Control of Notch-ligand endocytosis by ligand-receptor interaction

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Summary

In Notch signaling, cell-bound ligands activate Notch receptors on juxtaposed cells, but the relationship between ligand endocytosis, ubiquitylation and ligand-receptor interaction remains poorly understood. To study the specific role of ligand-receptor interaction, we identified a missense mutant of the Notch ligand Jagged1 (Nodder, Ndr) that failed to interact with Notch receptors, but retained a cellular distribution that was similar to wild-type Jagged1 (Jagged1^{WT}) in the absence of active Notch signaling. Both Jagged1^{WT} and Jagged1^{Ndr} interacted with the E3 ubiquitin ligase Mind bomb, but only Jagged1^{WT} showed enhanced ubiquitylation after co-culture with cells expressing Notch receptor. Cells expressing Jagged1^{WT}, but not Jagged1^{Ndr}, trans-endocytosed the Notch extracellular domain (NECD) into the ligand-expressing cell, and NECD colocalized with Jagged1^{WT} in early endosomes, multivesicular bodies and lysosomes, suggesting that NECD is routed through the endocytic degradation pathway. When coexpressed in the same cell, Jagged1^{Ndr} did not exert a dominant-negative effect over Jagged1^{WT} in terms of receptor activation. Finally, in *Jag1*$^{Ndr/Ndr}$ mice, the ligand was largely accumulated at the cell surface, indicating that engagement of the Notch receptor is important for ligand internalization in vivo. In conclusion, the interaction-dead Jagged1^{Ndr} ligand provides new insights into the specific role of receptor-ligand interaction in the intracellular trafficking of Notch ligands.

Key words: Notch, Delta-like, Jagged, Mind bomb, Endosome, MVB, Dynamin

Introduction

Endocytosis has an important role in the regulation of several intracellular signaling mechanisms, including the Notch signaling pathway (Brou, 2009; D'Souza et al., 2008; Le Borgne et al., 2005; Polo and Di Fiore, 2006). Notch signaling is a highly evolutionarily conserved cell-cell signaling mechanism required for normal development of many tissues and organs (Bray, 2006; Kopan and Ilagan, 2009). Notch receptors appear at the cell surface in a heterodimeric form, and interaction with transmembrane ligands presented on juxtaposed cells induces two proteolytic cleavage events in the Notch receptor. First, an ADAM metalloprotease activity cleaves the receptor in the juxtamembrane region (Brou et al., 2000; Mumm et al., 2000), which makes the membrane-bound receptor fragment a substrate for γ-secretase, an enzymatic complex that catalyzes a cleavage in the transmembrane region of Notch (De Strooper et al., 1999; Schroeter et al., 1998). This liberates the Notch intracellular domain (NICD), which translocates to the nucleus, where it interacts with the DNA-binding protein CSL (RBP-J) to regulate the transcription of downstream target genes (Kopan and Ilagan, 2009).

Endocytosis is required both on the receptor and ligand side in Notch signaling (Brou, 2009; Chitnis, 2006; D'Souza et al., 2008; Le Borgne et al., 2005). On the receptor side, monoubiquitylation of the Notch receptor results in endocytosis of the ADAM-cleaved, membrane-tethered form of Notch, an event that appears to be required for γ-secretase-mediated generation of Notch ICD (Gupta-Rossi et al., 2004). On the ligand side, Delta is found in endocytic vesicles (Kooh et al., 1993), and Shibire mutants, which carry a mutant form of dynamin, affect Notch signaling when expressed in the ligand-carrying cells (Seugnet et al., 1997). Further insights into ligand endocytosis came from genetic screens in *Drosophila* and zebrafish, which revealed a function for Liquid facets, a homolog of epsin that binds clathrin and the AP-2 adapter complex in coated pits (Overstreet et al., 2004).

The genetic screens also identified two E3 ubiquitin ligases that are important for Notch signalling: Neuralized and Mind bomb (Mib1), which ubiquitylate Notch ligands in their cytoplasmic C-terminal tails (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Le Borgne and Schweisguth, 2003; Pavlopoulos et al., 2001; Yeh et al., 2001) (for a review, see Chitnis, 2006). Ligand ubiquitylation by Mib is crucial for ligand endocytosis and for effective Notch signaling (Itoh et al., 2003; Le Borgne and Schweisguth, 2003; Overstreet et al., 2004; Wang and Struhl, 2004). The importance of the short cytoplasmic domain in Notch ligands is emphasized by mutational analysis, which revealed a motif that is required for ligand endocytosis but dispensable for Notch activation, and a second motif that is required for Mib interaction, ligand internalization and activation of Notch receptors (Glittenberg et al., 2006). Removal of the intracellular domain of Delta similarly inhibited endocytosis and Notch activation, but replacing this domain with the intracellular domain from the LDL receptor, which is known to be internalized by endocytosis, restored endocytosis and Notch activation by the hybrid Delta ligand (Wang and Struhl, 2004). As part of the receptor activation process, the Notch extracellular domain (NECD), which results

from furin-mediated cleavage, can be trans-endocytosed into ligand-expressing cells (Parks et al., 2000). NECD trans-endocytosis has more recently been shown to not require ADAM processing of the Notch receptor (Nichols et al., 2007), suggesting that NECD trans-endocytosis occurs before ligand-induced proteolytic processing.

Despite the characterization of a number of endocytic proteins that control ligand endocytosis, including dynamin, Epsin, Rab11, Arfophilin (Nuclear Fallout) and auxilin (for a review, see Brou, 2009), the precise role of endocytosis in the activation process remains to be established. Two principal, but not mutually exclusive, models have been put forward for the relationship between ligand endocytosis and Notch signaling. One model proposes an endocytosis-dependent maturation of the ligand. This model suggests that newly synthesized Notch ligands are not capable of binding to Notch receptors, and that endocytosis of ligand present at the cell surface would elicit a post-translational modification endowing the ligand with receptor-interacting and receptor-activating potential (Wang and Struhl, 2004; Wang and Struhl, 2005). The second model stipulates that endocytosis of ligand generates a shearing force required for shedding of the Notch extracellular domain interacting with ligand (Parks et al., 2000). This would, in turn, allow for ADAM-mediated cleavage (Nichols et al., 2007) and subsequent γ-secretase cleavage of the transmembrane receptor stub, generating a Notch ICD and active Notch signaling.

To gain further insight into the function of Notch ligands is important, not least because ligand mutations are linked to disease. For example, mutations in *JAG1*, the gene encoding Jagged1, are linked to Alagille syndrome (Spinner et al., 2001), and mutations in *DLL3* to spondylocostal dysostosis (Turnpenny et al., 2007). In this report, we identify a mutant form of Jagged1, Nodder, which has specifically lost its receptor-binding activity, but retains normal interaction with Mib1 and Hrs, and displays a normal cellular distribution in the absence of Notch signaling. Analysis of the Nodder mutant in vitro and in vivo allows us to dissect the specific role of receptor-ligand interaction for ubiquitylation and intracellular trafficking of Notch ligands.

