An Obligatory Role of Mind Bomb-1 in Notch Signaling of Mammalian Development

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Background. The Notch signaling pathway is an evolutionarily conserved intercellular signaling module essential for cell fate specification that requires endocytosis of Notch ligands. Structurally distinct E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), cooperatively regulate the endocytosis of Notch ligands in Drosophila. However, the respective roles of the mammalian E3 ubiquitin ligases, Neur1, Neur2, Mib1, and Mib2, in mammalian development are poorly understood.

Methodology/Principal Findings. Through extensive use of mammalian genetics, here we show that Neur1 and Neur2 double mutants and Mib2−/− mice were viable and grossly normal. In contrast, conditional inactivation of Mib1 in various tissues revealed the representative Notch phenotypes: defects of arterial specification as deltaklike4 mutants, abnormal cerebellum and skin development as jagged1 conditional mutants, and syndactylyism as jagged2 mutants. Conclusions/Significance. Our data provide the first evidence that Mib1 is essential for Jagged as well as Deltalike ligand-mediated Notch signaling in mammalian development, while Neur1, Neur2, and Mib2 are dispensable.

INTRODUCTION

The Notch signaling pathway is an evolutionarily conserved signaling module from nematode to human, which plays essential roles in pattern formation and cell fate determination through local cell-cell interactions [1]. Notch signaling is initiated by the interaction of the Notch receptors with their ligands, Delta [Deltalike (Dll) in mammals] and Serrate [Jagged (Jag) in mammals] [2,3]. These interactions induce two sequential proteolytic cleavages of Notch receptor (S2 and S3 cleavages), and generate a soluble intracellular domain (Nici) that translocates to the nucleus to form a transcriptional activator complex with Su(H)/CBF1/RBP-J. This complex activates the anti-neurogenic basic helix-loop-helix (bHLH) repressors.

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 receptors and trigger their cleavage is less understood. It has been suggested that the endocytosis of Notch ligands in the signal-sending cell is required for the effective activation of Notch signaling [4]. Two structurally distinct E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), regulate the endocytosis of the Notch ligand, Delta, in Drosophila and zebrafish, respectively [5–9]. Both proteins have been shown to interact with Delta and to promote its ubiquitination, endocytosis, and signaling. Interestingly, loss-of-function mutations in zebrafish mib and Drosophila neur cause similar expansions of the neural precursors, at the expense of the epidermisis, through the loss of lateral inhibition [6,9]. Since Mib and Neur share biochemical functions and their mutants show similar phenotypes, these two proteins were suggested to be functional homologues between species [10]. However, since the homologues of Mib and Neur are evolutionarily conserved, respectively, it has been suggested that a more complex interplay may exist between these E3 ubiquitin ligases and the endocytosis of Delta.

There are one neur (drear) gene and two mib (dmib1 and dmib2) homologue genes in Drosophila. Both dMib1 and dNeur are essential for a subset of the developmental events that are regulated by Notch signaling [10,11]. dmib1 mutants showed defects in wing margin specification, leg segmentation, and vein determination, while dneur mutants displayed increased numbers of neuroblasts and sensory organ precursors [9,10]. Although dMib1 and dNeur regulate both Delta and Serrate [12], it has been suggested that dMib1 and dNeur are required for a distinct subset of Notch signaling events, primarily because of their differential expression patterns [10]. Interestingly, ectopic overexpression of dMib1 and dNeur in each other’s mutants revealed their complementary functions, suggesting that they have similar molecular activities in Drosophila [10,11]. Likewise, each mutant shows specific phenotypes, but also has a redundant phenotype, depending on the expression profiles. In fact, the dneur and dmib double mutant fly showed a complete block of lateral inhibition in sensory organ precursors [12]. Thus, in Drosophila, both the Delta and Serrate ligands require E3 ubiquitin ligases for their activity, and the requirement of dmib and dneur appears to depend on the context.

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In vertebrates, there are two Mib homologues, Mib1 and Mib2, and two Neur homologues, Neur1 and Neur2 [5, 6, 13–15]. All of them are implicated in the endocytosis of Notch ligands [5, 6, 13–15]. Mib1 is abundantly expressed in both embryos and adult tissues, while Mib2 is highly expressed in adult tissues, but only slightly in embryos [14]. Both Neur1 and Neur2 are expressed in the brain, but also specifically expressed in skeletal muscle and kidney, respectively [13–17]. Their differential expression profiles suggest that these E3 ligases may have redundant and/or specific functions in distinct contexts. Loss-of-function genetic studies revealed that, unlike the Drosophila neur mutants, Neur1−/− mice have no defect in neurogenesis and embryonic development, possibly due to a functional overlap with Neur2 [16, 17]. In contrast, Mib1−/− mice exhibited a neurogenic phenotype as well as pleiotropic Notch-related defects on embryonic day 9.5, suggesting the possible obligatory role of Mib1 in the regulation of multipleDll and Jag ligands in mammalian development [13]. However, genetic evidence for these matters is still lacking, and other E3 ligases might have compensatory roles in the regulation of Notch ligands [14, 15]. To elucidate the interplay between the four E3 ubiquitin ligases and the Notch ligands in mammalian development, more comprehensive genetic studies of these four E3 ubiquitin ligases are required in a single organism.

There are five canonical Notch ligands, Dll1, Dll3, Dll4, Jag1, and Jag2 in mammals. Since Dll3 has no lysine residue for ubiquitin ligation [18], the others, Dll1, Dll4, Jag1, and Jag2, would be regulated by E3 ubiquitin ligases. The specific functions of each ligand have been identified through many genetic studies. Dll1 is required in somitogenesis and marginal zone B cell development, and Dll4 regulates arteriogenesis and angiogenesis [19–21]. Jag1 conditional knockout mice revealed its role in cerebellum development and hair maintenance [22, 23]. Jag2 mutant mice are well known to have syndactylysm [24, 25]. These mutant studies have suggested the specific roles of each ligand in various Notch-mediated fate decisions. However, which E3 ligases are implicated in the distinct contexts regulated by each Notch ligand in mammalian development is largely unknown.

To establish the respective roles of these four E3 ubiquitin ligases in the various contexts of a single organism, we have generated Neur2−/−, Neur1−/−Neur2−/− (Neur1&2DKO), Mib2−/−, and Mib1 conditional mutant (Mib1f/f) mice. Both the Neur2−/− and Mib2−/− mice are viable, fertile, and grossly normal. Surprisingly, Neur1&2DKO mice are also viable and have no recognizable developmental defect, demonstrating the dispensable role of Neur1 and Neur2 in mammalian development. In contrast, the conditional inactivation of Mib1 in the endothelium, skin epithelium, cerebellum, and apical ectodermal ridge faithfully showed the representative Notch phenotypes, such as the defects in arterial specification, and cerebellar and skin development exhibited by Dll4, and Jag1 mutant mice, respectively, and the syndactylysm shown in Jag2−/− mice. Our results clearly demonstrate the obligatory role of Mib1 in the regulation of Notch ligands during mammalian development.

