Mind Bomb Is a Ubiquitin Ligase that Is Essential for Efficient Activation of Notch Signaling by Delta

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Introduction

Notch signaling mediates a wide range of essential cell-cell interactions (Bray, 1998; Lewis, 1998). It is best known for its role in selecting cells to become neuroblasts or sensory organ precursors in Drosophila by the process of lateral inhibition, where cells adopting a neural fate inhibit their neighbors from adopting a similar fate. Cells in proneural clusters initially acquire the potential to adopt a neural fate by expressing proneural genes that encode basic helix-loop-helix (bHLH) transcription factors. The proneural genes drive the expression of Delta, a membrane-bound ligand that interacts with its receptor, Notch, in neighboring cells. Activation of Notch by Delta leads to expression of other bHLH transcription factors, in the Enhancer-of-split family, which inhibit proneural gene function. Through this simple mechanism, the cells within a proneural cluster compete to inhibit one another from adopting a neural fate. Some cells eventually emerge as the winner and, through autoregulation, acquire high enough levels of proneural gene expression to permit adoption of a neural fate. The feedback loop mediated by Notch signaling plays a similar role during the development of many different organ systems and is a well-conserved metazoan invention (Artavanis-Tsakonas et al., 1999). Notch signaling also functions in other cellular contexts in an inductive role (Kimble and Simpson, 1997), and it plays an essential part in a cellular oscillator during vertebrate somitogenesis (Holley et al., 2002; Jiang et al., 2000; Oates and Ho, 2002).

In zebrafish embryos, Notch signaling mediates the selection of a subset of neural progenitors that will become early neurons. These cells are singled out in the neural plate from proneuronal domains where they express proneural transcription factors. The proneural genes drive the expression of proneural genes that encode basic helix-loop-helix (bHLH) transcription factors. The proneural genes drive the expression of Delta, a membrane-bound ligand that interacts with its receptor, Notch, in neighboring cells. Activation of Notch by Delta leads to expression of other bHLH transcription factors, in the Enhancer-of-split family, which inhibit proneural gene function. Through this simple mechanism, the cells within a proneural cluster compete to inhibit one another from adopting a neural fate. Some cells eventually emerge as the winner and, through autoregulation, acquire high enough levels of proneural gene expression to permit adoption of a neural fate. The feedback loop mediated by Notch signaling plays a similar role during the development of many different organ systems and is a well-conserved metazoan invention (Artavanis-Tsakonas et al., 1999). Notch signaling also functions in other cellular contexts in an inductive role (Kimble and Simpson, 1997), and it plays an essential part in a cellular oscillator during vertebrate somitogenesis (Holley et al., 2002; Jiang et al., 2000; Oates and Ho, 2002).

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Summary

Lateral inhibition, mediated by Notch signaling, leads to the selection of cells that are permitted to become neurons within domains defined by proneural gene expression. Reduced lateral inhibition in zebrafish mib mutant embryos permits too many neural progenitors to differentiate as neurons. Positional cloning of mib revealed that it is a gene in the Notch pathway that encodes a RING ubiquitin ligase. Mib interacts with the intracellular domain of Delta to promote its ubiquitylation and internalization. Cell transplantation studies suggest that mib function is essential in the signaling cell for efficient activation of Notch in neighboring cells. These observations support a model for Notch activation where the Delta-Notch interaction is followed by endocytosis of Delta and transendocytosis of the Notch extracellular domain by the signaling cell. This facilitates intramembranous cleavage of the remaining Notch receptor, release of the Notch intracellular fragment, and activation of target genes in neighboring cells.

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characterized by a severe neurogenic phenotype; they also have a wide range of additional defects in the development of somites, neural crest, and vasculature that have been interpreted as consequences of deficits in Notch signaling in all these tissues (Jiang et al., 1996; Lawson et al., 2001; Schier et al., 1996; van Eeden et al., 1998). Though these studies have suggested that mib is likely to encode an essential component of the Notch pathway, it was not known how it contributes to Notch signaling.

Notch is a one-pass transmembrane receptor that is synthesized as a single peptide. Furin-mediated cleavage of the peptide creates two fragments that are held together in the mature receptor as a heterodimer, through a noncovalent calcium-dependent linkage. The extracellular fragment with EGF repeats mediates interactions with DSL (Delta, Serrate, Lag-2) ligands. Its membrane-proximal end is essential for binding to the second fragment, which spans the membrane and contains an intracellular domain that mediates Notch signaling. A key step in the activation of Notch is the removal of the extracellular fragment (Rand et al., 2000). Binding to Delta makes Notch susceptible to the action of TACE metalloproteases that cleave at a second site outside the Notch transmembrane domain (Brou et al., 2000). In Drosophila, it has been shown that the Delta-Notch interaction is accompanied by endocytosis of Delta by the signaling cell, which carries with it the bound Notch extracellular domain (Parks et al., 2000). The membrane-bound Notch fragment that remains on the adjacent cell after the cleavage by TACE metalloproteases is a substrate for \( \gamma \)-secretases that cleave it at a third site, within the membrane, to release an intracellular fragment (NotchICD) that functions in a transcriptional activation process (NotchICD); functions in a transcriptional activator complex with Su(H)/CBF1/RBP-Jk (De Strooper et al., 1999) to activate Notch target genes.

Recent studies have emphasized the role of ubiquitination in regulating Notch signaling (Lai, 2002). Ubiquitination is a multistep process that results in the addition of a 76 amino acid polypeptide, ubiquitin, to a substrate protein (Weissman, 2001). First, a ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent manner; then a ubiquitin-conjugating enzyme (E2) receives its ubiquitin from an E1. Finally, a ubiquitin ligase (E3) that contains a substrate recognition domain and provides a docking site for an E2 facilitates transfer of ubiquitin from the E2 to the E3-defined substrate. Ubiquitylation was originally recognized for its role in tagging proteins for destruction in proteasomes (Weissman, 2001). More recently, it has been shown that addition of ubiquitin to proteins can play a key role in changing the behavior or distribution of a protein and can affect a variety of events including endocytosis (Hicke, 2001; Weissman, 2001).