Results

Identification of the embryonic-lethal Jagged1 missense mutant Nodder

In a search for Notch ligand mutants that affect specific ligand functions, we identified a Jagged1 mutant from a large-scale ethylnitrose urea (ENU) mutagenesis screen in the mouse. Mapping by backcrossing showed that the mice with mutant Jagged1 protein, referred to as Nodder because of a nodding behaviour and balance defects in the heterozygous (Jagged1$^{+/Ndr}$) state (data not shown), carried a *Jag1* allele that encoded a histidine-to-glutamine replacement at position 268 (JagH268Q) (Fig. 1A). The Jagged1 His268 residue is highly evolutionary conserved and located in the second EGF-like repeat of the extracellular domain (Fig. 1B), which together with the DSL domain and EGF-like repeats 1 and 3, form an extended structure that is likely to have numerous interactions with Notch receptors (Cordle et al., 2008). Jagged1$^{Ndr/Ndr}$ mice showed embryonic lethality around embryonic day 12, and at E12.5, Jagged1$^{Ndr/Ndr}$ mice were smaller, pale and often exhibited hemorrhages, particularly in the trunk region (data not shown). At E11.5, Jagged1$^{Ndr/Ndr}$ embryos were slightly smaller, exhibited paler yolk sacs and a dysmorphic vasculature (Fig. 1C), but otherwise appeared grossly normal.

Jag1$^{Ndr/Ndr}$ encodes an interaction-dead form of Jagged1

The Nodder phenotype in Jagged1$^{Ndr/Ndr}$ embryos was similar to that of Jagged1$^{-/-}$ mice (Xue et al., 1999), suggesting that the H268Q Jagged1 ligand is Notch signalling incompetent. To address this, we generated a full-length Jagged1 construct carrying the H268Q mutation for expression in cells, and because two other Jagged1 mutants with phenotypes similar to Nodder were previously identified in ENU screens (Slalom and Headturner; Slm and Htu) (Kiernan et al., 2001; Tsai et al., 2001), we also generated expression constructs of these mutations. To study signaling efficacy, we used a cellular co-culture system, where cells expressing full-length Notch1 receptors and the 12XCSL-luciferase Notch reporter plasmid were interfaced with cells expressing full-length ligand Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} or Jagged1^{Htu}. Jagged1^{WT} generated robust 12XCSL-luciferase activation, whereas Jagged1^{Ndr}, Jagged1^{Slm} and Jagged1^{Htu} did not elicit signaling in

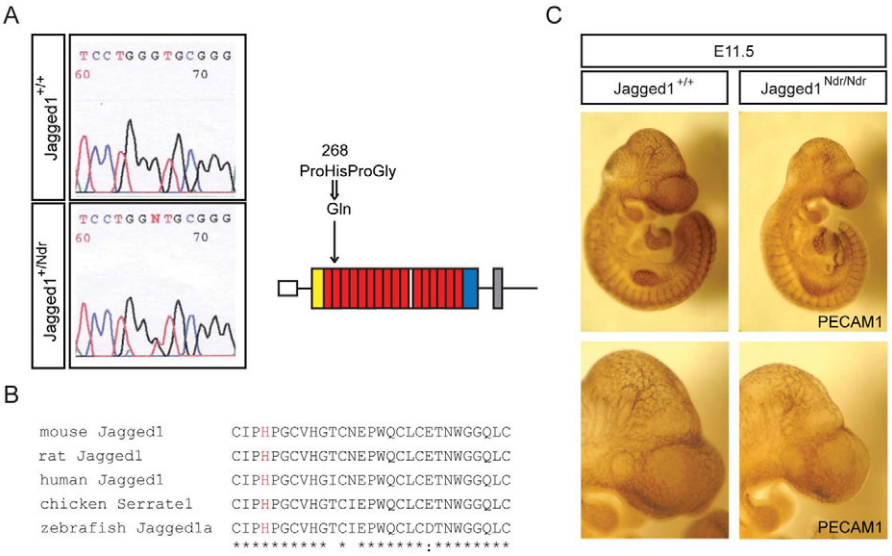

Fig. 1. Missense mutations in the extracellular domain of Jagged1 constitute loss-of-function alleles that are unable to bind Notch receptor. (**A**) Sequencing (reverse-strand primer) of the *Jag1* gene from Jagged1$^{+/+}$ (top trace) and Jagged1$^{+/Ndr}$ (bottom trace) mice reveals that the Nodder mutation results in a H268Q mutation in the second EGF-like repeat of Jagged1 (signal sequence, white; DSL domain, yellow; EGF-like repeats, red; CR domain, blue; transmembrane domain, grey). (**B**) Alignment of part of Jagged1 EGF-like repeat 2 in different species (His268 is red; * indicates conserved amino acid residue). (**C**) Whole-mount immunostaining for PECAM-1 in Jagged1$^{+/+}$ and Jagged1$^{Ndr/Ndr}$ embryos at E11.5. Jagged1$^{Ndr/Ndr}$ embryos exhibit a perturbed development of the vascular system (top panel), and defects in the branching of blood vessels in the head (bottom panel).