**RESULTS**

**Generation of Neur2−/− mice**

The murine Neur2 locus comprises 5 exons. The IRES-lacZ puro cassette was fused to exon 2, with the deletion of exon 3 (Figure 1A). The deleted region encodes amino acids 115 to 319 of the murine Neur2 protein, which includes about 40% of the Neur2 protein (Figure 1B). This insertion abrogates the two neutralized homology repeats that are required for the interaction with Notch ligands [15, 26]. Based on our targeting strategy, Neur2 transcripts will be fused with IRES-lacZ cDNA and lose the carboxy-terminal region of the Neur2 protein, which has the RING domain for ubiquitination (Figure 1A). After electroporation and drug selection, screening by Southern blotting with a 3′ external flanking probe using HindIII detected a homologous recombination event (Figure 1C). These clones were used to generate chimeric mice, and the heterozygous offspring were identified by PCR genotyping. When the heterozygous mice were intercrossed, the homozygous Neur2−/− offspring were viable and healthy. Using the primers specific for the undeleted 3′ region of the Neur2 cDNA, these homozygous mice were confirmed as null for Neur2 transcripts (Figure 1C). The homozygous mice were fertile and did not show any obvious Notch-related phenotypic abnormality up to now.

**Both Neur1 and Neur2 are dispensable for mammalian development**

In Drosophila, there is one dNeur that is essentially required for Notch signaling in lateral inhibition [9]. In vertebrates, however, there are two Neur homologues, Neur1 and Neur2, which have similar degrees of homology to dNeur [15]. The mutant mice with a disruption in either Neur1 or Neur2 were healthy and had few Notch-related phenotypes [16, 17], suggesting possible compensation by each other. To test this possibility, we generated Neur1&2DKO mice. Surprisingly, and unexpectedly, the Neur1&2DKO mice were born at the expected Mendelian ratios from intercrosses of Neur1G2 double heterozygous mice (Figure 1D). These double mutant mice were confirmed by the absence of both Neur1 and Neur2 transcripts in the adult brains (Figure 1C). Microscopic analyses of histologic tissue sections revealed that there were no observable abnormalities in various tissues, including the brain (Figure 1E, not shown). Interestingly, Neur1G2DKO mice exhibited normal morphology in the cerebral cortex and hippocampal region, demonstrating that both Neur1 and Neur2 are dispensable for mammalian cerebral development. Taken together, all of mouse Neur homologues are dispensable for many developmental processes that are regulated by Notch signaling, such as somitogenesis, neurogenesis, vasculogenesis and limb and skin development.

**Mib2 is dispensable in mammalian development**

Both Mib1 and Mib2 have similar biochemical activity to Notch ligand(s), and Mib2 can rescue the Notch phenotypes of zebrafish mib1 mutants [14]. In addition, Mib2 is highly expressed in adult tissues, but only slightly in embryos [14]. Thus, we speculated that Mib2 might function in adulthood, whereas Mib1 has an obligatory role in embryos. To test this possibility, we generated Mib2−/− mice. The murine Mib2 locus comprises 21 exons. The IRES-lacZ puro cassette was inserted within exon 5, and the remaining region from exon 5 to exon 15 was replaced with the IRES-lacZ puro cassette (Figure 2A). The deleted region encodes amino acids 152 to 656 of the murine Mib2 protein, encoding the Mib domain, the Mib repeat and the ankyrin repeat domain [14]. The deleted region includes over 50% of the Mib2 protein. The homozygous mutant (Mib2−/−) mice were generated as described for the Neur2−/− mice. Disruption of the Mib2 gene was confirmed by northern blot and RT-PCR analyses of brain tissues (Figure 2C, D).

Heterozygous intercrosses produced their offspring at the predicted Mendelian ratio, and the Mib2−/− mice did not show any recognizable differences in growth, body weight, and health up to 12 months, as compared to the control littermates. In addition, the mutant brains in embryos as well as in adults were indistinguishable in both size and appearance from their control littermates (not shown). A histological analysis of the Mib2−/− brains revealed no detectable abnormalities at both the embryonic...
and adult stages (Figure 2E, not shown). These results demonstrate that Mib2 is not essential for Notch signaling-dependent developmental processes, including somitogenesis, neurogenesis, vasculogenesis and limb and skin development.

To reveal the effects of the loss of Mib2 in more sensitized condition, we crossed Mib1\(+/-\) mice with Mib2\(-/-\) mice to generate Mib1\(+/-\);Mib2\(-/-\) mice. Although Mib1\(+/-\);Mib2\(-/-\) mice have only one Mib homologue, these mice are also viable and have no recognizable abnormalities, suggesting that a single allele of Mib1 gene is enough to regulate the mammalian Notch signaling (not shown). However, Mib1\(-/-\); Mib2\(+/-\) mice showed embryonic lethality with similar defects with Mib1\(-/-\);Mib2\(-/-\) or Mib1\(-/-\) mice (Figure 2F). Because the level of Mib2 transcripts are very low compared to Mib1 in the embryonic stages, this embryonic lethality are mainly caused by the loss of Mib1 not Mib2 [14]. Taken together, Mib1 is the dominant Mib homologue that regulates the mammalian Notch signaling, while Mib2 is dispensable.

Generation of Mib1 conditional knockout mice

Among the four E3 ubiquitin ligases for Notch ligands in mammals, only the Mib1\(-/-\) mice showed severe Notch-related phenotypes and early embryonic lethality (~E10.5) [13], while the Neur1\&2\(^{2\text{dKO}}\) and Mib2\(-/-\) mice did not show any obvious Notch-related phenotype until adulthood. Notch signaling is implicated in various cell fate decisions, not only in early embryogenesis but also in late organogenesis and the postnatal stages. Moreover, genetic

Figure 1. Generation of Neur1 and Neur2 double knockout mice and their dispensable role in mammalian cerebral development. (A) Targeted disruption of the murine neuralized-2 (Neur2) locus. Schematic drawings of the wild-type (wt) and recombinant (mt) loci and the targeting vector (tv) are shown. The homologous recombination event deletes exons 2-3 and places the IRES-LacZ gene within the exon 2. H, HindIII. (B) Schematic drawing of the Neur1 and Neur2 proteins with the deleted region (green). (C) Southern blot analysis showing the recombination event. The RT-PCR analysis shows the loss of Neur1 and Neur2 transcripts in each mutant indicated. \(\beta\)-actin was used for normalization. wt, wild-type band; mt, Neur2 mutant band. (D) Heterozygote intercrosses of Neur1\(^{+/+}\);Neur2\(^{+/+}\) mice. (E) H&E sections of the neocortex (left panel) and hippocampus (right panel) of wild-type and Neur1&2\(^{2\text{dKO}}\) mice. Note that there is no difference between the wild-type and Neur1&2\(^{2\text{dKO}}\) mice.