Several different ubiquitin ligases, or E3s, have been implicated in Notch signaling. Sel-10 and Suppressor of deltex/Ich are negative regulators of Notch identified for their role in promoting degradation of NotchICD (Qiu et al., 2000; Wu et al., 2001). LNX, in contrast, is a positive regulator of Notch signaling responsible for degradation of Numb, a membrane-associated protein that inhibits the function of the Notch receptor (Nie et al., 2002). Finally, neutralized (neur) was recently shown to encode a RING domain-containing E3 that monoubiquitylates and promotes endocytosis of Delta (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001). The neurogenic phenotype of the neur mutants has, in part, been accounted for by the role of Neur in degrading Delta in Notch-expressing cells, as Delta in cis with its receptor can diminish the ability to receive inhibition from neighboring cells (de Celis and Bray, 1997; Henrik et al., 1997). An absence of Neur, by permitting excessive accumulation of Delta on the cell surface, may thus make cells insensitive to lateral inhibition. An alternative account proposes that another, very different mechanism underlies the Neur phenotype. In Drosophila, neur is expressed most strongly in the cells that adopt a neuronal fate and escape lateral inhibition themselves while delivering it to their neighbors; thus, it has been suggested that Neur enhances the ability of Delta to activate the Notch receptor on neighboring cells (Pavlopoulos et al., 2001).

Our analysis of mib reveals that it encodes a previously uncharacterized ubiquitin ligase with Delta as one of its substrates, and that its function is similar to the latter function described above for Neur in Drosophila. We show that Mib has a primary role in protein trafficking, rather than simply in protein degradation: it promotes internalization of Delta and, in the process, paradoxically, increases the efficiency with which Delta activates Notch. Analysis of this component of the Notch signaling pathway provides fresh insight into poorly understood mechanisms that are required to make Delta an effective ligand.

**Results**

**mib Mutants Have a Neurogenic Phenotype Due to Reduced Notch Signaling**

**huc** expression at the three-somite stage in the zebrafish caudal neural plate reveals differentiating neurons in proneural domains (Kim et al., 1998). In mib mutants, a larger number of cells express **huc** within each of these domains, showing, in agreement with previous observations (Jiang et al., 1996; Schier et al., 1996), that too many cells have been permitted to become early neurons (Figure 1A). Cells are selected to become neurons within the lateral proneural domain a little earlier than in the intermediate domain, and comparison of these domains at the tailbud stage in wild-type embryos illustrates how expression of **ngn1** and **deltaA** (as well as **deltaB** and **deltaD**; see Haddon et al., 1998b) is progressively restricted to a smaller subset of cells as a consequence of lateral inhibition (Figures 1B and 1C, left panels). A comparison with mib mutants reveals that expression of **ngn1** and **deltaA** is not as effectively restricted in mutant embryos, suggesting that lateral inhibition mediated by Notch signaling is reduced in mib mutants (Figures 1B and 1C, right panels).

Ectopic expression of **ngn1** mRNA in the neuroectoderm of a wild-type zebrafish embryo creates a domain in which cells have the potential to become neurons; but as a consequence of lateral inhibition, only a subset of the cells within the ectopic **ngn1** domain is actually permitted to realize this potential (Blader et al., 1997; Chitnis and Kintner, 1996; Kim et al., 1997; Ma et al., 1996). A comparison of the pattern of neurons in wild-type and mib mutant embryos after ectopic expression...
of ngn1 mRNA reveals a denser pattern of ectopic neurons in mib mutants, adding to the evidence that lateral inhibition is reduced in mib mutants (Figures 1D and 1E). Furthermore, expression of the Notch target gene, her4, is reduced in mib mutants (Figure 1F), supporting the conclusion that Notch signaling is reduced.

The neurogenic phenotype in mib mutants is accompanied by an increase in the expression of all four delta genes (Figure 1C; Haddon et al., 1998b), presumably reflecting a loss of inhibition of delta gene expression. Therefore, the reduced her4 expression is not due to a failure to express Notch ligands but could be due to ineffectiveness of those ligands as activators of Notch. Injection of mRNA encoding the intracellular fragment of Notch5, notch5ICD, suppressed neurogenesis in mib mutants (Figure 1G), suggesting that steps in the Notch pathway that follow generation of NotchICD still function in mib mutants. Injection of Xdelta1 mRNA also suppressed neurogenesis in mib mutants (Figure 1H), supporting the possibility that although endogenous Delta...
is ineffective in mib mutants, the Notch pathway can still be effectively activated in these mutants when Delta is artificially expressed at very high levels.

**Failure of Lateral Inhibition Leads to Premature Differentiation of Neural Progenitors in the Spinal Cord**

At 24 hr, deltaA and notch5 are widely expressed in the spinal cord (Figures 1I and 1J; Appel et al., 2002). notch5 is expressed primarily in centrally located progenitors in a pattern that is complementary to differentiating neurons at the periphery of the spinal cord, while deltaA is preferentially expressed in progenitors being selected to become neurons (Appel et al., 2002). In mib mutants, by contrast, there is a dramatic reduction in deltaA and notch5 expression by 24 hr, suggesting loss of neural progenitors in the spinal cord (Figures 1I and 1J). Differentiating neurons are easily identified in huC-GFP transgenic embryos where the huC promoter drives GFP expression (Park et al., 2000). A three-dimensional reconstruction of a segment of the spinal cord made from scanning confocal microscope sections of wild-type and mib mutant huC-GFP embryos shows an excess of GFP-expressing cells in mib mutants (Figure 1K). Rotation of the reconstruction to reveal a cross-section shows that differentiating neurons are normally restricted to the periphery of the neural tube in wild-type embryos (Figure 1L, left panel; Appel et al., 2002). In contrast, in mib mutant transgenic huC-GFP fish, the GFP-expressing cells progressively fill the central region of the spinal cord (Figure 1L, right panel), suggesting that the loss of cells expressing deltaA and notch5 reflects a loss of neural progenitors as a result of their premature differentiation into neurons. In this way, apparently, failure of lateral inhibition brings neurogenesis to an early end and eventually disrupts the normal architecture of the spinal cord.

**Positional Cloning of mib Reveals that It Encodes a Previously Uncharacterized Protein with RING Domains**

Because functional analysis of mib mutants suggested it might correspond to a component of the Notch signaling pathway, homologs of Delta, Notch, presenilin, Su(H), deltex, neur, and kuzbanian were tested and eliminated as candidates for mib through linkage analysis (data not shown). mib was mapped to linkage group 2 within 0.34 cM of a polymorphism linked to GATA6 3’UTR (Figure 2A). This polymorphism led to the identification of a YAC (y80g9) and two BAC (b133o42 and b84g2) clones, which further identified two PACs (p228p21 and p227p15) predicted to contain the mib mutation based on progressive lack of recombination (Figure 2A). A cosmid library was made from these PACs, and the sequence from one of the cosmids (Cos2) led to the identification of a coding sequence with strong homology to an uncharacterized gene, CG5841, in Drosophila. 5’ and 3’ RACE with an embryonic RT-PCR library identified the remaining putative mib coding sequence. Sequencing of this gene in two mib alleles revealed point mutations that were predicted to result in truncated protein products. This provided initial confirmation that the gene was indeed mib (see below).

