.

RING-type E3 ubiquitin ligases regulate diverse cellular functions, and a RING ubiquitin ligase may have multiple substrates (Metzger et al., 2014). Indeed, previous studies suggested that Mib1/Mib2 family ubiquitin ligases have multiple substrates other than the Notch ligand; thus, Mib2 may also play Notch signalling-independent roles in vivo (Tseng et al., 2014). Accordingly, Mib2 is involved in maintaining muscle integrity in vivo in Drosophila, although the Mib2 substrate responsible for this function is not known (Nguyen et al., 2007; Carrasco-Rando and Ruiz-Gomez, 2008). In contrast, the conservation of this function of Mib2 among different species is not well understood. Conversely, the N-methyl-D-aspartate receptor (NMDAR) NR2B subunit was identified as a substrate for Mib2 in vitro, but the role of Mib2 in regulating NMDAR activity has not been investigated in vivo (Jurd et al., 2008).

Here, we generated zebrafish mib2 mutant lines using Transcription activator-like effector nucleases (TALENs) to better understand the in vivo role of Mib2. An analysis of the mib2 mutants did not reveal obvious contributions of Mib2 to Notch signalling,
muscle integrity, and NMDAR activity. Furthermore, redundant roles between Mib1 and Mib2 in Notch signalling were not explicitly evident, at least during early development.

RESULTS

Generation of Mib2 mutant zebrafish

To understand the functions of Mib2 in vivo, we generated three mutant alleles of zebrafish mib2 using transcription activator-like effector nucleases (TALENs), i.e. mib2<sup>cd1</sup>, mib2<sup>cd2</sup> and mib2<sup>cd3</sup> (Fig. 1A,B). Two of the mutant alleles, mib2<sup>cd1</sup> and mib2<sup>cd3</sup>, feature 8 bp deletions, whereas mib2<sup>cd2</sup> features one bp insertion and 8 bp deletions (Fig. 1). All mutations created stop codons in the first ankyrin repeat domain (Fig. 1A,B) (Mib2<sub>cd1</sub>, A538stop; Mib2<sub>cd2</sub>, Q531stop; and Mib2<sub>cd3</sub>, I536stop). We selected mib2<sup>cd1</sup> and mib2<sup>cd3</sup> for further analysis because their mutation can be explicitly evident, at least during early development.

Roles between Mib1 and Mib2 in Notch signalling were not explicitly evident, at least during early development. In the neural cells of mib1 mutant (mib1<sup>ta52b</sup>) and mib2 mutant embryos (Takke et al., 1999). The expression of her4.1 also remained unchanged in mib2<sup>cd3</sup> mutant embryos (Fig. 3B) (mib2<sup>cd3</sup>, 100% with normal expression, n=21), whereas it was reduced in both mib1 mutant (mib1<sup>ta52b</sup> and mib1<sup>cd3</sup>) embryos. However, the phenotype was slightly more pronounced in mib1<sup>ta52b</sup> embryos than in mib1<sup>ta52b</sup> mutant embryos (Fig. 3B) (mib1<sup>ta52b</sup>, 89% with low-level expression, n=9; mib1<sup>ta52b</sup> and mib1<sup>cd3</sup>, 100% with low-level expression, n=6). Furthermore, the collaborative functions of mib1 and mib2 were examined using mib1/mib2 double mutants. The expression levels of elavl3 (Fig. 3A) (mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 92% with high-level expression, n=12; mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 75% with high-level expression, n=8) and her4.1 (Fig. 3B) (mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 83% with low-level expression, n=12; mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 100% with low-level expression, n=7) did not significantly differ between the mib1 mutants and mib1/mib2 double mutants. These data suggest that Mib2 does not play a significant role in Notch signalling during early neurogenesis, alone or in combination with Mib1.

