Mind bomb 1 is essential for generating functional Notch ligands to activate Notch

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Summary

The Delta-Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism essential for cell fate specification. Mind bomb 1 (Mib1) has been identified as a ubiquitin ligase that promotes the endocytosis of Notch ligands. In accordance with the endocytosis of Delta. We now report that Mib1 interacts with and regulates all of the Notch ligands, jagged 1 and jagged 2, as well as Dll1, Dll3 and Dll4. Our results show that Mib1 is an essential regulator, but not a potentiator, for generating functional Notch ligands to activate Notch signaling.

Key words: Notch signaling, Mind bomb, Endocytosis, Notch ligand, Mouse

Introduction

The Notch signaling pathway controls embryonic cell-fate decisions in a variety of cell lineages in flies, worms and mammals (Artavanis-Tsakonas et al., 1999). Improper Notch signaling by genetic alteration often leads to developmental defects or cancer in humans and rodents (Harper et al., 2003; Radtke et al., 2002). Thus, it is essential to identify and understand the key components of the Notch signaling pathway.

The core components in Notch signaling include the ligands Delta and Serrate, the receptor Notch, and the transcription factor Suppressor of Hairless [Su(H)] in Drosophila. Notch signaling is initiated by the interaction of the Notch receptor with its ligands (Lai, 2004; Schweigskuth, 2004). These interactions induce proteolytic cleavage (S2) of the Notch receptors, which results in membrane-bound Notch fragments (Brou et al., 2000). After the S2 cleavage by metalloproteases, the remaining receptor fragments are cleaved at a third site (S3) within the membrane, by γ-secretase complexes containing presenilin 1 and presenilin 2, nicastrin and Aph1 (De Strooper, 2003; Mumm et al., 2000). The released intracellular fragments of Notch (N TLCd) translocate to the nucleus to form transcriptional activator complexes with Su(H)/CBF1/RBP-Jκ. These complexes activate Notch target genes, such as Hairy/E(spl)-related basic helix-loop-helix (bHLH) repressors (Iso et al., 2003).

Although much is known about Notch signal transduction after the receptor undergoes the ligand-dependent S2 cleavage, the mechanism by which the Notch ligands engage Notch and trigger its cleavage is less understood. It has been proposed that the endocytosis of Notch ligands on signal-sending cells that are bound to Notch on adjacent signal-receiving cells induces the S2 and S3 cleavage of the receptor, thus activating signal transduction (Parks et al., 2000). Delta-Notch interactions result in the endocytosis of Delta in the signaling cell, which carries along the bound Notch extracellular domain, and endocytosis-defective Delta mutants have reduced signaling capacity (Parks et al., 2000). These studies in Drosophila suggested that the endocytosis of Notch ligands might be important for effective Notch signaling.

To date, there are two candidate genes, neuralized (Neur; Neurl in mouse) and mind bomb 1 (Mib1) that promote the ubiquitination and the endocytosis of Notch ligands. The neur and mib1 mutants have defects in Notch activation in Drosophila and zebrafish, respectively (Boulianne et al., 1991; Itoh et al., 2003). However, disruption of the Neur1 gene in mice did not generate the characteristic Notch phenotypes displayed by Drosophila neur mutants, suggesting that unknown murine Neur1 homologues might compensate for the loss of Neur1 expression in mammals (Ruan et al., 2001; Vollrath et al., 2001). This discrepancy also raised the possibility that Mib1 is the functional homologue of Neur1 in
mice, because both Neur and Mib1 interact with Delta and promote its endocytosis through the ubiquitination (Le Borgne and Schweisguth, 2003).

Zebrafish mib1 mutants exhibit not only a severe neurogenic phenotype, but also a wide range of additional defects in the development of somites, neural crest and vasculature, all indicative of defective Notch signal transduction (Jiang et al., 2000; Jiang et al., 1996; Lawson et al., 2001). The phenotypes of zebrafish mib1 mutants are much more severe than those of deltaA (dx2), deltaD (after eight) and notch1 (deadly seven) (Bingham et al., 2003; Gray et al., 2001; Riley et al., 1999). These remarkable phenotypes have suggested that mib1 is likely to encode a core component of the Notch pathway in zebrafish. However, the lack of other zebrafish mutants with pan-Notch defects prevents a comparative study between mutants. We reported that Mib1 promotes the ubiquitination of zebrafish DeltaD and DeltaB, suggesting that Mib1 might regulate multiple ligands (Itoh et al., 2003). However, there are four Delta homologues (deltaA, deltaB, deltaC and deltaD) and three jagged (Jag) homologues (jagged1, jagged2 and jagged3) in zebrafish. Thus, it is necessary to determine whether Mib1 regulates other ligands, such as jagged homologues and other Delta homologues, and whether Mib1 is an essential core component in Notch signaling from nematodes to mammals.

In this study, we examined whether Mib1 plays an essential role in Notch signaling pathways by generating Mib1-gene targeted mice. Mib1-deficient mice exhibited pan-Notch defects, such as a lack of somitogenesis, impaired vascular remodeling and accelerated neurogenesis. Consistent with these findings, Mib1−/− embryos showed completely defective Notch activation, in terms of Nicd generation and Notch-target gene expression. Interestingly, Mib1 directly interacts with all of the known canonical Notch ligands [Delta-like (Dll) 1, 3 and 4, Jag1 and Jag2]. These data show that Mib1 is an essential core component of the mammalian Notch pathway that controls the function of multiple Notch ligands.