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studies of Notch ligands revealed the specific roles of each ligand in various Notch-mediated fate decisions. However, it is not known which E3 ligase is involved in these Notch signaling events. To clarify this issue, we generated Mib1 conditional knockout mice carrying two loxP sites between exons 1 and 2 and between exons 3 and 4 (Figure S1). The homozygous mice (Mib1f/f) were generated as described for the Neur2f/f mice. The Mib1f/f mice were fully fertile and had no abnormalities. Moreover, Mib1f/f mice also had no abnormalities. To verify the generation of Mib1f/f mice, the null mice (Mib1D/D) were generated using protamine-cre transgenic mice that produce a germ line deletion of the loxP-floxed region. These Mib1D/D mice showed the same phenotypes as the Mib1f/f mice (not shown) [13], indicating that the Mib1 conditional allele is fully functional and became a null allele by the Cre recombinase.

Figure 2. Generation of Mib2f/f mice and its dispensable role for mammalian development. (A) Gene targeting of the murine Mib2 locus. The homologous recombination event deletes exons 5–15 and places the IRES-LacZ gene within exon 5. Schematic structures of the wild-type (wt) and recombinant loci (mt) and the targeting vector (tv) are shown. E, EcoRI; B, BamHI; K, KpnI; spe, SpeI. (B) Southern blot analysis of tail DNA after EcoRI digestion with the flanking probe shown in (A). The positions of the wild-type (23kb) and the targeted (6.3kb) allele are indicated. (C) Northern blot analysis of Mib2 gene expression in adult brain mRNA from wild-type (+/+ ) and Mib2f/f (−/− ) mice. Loading and integrity of the RNA were assessed by ethidium bromide staining of the 28S RNA in the gel prior to membrane transfer. (D) RT-PCR analysis of adult brain using Mib2-specific primers. No PCR product was detected from Mib2f/f cDNA. β-actin was used for the normalization. (E) H&E sections of the hippocampus of wild-type (left) and Mib2f/f (right) mice. Note that there is no difference between the wild-type and Mib2f/f mice. (F) Whole-mount images of E9.5 embryos. Mib1f/f;Mib2f/f (i), Mib1f/f;Mib2f/f (ii), and Mib1f/f;Mib2f/f (iii). doi:10.1371/journal.pone.0001221.g002
Conditional inactivation of Mib1 in endothelial cells generates a Dll4 mutant phenotype

Dll4 is responsible for arterial cell specification in vascular development [19,20,27]. Mib1 interacts with Dll4 and induces its endocytosis [13]. Therefore, we examined whether Mib1 is required for vascular development. The Mib1f/f mice were bred with Tie2-cre transgenic mice, which express Cre recombinase under the control of the Tie2 promoter [28]. The Tie2-cre;Mib1f/f mice were embryonic lethal around E11.5. Therefore, the Tie2-cre;Mib1f/f embryos were dissected at E7.5-E10.5. At E8.5, there were no obvious differences between the wild-type and Tie2-cre;Mib1f/f embryos (not shown). Between E9.5 and E10.5, however, the Tie2-cre;Mib1f/f embryos exhibited characteristic vascular remodeling defects, such as a mottled avascular yolk sac, growth retardation, and pericardial effusion (Figure 3A, B). Staining of endothelial cells with an anti-PECAM1 antibody demonstrated the presence of a vessel structure consisting of endothelial cells in the Tie2-cre;Mib1f/f embryos, indicative of intact vasculogenesis (Figure 3D, F). However, the Tie2-cre;Mib1f/f mice displayed a complete absence of vascular remodeling. The capillary network was less extensive and more primitive than that of the wild-type littermates (Figure 3D, F). Transverse sections of PECAM stained embryos revealed that the paired dorsal aorta in the Tie2-cre;Mib1f/f embryos were frequently narrower (Figure 3H). The observed mutant phenotypes were reminiscent of the Dll4f/f and Dll4-/- mice, suggesting that Mib1 regulates Dll4 in endothelial cells.

We further examined whether arterial cell fate is also impaired in the Tie2-cre;Mib1f/f embryos. EphrinB2 is one of the earliest arterial-specific endothelial cell markers [29]. In wild-type embryos, ephrinB2 was normally expressed in arteries, but not in the Tie2-cre;Mib1f/f embryos, whereas its expression was retained in other tissues (Figure 3J). In addition, CD44 expression in the dorsal aorta, another arterial marker [30], was down-regulated in the Tie2-cre;Mib1f/f embryos (Figure 3L). When the recruitment of smooth muscle cells to nascent vessel walls was examined, the expression of both sn22 mRNA and z-smooth muscle actin protein (zSMA) were almost completely absent in the dorsal aorta of the Tie2-cre;Mib1f/f embryos (Figure 3N, P). Finally, the transcript of key1, one of the Notch target genes responsible for arterial cell specification, was down-regulated in the Tie2-cre;Mib1f/f embryos (Figure 3N, P). The Dll4 transcripts in the Tie2-cre;Mib1f/f para-aortic splanchopleura (P-Sp) were similar to those of the wild-type (Figure 3Q). These results demonstrate that Mib1 is required for arterial specification, which might be due to defective Dll4 activity.

Conditional inactivation of Mib1 in skin epithelial cells generates a Jag1 mutant phenotype

Notch signaling plays important roles in epidermal differentiation and hair follicle maintenance [31,32]. Jag1 is expressed in the differentiated interfollicular epidermis (IFE), outer root sheath (ORS), pre-cortex, and matrix of hair follicles, and a genetic study revealed that Jag1 is the Notch ligand responsible for the maintenance of hair [22]. Since Mib1 is also expressed in IFE and hair follicles and regulates the endocytosis of Jag1 [13,14], we examined whether conditional inactivation of Mib1 in the skin also reproduces the Jag1 mutant phenotype in the skin. We bred Mib1f/f mice with Msx2-cre transgenic mice, in which the Cre recombinase is active not only in the apical ectodermal ridge at E11.5 but also in the dorsal ectoderm of early embryos and the matrix of hair follicles after birth [31,33]. The Msx2-cre;Mib1f/f mice were smaller than their littermate controls and progressively lost their hair from the dorsal midline. They became completely bald at about 4 weeks after birth (Figure 4A).