The mib gene encodes a predicted 1030 amino acid protein with five characteristic motifs in its N-terminal, middle, and C-terminal sections (Figure 2B). The C-terminal section contains three RING domains; the first two are atypical while the third is a prototypical RING finger, associated with catalytic function in RING E3 ubiquitin ligases. In the middle section, depending on the stringency of the criteria used, there are six to eight ankyrin repeats, known for their role in mediating protein-protein interactions (Sedgwick and Smerdon, 1999). The N-terminal section has a ZZ zinc finger (Ponting et al., 1996), a domain seen in a number of proteins including HERC2, a ubiquitin ligase thought to be involved in protein trafficking (Li et al., 1999). A characteristic domain is present on either side of the ZZ zinc finger. It is similar to a domain in HERC2, and we have named it the Mib/HERC2 (M-H) domain. Between the M-H domain and the ankyrin domain Mib has a unique repeated sequence that we have called the Mib repeat.

Sequencing of mib cDNAs from five mutant alleles revealed that tfi101 and ta52b (previously known as white tail, wti) have point mutations that predict amino acid substitutions in the prototypical RING domain (ta52b, M1013R and tfi101, C1009S). Point mutations in the remaining alleles create premature stop codons after the ankyrin domains in m132 (C785stop), before the ankyrin repeats in m178 (G412stop), and within the N-terminal M-H domain in tfi91 (Y60stop), all of which eliminate the RING domain with predicted catalytic activity. All the alleles have a neurogenic phenotype, suggesting that this represents a loss-of-function phenotype.

By searching EST and genomic databases, we found that the human and mouse genomes each contain one mib ortholog. mib is a previously uncharacterized gene, showing remarkably high conservation. At the amino acid level, the fish and human genes are 93.9% identical, fish and mouse are 94% identical, and fish and Drosophila are 67.5% identical (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/4/1/67/DC1). The insect and vertebrate genomes contain other uncharacterized genes that are clearly related to mib in domain structure but have markedly lower percent identity (Drosophila: CG17492; human and mouse: skeletrophin; data not shown). Similarities in the genomic organization of the human and zebrafish mib gene helped define exon-intron boundaries of the zebrafish gene (data not shown). Antisense morpholinos were made complementary to exon-intron boundaries 1 and 3 (ex/int 1 MO and ex/int 3 MO), and injection of these morpholinos in embryos at the one-cell stage produced a neurogenic phenotype similar to that seen in mib mutants (Figure 2C), confirming that this gene is responsible for the mutant phenotype.

$mib$ transcripts are expressed maternally at low levels that can be detected by RT-PCR, and they continue to be expressed zygotically throughout the embryo (Figure 2D). Though broadly expressed at relatively low levels, their expression appears a little higher on the dorsal side at the shield stage and in the prospective neural plate as gastrulation proceeds (Figure 2E). At the one-somite stage, expression in the neural plate remains...
Mib-Mediated Delta Internalization Activates Notch

Figure 2. Molecular Characterization of mib
(A) Meiotic and physical map of mib on LG2.
(B) Structure of Mib protein with predicted domains and point mutations in five alleles.
(C) Effect of mib splice junction morpholinos on huC expression at the 3 ss. Morpholinos are predicted to generate protein truncations at sites indicated by arrows.
(D) RT-PCR analysis of mib expression from maternal (unfertilized) stage to 12 hr. EF-1 mRNA is amplified as a control.
(E) Expression of mib. All stages are viewed from the side except for 1 ss (dorsal view). Dorsal is to the right in shield stage and 90% epiboly embryos. Anterior is to the left in tailbud, 1 ss, 20 ss, and 24 hr embryos, at the top in shield stage and 90% epiboly embryos.

Figure 2E, arrow). At the 20-somite stage, expression remains broad throughout the embryo (Figure 2E). By 24 hr post-fertilization (hpf), the transcripts are most easily seen throughout the CNS (Figure 2E). The broad expression of mib during early embryogenesis is consistent with its suggested role as an essential component of the Notch pathway that is required in many different tissues throughout development.

Mib Is a Ubiquitin Ligase that Ubiquitylates Delta
To examine the potential function of Mib as a ubiquitin ligase, a GST-RING fusion protein made with the wild-type mib RING domain was combined with ATP, ubiquitin, E1, and an E2 in vitro. The wild-type Mib GST-RING fusion protein promoted self-ubiquitylation in an E2 (Ubc8 or UbcH5b)-dependent manner (Figure 3A). In contrast, GST fusion proteins made with mutant RING domains, designed either to mimic the ta52b point mutation (GST-M1013R-RING) or to disrupt the integrity of
Figure 3. Mib Is a Ubiquitin Ligase with Delta as a Substrate

(A) E2-dependent self-ubiquitylation by the Mib RING domain. GST fusion proteins of Mib wild-type RING or mutant RING (M1013R, C1001S) were incubated in a reaction mixture in the absence or presence of E2 (Ubc8 or UbcH5B). The bracket shows 32P-labeled polyubiquitylated GST-RING proteins.

(B) Xdelta1 ubiquitylation by Mib. Xdelta1-myc was immunoprecipitated (IP) with anti-Myc and detected by immunoblotting (IB) with anti-Myc or anti-HA to detect total and ubiquitylated Xdelta1.

(C) IP of Xdelta1 or Xdelta1\(\Delta ICD\) by various Mib constructs. The top panel shows IP of Delta by Mib constructs, and the middle and bottom panels are blots showing expression of Delta and Mib constructs, respectively, in cell lysates.

(D) A comparison of Xdelta1\(\Delta ICD\) and zebrafish DeltaD as substrates for Mib-mediated ubiquitylation.

(E) Schematic drawing of Mib constructs summarizing their ability to IP Xdelta1 or Xdelta1\(\Delta ICD\).

the RING structure by replacing a critical cysteine (GST-C1001S-RING), showed reduced self-ubiquitylation (Figure 3A). Because mib appears to play a critical role in Notch signaling, a number of proteins in this pathway were tested and Delta was identified as a potential substrate for Mib. COS7 cells were cotransfected with plasmids encoding FLAG-Mib, HA-ubiquitin, and Xdelta1-myc (Figure 3B). Immunoprecipitation with a Myc antibody and detection with anti-HA antibody showed that Xdelta1-myc ubiquitylation was enhanced in the presence of FLAG-Mib but not by mutant forms of Mib (Figure 3B). FLAG-tagged middle and C-terminal sections of Mib were also unable to promote ubiquitylation of Xdelta1-myc (Figure 3B).