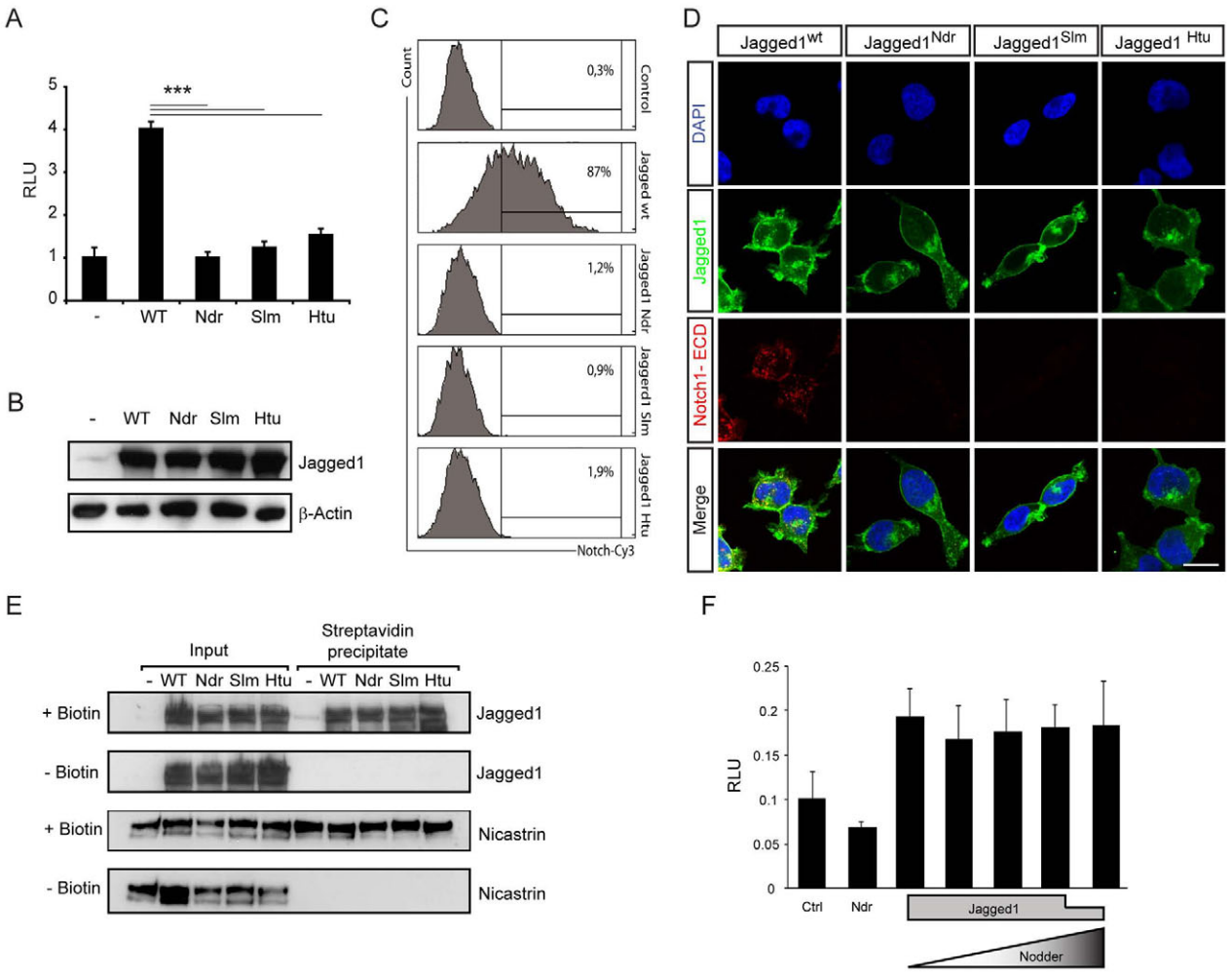

Fig. 2. Characterization of the signaling capacity of Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} and Jagged1^{Htu}. (**A**) 12XCSL-luciferase reporter activity in co-cultures of Notch-expressing cells (carrying 12XCSL-luc) and cells expressing wild-type or mutated Jagged1. Bar graphs show luciferase activity (arithmetic mean) from triplicates of each co-culture. Error bars indicate s.d.; ***P<0.001. (**B**) Western blot analysis of lysates from stable cell lines (Flp-in HEK293 cells) expressing no Jagged1 (–), Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm}, and Jagged1^{Htu} show comparable levels of Jagged1 (reprobed with β-actin as loading control). (**C**) Cell lines expressing Jagged1^{WT}, but not Jagged1^{Ndr}, Jagged1^{Slm} and Jagged1^{Htu}, bind N1ECD-Fc-Cy3 as assessed by FACS analysis of cells exposed to N1ECD-Fc-Cy3 for 1 hour. (**D**) Cells expressing Jagged1^{WT}, but not Jagged1^{Ndr}, Jagged1^{Slm} and Jagged1^{Htu}, bind N1ECD-Fc-Cy3 (red), whereas all four cell lines express Jagged1 (green) at the cell surface. Scale bar: 20 µm. (**E**) Streptavidin-precipitation of cell-surface biotinylated proteins (top panel; without biotin as control, bottom panel) from HEK293 cells stably expressing Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} and Jagged1^{Htu}. Reprobing for nicastrin shows that only the mature, cell-surface-bound form, is detected in the streptavidin-precipitated material (the five lanes to the right). (**F**) 12XCSL-luciferase reporter activity from Notch-expressing cells (carrying 12XCSL-luc) co-cultured with cells expressing wild-type Jagged1 and increasing levels of Jagged1^{Ndr}. No dominant-negative effect by Jagged1^{Ndr} is observed.

the neighboring cells (Fig. 2A), demonstrating that they are loss-of-function versions of Jagged1.

Next, we addressed whether ligand-receptor interaction was affected in the Jagged1 mutants compared with Jagged1^{WT}. In stable cell lines that expressed similar levels of Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} or Jagged1^{Htu} as a result of Flp-mediated targeting into the same homing locus (Fig. 2B), we explored whether the cells expressing the various Jagged1 forms could bind a soluble form of NECD (N1ECD-Fc-Cy3), containing EGF repeats 1-13 and used as a mimic of the NECD generated after the constitutive furin-based S1 cleavage (Brou et al., 2000; Mumm et al., 2000). We observed robust binding of N1ECD-Fc-Cy3 to more than 90% of cells expressing Jagged1^{WT}, but no significant binding (less than 2%) to the Ndr, Slm or Htu mutants (Fig. 2C,D and supplementary material Fig. S1). To rule out that this difference in binding was due to differences in levels of Jagged1 at the cell surface, we verified, by cell-surface biotinylation experiments, that the wild type, as well as the mutated forms of Jagged1, was readily detected at the cell surface (Fig. 2E).

To test whether the Ndr allele might act in a dominant-negative manner, we expressed Jagged1^{WT} with increasing amounts of Jagged1^{Ndr}. Jagged1^{Ndr} was unable to decrease the low but reproducible activation of Notch signaling by Jagged^{WT} in neighboring cells (Fig. 2F), arguing against a dominant-negative effect of the mutation. Together, these experiments show that the Ndr, Slm and Htu Jagged1 mutations abolished the capacity of the ligand to bind receptor and activate Notch signaling.

We next addressed the roles of Mib1 and ligand endocytosis for receptor-ligand interaction and signaling. To this end, HEK293T cells stably expressing full-length Notch1 were transfected with a 12XCSL-luc reporter plasmid and co-cultured with HEK293T cells transiently transfected with Jagged1^{WT}. Endocytosis was blocked in the ligand-carrying cells by co-expressing Jagged1^{WT} with a dominant-negative dynaminII (K44A) (van der Bliek et al., 1993). Mind bomb function was blocked by siRNA for specific knockdown of human Mind bomb1 (MIB1), and *MIB1* mRNA levels were reduced by approximately 85% by the siRNA (supplementary material Fig. S2). Blockade of Jagged1 endocytosis by K44A dynaminII or Mib1 activity by siRNA strongly reduced the capacity of Jagged1 to elicit Notch signaling in neighboring Notch1-expressing cells (Fig. 3A,B), extending previous observations for Delta-like 1 (Nichols et al., 2007). To verify the efficacy of dynaminII K44A, binding of transferrin was greatly reduced, and uptake into the cell virtually abolished, in dynaminII K44A-transfected cells (Fig. 3C, top cell), but not in cells not transfected with dynaminII K44A (Fig. 3C, bottom cell). To study the effects on receptor-ligand interaction we compared how HEK293T cells transiently transfected with Jagged1 and K44A dynaminII or siRNA against *MIB1*, bound N1ECD-Fc-Cy3. N1ECD-Fc-Cy3 binding was readily detected in Jagged1-expressing cells, whether they expressed dynaminII K44A (Fig. 3C, top cell) or not (data not shown). In cells not expressing Jagged1, no N1ECD-Fc-Cy3 binding was detected (Fig. 3C, bottom cell). FACS analysis for N1ECD-Fc-Cy3 cell-surface labeling revealed that binding of N1ECD-Fc-Cy3 was similarly enhanced in response to Jagged1 expression, irrespective of whether the cells were transfected with EGFP, dynaminII K44A, control siRNA or siRNA against *MIB1* (Fig. 3D,E; for FACS plots see supplementary material Fig. 3). In conclusion, these results show that, in this system, endocytosis of Jagged1 must occur to elicit Notch signaling in the neighboring cell but that endocytosis is not required for receptor binding per se.