At postnatal day 28 (P28), the back skin of the wild-type contained hair follicles in an anagen state (Figure 4B). In contrast, the hair follicles in the Msx2-cre;Mib1f/f back skin were barely detected, and large epidermal cysts below the interfollicular epidermis were formed as the hairs were progressively lost (Figure 4C), which is a representative phenotype of Jag1 mutant mice [22]. In the Msx2-cre;Mib1f/f mice, the epidermal cell layers were thickened and lots of squames were observed (Figure 4C). In order to characterize the phenotype further, we examined the expression of various epidermal markers, such as undifferentiation (K14) and differentiation markers (K10, Loricrin), with proliferation marker (PCNA). The numbers of K14-positive basal or ORS cells were markedly increased in the Msx2-cre;Mib1f/f skin, as compared to the wild-type (Figure 4E), whereas the numbers of differentiated epidermal cells, such as K10- or Loricrin-positive cells, were reduced (Figure 4G, I). Most of the K14-positive cells were PCNA-positive, indicating that they are proliferating (Figure 4E). The enlarged cysts observed in the Msx2-cre;Mib1f/f skin were filled with cornified materials (Figure 4C), reminiscent of the enlarged cysts in other Notch-related mutants, such as Numb, Numb1/2, RBPjk, presenilin1/2, or Jag1 deficient mice [22,31,32]. Taken together, the Msx2-cre;Mib1f/f mice showed peculiar skin epithelial phenotypes as in other Notch mutants, demonstrating that Mib1 is an essential E3 ubiquitin ligase that regulates epidermal differentiation and hair follicle maintenance.

Conditional inactivation of Mib1 in cerebellum generates a Jag1 mutant phenotype

Previous analyses of Notch1, Numb and Jag1 conditional mutant mice revealed that Notch signaling is important in cerebellar development [23,34,35]. Loss of Jag1 results in delayed granule cell migration, potentially because of the mis-organized Bergmann glial fibers [23]. In order to examine whether Mib1 is required for cerebellar development, we bred Mib1f/f mice with hGFAP-cre transgenic mice, in which Cre recombinase is expressed in the Bergmann glia and granule cells, but not in the Purkinje cells, in the cerebellum [36]. A western blot analysis of a cerebellar lysate confirmed the ablation of Mib1 in these mice (Figure 5A).

The hGFAP-cre;Mib1f/f mice were smaller than the wild-type littermates, and died between 2 to 4 months. While the cerebellum sizes were indistinguishable at P4, reductions in both the cerebellum size and foliation were recognizable in the mutant mice from P9 (Figure 5B). A histological analysis of the P15 cerebellum revealed the accumulation of granule cells in the external germinall layer (EGL), as compared to the wild-type (Figure 5C, D). At P17, granule cells in the EGL were still retained in the mutant cerebellum, but not in the wild-type (Figure 5E, F). At P21, however, all of the granule cells had finally migrated into the internal granule cell layer (IGL) in the mutant mice (not shown). These results suggest that granule cell migration is delayed in the hGFAP-cre;Mib1f/f mice. An immunohistochemical analysis using Ki67, a proliferation marker, revealed a similar level of proliferation in the EGL at P4 (Figure 5I, J), suggesting that the accumulation of granule cells in the mutants is not caused by the increased proliferation. Consistent with this finding, an immunohistochemical analysis using a postmitotic neuronal marker, NeuN [37], showed the delayed migration of the postmitotic granule cell precursors from the EGL to the internal granule cell layer (IGL) in the hGFAP-cre;Mib1f/f mice (Figure 5N).

A previous study suggested that the delayed migration of granule cells in the E12.5-Jag1f/f conditional knockout cerebellum
might be due to the mis-organized Bergmann glial fibers [23]. When we examined the integrity of the Bergmann glial fiber in the developing cerebellum with the glial markers, GFAP and BLBP, the Bergmann glial processes were severely disorganized in the P15 hGFAP-cre;Mib1f/f cerebellum, while the wild-type cerebellum showed radially organized processes of the Bergmann glia (Figure 5O–R). Taken together, the hGFAP-cre;Mib1f/f mice displayed the exact phenocopy of the Jag1 conditional knockout

Figure 3. Conditional inactivation of Mib1 in endothelial cells generates a Dll4 mutant phenotype. (A, B) External view of embryonic day 9.5 (E9.5) yolk sacs (A) and embryos (B). The Tie2-cre;Mib1f/f yolk sac (A, right) has failed to remodel the primary vascular plexus to form large vitelline blood vessels. The Tie2-cre;Mib1f/f embryo (B, right) exhibits growth retardation and pericardial effusion. (C–F) Whole-mount PECAM staining of E9.5 wild-type (C, E) and Tie2-cre;Mib1f/f (D, F) embryos. The large cranial vessels appear truncated and degenerated in the mutants (D, arrows). Intersomitic vessels are present, but the angiogenic sprouts are disorganized in the mutants (F). (G, H) Sections of PECAM-1 stained embryos. The dorsal aortas of Tie2-cre;Mib1f/f embryos are reduced in diameter (H). ACV, anterior cardinal vein; DA, dorsal aorta. Scale bar, 200 μm. (I–L) Sections of ephrinB2 (I,J) and CD44 (K,L) stained embryos. The large cranial vessels appear truncated and degenerated in the mutants (D, arrows). Intersomitic vessels are present, but the angiogenic sprouts are disorganized in the mutants (F). (M–P) Sections of sm22 (M,N) and αSMA (O,P) stained embryos. The smooth muscle cell markers, sm22 and αSMA, are lost in the dorsal aortas of Tie2-cre;Mib1f/f embryos (N,P). (Q) Semi-quantitative RT-PCR in the yolk sac (ys) and para-aortic splenchnopleura (P-Sp) of wild-type (wt) and Tie2-cre;Mib1f/f (mt). Hey1 is down-regulated in the mt yolk sac (left panel). Dll4 expression in the mt P-Sp is similar to that of wt (right panel). β-actin was used for normalization. doi:10.1371/journal.pone.0001221.g003
mice, suggesting that Mib1 is an essential E3 ubiquitin ligase for Jag1 in the developing cerebellum.