To identify the requirements for interactions between Mib and Delta, we cotransfected HA-tagged Xdelta1 with Myc-tagged wild-type, mutant, or truncated forms of Mib. These forms of Mib were immunoprecipitated with Myc antibody and immunoblotted with anti-HA to detect coprecipitation of Delta. All forms of Mib that included either its N-terminal section or its middle section coimmunoprecipitated Xdelta1-HA (Figures 3C and 3E), suggesting that the N-terminal and middle domains can independently interact with Delta. However, these same forms of Mib were unable to pull down Xdelta1 radical, the truncated form of Xdelta1 that lacks its intracellular domain, suggesting that this domain is essential for interaction with Mib (Figures 3C and 3E). Consistent with this observation, Mib was unable to promote ubiquitylation of Xdelta1 radical (Figure 3D). Mib was, however, able to promote ubiquitylation of zebrafish DeltaD (Figure 3D) and DeltaB (not shown), confirming that multiple zebrafish Deltas can serve as natural substrates for this ubiquitin ligase.

Mib Interaction with Delta Promotes Its Internalization

To examine the cellular distribution of Mib and visualize the consequences of its interaction with Delta, we studied COS7 cells transfected with Myc-Mib and Xdelta1-HA. When Myc-Mib was transfected alone into COS7 cells, it was broadly distributed in the cytoplasm and typically excluded from the nucleus; some punctate expression was also seen within the cytoplasm (Figure 4A). Xdelta1-HA is a membrane-bound protein and when it was transfected alone, its expression clearly defined the surface and membranous extensions of transfected cells (Figure 4B). Some vesicular expression was also seen in some cells (not shown). When Myc-Mib and Xdelta1-HA were cotransfected, much less Xdelta1 expression was seen on the cell surface and most of it tended to accumulate in vesicles and in a perinuclear structure, where it colocalized with Myc-Mib (Figure 4D). In contrast, when Xdelta1-HA was transfected with Myc-mib radical, whose RING domain is nonfunctional, Xdelta1-HA remained on the cell surface where it colocalized with Myc-mib radical (Figure 4E). As might be predicted from the observation that the Delta intracellular domain is essential for interaction with Mib, Xdelta1 radical-HA did not colocalize with, nor did it have its distinct subcellular distribution altered by, Myc-Mib (Figure 4F). Together, these observations suggest that Mib normally associates with Delta and promotes its accumulation in intracellular vesicles. In the absence of a functional RING domain, however, Mib fails to promote internalization of Delta and instead remains associated with it at the cell surface.

To determine the nature of the vesicles in which Xdelta1 and Mib were colocalized, we examined a number of organelle markers. We found Mib colocalized with GFP-tagged RabB, a GTPase typically seen in late endosomes and lysosomes (Barbero et al., 2002), suggesting that reduction of cell surface Delta may be due to increased endocytosis (Figure 4G). Colocalization was also seen, though not as clearly, with GFP-tagged Rhod, another GTPase involved in trafficking of endosomes (data not shown).

A surface ubiquitylation assay was used to confirm that Mib reduces cell surface Delta (Figure 4H). Xdelta1-HA DNA was transfected into cells alone or together with wild-type mib or mib radical, and cell surface proteins were biotinylated prior to harvesting of the cells. Xdelta1-HA was immunoprecipitated from total cell lysate and blotted with streptavidin-HRP to detect biotinylated Xdelta1-HA. Cells cotransfected with wild-type mib showed a clear reduction in cell surface Xdelta1-HA compared to control cells, while no significant difference was seen in cells transfected with mib radical (Figure 4H). These observations provide additional evidence that Mib reduces cell surface Delta.

Finally, we looked to see whether Mib acts similarly on Delta under normal physiological conditions in the zebrafish embryo. Using a monoclonal antibody (zdd2) raised against the DeltaD extracellular domain, we compared the distribution of endogenous DeltaD in homozygous mib mutant siblings. In the wild-type, DeltaD staining (in the neural tube and sensory epithelia, at least) is punctate and predominantly intracellular. In the mib mutant, by contrast, there is strong staining at cell surfaces. The hair cells of the developing inner ear, which transiently express the four zebrafish delta genes and are overproduced in mib mutants (Haddon et al., 1998a, 1999), illustrate this well (Figure 4I). Thus, wild-type Mib acts in the normal embryo to keep cell surface levels of Delta protein low.

By ubiquitylating Delta, Mib could reduce cell surface Delta either by promoting its endocytosis or by sorting it directly from the Golgi network to late endosomes and lysosomes. To confirm that Mib actually promotes endocytosis of Delta, we specifically examined accumulation of internalized cell surface DeltaD using the zdd2 antibody. COS7 cells were transfected with either DeltaD alone or with DeltaD plus a myc-tagged wild-type or RING mutant mib. The live cells were then incubated with the zdd2 antibody for 9 hr to label cell surface DeltaD and follow its internalization over this extended period (Figure 5; Table 1).

In cells that had only been transfected with DeltaD, the zdd2 antibody was primarily on the cell surface in 54% (30/56) of the cells (Figure 5A, class I); it was on both the surface and in intracellular vesicles in 30% (17/56; Figure 5B, class II); and it was primarily associated with prominent intracellular vesicles that often had a perinuclear distribution in the remaining 16% (9/56; Figure 5C, class III). The class II and class III patterns suggest that COS7 cells contain endogenous factors that promote internalization of cell surface Delta, but only in
Figure 4. Ubiquitylation Reduces Cell Surface Delta Expression

(A–F) The Myc and HA epitopes were detected with Alexa 488 and Alexa 594, and images were pseudocolored green and magenta, respectively. Overlapping expression is white.

(A–C) Expression of Myc-Mib (A), Xdelta1-HA (B), and Xdelta1.1ICD-Ub-HA (C) in COS7 cells.

(D) Cotransfection of Myc-Mib and Xdelta1-HA.

(E) Cotransfection of Myc-mibC1001S and Xdelta1-HA.

(F) Cotransfection of Myc-Mib and Xdelta1.1ICD-HA.

(G) Cotransfection of FLAG-mib (green), Xdelta1-HA (red), and Rab9-GFP (blue). Arrows show colocalization of all three proteins.

(H) Surface expression of Xdelta1 but not Xdelta1.1ICD is reduced by wild-type Mib. Surface-biotinylated Xdelta1 or Xdelta1.1ICD is shown in the upper panel; total Xdelta1 or Xdelta1.1ICD (detected by reprobing the same blot with anti-HA) is shown in the lower panel.

(I) Distribution of endogenous DeltaD, detected with zdd2 antibody, in cryosections of sensory patches in zebrafish ear at 72 hr. Newly formed hair cells express DeltaD in intracellular apical granules in wild-type, but at the cell surface in mib (tfi91); green counterstain shows actin. Note that in mib, supporting cells are missing and hair cells are abnormally tall, spanning the epithelium (Haddon et al., 1998a).