Mib2 is not involved in Notch signalling during early neurogenesis and is not redundant to Mib1

Because Mib2 exhibits sequence homology with Mib1, we investigated the functions of Mib2 alone and in collaboration with Mib1 during early neurogenesis. The expression of an early neuronal cell marker, elavl3, was not dramatically changed in mib2<sup>cd3</sup> mutant embryos at the 3 somite stage (Fig. 3A) (100% with normal expression, n=20). In contrast, embryos homozygous for two alleles of mib1, mib1<sup>ta52b</sup> and mib1<sup>cd3</sup>, showed increased levels of elavl3 expression (Fig. 3A) (mib1<sup>ta52b</sup>, 88% with high-level expression, n=16; mib1<sup>ta52b</sup> and mib1<sup>cd3</sup>, 89% with high-level expression, n=9). We next examined the expression of a Notch target gene, her4.1, in the neural cells of mib1 and mib2 mutant embryos at the 3 somite stage (Takke et al., 1999). The expression of her4.1 also remained unchanged in mib2<sup>cd3</sup> mutant embryos (Fig. 3B) (mib2<sup>cd3</sup>, 100% with normal expression, n=21), whereas it was reduced in both mib1 mutant (mib1<sup>ta52b</sup> and mib1<sup>cd3</sup>) embryos. However, the phenotype was slightly more pronounced in mib1<sup>ta52b</sup> embryos than in mib1<sup>ta52b</sup> mutant embryos (Fig. 3B) (mib1<sup>ta52b</sup>, 89% with low-level expression, n=9; mib1<sup>ta52b</sup> and mib1<sup>cd3</sup>, 100% with low-level expression, n=6). Furthermore, the collaborative functions of mib1 and mib2 were examined using mib1/mib2 double mutants. The expression levels of elavl3 (Fig. 3A) (mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 92% with high-level expression, n=12; mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 75% with high-level expression, n=8) and her4.1 (Fig. 3B) (mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 83% with low-level expression, n=12; mib1<sup>ta52b</sup>; mib2<sup>cd3</sup>, 100% with low-level expression, n=7) did not significantly differ between the mib1 mutants and mib1/mib2 double mutants. These data suggest that Mib2 does not play a significant role in Notch signalling during early neurogenesis, alone or in combination with Mib1.
of Mib2 during early somitogenesis, but the pattern of somite segmentation, as revealed by xirp2a expression, was not affected in MZ mib2<sup>cd3</sup> mutant embryos at 24 hpf (Fig. 4B) (number of somite boundaries; mib2<sup>cd3/</sup>, 19.1±0.5, n=11; MZ mib2<sup>cd3</sup>, 19.3±1.4, n=11). In contrast, her4.1 expression was reduced in mib1<sup>1091</sup>, mib2<sup>cd3/</sup> embryos (Fig. 4A) (mib1<sup>1091</sup>, mib2<sup>cd3/</sup>, 100% with low-level expression, n=10), and the regular spacing of the somites was disrupted (Fig. 4B) (number of boundaries: mib1<sup>1091</sup>, mib2<sup>cd3/</sup>, 16.4±2.3, n=16) because these deficits are due to the disruption of Notch function. However, mib1<sup>1091</sup>: MZ mib2<sup>cd3</sup> double mutant embryos did not show more severe phenotypes than mib1<sup>1091</sup>, mib2<sup>cd3/</sup> embryos, both in her4.1 expression (Fig. 4A) (her4.1: mib1<sup>1091</sup>, mib2<sup>cd3</sup>, MZmib2<sup>cd3</sup>, 100% with a similar level to that in mib1<sup>1091</sup>, mib2<sup>cd3/</sup>, n=5) and somite numbers (Fig. 4B) (mib1<sup>1091</sup>, MZmib2<sup>cd3</sup>, 16.1±1.1, n=14). Therefore, maternal mib2 expression does not compensate for the loss of zygotic Mib2 function.