**Conditional inactivation of Mib1 in the apical ectodermal ridge generates a Jag2 mutant phenotype**

Syndactyly (digit fusions) is a well known Jag2 mutant phenotype [24,25]. Especially, the loss of Notch signaling in the apical ectodermal ridge (AER) of the embryonic limb bud causes this phenotype [38]. Jag2 is expressed in the AER from E10.5, and its mutant mice show severe syndactylyism that is equivalent to the phenotypes of Notch1<sup>2DKO</sup> and presenilin1<sup>2DKO</sup> mice, suggesting the exclusive role of Jag2 in this process. Interestingly, Mib1 is also highly expressed in the AER and in the underlying mesenchyme at E10.5 (Figure 6A). To examine whether Mib1 functions as an essential E3 ubiquitin ligase of Jag2 in the developing AER, we crossed Mib1<sup>f/f</sup> mice with Msx2-cre transgenic mice. At E13.5, the developing hind limb showed primary digit identity, with 5 digit tips in the wild-type embryos (Figure 6B). In contrast, the Msx2-cre;Mib1<sup>f/f</sup> embryos had a narrower interdigital space between digits 2 and 3 (Figure 6C). At E15.5, the Msx2-cre;Mib1<sup>f/f</sup> mice had no digit separation (Figure 6E). Eventually, the Msx2-cre;Mib1<sup>f/f</sup> mice had fused digits (2, 4) in adulthood (Figure 6G). All of the
mutant mice examined exhibited soft tissue fusions and fused nails of digits 2 and 3. Skeletal staining also revealed the fusion of the distal phalanges of digits 2 and 3 (Figure 6I).

To examine whether Msx2-cre;Mib1f/f embryos have similar molecular changes in the developing limb bud as in other Notch-related mutants, we analyzed the expression patterns of Fgf8 (fibroblast growth factor 8; AER marker) and Bmp2 (bone morphogenetic protein 2; interdigital cell marker at E13.5). Other notch-related mutant mice exhibit a hyperplastic AER and an expanded Fgf8 expression domain [24,25,38]. As expected, the Fgf8 expression domain in the newly formed AER of the hind limb bud was expanded in the Msx2-cre;Mib1f/f embryos, as compared to the wild-type (Figure 6J, K). Bmp signaling regulates apoptotic cell death in the interdigital regions, and Bmp expression is restricted to interdigital apoptotic cells [39,40]. At E13.5, the Msx2-cre;Mib1f/f embryos exhibited reduced Bmp2 expression in the interdigital region, especially between digits 2 and 3, suggesting decreased interdigital cell death (Figure 6M). Interestingly, the expression of Jag2 in the AER of E11.5 embryos was not altered in the msx2-cre;Mib1f/f embryos, as compared to the wild-type (Figure 6N, O). This excludes the possibility that the loss of Jag2 expression causes the syndactyly of the Msx2-cre;Mib1f/f mice. These data suggest that Mib1 functions as an essential E3 ubiquitin ligase of Jag2 that regulates the separation of digits in the developing limb.

**DISCUSSION**

Many E3 ubiquitin ligases are involved in regulating the Notch signaling pathway [41]. They are divided into two groups, one that ubiquitinates Notch receptors and the other that regulates Notch ligands. Two kinds of E3 ubiquitin ligases, Neur and Mib1, are important for the endocytosis of Notch ligands in Drosophila and zebrafish/mice, respectively [5–8,13]. In Drosophila, depending on the developmental context, either dMib1 or dNeur is required for
the activation of two Notch ligands, Delta and Serrate [10–12]. In mammals, there are two Mib homologues, Mib1 and Mib2, and two Neur homologues, Neur1 and Neur2, along with five Notch ligands, Dll1, Dll3, Dll4, Jag1, and Jag2, in the genome. However, the requirement of each E3 ubiquitin ligase in the developmental contexts where Notch signaling is active was largely unknown. In this report, we have analyzed the phenotypes of Neur2−/−, Neur1&2DKO, Mib2−/−, and Mib1f/f mice expressing the Cre enzyme by various promoters. Unexpectedly, only the Mib1 conditional mutant mice faithfully exhibited the various Notch phenotypes representing each of the Dll4, Jag1, and Jag2 mutant mice. Collectively, our extensive genetic studies reveal that Mib1 is the E3 ubiquitin ligase responsible for the regulation of Notch ligands in mammalian development.

**Figure 6. Conditional inactivation of Mib1 in apical ectodermal ridge generates a Jag2 mutant phenotype.** (A) Whole-mount in situ hybridization of Mib1 in E10.5 apical ectodermal ridge (AER). The image on the left is an enlarged view of the boxed area in the right image. (B–G) Hind limbs of E13.5 (B, C), E15.5 (D, E), and P21 (F, G) of wild-type (B, D, F) and Msx2-cre;Mib1f/f (C, E, G) mice. Note that the mutants show the fusion of digits 2 and 3 (C, E, G, asterisk). Numbers indicate the digit identity. (H, I) Stained skeletal preparations of neonatal hind limbs of wild-type (H) and Msx2-cre;Mib1f/f (I) mice. Numbers indicate the digit identity. (J–O) Whole-mount in situ hybridization of Fgf8 (J, K, E11.5), Bmp2 (L, M, E13.5), and Jag2 (N, O, E11.5) in wild-type (J, L, N) and Msx2-cre;Mib1f/f (K, M, O) embryonic hind limbs. Msx2-cre;Mib1f/f embryos have broad Fgf8 expression (K) and show the loss of interdigital Bmp2 expression (M, arrow).

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**An obligatory role of Mib1 in mammalian development**

In *Drosophila*, dneur mutants were initially identified by the loss of lateral inhibition in neurogenesis, which results in the overproduction of neuroblasts at the expense of the epidermoblast [9]. Although dMib1 and dNeur have similar functions and are exchangeable in some developmental contexts, the endogenous level of dNeur is important for the lateral inhibition that inhibits the neuronal fate in the epidermis [10,11]. Moreover, the overexpression of dMib1 in dneur mutant *Drosophila* cannot rescue the cuticular neurogenic phenotype of dneur mutant embryos, indicating a non-redundant function of Neur in this context [10]. Consistently, dneur is expressed throughout the ectoderm during neuronal cell fate specification [42]. Likewise, both murine *Neur1* and *Neur2*...
and Neur2, which have a similar phylogenetic distance from dneur, are expressed in the embryonic and adult brain [13]. In this study, however, the Neur1&2.DKO mice did not show any obvious developmental defects in brain development and their other tissues were grossly normal, demonstrating that both Neur1 and Neur2 are dispensable in the neurogenesis where Notch-mediated lateral inhibition controls the number of neurons in mammalian development. Although we did not observe an obvious Notch phenotype in the Neur1&2.DKO mice, we cannot exclude a possible role in the fine regulation of Notch ligands. Since Neur1&2.DKO mice have olfactory discrimination defects and ethanol hypersensitivity [16], the Neur1&2.DKO mice could have more severe defects in their behavior, although they did not exhibit a prominent developmental defect.