Cell surface (Figure 5E), providing additional support to the conclusion that the RING mutant Mib does not promote internalization of Delta. Because 41% of the cells cotransfected with DeltaD and MibC1001S had zdd2 primarily at the cell surface, it seems that MibC1001S does not have strong inhibitory effects on endogenous factors that promote internalization of DeltaD. No zdd2 labeling was observed in cells that had not been transfected with DeltaD, showing that binding and internalization of zdd2 only took place in the presence of cell surface DeltaD (data not shown).

Together, these observations show that Mib promotes
internalization of Delta, although they do not rule out the possibility that Mib also regulates the amount of Delta at the cell surface by promoting the sorting of newly synthesized Delta directly to late endosomes and lysosomes.

Ubiquitin Alters the Function and Distribution of XDelta1ICD

Because the Delta intracellular domain is essential for Mib-mediated ubiquitylation, and its removal creates a form of Delta that hinders Notch signaling (Chitnis et al., 1995; Sun and Artavanis-Tsakonas, 1996), we asked whether addition of the ubiquitin peptide to Xdelta1ICD would restore its ability to promote lateral inhibition and/or abolish its ability to block lateral inhibition. A fusion protein, Xdelta1ICD-Ub-HA, was created with ubiquitin-HA fused in-frame to the C-terminal of Xdelta1ICD. Embryos were injected on one side at the two-cell stage with mRNA encoding Xdelta1-HA, Xdelta1ICD-HA, or Xdelta1ICD-Ub-HA, and effects on neurogenesis were assayed by examining huC expression at the three-somite stage. Ectopic expression of Xdelta1-HA inhibited huC expression (Figure 6A) and Xdelta1ICD produced an increased density of huC-expressing cells (Figure 6B), but ectopic expression of Xdelta1ICD-Ub-HA inhibited neurogenesis, producing a phenotype similar to that seen with Xdelta1-HA (Figure 6C). Addition of ubiquitin not only restored the ability of Xdelta1ICD to inhibit neurogenesis, it also altered its cellular distribution. XDelta1ICD-HA is expressed broadly on the cell surface with some perinuclear expression (Figure 4F, magenta), while XDelta1ICD-Ub-HA does not appear to be expressed significantly at the cell surface, but instead has a predominantly perinuclear distribution (Figure 4C). This observation is consistent with the possibility that addition of ubiquitin restores the mechanism of Delta internalization. This experiment by itself does not directly show that addition of ubiquitin promotes internalization, because it is not known whether XDelta1ICD-Ub-HA ever gets to the cell surface, but it is likely that XDelta1ICD-Ub-HA does reach the surface, as it is effective at mediating lateral inhibition and inhibiting neurogenesis in embryos.

mib Mutant Cells Are Less Effective at Producing an Inhibitory Signal during Lateral Inhibition

We used transplantation experiments to determine whether loss of mib function primarily affects a cell’s ability to produce an inhibitory signal during lateral inhibition or its ability to receive such a signal. If mib function were essential for producing an inhibitory signal but not for receiving inhibition from neighboring cells, then in proneuronal domains, cells with reduced mib function would be less likely to become neurons: they would be refractory to the neighbors’ inhibitory signals. On the other hand, if loss of mib function were to interfere primarily with a cell’s ability receive lateral inhibition signals, then cells with reduced mib function would be more likely to become neurons: they could effectively inhibit the neighbors while remaining refractory to the neighbors’ inhibitory signals. The huC-GFP

Table 1. Effect of Mib and MibC1001S on Internalization of zdd2

<table>
<thead>
<tr>
<th></th>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
</tr>
</thead>
<tbody>
<tr>
<td>DeltaD</td>
<td>54% (30/56)</td>
<td>30% (17/56)</td>
<td>16% (8/56)</td>
</tr>
<tr>
<td>DeltaD + Mib</td>
<td>2% (1/43)</td>
<td>26% (11/43)</td>
<td>72% (31/43)</td>
</tr>
<tr>
<td>DeltaD + MibC1001S</td>
<td>52% (22/42)</td>
<td>41% (17/42)</td>
<td>7% (3/42)</td>
</tr>
</tbody>
</table>
transgenic line was used in transplantation experiments to test these predictions.

Rhodamine-dextran–labeled cells from huC-GFP transgenic donor embryos were transplanted into unlabeled huC-GFP transgenic hosts before the shield stage. At around 24 hpf, the embryos were examined to determine what fraction of donor cells within the neural tube had become GFP positive (GFP+), indicating that they had differentiated as neurons (Figure 7A). To determine how reduction of mib function influences the probability of becoming a neuron, the donor huC-GFP transgenic embryos were injected with either a control MO or the mib ex/int-1 MO described earlier. In a smaller subset of experiments, homozygous mibm178 mutants obtained from m178/+; huC-GFP/huC-GFP parents provided the donor cells. When cells from control MO–injected embryos were transplanted into wild-type embryos, 27% (166/619) of donor cells became GFP+, indicating that this is the likelihood of a wild-type donor cell becoming a neuron when it is incorporated in a wild-type host neural tube (see Table 2). When cells from mib ex/int-1 MO–injected embryos were transplanted into wild-type embryos, however, only 17% (92/531) of donor cells became GFP+. When mibm178 mutant cells were transplanted into wild-type embryos, even fewer donor cells, 7% (10/172), became GFP+. In contrast, when cells with wild-type mib function were transplanted into hosts injected with mib ex/int-1 MO, 39% (87/243) of the cells became GFP+. The lower probability of mib-deficient cells becoming neurons in a wild-type environment and conversely, the higher probability of wild-type cells becoming neurons in a mib-deficient environment, suggests that while mib-deficient cells are capable of receiving lateral inhibition from neighbors, they make ineffective inhibitory signals. Not that by 24 hr, it may also become easier for wild-type cells to become neurons in mib mutant hosts because by this stage, expression of both Delta and Notch is reduced in the host spinal cord.

We have shown here that Mib interacts with the intracellular domain of Delta to promote internalization of Delta and that loss of mib function reduces a cell’s ability to produce an effective inhibitory signal during lateral inhibition. Previous studies have shown, however, that DeltaICD produces a neurogenic phenotype primarily by cell autonomously interfering with receptor, rather than presentation, of the inhibitory signal (Dorsky et al., 1997; Henrique et al., 1997). Consistent with this observation, we found that cells expressing XDeltaICD,HA were more likely to become GFP+ (34%, 141/420) when transplanted into wild-type hosts, suggesting that they were less likely to be inhibited from becoming neurons (Table 2). This observation raised the question of whether Mib’s interaction with the Delta intracellular domain also influences Delta’s ability to cell autonomously inhibit Notch function, as has previously been suggested for the ubiquitin ligase Neur.