Materials and methods
Generation of Mib1 knockout mice
The IRES-lacZ-puro cassette was fused to exon 6, with the deletion of exon 7 encoding amino acid 303 to 364 of the murine Mib1 protein (see Fig. S1A in the supplementary material). E14K ES cells were screened, and six clones showed homologous recombination. Three clones were used to generate chimeric mice after injection into C57BL/6 blastocysts. Subsequent breeding was carried out with C57BL/6 mice to generate congenic mice and with FVB/N to test the effects of the genetic background.

In situ hybridization
Details of the RNA in situ hybridizations on whole-mount or sectioned embryos were described (de la Pompa et al., 1997). Antisense DIG-labeled (digoxigenin) riboprobes were generated from pGEM-T vectors (Promega) containing amplified cDNA fragments (about 700–800 bp). Staining patterns were confirmed by comparisons with previously published data, except for Mib1. Probe information can be provided on request.

Histology and immunohistochemistry
For histological analysis, embryos and tissues were fixed in 4% paraformaldehyde overnight at 4°C and 4 μm sections were cut and stained with Hematoxylin and Eosin. Sections were incubated with antibodies (Abs) for Nestin (Chemicon) and huC/D (Molecular Probes) and then with Alexa-546-conjugated anti-mouse IgG Ab (Molecular Probes). For BrdU labeling, pregnant mice were injected with BrdU (150 μg/g) 2 hours before they were sacrificed. BrdU-incorporation was analyzed with an anti-BrdU FITC-conjugated Ab (BD). Apoptotic cells were detected by an in situ Cell Death Detection Kit (Roche). For Dll1 staining, 10 μm cryosections were stained with anti-Dll1 (T-20; Santa Cruz Biotechnology) Ab, followed by an Alexa-594-conjugated secondary Ab. Endothelial cell staining of whole-mount preparations was performed with a Flik1 antibody (Avas 12x1, BD) and a PECAm antibody (MEC13.3, BD), using the Vectastain Elite ABC kit (Vector Laboratories).

RT-PCR analysis
Total RNA was extracted from complete yolk sacs and embryos, using an RNasey Micro kit (Qiagen) according to the manufacturer’s instructions. Aliquots of 1 or 2 μg RNA were used for reverse transcription (Omniscript RT, Qiagen) with oligo-dT priming. Real-time RT-PCR reactions with SybrGreen quantification were set up with 1/25 of each cDNA preparation in a Roche LightCycler. Relative expression levels and statistical significance were calculated based on a β-actin standard, using the LightCycler software. All amplicons (100–200 bp) showed efficient amplification that allowed us to equate one threshold cycle difference. Primer information can be provided on request.

cDNA cloning and plasmid construction
The mouse Mib1, Dll1, Dll3, Dll4, Jag1 and Jag2 cDNAs were cloned into the pGFP-N3 (Clontech) or pCS-MT3 vectors. The AEN1 and Dll1 cDNAs were cloned into the HpaI site of pMSCV. D. Hayward kindly provided the 8× wild-type and 8× mutant CBF Luc. All of the cDNAs amplified by PCR were sequenced and tested for expression by Western blotting.

Western blot analysis and co-immunoprecipitation assay
Embryos were homogenized in lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 M EDTA] containing a protease inhibitor mixture (Roche). Generally, 25–40 μg of protein containing supernatants were separated by size, blotted with primary and secondary Abs and visualized with ECL plus (Amersham Biosciences). The primary Abs used were as follows: rabbit anti-mouse DIP-1/Mib1 (gift from Dr Gallagher), rabbit anti-actin (Sigma), rabbit anti-mouse Hes5 (Chemicon), rabbit anti-N1icd (Cell Signaling) and mouse anti-Notch1 (mN1A; Chemicon). Immunoprecipitation was performed previously described (Koo et al., 2005).

Isolation of embryonic fibroblasts, MSCI infection, Luc assay, and neurosphere forming assay
Embryonic fibroblasts were isolated from Trypsin/EDTA digested E9.5 embryos. For MSCI virus infection, a high titer virus soup was produced with gp2-293 cells transfected with pMSCV (Clontech) and VSV-G vectors. Embryonic fibroblasts were infected for 24 hours and selected to eliminate the uninfected cells. For the CBF-Luc assay, the 8× wild-type and mutant CBF luc cassettes were transfected with pRL-TK, using Lipofectamine 2000 (Invitrogen). Luciferase activities were measured with a Dual Luciferase kit (Promega). Neurospheres were generated as described (Grandbarbe et al., 2003).

Subcellular localization analysis and flow cytometry
COS7 cells were transiently transfected with various cDNAs. Subcellular localization analysis was performed previously described (Koo et al., 2005). To detect the internalization of XD, the cells were detached with dissociation buffer (Sigma) and stained with anti-HA Ab (Santa Cruz Biotechnology) followed by anti-mouse Ab conjugated with PE (BD). All samples were analyzed by flow cytometry using a FACSscan (BD).
Results

Generation of Mib1−/− mice

Mib1−/− mice were generated as described in the supplementary material (see Figs S1, S2). At E9.5, homozygous Mib1−/− embryos were severely growth retarded, but they were present in the expected Mendelian ratio (see Table S1 in the supplementary material). Mib1−/− embryos that approximately resembled their littermates with respect to size and developmental stage could be found at E8.5, but only dead and resorbed embryos were found at E11.5-E12.5.