Since Notch ligands, similarly to Notch receptors, are processed by ADAM proteases (Qi et al., 1999), we studied the consequences of interfering with the ADAM-mediated cleavage of Jagged1 for receptor-binding capacity and Mib1-mediated internalization. Treatment of the cells with the ADAM inhibitors GM6001 and TAP-1 inhibited generation of a processed Jagged1 (Jagged1 CTF), but did not affect internalization in response to binding of Mib1 or NECD to the cells (supplementary material Fig. S4A). In the converse experiment, transfection of dynaminII K44A did not affect the ratio between full-length or the C-terminal form of Jagged1, indicating that ADAM processing does not require endocytosis (supplementary material Fig. S4B).

Jagged^{Ndr} undergoes intracellular trafficking and interacts with MIB1

To test how the specific loss of receptor interaction in Jagged1^{Ndr} affected the intracellular distribution and ubiquitylation of the ligand, we analyzed whether the intracellular distribution of Jagged1 ligand and Notch1 receptor was altered in neighboring cells engaged in productive Notch signaling. To measure Notch signaling in the signal-receiving cell with single-cell resolution, we co-transfected the Notch1-carrying cells with a fluorescent reporter construct (12XCSL-EGFP or 12xCSL-dsRed) (Hansson et al., 2006). We observed higher EGFP or dsRed expression in cells expressing Notch1 receptor that were in close proximity to cells expressing

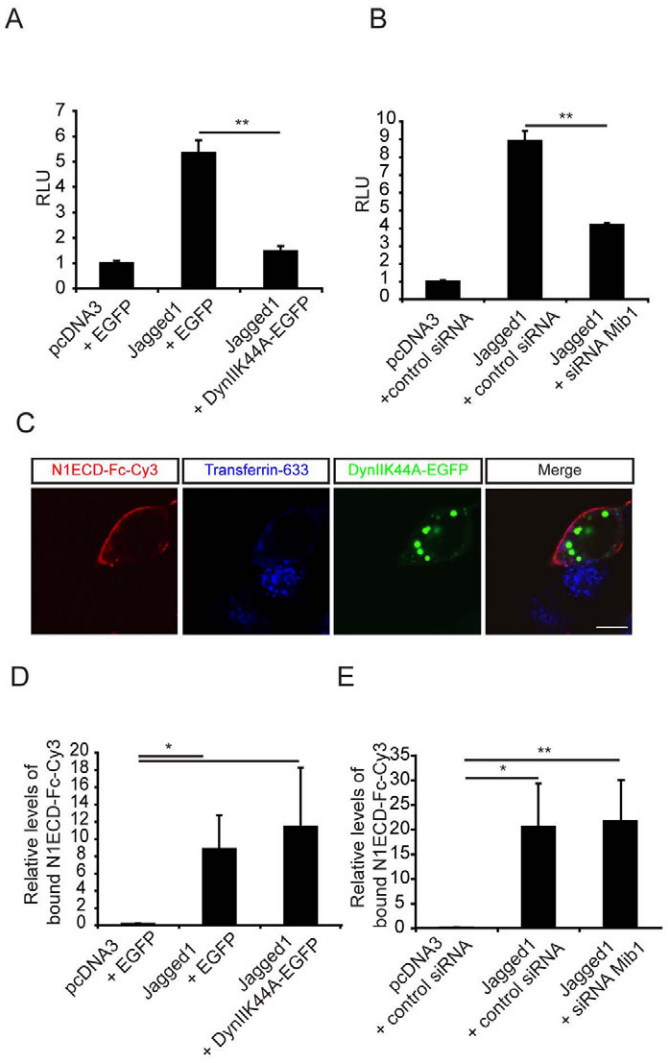

Fig. 3. Mib1 and endocytosis of Jagged1 are required for activation of, but not binding to, Notch receptors. (**A**,**B**) 12XCSL-luciferase reporter activity in full-length Notch1-expressing cells co-cultured with cells transiently transfected with (A) EGFP, Jagged1+EGFP or Jagged1+EGFP-tagged DynaminII K44A as indicated or (B) control siRNA, Jagged1+control siRNA or Jagged1+siRNA specific for *MIB1* as indicated. Bar graphs show luciferase activity (arithmetic mean) from triplicates of each co-culture. Luciferase activity was normalized to control cultures transfected with EGFP (A) or control siRNA (B). Error bars indicate s.d.; $^{**}P<0.01$.
(**C**) Immunocytochemistry demonstrating that cells transfected with DynaminII K44A and Jagged1 (top cell) can bind N1ECD-Fc-Cy3, whereas cells not transfected with DynaminII K44A and Jagged1 (the bottom cell) does not bind N1ECD-Fc-Cy3. As a control for block of endocytosis by DynaminII K44A, there is no transferrin uptake in the top (DynaminII K44A expressing) cell, whereas transferrin is internalized in the bottom cell. Scale bar: 10 µm. Immunofluorescence images were adjusted using the levels function in Photoshop. In each case, adjustments were applied to the entire image.
(**D**) Detection of ligand-receptor interaction by flow cytometry in cells transiently transfected with empty plasmid and EGFP, Jagged1 and EGFP or DynaminII K44A and EGFP, as indicated. (**E**) Detection of ligand-receptor interaction by flow cytometry in cells transiently transfected with empty plasmid and control siRNA, Jagged1 and control siRNA or Jagged1 and *MIB1* siRNA, as indicated. Percentages of fluorescently labeled cells (arithmetic mean) is shown (bar graphs). Error bars indicate s.d.; $^{*}P<0.05$, $^{**}P<0.01$.