**Cell-Autonomous Inhibition of Notch Signaling by Delta Is Not Effectively Reversed by Mib**

A CBF activity assay was used to determine how Mib influences Delta’s ability to cell autonomously inhibit Notch function. In this assay, a luciferase reporter with Notch-responsive elements was transfected into a stable Notch1-expressing C2C12 cell line. The reporter was then used to quantify activation of Notch when these cells were cocultured with D19 Delta1-expressing cells. Approximately 7.5-fold higher luciferase activity was induced when the Notch-expressing cells were cocultured with Delta-expressing D19 cells instead of control L cells (Figure 7B). Transfection of the Notch-expressing C2C12 cells with increasing amounts of Xdelta1 progressively reduced relative luciferase induction (Figure 7B, solid bars), confirming that Xdelta1 can cell autonomously inhibit Notch function. Cotransfection of increasing amounts of Mib with Xdelta1, however, did not significantly reduce the ability of Xdelta1 to inhibit Notch function (Figure 7B). We also compared the ability of Delta1, Xdelta1ICD, and Xdelta1ICD-Ub to inhibit Notch function. We found that all three forms of Delta produce a dose-dependent reduction in luciferase induction, suggesting that all these forms of Delta have a similar ability to cell autonomously inhibit Notch signaling (Figure 7C). These data imply that in such in vitro experiments, internalization of Delta by Mib was not able to reduce cell-autonomous inhibition of Notch function.

It is important to note that degradation of Delta was not necessarily increased in the presence of Mib in these in vitro experiments. In independent, pulse chase experiments (data not shown), mib did not have a consistent effect on Delta stability. Mib did, however, promote its own polyubiquitylation and turnover in proteasomes, and Mib degradation could be reduced by proteasome inhibitors such as MG132 in in vitro assays (data not shown).

**Discussion**

In zebrafish mib mutants, reduced lateral inhibition mediated by Notch signaling permits excessive numbers of cells to become neurons and depletes the population of progenitors needed for neurogenesis in the CNS to continue. Analysis of mib revealed that it encodes an E3 that promotes ubiquitylation and endocytosis of Delta.

There are two models that could explain why an E3 that is responsible for ubiquitylation and internalization of Delta would be required for effective Notch signaling. One possibility is based on the proposition that Mib is
Figure 7. Mib Is Essential for Sending and Not Receiving an Inhibitory Signal

(A) Confocal image of host spinal cord showing huC promoter-driven GFP+ neurons (green) and transplanted donor cells (red); transplanted cells that have become GFP+ neurons are yellow. Neural tube is indicated by a bracket.

(B) Notch pathway activation, assayed by a CBF-luciferase reporter, is reduced by expression of Xdelta1 in the Notch-expressing cells, but this inhibition is not relieved by coexpression of Mib. The first open column shows basal reporter activity in Notch-expressing cells cultured with L cells; the next three groups of black to light gray columns show reporter activity induced by coculture with Delta-expressing D19 cells, when the Notch-expressing cells were cotransfected with varying amounts of Xdelta1 (0, 0.1, or 1.0 μg per well). Each group transfected with a particular amount of Xdelta1 was cotransfected with increasing doses (triangles) of Mib (0, 50, 150, or 450 ng per well).

(C) The effect of increasing doses (triangles) of Xdelta1, Xdelta1ΔICD, and Xdelta1ΔICD-Ub on reporter activity in the Notch-expressing cells. Reporter activity was measured in each case relative to the value seen on L cells, defined as 1. Error bars represent standard deviations.

Table 2. Transplantation Results

<table>
<thead>
<tr>
<th>Donor → host</th>
<th>Transplanted GFP+ cells</th>
<th>Transplanted cells in neural tube</th>
<th>GFP+ cells</th>
<th>Number of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con MO → wt</td>
<td>166</td>
<td>619</td>
<td>27%</td>
<td>n = 44</td>
</tr>
<tr>
<td>Mib MO → wt</td>
<td>92</td>
<td>531</td>
<td>17%</td>
<td>n = 48</td>
</tr>
<tr>
<td>m178 → wt</td>
<td>10</td>
<td>172</td>
<td>7%</td>
<td>n = 9</td>
</tr>
<tr>
<td>Wt → mib MO</td>
<td>87</td>
<td>243</td>
<td>39%</td>
<td>n = 16</td>
</tr>
<tr>
<td>Xdelta1ΔICD</td>
<td>141</td>
<td>420</td>
<td>34%</td>
<td>n = 13</td>
</tr>
</tbody>
</table>
required in the cell that delivers signals; the other assumes that it is required in the cell that receives them. In the first model, Mib promotes the transendocytosis of the Notch extracellular domain by promoting endocytosis of Delta and, in doing so, facilitates proteolytic events that generate the transcriptionally active NotchICD fragment (Figure 8). This proposal comes from studies of the neurogenic phenotype of shibire and neur mutants in Drosophila, suggesting that transendocytosis of the Notch extracellular domain by the adjacent Delta-expressing cell is essential for efficient Notch activation (Parks et al., 2000; Paviopoulo et al., 2001). In our system, transplantation experiments show that cells with reduced mib function are less likely to become neurons when surrounded by wild-type cells. This supports the idea that loss of mib function primarily reduces a cell’s ability to produce an effective inhibitory signal in the competition to become a neuron.

The other model that explains why ubiquitylation and internalization of Delta might be essential for Notch signaling postulates a cell-autonomous role for mib in signal reception, as has also been suggested previously for neur (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001). According to this model, mib-mediated Delta turnover would limit Delta’s ability to inhibit Notch function cell autonomously. However, the cell transplantation results argue against a significant deficit in reception of the inhibitory signal. Furthermore, the luciferase experiments, in which cells were cotransfected with notch and various delta constructs, show that, while Delta does indeed have a cell-autonomous effect in blocking signal reception, mib does not significantly influence this action of Delta. Moreover, this action of Delta does not seem to be ubiquitin dependent: the recombinant addition of ubiquitin does not significantly reduce DeltaICD’s ability to inhibit Notch function.

It is possible that in these assays, Mib is ineffective at reducing cell-autonomous inhibition of Notch by Delta because very high levels of artificially expressed Delta in the transfected cells in vitro may overwhelm the capacity of the Mib-dependent machinery. However, in the studies in COS7 cells, at least, Mib was effective in removing artificially expressed Delta from the cell surface, suggesting that the inhibitory effect of Delta may be independent of delivery of Delta to the cell surface: it may result from Delta-Notch interactions within the secretory pathway, as previously suggested (Sakamoto et al., 2002). In short, our observations do not support a significant role for Mib in limiting Delta’s ability to cell autonomously inhibit Notch function as has been described for Neur, but we cannot as yet completely rule out such a role.