Pleiotropic Notch defects in embryos lacking Mib1

At E9.5, the Mib1−/− embryos always lacked blood circulation and were posteriorly truncated. Heart looping did not occur, and the Mib1−/− embryos displayed an enlarged balloon-like pericardial sac (Fig. 1B). Somitogenesis had begun albeit irregular and fused, and embryonic turning occurred in the majority of the mutants. The optic vesicles, the otic vesicles and the first branchial arches were also formed. However, the second branchial arches were completely absent (Fig. 1D). Although an initial vascular plexus and primitive red blood cells formed, the organization into a discrete network of vitelline vessels did not occur. Furthermore, the yolk sacs had a blistered appearance. Toluidine Blue staining of semi-thin sections from wild-type and mutant yolk sacs revealed that the mutants had only small capillaries, and lacked large vitelline collecting vessels (Fig. 1F).

Transverse sections showed that the Mib1−/− embryos had smaller and thinner hearts, with broaden pericardial cavity...
when compared with the wild-type embryos (Fig. 1H). These sections also revealed that the mutants had a smaller dorsal aorta, and displayed loss of mesenchyme cells and fusion of the notochord to the neural tube (Fig. 1H). These data suggest that Mib1 might be essential for somitogenesis, vasculogenesis and cardiogenesis, which are reminiscent of the Notch-related phenotypes.

**Impaired somitogenesis in Mib1<sup>−/+</sup> embryos**

At E8.5, the Mib1<sup>−/+</sup> embryos were of normal size and appearance, but failed to show normal somite segmentation (not shown). Transverse sections of wild-type embryos and coronal sections of Mib1<sup>−/+</sup> embryos at E9.5 also revealed unevenly divided somites, with kinked neural tubes in Mib1<sup>−/+</sup> embryos (Fig. 1I,J), while six or seven irregular somites were present in anterior region of the E9.0 wild-type and Mib1<sup>−/+</sup> embryos (Fig. 1K). To characterize the somitogenesis defects in the Mib1<sup>−/+</sup> embryos, we analyzed the expression of Uncx4.1, Dll1, Hes7, lunatic fringe (Lfng) and Heyl. The expression of Uncx4.1, a homeobox gene expressed in the posterior half of each somite (Leitges et al., 2000), was undetectable in the segmental plate of E8.5 Mib1<sup>−/+</sup> embryos (Fig. 1L). Consistently, the expression of Dll1 in the posterior half of each somite was also absent (Fig. 1M). Hes7 and Lfng expression normally oscillates in the presomitic mesoderm (PSM) of E8.5 wild-type embryos, but the Hes7 expression was disturbed and Lfng expression was lost in the Mib1<sup>−/+</sup> embryos (Fig. 1N,O). Interestingly, the expression of Heyl, another Notch target gene, was completely absent (Fig. 1P). Taken together, the Mib1<sup>−/+</sup> embryos show a lack of somatic polarity and oscillation, whereas their somitic myogenesis is not altered in very early embryogenesis.

**Premature neurogenesis in Mib1<sup>−/+</sup> embryos**

As in zebrafish, murine Mib1 mutants also exhibit a severe neurogenic phenotype. A histological analysis of Mib1<sup>−/+</sup> embryos isolated at E9.5 revealed localized areas of cell death, which did not seem to be random, but instead appeared preferentially in regions of the brain tissue. Picnotic cells were found in the neuroepithelium of the central nervous system, particularly in the optic lobe and the hindbrain (Fig. 2B,D,H).

At E9.0~9.5, the forebrain and hindbrain of wild-type embryos mostly consisted of nestin-positive neural precursor cells, and only a small population of cells became huC/D

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**Fig. 2.** Premature neurogenesis in Mib1<sup>−/+</sup> embryos. (A,B) Sagittal sections of wild-type (A) and Mib1<sup>−/+</sup> (B) embryos at E9.5. The Mib1<sup>−/+</sup> embryo has a smaller head, with picnotic cells in the brain and inside the ventricles. Enlarged views of the forebrain are shown in insets. (C-H) Sagittal and transverse sections of E9.0-E9.5 brain regions from wild-type (C,E,G) and Mib1<sup>−/+</sup> (D,F,H) embryos. Sections were stained for nestin (C,D; in red) and huC/D (E-H; in red). BrdU incorporation (E,F; in green) and TUNEL staining (C,D,G,H; in green) were used for the detection of proliferative and apoptotic cells, respectively. Nuclear DNA was stained with Hoechst (in blue). (I) Impaired neurosphere formation in Mib1<sup>−/+</sup> forebrains. Neural stem cells are absent in E9.5 (n=3) and E9.75 (n=5) Mib1<sup>−/+</sup> forebrains. (J) Expression of neurogenin 1 (Ngn1), Dll1, Hes5 and Lfng in the neural tubes of E9.5 wild-type and Mib1<sup>−/+</sup> embryos. (K,L) Neurogenin 1 (Ngn1) (K) and NeuroD1 (L) expression in E9.5 wild-type (left) and Mib1<sup>−/+</sup> (right) embryos. Note that the expression of both Ngn1 and NeuroD1 in the trigeminal ganglia is increased in the Mib1<sup>−/+</sup> embryos (arrows), and Ngn1 expression is also increased in the neural tube of the Mib1<sup>−/+</sup> embryos (asterisk).
positive committed early neurons of the pial surface (Fig. 2C,E,G). By contrast, most of the brain cells in mutant embryos became huC/D positive (Fig. 2F,H). Interestingly, in spite of the massive neurogenesis, most of the cells still remained nestin positive in the mutant brains (Fig. 2D), which is similar to their phenotype in the Hes1/5DKO mutant brain (Ohtsuka et al., 1999). In the Mib1−/− mutant brains, most of the huC/D and nestin double-positive neuronal cells might be postmitotic differentiating neurons. Consistent with the premature differentiation into immature neurons in Mib1−/− mice, the mutant brains had virtually no BrdU-positive cells, whereas the wild-type brains had proliferative zones along with the ventricle (Fig. 2E,F), indicating that the nestin-positive cells in the mutant brain were not proliferative. Moreover, the E9.5 and E9.75 Mib1−/− embryos lacked neosphere-forming cells, suggesting the absence of a neuronal stem cell population in these stages (Fig. 2I). The mutant embryos had many TUNEL-positive cells, and large numbers of detached apoptotic cells were often visible as a mass in the ventricle (Fig. 2D,F,H).