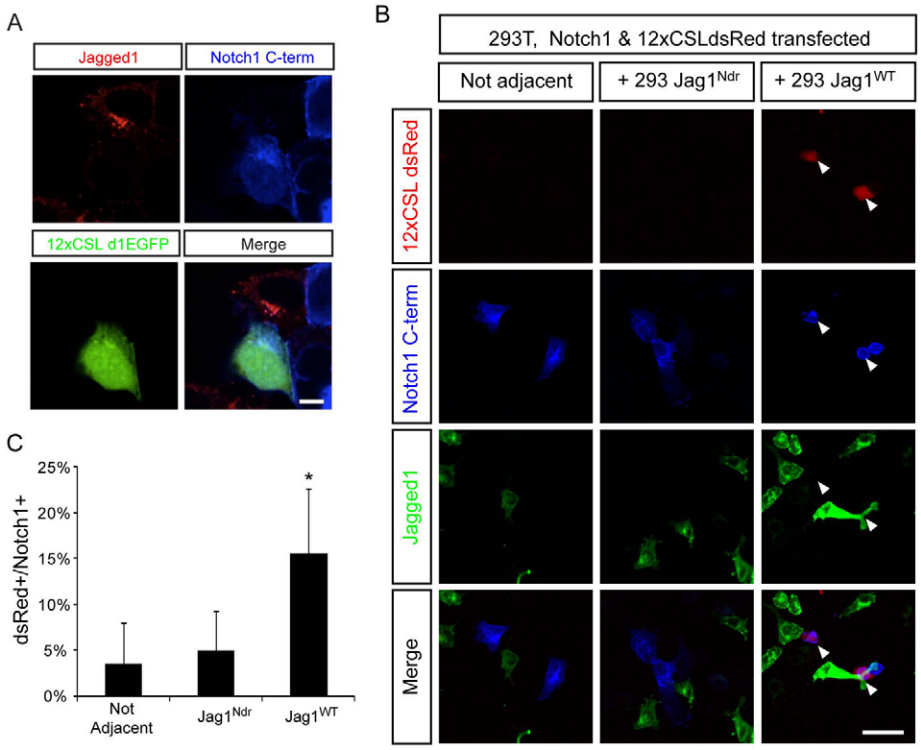

Fig. 4. Analysis of ligand and receptor localization following ligand-receptor interaction. (**A**) Immunocytochemistry of co-cultured HEK293T cells expressing Jagged1 and Notch1. In a cell with ongoing Notch signaling, as determined by a fluorescent 12XCSL-EGFP reporter of Notch signaling (bottom cell), Notch immunoreactivity (C-terminal) was seen in the nucleus and intracellular vesicles (in more than 95% of the cells), whereas Notch immunoreactivity was more predominant at the cell surface in a cell not engaged in active Notch signaling (cell in top-right corner). Jagged1 immunoreactivity (C-terminal) was found in intracellular vesicles in the ligand-expressing cell (middle cell), which is interfaced with the cell with active Notch signaling (bottom cell). Scale bar: 10 µm. (**B**) HEK293 cells stably expressing Jagged1^{WT} or Jagged1^{Ndr} were co-cultured with HEK293T cells transfected with Notch1 and 12xCSL-dsRed. Notch1-expressing cells adjacent to Jagged1^{WT}-expressing cells displayed red fluorescence and nuclear Notch1 C-terminus staining (arrowheads). Scale bar: 50 µm. (**C**) Quantification of dsRed- and Notch1-positive cells shows that significantly more Notch1-positive cells adjacent to Jagged1^{WT} cells manifest red fluorescence compared with cells not adjacent to Jagged1^{WT} cells in the same culture or Notch1-positive cells adjacent to Jagged1^{Ndr} cells.

Jagged1^{WT}, but not in cells not in contact with Jagged1^{WT}-expressing cells or in cells juxtaposed to Jagged1^{Ndr}-expressing cells (Fig. 4A,B,C). A low background level of 12xCSL activation was seen (Fig. 4C), which probably results from the fact that wild-type HEK293 and HEK293T cells express low levels of endogenous Jagged1 and Notch1 (data not shown). Activated Notch signaling was accompanied by internalization of the C-terminal portion of the Notch1 receptor into the receptor-expressing cell and by increased internalization of ligand into the Jagged1^{WT}-expressing cells (Fig. 4A). Internalization of the Notch1 C-terminus was less frequently seen in cells juxtaposed to Jagged1^{Ndr}-expressing cells or in cells not in contact with ligand-expressing cells (Fig. 4B). In cells not engaged in signaling, staining for both ligand and receptor was more pronounced at the plasma membrane, although some Jagged1 immunoreactivity, both for Jagged1^{WT} and Jagged1^{Ndr}, was observed in intracellular vesicles (Fig. 4B).

To study whether the receptor-interaction-dead Jagged1 forms could interact with Mib1, the cell lines stably expressing Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} or Jagged1^{Htu} were transfected with Mib1. Co-immunoprecipitation experiments showed that both Jagged1^{WT} and all three mutated ligands readily interacted with Mib1 (Fig. 5A). To study whether the intracellular localization of Jagged1^{WT} or Jagged1^{Ndr} was altered by reduced Mib1 activity, we compared the proportion of EEA1-positive intracellular vesicles that also contained Jagged1 with control cells and with cells that expressed a mutated form of Mib1, Mib1(M989R). This mutated form of Mib1 corresponds to the ta52b (white tail) mutation in zebrafish Mind bomb, which has been shown to act as an antimorphic allele and, when overexpressed, can inhibit endogenous Mind bomb activity (Zhang et al., 2007). In Mib1(M989R)-transfected cells, there was a reduction in the proportion of EEA1-positive vesicles that contained Jagged1, both for Jagged1^{WT} and Jagged1^{Ndr} cells (Fig. 5B). In conclusion, receptor-interaction-dead ligands show a similar cellular localization, Mib1 interaction and response to reduced Mib1 activity as Jagged1^{WT}, establishing that intracellular trafficking is not significantly affected by the loss of the ability to bind receptor in the Jagged mutants.

As receptor engagement led to increased ligand internalization, we analyzed whether receptor-induced Jagged1 internalization correlated with increased Jagged1 ubiquitylation. The ubiquitylation status of Jagged1 was analyzed in HEK293T cells stably expressing Jagged1 and transfected with EGFP-tagged ubiquitin and co-cultured with Notch1-expressing or control cells. Higher levels of Jagged1 ubiquitylation were observed in cells transfected with Jagged1^{WT} than with Jagged1^{Ndr} when they were co-cultured with Notch1-expressing cells (Fig. 6). Low ubiquitylation levels were observed when Jagged1^{WT}- or Jagged1^{Ndr}-transfected cells were co-cultured with control HEK293T cells (Fig. 6). Simultaneous

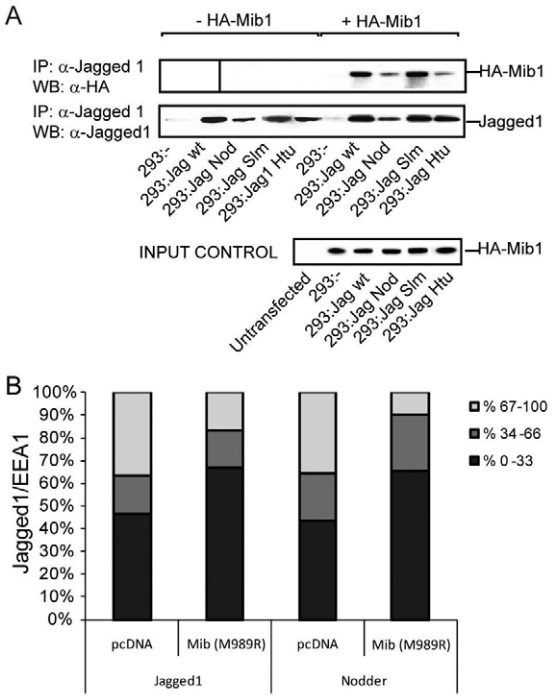

Fig. 5. Interaction of Jagged1 mutants with Mib1. (**A**) HEK293 cells or HEK293 cells stably expressing Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} or Jagged1^{Htu} were transfected with HA-Mib1. Immunoprecipitation of Jagged1 was followed by western blotting for the HA-tag on HA-Mib1. HA-immunoreactive material was precipitated only in cells transfected with HA-Mib1 and expressing Jagged1^{WT}, Jagged1^{Ndr}, Jagged1^{Slm} or Jagged1^{Htu} (the four right-hand lanes). Immunoprecipitation of Jagged1 and western blotting for Jagged1 (top panel) showed that Jagged1 was immunoprecipitated in both *Mib1*-transfected (five right lanes) and non-transfected cells (five left lanes). In the top panel, for technical reasons, the HA-Mib1 western blot was made from a separate gel to the Jagged1 western blot. An input control for HA-Mib1 is shown in the bottom panel. (**B**) Jagged1^{WT} and Jagged1^{Ndr} cells transfected with Mib1(M989R) showed a reduced percentage of Jagged1-positive EEA1-positive vesicles.