**Mib2 is not involved in melanophore development and does not antagonize Mib1<sup>ta52b</sup> protein**

A loss of Mib1 function results in a white tail phenotype caused by a decrease in neural crest-derived black melanophores. As reported earlier, we observed the white tail phenotype in the mib1 mutants 2 days post fertilization (dpf), and the phenotype was more severe in mib1<sup>ta52b</sup> than in mib1<sup>1091</sup> embryos (Fig. 5A,B) (mib1<sup>1091</sup>, 100% with mild phenotype, n=7; mib1<sup>ta52b</sup>, 100% with severe phenotype, n=10). In contrast, the tail pigmentation was normal in mib2<sup>cd3</sup> mutant embryos, and the pigment phenotype was not enhanced in mib1<sup>1091</sup>: mib2<sup>cd3</sup> double mutant embryos (Fig. 5A,B) (mib2<sup>cd3</sup>, 100% with normal phenotype, n=18: mib1<sup>1091</sup>; mib2<sup>cd3</sup>, 100% with similar phenotype to mib1<sup>1091</sup>, n=11).

**Notch-independent roles of Mib2 in muscle and NMDAR activity are not apparent in vivo**

Mib2 is assumed to be involved in muscle integrity in zebrafish, as reported for Drosophila (Nguyen et al., 2007; Carrasco-Rando and Ruiz-Gomez, 2008). Therefore, we examined slow and fast muscle formation by detecting the levels of myoD mRNA and myosin heavy chain protein at 24 hpf. The level of myoD mRNA, which is expressed in both slow and fast muscles, was not dramatically changed in MZ mib2<sup>cd3</sup> mutant embryos (Fig. 6A) (100% with normal expression, n=18). Likewise, slow muscle formation was not dramatically affected in mib2<sup>cd3</sup> mutants, as detected by F59 antibody, which recognizes myosin heavy chain protein (Fig. 6B) (100% with normal expression, n=5).

Previously, Jurd et al. suggested that Mib2 negatively regulates the functional activity of NMDAR by ubiquitinating its NR2B
subunit (Jurd et al., 2008). Unfortunately, we could not measure the NR2B protein level in the mib2cd3 mutant due to the unavailability of zebrafish NR2B-specific antibody. Therefore, we utilized an ammonia toxicity assay to evaluate NMDAR activity in zebrafish. Hyperammonaemia leads to elevated extracellular glutamate concentrations, which hyperstimulates NMDAR (Kosenko et al., 2003). Several NMDAR antagonists prevent hyperammonaemia-induced death in zebrafish (Feldman et al., 2014). As reported previously, we observed that ammonium acetate (NH4Ac) treatment significantly decreased survival compared with the control sodium acetate (NaAc) treatment (Fig. 7) (NaAC WT vs NH4Ac WT; P<0.001). However, NH4Ac treatment did not reduce the survival of mib2cd3 embryos compared with that of wild type control embryos (Fig. 7) (NH4Ac wt vs NH4Ac mib2cd3; P=0.61).

These results suggested that Mib2 might not regulate muscle integrity and NMDAR activity in zebrafish during embryogenesis.

**DISCUSSION**

**Mib2 does not play important roles in Notch signalling during development**

Previous studies show that Mib2 ubiquitinates the Delta and Jagged proteins to enhance their endocytosis *in vitro* (Koo et al., 2005; Takeuchi et al., 2005). These actions are similar to that of Mib1, a paralogue of Mib2, which is known to be essential for activating Notch signalling *in vivo*. Here, we show that Mib2 is dispensable, at least during neurogenesis, somitogenesis, and pigment cell development, and for Notch signalling in zebrafish. Similar observations have been made in the mouse and Drosophila (Koo et al., 2007; Nguyen et al., 2007; Wu et al., 2007). Furthermore, our data suggest that the maternal gene product of Mib2 does not play a critical role during zebrafish development. These studies suggest that Mib2 is not essential for activating Notch signalling via the ubiquitination of Notch ligands during metazoan development.
Redundancy between Mib1 and Mib2 functions

Because Mib1 and Mib2 share substrates and form hetero-oligomers in vitro, they may act in a redundant fashion (Koo et al., 2005; Takeuchi et al., 2005; Zhang et al., 2007a). However, functional redundancy between Mib1 and Mib2 in vivo has been controversial in different species. Koo et al. reported that Mib1 and Mib2 do not act redundantly to control mouse embryonic development because a Mib1/Mib2 double knockout does not enhance the phenotypes of Mib1 knockout mice (Koo et al., 2007). In contrast, Zhang et al. suggested that these proteins play redundant roles based on the characterization of zebrafish antimorphic mib1 alleles (Zhang et al., 2007b).