To further examine neurogenesis in Mib1−/− embryos, we used in situ hybridization to analyze the expression of neurogenin 1 and Neurod1, which are bHLH transcription factors that are expressed during neuronal determination and neuronal differentiation, respectively (Ross et al., 2003). In E9.0 wild-type embryos, both neurogenin 1 and Neurod1 were expressed mainly in the developing trigeminal ganglia (Fig. 2K,L). In E9.0 Mib1−/− embryos, neurogenin 1 was ectopically overexpressed in the neural tube, and both markers were highly induced in the trigeminal ganglia (Fig. 2K,L). To test whether the massive neurogenesis in Mib1−/− embryos is caused by a lack of Notch signaling, we examined the Hes5 and Lfng expression in the neural tube. As expected, the Mib1−/− embryos lacked Hes5 and Lfng expression, whereas Dll1 expression was upregulated (Fig. 2J). Thus, Mib1 is a critical component of Notch-mediated lateral inhibition in neurogenesis (de la Pompa et al., 1997; Ma et al., 1998).

Vascular defects with the loss of arterial fate in Mib1−/− embryos

Dll4+/, Notch1+/- and Hey1/2DKO mice comprise the molecular cascades for suitable arterial fate decision (Dll4→Notch1→Hey1/2) (Duarte et al., 2004; Fischer et al., 2004; Gale et al., 2004; Krebs et al., 2000; Krebs et al., 2004). We examined whether Mib1−/− embryos also have similar vascular defects, with the loss of arterial fate decision. For a detailed analysis of the vasculature, the E9.5 wild-type and Mib1−/− embryos were immunostained with an anti-Flikl antibody to detect endothelial cells and endothelial precursors (Fig. 3A-D). The Flikl expression revealed that vasculogenesis had occurred in both the wild-type and Mib1−/− embryos. In the Mib1−/− embryos, however, the development of the vasculature was impaired. In the head region, the vessels were thin and

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Fig. 3. Vascular defects in Mib1−/− embryos. (A-D) Whole-mount Flik1 antibody staining of E9.0 wild-type (A,C) and Mib1−/− (B,D) embryos. Mib1−/− embryos have relatively thin and disorganized blood vessels. (A,B) Lateral view of the head; (C,D) Dorsal view of the trunk. (E-N) Transverse sections of E9.0 wild-type (E,G,I,K,M) and Mib1−/− (F,H,J,L,N) embryos, stained with an anti-PECAM antibody (E,F) or labeled by in situ hybridization with specific probes for ephrin B2 (G,H), Sm22 (I,J), Dll4 (K,L) and Hey1 (M,N). The PECAM-stained sections revealed the marked reduction or loss of the dorsal aorta (da) in the Mib1−/− embryos. The lack of vascular ephrin B2 expression (H; inset), smooth muscle cell recruitment (J; inset), and Hey1 expression (N; inset) in the Mib1−/− embryos is evident, whereas the Dll4 expression is normal (L; inset). (O,P) Expression of vascular Notch target genes (Hey1 and Hey2) and genes for vasculogenesis (ephrin B2, EphB4, Vegf and Shh). Total RNA from E9.0 wild-type and Mib1−/− yolk sacs was analyzed by semi-quantitative RT-PCR (O). The expression of Hey1 and Hey2 was analyzed by real-time quantitative RT-PCR (P). The numbers on each bar indicate the mean fold of induction, and the error bars indicate the standard deviation. β-actin was used for normalization. The results are representative of three independent experiments. ***P<0.0001, #P<0.01.
truncated, and did not form a finely branched tree (Fig. 3B). The intersomitic vessels that form through angiogenic sprouting were not present (Fig. 3D). This might be caused by the lack of somitic structures. However, the anterior part of the intersomitic vessels also showed an irregular ladder shape, where the somitic defects were rather mild.