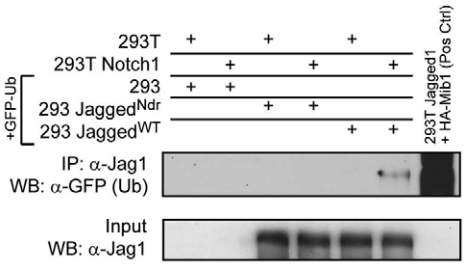

Fig. 6. Jagged1 is ubiquitylated by Mind bomb in response to Notch interaction. HEK293T or HEK293T cells expressing Notch1 were co-cultured with HEK293 cells or HEK293 cells expressing Jagged1^{WT} or Jagged1^{Ndr} (which were also co-transfected with EGFP-ubiquitin) in various combinations, as indicated. Immunoprecipitation of Jagged1 was followed by blotting for EGFP-tagged ubiquitin to reveal ubiquitylation of Jagged1. Increased levels of ubiquitylation were observed only when HEK293T cells expressing Notch1 were co-cultured with Jagged1^{WT} cells. As a control, co-transfection of HA-Mib1 significantly enhanced ubiquitylation (rightmost lane). Note that four-times less lysate was loaded in the right-most lane, which explains the low level of Jagged1 seen in the Jagged1 control blot. Longer exposure times reveal a weak Jagged1 band in this lane (data not shown).

transfection of Mib1(M989R) into the ligand-expressing cells resulted in a very low level of ubiquitylation when Jagged1^{WT}-transfected cells were co-cultured with Notch1-expressing cells (data not shown). In summary, this suggests that interaction with receptor leads to enhanced ligand ubiquitylation.

NECD is trans-endocytosed into the ligand-expressing cells and co-transported with ligand throughout the endocytic degradation pathway

We next addressed whether NECD was trans-endocytosed into Jagged1-expressing cells. HEK293T cells transfected with a full-length HA-tagged Notch1 expression construct were co-cultured with Jagged1^{WT}- or Jagged1^{Ndr}-expressing HEK293T cells transfected with EGFP-EEA1 to visualize early or sorting endosomes. HA immunoreactivity colocalized with EGFP and Jagged1 in the Jagged1^{WT}-expressing cells and only when these cells were juxtaposed to receptor-expressing cells (Fig. 7A). This shows that the Notch1 extracellular domain can be transferred to early or sorting endosomal vesicles in neighboring Jagged1^{WT}-expressing cells, in keeping with previous reports (Klueg and Muskavitch, 1999; Nichols et al., 2007; Parks et al., 2000), while in the Jagged1^{Ndr}-expressing cells, much lower levels of internalized NECD were observed (Fig. 7B), presumably because of the endogenous levels of Jagged1 in these cells.

To study the possible routing of NECD through the intracellular degradation pathway, i.e. via early or sorting endosomes, late endosomes, multivescular bodies (MVBs) and eventually to lysosomes (Bonifacino and Traub, 2003; Gruenberg and Stenmark, 2004), we incubated N1ECD-Fc-Cy3 with Jagged1^{WT}-expressing cells, which allowed us to directly visualize ligand-dependent uptake and trafficking of the NECD. The localization of internalized NECD-Fc-Cy3 was followed at different time points after co-culture. Immediately after addition of NECD-Fc-Cy3 to the culure N1ECD-Fc-Cy3 colocalized with EGFP-F, a farnesylated form of EGFP that is targeted to the plasma membrane (Fig. 7C). At later time points, N1ECD-Fc-Cy3 was increasingly internalized, and after 60 minutes, a substantial fraction was found in EEA1-positive early or sorting endosomes (Fig. 7D). Finally, 4 hours after labeling, a significant fraction of N1ECD-Fc-Cy3 and Jagged1 was observed in lysosomal vesicles, as visualized by Lysotracker Red (Fig. 7E). No uptake of N1ECD-Fc-Cy3 could be observed in cells expressing Jagged1^{Ndr} (data not shown; see also Fig. 2C, Fig. 7B and supplementary material Fig. S1). Together, this shows that NECD is trans-endocytosed into cells expressing functional ligand and is trafficked through the degradation pathway to lysosomes.

Jagged1 interacts with Hrs

To test for ligand internalization in a different manner, we analyzed whether Jagged1 interacted with Hrs. Interaction with Hrs, which resides in sorting endosomes, is believed to result in the sorting of ubiquitylated transmembrane proteins into newly formed MVBs, a key step in the transport down the degradation pathway to the lysosome (Raiborg et al., 2003). Transfection of Myc-tagged Hrs into HEK293T cells stably expressing Jagged1, followed by immunoprecipitation with an antibody specific for Jagged1 readily co-precipitated Hrs (Fig. 8A). Simultaneous expression of Jagged1^{WT} with Mib1(M989R) abolished the interaction between Hrs and Jagged1 (Fig. 8A), suggesting that ubiquitylation of