In contrast, we previously reported that Mib1−/− mice exhibit a severe neurogenic phenotype in the developing brain and neural tube in E9.5 embryos [13], demonstrating that Mib1 is essential for the lateral inhibition of embryonic neurogenesis, and the endogenous levels of both Neur1 and Neur2 cannot compensate for the loss of Mib1. In addition, the endocytosis of Dll1 was completely impaired in the Mib1−/− mice, suggesting that Mib1 is essentially required for Dll1 endocytosis [13]. Consistently, the overexpression of either mouse Neur1 or Neur2 alone does not promote the endocytosis of Xenopus Delta in Cos7 cells, while mouse Mib1 does [15]. Furthermore, zebrafish mib1 mutants also showed DeltaD accumulation in the plasma membrane and a neurogenic phenotype that is a representative Notch defect of lateral inhibition in neurogenesis [6]. Consistent with these findings, the overexpression of mouse Neur2 in the zebrafish mib1 mutants could not reduce the increased number of zoth-1-positive hair cells, which results from the failure of lateral inhibition in the mechanosensory organs [15]. Abundant evidence suggested that Mib1, but not Neur1/2, might be the E3 ubiquitin ligase that regulates Notch ligands in mammalian development.

Previously reported Mib1−/− mice exhibited multiple defects in neurogenesis, somitogenesis, vasculogenesis, and cardiogenesis [13]. The developmental defects shown in Mib1−/− mice might be caused by the loss of Dll1 (neurogenesis, somitogenesis), Dll4, (vasculogenesis, cardiogenesis) signaling [13]. In this study, the Tie2-cre;Mib1f/f embryos also exhibited the vascular defects characteristic of Dll1−/− and Dll2−/− mice, such as defective remodeling and impaired arterial fate specification. Since the Dll4-Notch1 signaling pathway is essential for arterial fate specification, our data strongly suggest that Mib1 is the E3 ligase that regulates Dll4 activity in endothelial cells. Moreover, conditional inactivation of Mib1 in hematopoietic system also exhibited the characteristic phenotype of Dll1 mutants, the defect in the formation of marginal zone B cells (Song et al., our unpublished observation).

In addition to the regulation of Dll ligands, our data suggested that Mib1 is also the E3 ubiquitin ligase that regulates Jag ligands in mammalian development. The hGEAP-cre,Mib1f/f mice exhibited delayed granule cell migration in the cerebellum. In addition, the Mx2-cre,Mib1f/f mice showed hair follicle loss and epidermal cyst formation in the skin. These are both representative phenotypes of Jag1 conditional knockout mice [22,23]. Furthermore, the Mx2-cre,Mib1f/f mice also showed syndactylysm, which is a well known Notch phenotype of Jag2 null mice [24,25]. Among the five canonical Notch ligands in mammals, Dll3 lacks a lysine residue in the cytoplasmic domain for ubiquitination, suggesting that it might not be a direct substrate of the E3 ubiquitin ligases. Except for Dll3, the other four Notch ligands, Dll1, Dll4, Jag1, and Jag2, should be regulated by E3 ligases for their endocytosis, because a plethora of evidence suggested that ligand endocytosis in the signal-sending cells is required for proper Notch activation in the signal-receiving cells, and that E3 ligases regulate their endocytosis. While Neur1&2.DKO and Mib2−/− mice did not show any obvious Notch phenotype, the Mib1−/− mice in the previous study and the Mib1 conditional knockout mice in various tissues in the present study clearly displayed the representative Notch phenotypes of each Notch ligand-null strain [13]. Thus, we concluded that Mib1, but not Neur1/2, plays an exclusive role in mammalian development, by potentially regulating the endocytosis of Jag ligands as well as Dll ligands.

**Evolutionary flux in the regulation of Notch ligands by E3 ligases**

The Notch signaling pathway is evolutionarily conserved from nematode to human. In terms of the requirement of E3 ligase for Notch ligand endocytosis, however, evolutionary divergence may exist. In *C. elegans*, in which the Notch signaling pathway is conserved as in *Drosophila* and vertebrates, there is a new homologue, but no *mib* homologue. However, in contrast to the strict requirement of Notch ligand endocytosis in *Drosophila*, DSL ligand endocytosis does not seem to be conserved in *C. elegans*. The extracellular domains of DSL ligands can fully rescue the lag-2 mutant and activate Notch signaling, while the intracellular domain-deleted mutants of Delta have a dominant-negative activity in *Drosophila* [43–45]. In zebrafish, mib1 mutants accumulate DeltaD in the plasma membrane, and in a transplantation experiment, mib1 null cells could not activate Notch signaling in the adjacent signal-receiving cells, indicating a strong requirement for ligand endocytosis in the Notch activation in zebrafish as well [6]. Consistently, Mib1−/− mice, in which the endocytosis of Dll1 was completely impaired and Dll1 accumulated in the plasma membrane, exhibit reduced expression of Notch target genes and loss of N1icd generation [13]. Furthermore, Mib1−/− cells cannot activate Notch signaling in a coculture system with C2C12-Notch1 cells containing a CBF-Luciferase reporter gene [13]. Collectively, the requirement of E3 ligase in Notch ligand endocytosis appears to be well conserved from *Drosophila* to mammals.

In the regulation of Notch ligand endocytosis, however, two structurally distinct E3 ligases, Mib and Neur, might have evolved differently. In *Drosophila*, both Notch ligands, Delta and Serrate, require an E3 ubiquitin ligase, either dMib or dNeur, for their activity in Notch-mediated cell fate decisions dependent on expression patterns [10–12]. In mammals, however, Mib1 is essential for Notch-mediated cell fate decisions in all contexts of mammalian development examined in this study and the previous studies [13]. In contrast, the Neur1−/− mice generated by two independent groups did not show any apparent developmental Notch defects [16,17]. In this study, the Neur2−/− and Neur1&2.DKO mice also were grossly healthy without any observable abnormality. Consistent with this finding, Neur1 and Neur2 do not induce endocytosis of Notch ligands in various cells, including Cos7 cells [15], whereas Mib1 and Mib2 readily do [13,14]. Although Xenopus Neur1 has been suggested to regulate Notch ligands in lateral inhibition, as revealed by an overexpression experiment [5], the loss-of-function studies using zebrafish mib1 mutants and Mib1−/− and Mib1/DKO mice indicated that Mib1 is required for various Notch-dependent cell fate decisions [6,13]. Thus, the two structurally distinct E3 ligases, Mib and Neur, might have evolved differently in the Notch signaling pathway during evolution, especially in insects and vertebrates.