In zebrafish, a phenotypic comparison of different mib1 alleles revealed that mib1as2b (Mib1-M1013R) results in more severe defects than those of other alleles that produce a premature stop codon, such as mib1604 (Zhang et al., 2007b). Therefore, mib1as2b is an antimorphic but not dominant-negative allele because it is inherited in a recessive manner. This recessive antimorphism is a rare genetic phenomenon and was recently reported for the strongest allele of the TSO1 gene in Arabidopsis (Sijacic et al., 2011). The recessive antimorphic allele can produce a phenotype more severe than null by interfering with the function of family genes. In accordance with this function, Zhang et al. suggested that Mib2 may be involved in the more pronounced phenotype in mib1as2b, i.e. the roles of Mib2 and Mib1 are redundant, because mib1-null mutants (mib1604) with mib2 knockdown phenocopy mib1as2b mutants and mib2 mRNA injection partially rescues mib1as2b mutant phenotypes. On the contrary, our study showed that the genetic ablation of mib2 neither enhances mib1604 phenotypes nor alleviates mib1as2b phenotypes, suggesting that the actions of endogenous Mib2 and Mib1 are not redundant.

Two possibilities may account for this discrepancy. First, the truncated Mib2 protein, which is produced by the mib2a allele but not by the mib2-MO knockdown, might exert residual functions to support Mib1/Mib2 hetero-oligomer activity. However, this possibility is unlikely because the level truncated mib2a protein itself may be reduced due to its mRNA decay. Second, other protein(s) may compensate for a loss of Mib2 function in the mib2a allele during development, but this developmental compensation is precluded by morpholino-mediated mib2 knockdown. Supporting this notion, Rossi et al. recently reported the activation of a compensatory network to buffer against genetic deleterious mutations, which was not observed after translational or transcriptional knockdown (Rossi et al., 2015). Future studies should investigate the compensatory activation of genes in mib2a mutants.

Notch signalling-independent functions of Mib2

Several Notch-independent functions of Mib2 have been reported (Nguyen et al., 2007; Jurd et al., 2008; Stempin et al., 2011). In Drosophila, Mib2 plays a Notch-independent role in muscle integrity and survival (Nguyen et al., 2007; Carrasco-Rando and Ruiz-Gomez, 2008). However, our study and others show that in zebrafish, Mib1 but not Mib2 is sufficient for the maintenance of somite integrity (Pascoal et al., 2013). Therefore, the role of Mib family proteins in the muscular system may differ by species.

The NR2B subunit of the NMDAR is a potential substrate for Mib2 and is negatively regulated by Mib2 in a ubiquitin-proteasome-dependent manner in vitro (Jurd et al., 2008). In the absence of Mib2 function, NMDAR activity might be upregulated due to the increased NR2B protein level. However, Mib2 deficiency does not affect NMDAR activity in zebrafish embryos, as assessed based on the neurotoxic effects of ammonia, suggesting that Mib2 may not be involved in NMDAR activity. However, one caveat associated with this interpretation is that the ammonia toxicity assay is not sufficiently sensitive to determine the effect of Mib2 on NMDAR activity in vivo. Future studies should address this issue.