To examine the defects in arterial fate decision, we analyzed the expression of PECAM, ephrin B2 (mRNA) and sm22 (mRNA) in transverse sections of E9.0 wild-type and Mib1−/− embryos. PECAM and ephrin B2 were used as a pan-endothelial cell marker and an arterial endothelial marker, respectively (Fischer et al., 2004). sm22 is a marker for smooth muscle cells that are recruited to the arterial endothelium (Fischer et al., 2004). All of the mutant embryos had markedly smaller dorsal aorta compared with the anterior cardinal veins (Fig. 3F). Similar to the Dll4−/−, Notch1−/− and Hey1/2DKO mutants, the Mib1−/− embryos had no or significantly reduced expression of ephrin B2 in the endothelium of the dorsal aorta (Fig. 3H) (Fischer et al., 2004). Smooth muscle cell recruitment to the dorsal aorta was also dramatically reduced, which might be caused by the loss of arterial identity (Fig. 3I).

To test whether the defect in artery formation is caused by the lack of Notch activation, we examined the expression of Dll4 and Hey1, a Notch ligand and a downstream target gene for the arterial fate decision, respectively. Interestingly, Dll4 expression was unaffected in Mib1−/− dorsal aorta, but Hey1 expression was undetectable (Fig. 3L,N; see Fig. S3 in the supplementary material), indicating that Dll4 expression itself is not sufficient for the activation of the Notch target gene in the absence of Mib1. To further test the notion that Hey1 and Hey2 are downstream of Mib1-regulated Notch activation, RNA from the embryonic yolk sacs of E9.0 wild-type and mutant embryos was analyzed by RT-PCR and real-time quantitative RT-PCR. Both Hey1 and Hey2 were expressed in the wild-type yolk sacs, but the amounts of these transcripts from the Mib1−/− yolk sacs were strongly reduced, by factors of 3.7 and 1.6, respectively (Fig. 3O,P). We also detected downregulation of the arterial-specific marker ephrin B2 in the yolk sacs of Mib1−/− embryos, while the transcript contents of its receptor, Ephb4, which is highly expressed in veins, was upregulated (Fig. 3O). Taken together, these results strongly suggest that Mib1 is an essential component involved in arterial fate decisions.

**Notch signaling defects in Mib1−/− mice**

Based on the multiple Notch-related phenotypes observed in Mib1−/− embryos, we tested the expression patterns of Notch-related genes. Previous studies of mutants lacking presenilin 1/2, RBP-Jk and POFT1 revealed the marked upregulation of Dll1 in the neural tube and brain, with the combined loss of Hes5 expression in the neural tube (de la Pompa et al., 1997; Donoviel et al., 1999; Shi and Stanley, 2003). In E9.0 Mib1−/− embryos, Dll1 expression was strongly upregulated in the neural tube (Fig. 4A, part a’) with the loss of Notch target genes, such as Hes5 and Hey1 (Fig. 4A, parts b’,c’). Similar up- and downregulation of these genes were also detected using RT-PCR and quantitative real-time RT-PCR of E8.5 wild-type and mutant embryos (Fig. 4B,C). Dll1 transcripts in mutant embryos were upregulated about sevenfold and Hes5 transcripts were reduced about fivefold, when compared with their wild-type counterparts. Hes1 was downregulated in the first branchial arches (Fig. 4A, part f). Hey1 was also downregulated in the branchial arches and the forming somites (Fig. 4A, part h’). In situ hybridization of Jag1 and Lfng in E9.0 embryos showed their reduced expression levels in the branchial arch and the PSM, respectively, of mutant embryos (Fig. 4A, parts b’,e’), while Notch1 and Notch2 showed comparable expression levels and patterns except in the PSM and somites (Fig. 4A, parts c’,d’). All of these results are indicative of Notch signaling defects.

To investigate the possibility that the Notch components (Notch1, Notch2, Dll1, Jag1, presenilins, mastermind 1, RBP-Jk and neur) are defective in Mib1−/− embryos, RT-PCR analyses were performed. In short, all of these molecules were normally expressed or upregulated in E8.5 Mib1−/− embryos (Fig. 4B,C). Thus, we excluded the possibility that the Mib1−/− embryos lack essential components for Notch signaling, except Mib1 itself.

To evaluate directly whether these remarkable changes in gene expression are caused by the lack of Notch activation, we examined the generation of the Notch1 intracellular domain (N1icd) and its target gene product, Hes5. In E9.0 wild-type whole-embryo lysates, N1icd was readily detected by western blotting. By contrast, N1icd was not observed in E9.0 Mib1−/− whole-embryo lysates (Fig. 5A). In accordance with the defective generation of N1icd, Hes5 expression was markedly reduced in Mib1−/− embryos (Fig. 5A). These defects in N1icd generation and Hes5 expression in Mib1−/− embryos were not due to the lack of Notch1 expression, as the Notch1 expression in the Mib1−/− embryos was comparable with that in the wild-type embryos (Fig. 5A). These results indicate that the Mib1−/− embryos have defects in Notch activation, especially upstream of the γ-secretase-mediated S3 cleavage.