The role of Mib in signal delivery is strongly supported and tightly correlated with Delta ubiquitylation. Ectopic expression of XDelta1ICD, which cannot be ubiquitylated, permits too many cells to become neurons, while XDelta1 and XDelta1ICD-Ub, ectopically expressed in the embryo in a similar way, both inhibit cells from becoming neurons. These effects correlate with the ubiquitin-dependent reduction of cell surface Delta. The internalization of XDelta1ICD-Ub is consonant with previous studies that have shown that in-frame addition of ubiquitin to stable plasma membrane proteins can serve to target their entry into the endocytic pathway (Shih et al., 2000). The obvious suggestion, therefore, is that Mib-induced ubiquitylation drives internalization of Delta by endocytosis, and that this process is critical for effective signaling by Delta.

An additional possibility that we cannot yet exclude is that Mib-dependent ubiquitylation of Delta also decreases the amount of Delta that reaches the cell surface by sorting Delta directly from the Golgi complex to late endosomes. Such a dual role has been shown in yeast for the E3 ligase Rsp5p, which ubiquitylates its substrate, Gap1p, to regulate the total amount of Gap1p at the cell surface (Hellwell et al., 2001; Soetens et al., 2001). Ubiquitylation of Gap1p by Rsp5p promotes endocytosis of Gap1p and favors sorting of Gap1p from the Golgi to the vacuole, where it is degraded. Mib may also have dual roles in endocytosis of Delta and in direct sorting of Delta to late endosomes/lysosomes; however, it is not clear at this time how the later function might contribute to Notch signaling.

Although mib mutants express unusually high levels of cell surface Delta, it is unlikely that this is per se the
cause of the neurogenic phenotype, as artificial expression of even higher levels of Delta in mib mutants following injection of delta mRNA suppresses the neurogenic phenotype.

From our observations, it seems that while all the forms of Delta that we examined can cell autonomously inhibit Notch function, only the forms of Delta that are ubiquitylated and endocytosed can effectively activate Notch in neighboring cells. It is likely that when Delta is driven to high levels in a group of cells, the effect of Delta in trans, as an activator of the Notch pathway, dominates over its effect in cis, as an inhibitor, accounting for the ability of Xdelta1 and Xdelta1\textsubscript{ICD-Ub} to inhibit neurogenesis in the embryo.

The opposing cell-autonomous and nonautonomous effects on Notch signaling define two synergistic mechanisms by which a cell expressing more Delta than its neighbors gains an enhanced ability to become a neuron. By activating Notch in neighboring cells, Delta reduces the neighbors’ ability to express the Notch ligand Delta at high levels. At the same time, Delta interferes with Notch function in the cell where Notch and Delta are coexpressed, making it harder for this cell to be inhibited from becoming a neuron by Delta in neighboring cells.

The role for mib in promoting endocytosis in the signal-delivering cell, as demonstrated in this study, is similar to one role proposed for neur in Drosophila. In vertebrates, however, neur seems to have a much more limited role than has been demonstrated for it in Drosophila. Mice that are homozygous for a neur function mutation have restricted defects: one study revealed the dramatic neural phenotype of these studies revealed the dramatic neural phenotypes or defects in somitogenesis that are seen when there is broad loss of Notch signaling. In contrast, mib mutants do show widespread abnormalities, suggesting a deficit in many more Notch-dependent developmental events. We are currently investigating whether mib has assumed some roles that were originally played by neur in Drosophila or whether a cooperative role for neur and mib in Notch signaling limits the deficit caused by loss of neur alone in vertebrates.

In summary, the analysis of the zebrafish mib mutant has led to the identification of a gene that is essential for effective Notch signaling in many different tissues during development. The function of Mib as a ubiquitin ligase in the internalization of Delta provides new avenues for clarifying the mystery of how endocytosis may increase the ability of cell surface Delta to deliver lateral inhibition signals.

**Experimental Procedures**

**Fish Maintenance and Mutants**

Zebrafish were raised and maintained under standard conditions. Three alleles of mib, m178, m132, and ta52b were previously described (Jiang et al., 1996; Schier et al., 1996; van Eeden et al., 1996). mib\textsuperscript{m178} and mib\textsuperscript{m132} were independently identified in noncomplementing allele screening by Y.-J. Jiang.

**Positional Cloning and cDNA Cloning of mind bomb**

Map crosses were generated by outcrossing m178 heterozygotes (AB background) to the TL strain. Genomic DNA was prepared by digestion of embryos for meiotic mapping with proteinase K and used for PCR. BAC and YAC clones were identified from DNA pools by PCR as described by the supplier (Genome Systems and Research Genetics). PAC clones were provided by Chris Amemiya and identified by a combination of PCR and Southern blotting. PCR primers designed against nonrepetitive regions of YAC, BAC, and PAC clones and markers shown to be present on LG2 by radiation hybrid mapping were used to establish physical contigs and identify polymorphisms for use in meiotic mapping. A cosmid library was made from the PAC clones using SuperCos I Cosmid vector kit (Stratagene). A 5’ RACE and 3’ RACE library was made from tailbud stage embryos and used to clone mib cDNA (Clontech). Sequencing of RT-PCR or genomic PCR products identified mutations in the mib alleles.

**Plasmids**

All plasmids for in vivo expression were made by subcloning either PCR-amplified or restriction enzyme-digested fragments of mib into a pc52-FLAG or pc53-MT vector. N-terminal, middle, and C-terminal regions correspond to amino acids 1–341, 342–739, and 740–1030, respectively. Different forms of the mib RING domain (amino acids 740–1030) were subcloned into pGEX-5T for the production of GST fusion proteins. Xdelta1, Xdelta1\textsuperscript{ICD}, and XdeltaD\textsuperscript{ICD-Ub} were amplified by PCR and subcloned into pCS2+HA to add C-terminal HA tags. Xdelta1\textsuperscript{ICD-Ub} was generated by addition of the Ub sequence (76 amino acids) in-frame at the C terminus of Xdelta1\textsuperscript{ICD}. The deltaD expression construct was made by subcloning full-length deltaD cDNA into pcDNA3.1.

**Whole-Mount In Situ Hybridization, Antibody, and α-Galactosidase Staining**

Plasmids that have been used to make in situ probes have been published previously: ngn\textsuperscript{r} (Blader et al., 1997; Kim et al., 1997), huc (Kim et al., 1996), deltaA (Appel and Eisen, 1998; Haddon et al., 1998b), notch5 (Itoh and Chitnis, 2001; Lawson et al., 2001), and herd (Takke et al., 1999). To detect α-galactosidase conjugated with various synthesized mRNAs, embryos were fixed in 4% paraformaldehyde overnight at 4°C and stained by either X-gal or salmon-β-galactoside (Biosynth). Myc monoclonal antibody (9E10) was used for detecting Myc-tagged Xdelta1 protein.