There are two Mib homologues, Mib1 and Mib2, from *Drosophila* to human [14]. As described above, Mib1 is functionally
well conserved from *Drosophila* to mice. However, a substantial disparity exists, at least between zebrafish and mice. The zebrafish *mib1* mutants accumulated DeltaD in the plasma membrane and have various Notch mutant phenotypes, affecting neurogenesis, somitogenesis, vasculogenesis, and hematopoiesis. Interestingly, overexpression of *Mib2*, but not *Neur1* and *Neur2*, rescues the neurogenic and vasculogenic defects in these mutants [14,15], suggesting that *Mib2* can regulate Notch ligands in these contexts. In fact, a recent comparative analysis of zebrafish *mib1* mutants with an antimorphic allele, *mib1*<sup>plα2b</sup>, and a null allele, *mib1*<sup>tfi91</sup> suggested that *Mib1* and *Mib2* might have a redundant role in Notch signaling [46]. The *mib1*<sup>tfi91</sup> mutants exhibited no obvious somite defects, while the *mib1*<sup>plα2b</sup> mutants have a severe somite phenotype, suggesting that the antimorphic *mib1*<sup>plα2b</sup> mutant with a mutation in the RING domain might suppress the *Mib2* function in the *mib1*<sup>plα2b</sup> mutants, and that both *Mib1* and *Mib2* might function in somite formation. In contrast, two independently generated *Mib1<sup>−/−</sup>* mouse strains [13,47] and the *Mib<sup>jfl-A</sup>* mice conditionally deleted by the *prostamine-cre* system in this study display defects in somitogenesis, with only a few anterior somites with irregular shapes. Thus, the dependency of *Mib1* and *Mib2* in species might have evolved differently.

We cannot completely exclude the possibility that other E3 ligases, *Mib2*, *Neur1*, and *Neur2*, might play a critical role in the fine regulation of Notch signaling, which could not be recognized in the present assay systems that have been developed in order to elucidate Notch contexts, because of technical limitations. However, the inactivation of *Mib1* faithfully reproduced well-known Notch phenotypes, such as defects in neurogenesis, somitogenesis, vasculogenesis, hematopoiesis, skin morphogenesis, etc., in all contexts of mammalian development examined, without exception. Thus, our study strongly suggests that *Mib1* is the core E3 ubiquitin ligase that regulates Notch ligands. This provides the first genetic evidence that *Mib1* is required for the Notch-mediated cell fate decisions in a single organism, especially in mammals.

**METHODS**

**Mice**

The *Mib1<sup>jfl</sup>*, *Mib2<sup>jfl-A</sup>*, and *Neur2<sup>jfl-A</sup>* mice were generated through the germ-line transmission of chimeric C57BL/6 mice with targeted E14K ES cells, and the *Neur1<sup>jfl-A</sup>* mice were previously generated [16]. The transgenic mouse lines, *Tie2-cre*, and *MGFAP-cre*, were purchased from Jackson Laboratories, and the *Mx2-cre* mouse line was kindly provided by B. Harfe and G.R. Martins. All of the mice were maintained in our animal colony under institutional guidelines.

**Southern and northern blot analyses and RT-PCR analysis**

For the Southern blot analysis, genomic DNA from ES cells or mouse tail tip cells was digested by the appropriate restriction enzymes and fractionate by agarose gel electrophoresis. The DNA was transferred to a nylon membrane and analyzed with ³²P-labeled DNA probes. For the northern blot analysis, freshly isolated RNA from mouse brain tissues was separated by agarose gel electrophoresis, transferred to a nylon membrane, and analyzed with ³²P-labeled DNA probes. For the RT-PCR analysis, total RNA samples were extracted from mouse brains, using TRI-reagent (Sigma) 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. Primer information will be provided upon request.

**Western blot analysis**

For the western blot analysis, equal amounts of tissue extracts were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with antibodies to *Mib1* (gift from Dr. P. Gallagher) and β-actin (Sigma). Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

**Histology and Immunohistochemistry**

For histological analysis, tissues were fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin wax or O.C.T. for sectioning. The sections were subjected to hematoxylin and eosin (H&E) or Nissle staining. For immunohistochemistry, paraffin-embedded or O.C.T.-embedded sections were used, and the antigenic epitopes were exposed using 10 mM citrate buffer and micro-waving. Sections were incubated in blocking solution (3% BSA, 5% goat serum or horse serum, and 0.5% Tween-20 in PBS) at RT for 4 hr, followed by an additional incubation with various antibodies (Abs). Specific binding was detected with an ABC kit (Vectastain), an Envision kit (DAKO) or Alexa-488 (green) and/or -594 (red)-labeled Ab (Molecular Probes). Antibody probes included PEGAM (1:2000, MEC13.3, BD), CD44 (1:200, BD), sMA (1:200, NeoMarker), K14 (1:1000, Covance), K10 (1:500, Covance), Loricrin (1:500, Covance), PCNA (1:200, Santa Cruz Biotechnology), K167 (1:200, Novocasta), NeuN (1:100, Chemicon), BLBP (1:2000, gift from Dr. N Heintz), and GFAP (1:3000, Daco).

**In situ hybridization and bone staining**

Details of the RNA *in situ* hybridizations on whole mount or sectioned embryos were described [13]. The *splend42b*, *sm22*, *Fgβ2*, and *Jag2* 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. The *bmp2* probe was kindly provided by M. Logan. For the skeletal analysis, following removal of the skin, the specimen was fixed overnight in ethanol, and stained with Alcian blue and Alizarin red.

**SUPPORTING INFORMATION**

**Figure S1** Generation of *Mib1* conditional knockout mice. (A) Schematic drawing of the targeting strategy. The wild-type allele (*wt*) was recombined with the conditional targeting vector (*tv*) to generate the floxed allele (*fl*). As a result, one *loxP* sequence and a *loxP-neomycin* cassette are inserted between exons 1 and 2 and exons 3 and 4 of the mouse *Mib1* locus, respectively. Upon Cre expression, the floxed allele loses its exons 2 and 3 and becomes a null allele (*deltav*). *Ev*, *EosR*V; *H*, *Hind*III; DTA, *Diphtheria toxin* A; neo, *neomycin* resistance gene. (B) The genomic Southern blot analyses by *EosR*V (*Ev*) with a flanking probe and *Hind*III (*H*) with an internal probe show targeted embryonic stem cell clones (middle lane). Found at: doi:10.1371/journal.pone.0001221.s001 (8.74 MB TIF)

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**Author Contributions**

Conceived and designed the experiments: YJ YK BK MY KY CK PS. Performed the experiments: BK MY KY SI YK. Analyzed the data: YK BK MY KY SI YK. Contributed reagents/materials/analysis tools: YJ. Wrote the paper: YK BK MY KY SI YK.
REFERENCES

1. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284: 770–776.

2. Lai EC (2004) Notch signaling: control of cell communication and cell fate. Development 131: 965–973.

3. Schweigart F (2004) Notch signaling activity. Curr Biol 14: R129–138.

4. Parks AL, Khleg KM, Stout JR, Muskavitch MA (2000) Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development 127: 1373–1385.

5. Deblonde GA, Lai EC, Kintner C (2001) Xenopus neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. Dev Cell 1: 795–806.