To investigate whether the γ-secretase-mediated S3 cleavage and its downstream signaling are intact in Mib1−/− embryos, the Notch1 deleted extracellular domain (ΔEN1) was expressed in embryonic fibroblasts (EF) from wild-type and Mib1−/− embryos. ΔEN1 is readily cleaved by the γ-secretase complex to release the active N1icd, independent of a ligand/receptor interaction. In short, ΔEN1 was cleaved in both the wild-type and Mib1−/− EFs, and the cleaved N1icd was readily translocated to the nucleus to activate the transcriptional activity of downstream target genes (Fig. 5B,C,D). These results clearly show that the Mib1−/− embryos have no defect in the downstream signaling of S3 cleavage or in S3 cleavage itself. To evaluate directly the ligand function of the Mib1−/− embryos, Xdelta1-Myc (XD-Myc) was expressed in the EFs from wild-type and Mib1−/− embryos. XD-Myc-expressing EFs were co-cultured with C2C12-Notch1 cells containing CBF-Luciferase reporter gene (CBF-Luc). Notch activation was readily observed in the co-culture with XD-Myc-labeled wild-type and Mib1−/− EFs, and the cleaved N1icd was readily translocated to the nucleus to activate the transcriptional activity of downstream target genes. When the mutant CBF-Luc reporter was used, both co-cultures did not induce luciferase activity. Furthermore, when Dll1-Myc and Jag1-Myc were used instead of XD-Myc, the Notch activation was observed only in the co-culture with wild-type EFs (Fig. 5F). To test whether murine Mib1 directly induces the internalization of ligand, HA-tagged Xdelta1 (HA-XD-Myc) was co-expressed with Mib1-GFP in COS7 cells. As expected, Mib1-GFP induced internalization of Xdelta1 (Fig. 5E). Thus, the Notch signaling defects in the Mib1−/− embryos might be due to the defective endocytosis of Notch ligands.
Mib1 is essential for Notch signaling

Interactions between Mib1 and all known Notch ligands

Based on molecular interactions between Mib1 and Delta, we speculated that the pan-Notch phenotypes of Mib1–/– embryos might be caused by the lack of multiple Notch ligand-mediated signaling. To test this possibility, we examined the interaction of each murine Notch ligand (three Dll and two Jag homologues) with Mib1. HA-tagged Mib1 (HA-Mib1) protein was co-immunoprecipitated with all of the Myc-tagged Delta-related Notch ligands (Dll1, Dll3 and Dll4) in HEK-293A cells (Fig. 6A). Surprisingly, Jag1 and Jag2, the Serrate-related Notch ligands, also co-immunoprecipitated with HA-Mib1 under the same conditions (Fig. 6A).

To characterize the consequences of the interactions between Notch ligands and Mib1, we tested whether murine Mib1 promotes the endocytosis of Notch ligands. Overexpression of HA-tagged Xenopus Delta (XD-Myc), murine Dll1 (Dll1-Myc) and murine Jag1 (Jag1-Myc) in COS7 cells resulted in the characteristic plasma membrane expression or cytoplasmic expression with mesh-like patterns (Fig. 6B, parts b, c). When GFP-tagged Mib1 (Mib1-GFP) alone was expressed in COS7 cells, it was localized in the cytoplasm as punctate structures (Fig. 6B, part a). However, when both XD-Myc and Mib1-GFP were co-expressed, the XD-Myc expression on the cell surface was decreased, and the XD-Myc accumulated in the cytoplasm as vesicular structures where it was co-localized with Mib1-GFP, as previously described (Itoh et al., 2003). Likewise, when Dll1-Myc and Mib1-GFP (Fig. 6B, part d) or Jag1-Myc and Mib1-GFP (Fig. 6B, part e) were co-expressed in COS7 cells, the expression patterns of Dll1-Myc and Jag1-Myc were also changed, as in XD-Myc. In addition, we found the similar localization of other Notch ligands, Dll3, Dll4 and Jag2, when those ligands were co-expressed with Mib1 (data not shown).

To directly evaluate whether Mib1 regulates the endocytosis of Notch ligand in vivo, sections from E9.0 wild-type and Mib1–/– embryos were stained with anti-Dll1 antibody. The
Dll1 in wild-type embryos was localized in the cytoplasm near the nucleus (Fig. 6C). Surprisingly, in Mib1–/– embryos, Dll1 exclusively accumulated in the plasma membrane (Fig. 6C). All of these observations indicate that murine Mib1 is essential for the endocytosis of Notch ligand.

Discussion

In large-scale mutagenesis screens, zebrafish Mib1 mutants were initially identified by their neurogenic phenotype, which is a hallmark for the disruption of Notch signaling (Jiang et al., 1996; Schier et al., 1996). Recently, Mib1 has been identified as an E3 ubiquitin ligase that interacts with Delta to promote its ubiquitination and internalization, and to potentiate its signaling activity in the signal-sending cells (Itoh et al., 2003). As the overexpression of Xdelta in zebrafish Mib1 mutants rescues the neurogenic defect, it has been suggested to be a potentiator in generating functional Delta ligands to activate Notch (Itoh et al., 2003). However, by generating Mib1–/– mice, we have clearly demonstrated that Mib1 is an essential regulator of Notch ligand activation, but not a potentiator, and this ligand activation is absolutely required for Notch signaling.