**mRNA and Morpholino Antisense Oligonucleotide Injection**

For microinjection of mRNA, constructs were linearized and transcribed with SP6 RNA polymerase using the mMessage mMachine (Ambion). Morpholinos (Gene Tools) were resuspended in DEPC water and stored at −20°C. The sequences of the morpholinos used were 5’-GCAGGCCTCACCTGAGGCGACGTG-3′ for Ex/int1 and 5’-GTGTTAGGGCCATTCCCTCCTT-3′ for Ex/int3. Two to five nanograms of oligonucleotides were injected into one- to two-cell stage embryos.

**In Vitro Ubiquitylation Assay**

GST fusion proteins were expressed in log phase Escherichia coli BL21-CodonPlus-RP (Stratagene) and purified with glutathione Sepharose 4 Fast Flow (Amersham Pharmacia). In vitro ubiquitylation assays were carried out as previously described (Lorick et al., 1999).

**Cell Culture, Transfection, Immunoprecipitation, and Western Blot Analysis**

COS7 cells were transiently transfected with 2–4 μg of plasmid DNA per 6 cm dish using either Fugene6 (Roche) or Gene Porter2 (Gene Therapy Systems). The total amount of plasmid DNA used for transfection was kept constant by adding an appropriate amount of the CS2+ vector plasmid. Two days after transfection, cells were harvested and lysed in TENT buffer (50 mM Tris-HCl [pH 8.0], 2 mM
EDTA, 150 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation and incubated with antibodies for 2 hr at 4°C, and then incubated with protein A or G Sepharose for 30 min with biotinylated antibodies (Vector). After removal of the biotinylation buffer, the cells were incubated for 20 min at 4°C in quenching buffer (100 mM glycine in PBS), washed once with ice-cold PBS, and lysed in 1 ml of TENT. Lysates were incubated with anti-HA-conjugated beads (Roche) for 1 hr and then the beads were washed five times with 1 ml of TENT and extracted in SDS sample buffer by boiling. The surface-biotinylated fraction was visualized using horseradish peroxidase-conjugated streptavidin (Vector Laboratory) by Western blot.

Immunocytochemistry
Transfected COS7 cells were fixed, 24 hr posttransfection, in MeOH at −20°C for 5 min and air dried. Fixed cells were then incubated in blocking solution (10% normal goat serum in PBS) for 1 hr, followed by staining with appropriate primary antibodies (rabbit anti-MyC (A14) or FLAG polyclonal, biotinylated rat anti-HA, all at 1:1000 dilution) in blocking solution for 1 hr at room temperature. Subsequently, cells on coverslips were washed three times with PBS and incubated with goat-antirabbit antibody conjugated with Alexa 488 or 594, or with Alexa 594- or 350-conjugated streptavidin, for 1 hr in the dark at room temperature. Coverslips were washed three times, mounted on glass slides, and analyzed on a Zeiss Axiophot fluorescent microscope. Images were collected on a Hamamatsu Orca camera and processed using Openlab and Photoshop software.

DeltaD Monoclonal Antibody zdd2
DNA coding for the entire extracellular region of zebrafish DeltaD was fused in-frame to the rat CD4 tag in the vector pECE-BOSS-XB and expressed as a soluble fusion protein by transient transfection of HEK293T cells and purified by immunoaffinity chromatography using the anti-tag monoclonal antibody OX-68 (Wright et al., 2000). Hybridomas were generated by fusing splenocytes from immunized mice to the SP2/0 cell line. Hybridize supernatants were screened by ELISA and Western blot for specificity and were further tested for specificity and formalin fixation sensitivity by FACS analysis of fixed HEK293T cells transfected with zebrafish deltaD (which the antibody recognized) or with deltaC (which it did not recognize).

Internalization of DeltaD Antibodies
After 24 hr of transfection, zdd2 antibody (10 μg/ml) and leupeptin (10 μg/ml) were added and the cells were further incubated for 9 hr. Following fixation and permeabilization, the zdd2 antibody was detected using a goat anti-mouse Alexa 594 secondary antibody. The Myc-tagged wild-type and RING mutant forms of Mib were detected by the rabbit A14 antibody followed by a goat anti-rabbit Alexa 488 secondary antibody.

Surface Biotinylation Assay
Transfected COS7 cells were washed three times with ice-cold PBS buffer. The cells were then incubated at 4°C for 30 min with biotinylation buffer (0.25 mg/ml EZ-link sulfo-NHS-LC-biotin [Pierce] in PBS). After removal of the biotinylation buffer, the cells were incubated for 20 min at 4°C in quenching buffer (100 mM glycine in PBS), washed once with ice-cold PBS, and lysed in 1 ml of TENT. Lysates were incubated with anti-HA-conjugated beads (Roche) for 1 h and then the beads were washed five times with 1 ml of TENT and extracted in SDS sample buffer by boiling. The surface-biotinylated fraction was visualized using horseradish peroxidase-conjugated streptavidin (Vector Laboratory) by Western blot.

Cell Transplantation and Analysis
Either wild-type or m178 heterozygous fish carrying the huC promoter-driven GFP transgene were crossed to produce embryos. Embryos were injected with either control morpholino or morpholino against mib (Ex/int1) with rhodamine-dextran (Molecular Probes). Twenty to thirty cells from host embryos were transplanted into donor embryos at 4 hpf, and embryos were developed until around 24 hpf as pairs of host and donor in each well of 24-well plates. Z series sections of embryos were generated by confocal laser scanning microscopy (BioRad) and 3D images were constructed using MetaMorph (Universal Imaging) software.

C6F1 Assay
Notch1-expressing C2C12 cells were plated into 24-well culture plates and the next day were cotransfected with 100 ng 8xCBF1-luciferase reporter construct (Zhou et al., 2000) and 2 ng prLTK (Promega)-expressing Renilla luciferase (to normalize levels of transfection) and mind bomb (50, 150, and 450 ng), Xdelta1 (0.1 and 1 μg [Figure 6B] or 5, 50, and 500 ng [Figure 6C]), Xdelta1-ICD (5, 50, and 500 ng), or Xdelta1-ICD-Ub (5, 50, and 500 ng). Cocultures and luciferase assays were carried out as previously described (Nofziger et al., 1999). Notch-induced activation of CBF1 is expressed as a ratio of normalized luciferase value induced by the Delta-expressing (D19) cells compared to that obtained with parental L cells. All experiments were performed in triplicate.

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References


Accession Numbers

Nucleotide sequences of the zebrafish, mouse, and human mind bomb cDNA have been submitted to GenBank under the accession numbers AF537301, AY149907, and AY149908, respectively.