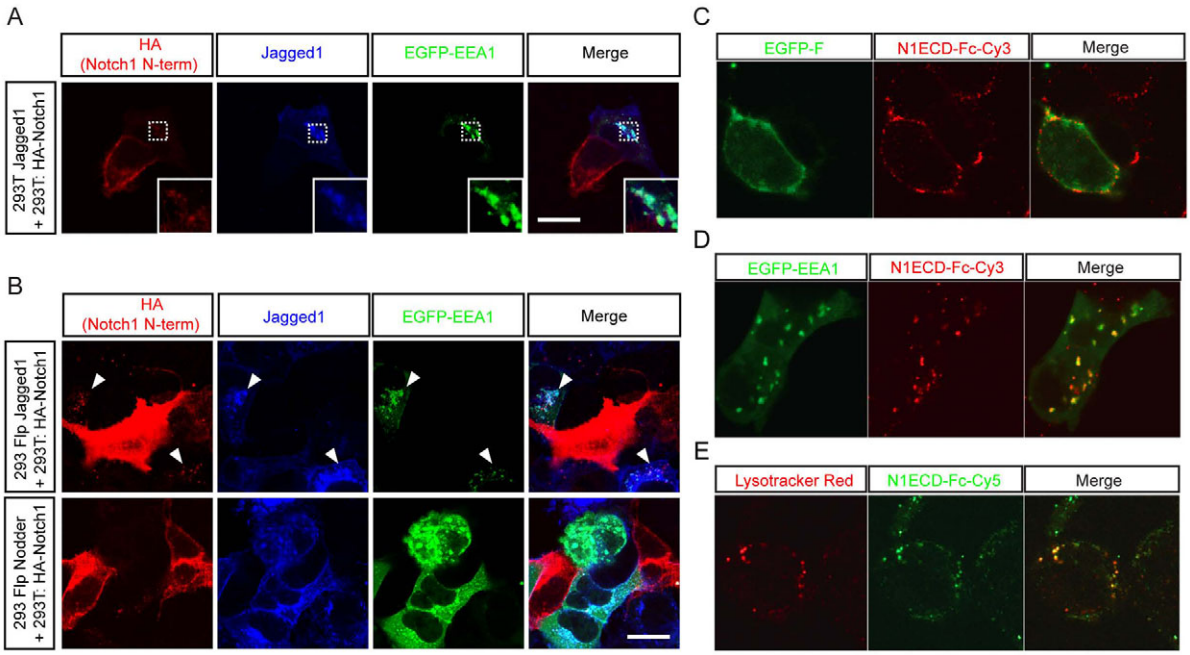

Fig. 7. NECD is transendocytosed into Jagged1^{WT}-expressing cells and transported through the endocytic degradation pathway. (**A**) HEK293T cells expressing full length HA-Notch1 (N-terminally tagged Notch1) were co-cultured with Jagged1^{WT}-expressing HEK293T cells transfected with EGFP-EEA1. HA immunoreactivity was observed in EGFP-positive and Jagged1-positive vesicles indicating trans-endocytosis of Notch1 ECD into EEA1-positive vesicles in Jagged1-expressing cells. Boxed region is magnified in inset. Scale bar: 20 µm. (**B**) Jagged1^{WT} cells exhibit high levels of trans-endocytosis of Notch1 ECD, whereas Jagged1^{Ndr} cells exhibit low-to-no trans-endocytosis of Notch1ECD into Jagged1- and EEA1-positive vesicles (arrowheads). Scale bar: 20 µm. (**C-E**) Immunocytochemistry of cells with fluorescently labeled subcellular markers and incubated with N1ECD-Fc-Cy3. (C) Immediately after incubation, N1ECD-Fc-Cy3 (red) colocalized with the plasma-membrane marker EGFP-F. (D) At 1 hour, N1ECD-Fc-Cy3 (red) was detected in EGFP-EEA1-positive vesicles. (E) At 4 hours, N1ECD-Fc-Cy5 (green) was detected in lysosomes, as visualized by the lysosome marker Lysotracker Red (red).

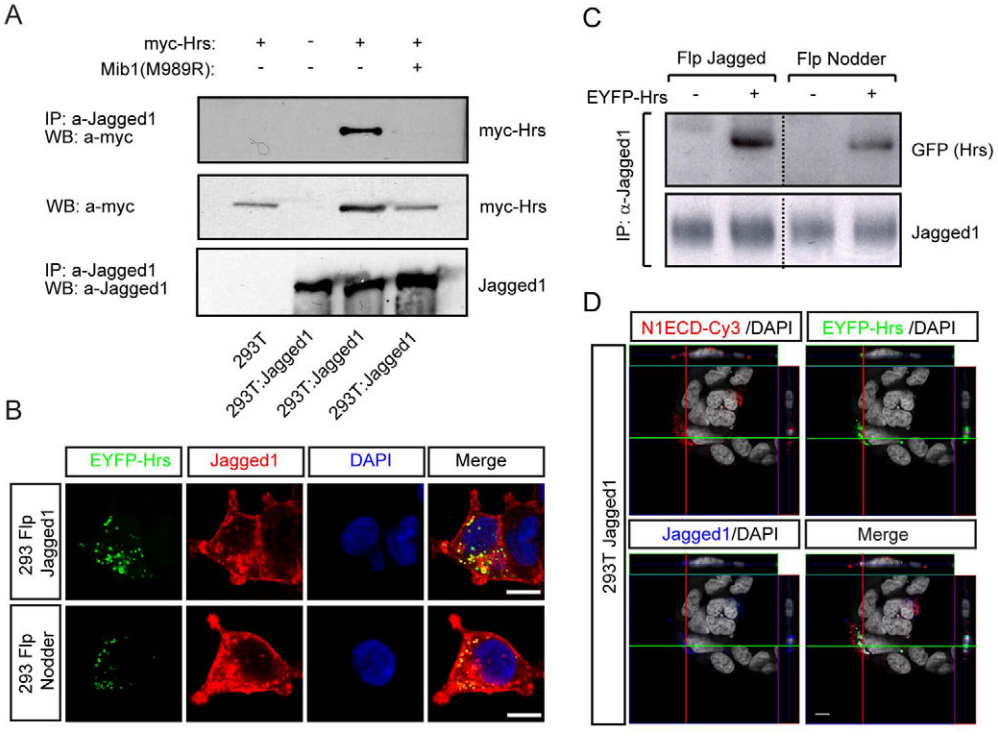

Fig. 8. Jagged1 and Hrs interact and colocalize in intracellular vesicles. (**A**) Jagged1 was immunoprecipitated from (from left to right): native HEK293T cells transfected with Myc-Hrs; HEK293T cells expressing Jagged1; HEK293T cells expressing Jagged1 and transfected with Myc-Hrs; HEK293T cells expressing Jagged1 and transfected with Myc-Hrs and Mib1(M989R). In the top panel, immunoprecipitation of Jagged1, followed by western blot for Hrs (α-myc) is depicted. The middle panel shows that similar amounts of Hrs were expressed in the Myc-Hrs-transfected cells (lanes 1, 3 and 4). In the bottom panel, the input was probed for Jagged1. (**B**,**C**) Jagged1^{Ndr} interacts with Hrs. (B) EYFP-Hrs was extensively colocalized with Jagged1 in Jagged1^{WT} and Jagged1^{Ndr}-expressing HEK293 cells. (C) Jagged1 was co-immunoprecipitated with EYFP-Hrs in cells expressing both Jagged1^{WT} and Jagged1^{Ndr}. (**D**) Confocal stack showing that both Jagged1 and NECD are present in Hrs-immunoreactive intracellular vesicles in Jagged1^{WT}-expressing HEK293T cells. Scale bar: 10 µm.

Jagged1 is required for interaction with Hrs. Co-expression of EYFP-tagged Hrs revealed a co-localization with Jagged1^{WT} and Jagged1^{Ndr} in intracellular vesicles (Fig. 8B) and immunoprecipitation of Jagged1 showed that Hrs was co-immunoprecipitated with both Jagged1^{WT} and Jagged1^{Ndr} (Fig. 8C). Furthermore, we found that in cells stably expressing Jagged1^{WT}, transfected with Myc-Hrs and exposed to NECD, there was a partial co-localization between NECD, Jagged1 and Hrs in intracellular vesicles (Fig. 8D).