The Mib1–/– mice exhibit multiple Notch-related phenotypes, such as defects in somitogenesis, neurogenesis, vasculogenesis and cardiogenesis. In addition, the Mib1–/– embryos showed an enlarged balloon-like pericardial sac, fusion of the notochord to the neural tube, disorganization of the trunk ventral neural tube, loss of mesenchyme cells, and abnormal heart and second branchial arch development. The phenotypes of the Mib1–/– embryos most closely resemble those of embryos that lack core Notch signaling components, such as Pofut, presenilins 1/2 and RBP-Jκ (de la Pompa et al., 1997; Donoviel et al., 1999; Oka et al., 1995; Shi and Stanley, 1997; Donoviel et al., 1999; Oka et al., 1995; Shi and Stanley,
Mib1 is essential for Notch signaling

Fig. 6. Interactions between Mib1 and all known Notch ligands. (A) Co-immunoprecipitation (Co-IP) of murine Mib1 with murine Notch ligands (Dll1, Dll3, Dll4, Jag1 and Jag2). HA-tagged Mib1 (HA-Mib1) or control vectors were co-expressed with Myc-tagged Notch ligands in HEK293A cells. The top panels show IP of Notch ligands by HA-Mib1, and the middle and bottom panels show the expression of HA-Mib1 and Notch ligand-Myc, respectively, in total cell lysates. (B) Subcellular localization of Mib1 (in green) and Notch ligands (Dll1, Jag1 and XD; in red). Mib1-GFP and/or Myc-tagged Notch ligand constructs were co-expressed in COS7 cells. Myc epitopes were detected with an anti-Myc antibody followed by a TRITC-labeled antibody. Nuclear DNA was stained with Hoechst (in blue). (a-c) Expression of Mib1-GFP (a), Dll1-Myc (b) and Jag1-Myc (c). (d-f) Co-transfection of Mib1-GFP with either Dll1-Myc (d), Jag1-Myc (e) or XD-Myc (f). Overlapping expression is yellow. (C) Transverse sections of E9.0 wild-type (wt) and Mib1–/– (mt) embryos stained with anti-Dll1 antibody. Dll1 is localized in the cytoplasm in wild-type neural tube, and accumulates in the plasma membrane in Mib1–/– neural tube (in red). Nuclear DNA was stained with Hoechst (in blue). The overall images are shown in insets. (D) Notch signal transduction. The pan-Notch phenotype in Mib1–/– embryos and the molecular interactions between Mib1 and multiple Notch ligands suggest a new core-Notch component that regulates the endocytosis of Dll and Jag ligands. The endocytosis of the Notch ligands by Mib1 stimulates the S2 and S3 cleavages of Notch receptors and the released Nicd translocates to the nucleus to express the Notch target genes, such as Hes5, Hey1 and HeyL.
Development (Barrantes et al., 1999). Moreover, the Notch phenotypes in vasculature, and for limb and craniofacial development, and Jag2 are essential for remodeling of the embryonic somite (Barrantes et al., 1999; Hrabe de Angelis et al., 1997; are required for the proper anteroposterior polarity of each ligand has redundant and non-redundant roles in the Notch partake in the trans-endocytosis of Notch to the ligand has not been investigated, although it has been shown that endocytosis of Serrate-related ligands by E3 ubiquitin ligases revealed that Mib1 directly regulates the endocytosis of Mib1 and Mib1 mutants. Considering the evolutionary conservation of the Notch ligand functions. Drosophila Mib1 and Drosophila Neur (Lai et al., 2005; Le Borgne et al., 2005). In contrast to our Mib1–/– mice exhibiting pan-Notch defects, Drosophila Mib1 mutants display part of the Notch phenotypes, suggesting functional redundancy between Drosophila Mib1 and Drosophila Neur. In our recent study, we identified a Mib1 parologue, Mib2, which has similar activities, but different expression patterns compared with those of Mib1 (Koo et al., 2005). Mib2 is mainly expressed in the adult tissues, but not in early embryonic stages, whereas Mib1 is abundantly expressed in both embryos and adult tissues, suggesting Mib1 might have a dominant role during early embryogenesis. However, it is not tested whether Drosophila mib2 (CG17492) has an essential or redundant role in the Drosophila Notch signaling pathway. Neur mutants in Drosophila are characterized by neurogenic phenotypes and have defects in Notch activation (Boulianne et al., 1991; Price et al., 1993). However, the disruption of the Neur gene in mice did not generate the characteristic Notch phenotypes as in Drosophila neur mutants (Ruan et al., 2001; Vollrath et al., 2001). This discrepancy suggests that unknown murine neur homologues and/or murine Mib1 can compensate for the loss of Neur in mammals. There are two murine neutralized genes, Neur1 and Neur2, which have similar phylogenetic distances from Drosophila neur gene. Although the Mib1–/– mutants, the expression of Dll1 was accumulated in the plasma membrane, while it was localized in the cytoplasm near the nucleus in the wild type, indicating that Mib1 is essential for the endocytosis of Dll1. Consistent with the endocytic defects of Notch ligand, Notch activation, such as the generation of Nicd and the activation of Notch target genes, was abolished. Thus, our data clearly show that Mib1 is an essential regulator of Notch ligand endocytosis and activation of Notch signaling.