MATERIAL AND METHOD

Zebrafish lines and maintenance

The zebrafish were raised and maintained under standard conditions with approval by the Institutional Animal Care and Use Committee at Chiba University. Zebrafish embryos were obtained from the natural spawning of wild-type adults or identified carriers, which were heterozygous for mib2w01, mib2a, mib1p001, mib2a, mib1ps2b, mib2a, and mib1p001 and homozygous for mib2a.

Construction of TALEN plasmids

The plasmids used to synthesize TALEN mRNAs were constructed as described previously (Hisano et al., 2015). RVD repeat arrays were cloned into pCS2TAL3DD and pCS2TAL3RR to generate left and right TALEN constructs (mib2-TALEN-F and mib2-TALEN-R). The amino acid sequences of the constructed TALENs are shown in Table S1.
mRNA and morpholino antisense oligonucleotide injection

To microinject TALEN mRNA, the mih2-TALEN-F and mih2-TALEN-R plasmid were linearized and transcribed with SP6 RNA polymerase using the mMessage mMachine Kit (Life Technologies). The mih2 forward and reverse TALEN mRNAs (400 pg each) were injected together into the blastomeres of zebrafish embryos at the one-cell stage. The Mib1 morpholino was obtained from Gene Tools and used as described previously (Itoh et al., 2003).

Whole-mount in situ hybridization and antibody staining

Whole-mount in situ hybridization was performed as described previously (Yamamoto et al., 2010). The zebrafish mih2 probe was generated from a cRNA TOPOII vector plasmid in which the mih2 CDNA fragment was subcloned. All probes have been previously published: hox4.1 (Takke et al., 1999), elaiv3 (Kim et al., 1996), mib2a (Schröter and Oates, 2010). Whole-mount antibody staining was performed using the following antibodies: anti-Myosin heavy chain (F59, DSHB) and Alexa-488 anti-mouse IgG (Invitrogen).

PCR-based restriction fragment length polymorphism typing

Genotyping was performed using following primers and restriction enzymes. Fragments were confirmed by electrophoresis.

\[
\text{csl1: } \text{Fw}, \text{CTGCTACGTCACAAGCATATAT} \text{ and Rev, ATAACGAT-}
\text{TTCGAGCAGGAAG. Restriction enzyme, } \text{SstI (TAKARA BIO, Japan).}
\]

Fragment size: wild type, 130 bp; heterozygote, 150+130 bp; homozygote, 150 bp.

\[
\text{cd1: } \text{Fw, GGTTGACATTAAGACCCAGAGAAAG and Rev, GCGGCCTGCTCCCTATCATTTGCTC. Restriction enzyme, } \text{HindIII (TOYOBO, Japan).}
\]

Fragment size: wild type, 100 bp; heterozygote, 130 bp+100 bp; homozygote, 130 bp.

Quantitative RT-PCR (q-PCR)

Total RNA was obtained using RNAiso Plus (TaKaRa Bio, Japan) according to the manufacturer’s protocol. Total RNA was reverse transcribed using ReverTra Ace (TOYOBO) according to the manufacturer’s protocol. The zebrafish mih2 gene sequence was retrieved from the UCSC Genome Browser for real time PCR (http://genome.ucsc.edu/cgi-bin/hgGateway). The primers were designed by Primer3web version 4.0.0 (http://primer3.ut.ee/). Primer specificity was confirmed by NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The transcript levels of mih2 and Rpl13α were confirmed by elavl3 and mib2a. Fragments were confirmed by electrophoresis.

Ammonia toxicity assay

One dfp embryos were arrayed in a 12-well plate with 3 ml of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄). At 4 dpf, the larvae were treated with sodium acetate (NaAc; Wako, Japan) or ammonium acetate (NH₄Ac; Wako). The survival time was assessed every 30 min based on the presence/absence of a visible heartbeat. The Mantel–Cox log-rank test was used to statistically analyse the data using the Prism 6 software (GraphPad).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.M. and M.I. conceived and designed the experiments, S.M., M.N., A.K. and T.M. performed the experiments, S.M., T.M. and M.I. analysed the data and wrote the paper.

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Supplementary information

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