6. Itoh M, Kim CH, Palardy G, Ota T, Jiang YJ, et al. (2000) Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev Cell 4: 67–82.

7. Lai EC, Deblonde GA, Kintner C, Rubin GM (2001) Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. Dev Cell 1: 783–794.

8. Pavlopoulos E, Pitouli C, Khleg KM, Muskavitch MA, Moschosnik NK, et al. (2001) neuralized Encodes a peripheral membrane protein involved in delta signaling and endocytosis. Dev Cell 1: 807–816.

9. Yeh E, Dermer M, Cominisco C, Zhou I, McClade CJ, et al. (2001) Neuralized functions as an E3 ubiquitin ligase during Drosophila development. Curr Biol 11: 1675–1679.

10. Le Bourge R, Renaud S, Hamel S, Schweigart F (2005) Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. PLoS Biol 3: e96.

11. Lai EC, Rojegers F, Qin X, Jan YN, Rubin GM (2005) The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. Development 132: 2319–2332.

12. Pitouli C, Delidakis C (2005) The interplay between DSL proteins and ubiquitin ligases in Notch signaling. Development 132: 4841–4850.

13. Koo BK, Lim HS, Song R, Yoon MJ, Yoon KJ, et al. (2005) Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. Development 132: 3459–3470.

14. Koo BK, Yoon KJ, Yoo KW, Lim HS, Song R, et al. (2005) Mind bomb-2 is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. J Biol Chem 281: 36391–36400.

15. Lai EC (2004) Notch signaling: control of cell communication and cell fate. Curr Biol 14: R129–138.

16. Sun X, Artavanis-Tsakonas S (1997) Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila. Development 124: R74–78.

17. Krebs LT, Shutter JR, Tamigaki K, Honjo T, Stark KL, et al. (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 18: 2469–2473.

18. Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, et al. (1999) Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 13: 295–306.

19. Weidler SC, Isacce GM, Crossley PH (1995) Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning. Development 119: 295–306.

20. Yanamamote N, Tamigaki K, Han H, Hsei H, Honjo T (2003) Notch/RBPJ signaling regulates epidermis/hair fate determination of hair follicular stem cells. Curr Biol 13: 333–338.

21. Sun X, Lewisds M, Meyers EN, Liu YH, Masson RE, et al. (2000) Conditional inactivation of Fgf1 reveals complexity of signalling during limb bud development. Nat Genet 25: 83–86.

22. Klein AL, Zilian O, Suter U, Taylor V (2004) Murine numb regulates granule cell maturation in the cerebellum. Dev Biol 265: 161–177.

23. Latolle S, Radke F, Aguet M, Suter U, Taylor V (2002) Numb1 is required for neuronal and glial differentiation in the cerebellum. Development 129: 373–385.

24. Zhao L, Then M, Alvarez-Mayor M, Bremmer M, Willecke K, et al. (2001) bGAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. Genesis 31: 85–94.

25. Lind D, Franken S, Kappler J, Jankowski J, Schilling K (2005) Characterization of the neuronal marker NeuN as a multiply phosphorylated antigen with discrete subcellular localization. J Neurosci Res 79: 295–302.

26. Zou H, Niswander L, Artzt K (2005) Mind bomb1 is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. Dev Cell 1: 783–794.

27. Commisso C, Boulianne GL, Artavanis-Tsakonas S, Warburton ME, Ros MA, et al. (1997) Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. Development 124: 1109–1117.

28. Zou H, Niswander L (1996) Requirement for BMP signaling in interdigital apoptosis and scale formation. Science 272: 738–741.

29. Lai EC (2002) Protein degradation: four E3s for the notch pathway. Curr Biol 12: R74–78.

30. Barsi JC, Rajendra R, Wu JI, Artzt K (2005) Mind bomb1 is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. J Biol Chem 281: 36391–36400.

31. Klein AL, Zilian O, Suter U, Taylor V (2004) Murine numb regulates granule cell maturation in the cerebellum. Dev Biol 265: 161–177.

32. Yamamoto N, Tamigaki K, Han H, Hsei H, Honjo T (2003) Notch/RBPJ signaling regulates epidermis/hair fate determination of hair follicular stem cells. Curr Biol 13: 333–338.

33. Sun X, Lewisds M, Meyers EN, Liu YH, Masson RE, et al. (2000) Conditional inactivation of Fgf1 reveals complexity of signalling during limb bud development. Nat Genet 25: 83–86.

34. Klein AL, Zilian O, Suter U, Taylor V (2004) Murine numb regulates granule cell maturation in the cerebellum. Dev Biol 265: 161–177.

35. Latolle S, Radke F, Aguet M, Suter U, Taylor V (2002) Numb1 is required for neuronal and glial differentiation in the cerebellum. Development 129: 373–385.

36. Zhao L, Then M, Alvarez-Mayor M, Bremmer M, Willecke K, et al. (2001) bGAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. Genesis 31: 85–94.

37. Lind D, Franken S, Kappler J, Jankowski J, Schilling K (2005) Characterization of the neuronal marker NeuN as a multiply phosphorylated antigen with discrete subcellular localization. J Neurosci Res 79: 295–302.

38. Zou H, Niswander L (1996) Requirement for BMP signaling in interdigital apoptosis and scale formation. Science 272: 738–741.

39. Lai EC (2002) Protein degradation: four E3s for the notch pathway. Curr Biol 12: R74–78.

40. Barsi JC, Rajendra R, Wu JI, Artzt K (2005) Mind bomb1 is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. J Biol Chem 281: 36391–36400.

41. Lai EC (2002) Notch signaling: control of cell communication and cell fate. Curr Biol 12: R74–78.

42. Artavanis-Tsakonas S, de la Concha A, Campos-Ortega JA, Jan YN, Jan YN (1991) The Drosophila neurogenic gene neuralized encodes a novel protein and is expressed in precursors of larval and adult neurons. Embio 10: 2975–2983.

43. Fitzgerald K, Greenwald I (1995) Interchangeability of Caenorhabditis elegans DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. Development 121: 4273–4282.

44. Sun X, Artavanis-Tsakonas S (1996) The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands. Development 122: 2463–2474.

45. Sun X, Artavanis-Tsakonas S (1997) Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila. Development 124: 3439–3448.

46. Zhang C, Li Q, Lim CH, Qu X, Jiang YJ (2007) The characterization of zebrafish antimorphic miB1 alleles reveals that Mib and Mind bomb-2 (MiB2) function redundant. Dev Biol 305: 14–27.

47. Barai GC, Rajenrudra R, Wu JJ, Artzt K (2005) Mind bomb1 is a ubiquitin ligase essential for mouse embryonic development and Notch signaling. Mech Dev 122: 1106–1117.