Although both Neur and Mib1 interact with Delta and regulate its endocytosis, it is not clear whether they have redundant or unique, non-redundant functions in Notch signaling. Consistent with previous studies in zebrafish Mib1 mutants, our Mib1–/– mice exhibited pan-Notch defects, indicating that Mib1 has non-redundant roles in Notch signaling in both zebrafish and mammals. Most recently, two groups have reported the serrate-related function of Drosophila Mib1 and they also showed replaceable function of Drosophila Mib1 and Drosophila Neur (Lai et al., 2005; Le Borgne et al., 2005). In contrast to our Mib1–/– mice exhibiting pan-Notch defects, Drosophila Mib1 mutants display part of the Notch phenotypes, suggesting functional redundancy between Drosophila Mib1 and Drosophila Neur. In our recent study, we identified a Mib1 parologue, Mib2, which has similar activities, but different expression patterns compared with those of Mib1 (Koo et al., 2005). Mib2 is mainly expressed in the adult tissues, but not in early embryonic stages, whereas Mib1 is abundantly expressed in both embryos and adult tissues, suggesting Mib1 might have a dominant role during early embryogenesis. However, it is not tested whether Drosophila mib2 (CG17492) has an essential or redundant role in the Drosophila Notch signaling pathway. Neur mutants in Drosophila are characterized by neurogenic phenotypes and have defects in Notch activation (Boulianne et al., 1991; Price et al., 1993). However, the disruption of the Neur gene in mice did not generate the characteristic Notch phenotypes as in Drosophila neur mutants (Ruan et al., 2001; Vollrath et al., 2001). This discrepancy suggests that unknown murine neur homologues and/or murine Mib1 can compensate for the loss of Neur in mammals. There are two murine neutralized genes, Neur1 and Neur2, which have similar phylogenetic distances from Drosophila neur gene. Although the Mib1–/– mutants, the expression of Dll1 was accumulated in the plasma membrane, while it was localized in the cytoplasm near the nucleus in the wild type, indicating that Mib1 is essential for the endocytosis of Dll1. Consistent with the endocytic defects of Notch ligand, Notch activation, such as the generation of Nicd and the activation of Notch target genes, was abolished. Thus, our data clearly show that Mib1 is an essential regulator of Notch ligand endocytosis and activation of Notch signaling.

There are five canonical Notch ligands (Dll1, Dll3, Dll4, Jag1 and Jag2) in mammals (Lai, 2004). A previous study revealed that Mib1 directly regulates the endocytosis of zebrafish DeltaB and DeltaD (Itoh et al., 2003). However, the endocytosis of Serrate-related ligands by E3 ubiquitin ligases has been investigated, even though it has been shown that Serrate, like Delta, is a transmembrane ligand that can participate in the trans-endocytosis of Notch to the ligand expressing cells (Klueg and Muskavitch, 1999). Each Notch ligand has redundant and non-redundant roles in the Notch activation pathway at various cell fate decisions. Dll1 and Dll3 are required for the proper anteroposterior polarity of each somite (Barrantes et al., 1999; Hrabe de Angelis et al., 1997; Kusumi et al., 1998). Dll4 is crucial in the arterial fate decision (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). Jag1 and Jag2 are essential for remodeling of the embryonic vasculature, and for limb and craniofacial development, respectively (Jiang et al., 1998; Xue et al., 1999). The pan-Notch phenotypes in Mib1–/– embryos could be explained by the combined loss of multiple ligands. The defects in somitogenesis, the loss of Uncx4.1 and Hey1 expression, and the impaired oscillations of Hes7 and Lfng can be explained by the combined loss of Dll1 and Dll3. The defects in vasculogenesis and the loss of Hey1 and ephrin B2 expression closely resemble the vascular phenotypes in Dll4–/– mice. Although the Mib1–/– embryos do not clearly explain the phenotypes in Jag1–/– and Jag2–/– mice, the thin vascular network and the defect in the second branchial arch development appear to represent the loss of Jag1 and Jag2 activity, respectively. In accordance with the pan-Notch phenotypes in Mib1–/– embryos, Mib1 interacts with all of the Notch ligands, Jag1 and Jag2 as well as Dll1, Dll3 and Dll4, and induces their endocytosis, suggesting that all of these defects might be due to the inactivation of most, if not all, of the Notch ligand functions.

A crucial step for efficient Notch signaling by Delta was revealed by an analysis of Drosophila neur and zebrafish Mib1 mutants (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Pavlopoulos et al., 2001). These two genes promote the endocytosis of Delta in a ubiquitination-dependent manner. It has been proposed that the endocytosis of Notch ligands, on signal-sending cells that are bound to Notch on adjacent signal-receiving cells, induces the S2 cleavage of the receptor, thus activating signal transduction (Parks et al., 2000). Surprisingly, in Mib1–/– mutants, the expression of Dll1 was accumulated in the plasma membrane, while it was localized in the cytoplasm near the nucleus in the wild type, indicating that Mib1 is essential for the endocytosis of Dll1. Consistent with the endocytic defects of Notch ligand, Notch activation, such as the generation of Nicd and the activation of Notch-target genes, was abolished. Thus, our data clearly show that Mib1 is an essential regulator of Notch ligand endocytosis and activation of Notch signaling.
unfertilized eggs (Itoh et al., 2003). Furthermore, Mib1 interacts with all of the murine Notch ligands, and genetic inactivation of Mib1 results in multiple developmental defects that are characteristic of impaired Notch signaling. Thus, our data provide the first evidence in mammals that the E3 ubiquitin ligase Mib1 is an essential core component of Notch signaling.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/15/3459/DC1

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References