Jagged1^{Ndr} protein shows an altered cellular localization in vivo

The Jagged1$^{Ndr/Ndr}$ mice presented an opportunity to specifically study the importance of ligand-receptor interaction for ligand endocytosis and intracellular localization in vivo. The cellular distribution of Jagged1 was analyzed in cells in the spinal cord of E10.5 Jagged1$^{+/+}$, Jagged1$^{+/Ndr}$ and Jagged1$^{Ndr/Ndr}$ mouse embryos. Jagged1 and Dll1 ligands are expressed in alternate dorso-ventral domains in the spinal cord (Lindsell et al., 1996), and we analyzed Jagged1 distribution in cells in the dP6 domain (Fig. 9A), an area where active Notch signaling is known to take place (Marklund et al., 2010). In Jagged1$^{+/+}$ mice, Jagged1 protein displayed a patchy, punctate intracellular distribution (Fig. 9B-E). By contrast, in sections from Jagged1$^{Ndr/Ndr}$ mice, Jagged1 immunoreactivity was stronger and largely co-localized with pan-cadherin immunoreactivity (Fig. 9J-L), supporting a predominant plasma membrane localization. Interestingly, ligand accumulation at the cell surface was seen not only in the Jagged1$^{Ndr/Ndr}$ (Fig. 9J-L), but also in Jagged1$^{+/Ndr}$ embryos (Fig. 9F-I). In a different CNS region, in the basal plate of the midbrain (Fig. 9N), accumulation of Jagged1^{Ndr} at the cell perimeter was even more pronounced (Fig. 9O-W). Together, these data demonstrate that the inability to interact with Notch receptors results in reduced ligand endocytosis in the Jagged1$^{Ndr/Ndr}$ embryos and ligand accumulation at the cell surface.

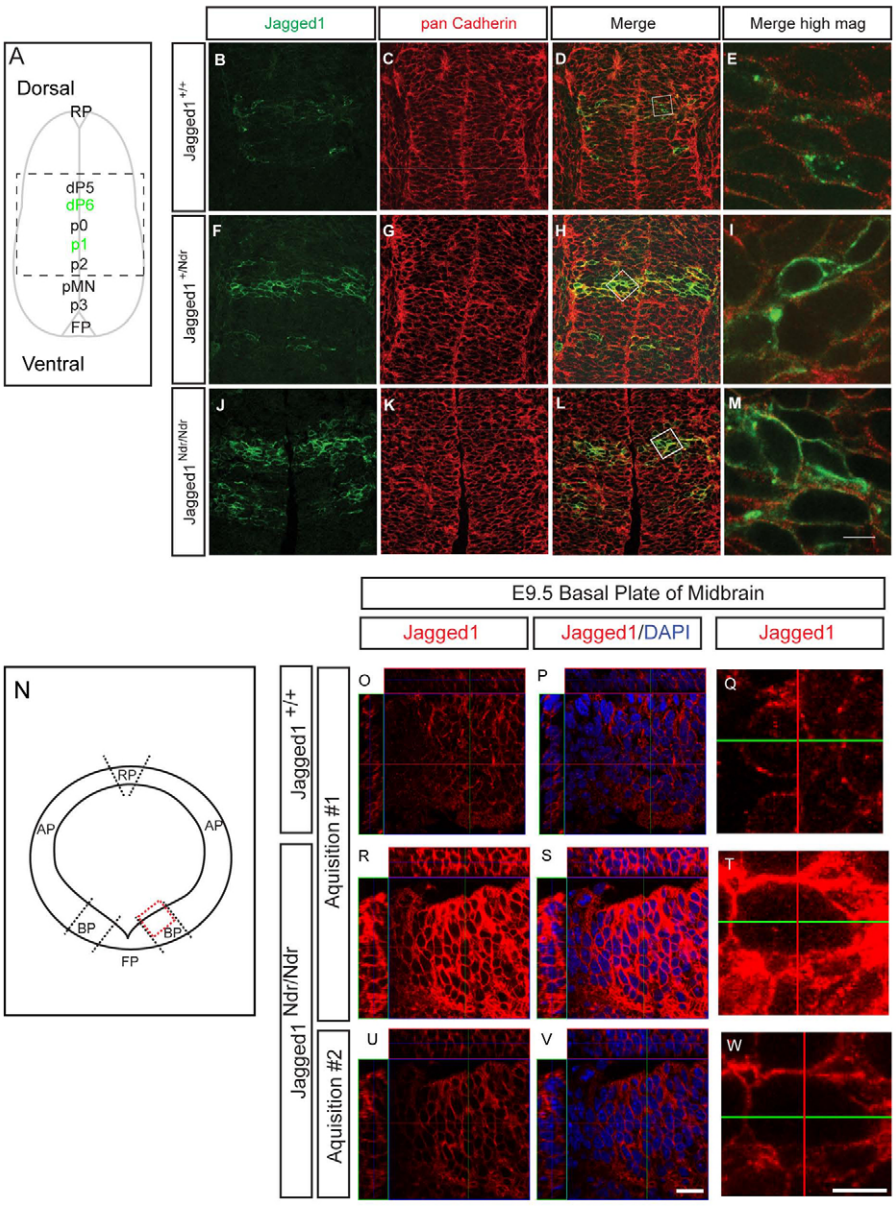

Fig. 9. The interaction-dead Jagged1^{Ndr} protein accumulates at the cell surface in vivo. Immunohistochemistry of E10.5 mouse spinal cord and E9.5 midbrain sections. (**A**) Schematic illustration of the embryonic spinal cord, where the area shown in other panels is indicated (dashed rectangle). p3-dP5 refers to the ventral and intermediate progenitor domains, and the floor plate is indicated as FP. (**B-M**) Jagged1 expression is confined to the p1 and dP6 domains. Low-magnification images (B-D, F-H and J-L) and corresponding high-magnification images (E,I and M) of dP6 cells (indicated by boxes in D,H and L). Wild-type embryos (B-E) show a predominantly intracellular, punctuate immunoreactivity for Jagged1 (E), whereas in Jagged$^{+/Ndr}$ (F-I) and Jagged$^{Ndr/Ndr}$ (J-M) embryos, there is an accumulation of Jagged1 at the plasma membrane (visualized with anti-pan cadherin immunoreactivity). (**N**) Schematic illustration of the embryonic midbrain, depicting the ventricular zone of the alar plate where the confocal stacks shown in O-W were acquired. AP, alar plate; BP, basal plate; RP, roof plate; VM, ventral midbrain. (**O-Q**) In the Jagged1$^{+/+}$ alar plate, Jagged1 expression was seen at the membrane and intracellularly throughout the cell body, spread out in punctae. Higher magnification of the cross-hair is shown in Q. (**R-W**) In Jagged1$^{Ndr/Ndr}$ mice, Jagged1 expression is much higher, as seen in images taken with the same acquisition settings (R-T), and very little Jagged1 was seen intracellularly, or in images acquired with lower gain and no offset (U-W), which reveal all background staining and still very little or no intracellular Jagged1. Scale bars: 50 µm (B-D,F-H,J-L), 7 µm (E,I,M), 20 µm (O-P,R-S,U-V) and 5 µm (Q,T,W).

Discussion

In the Notch signaling pathway, endocytosis is required both on the receptor and on the ligand side (Brou, 2009; Chitnis, 2006; D'Souza et al., 2008; Le Borgne et al., 2005). Previous studies have established that ligand ubiquitylation by Mind bomb and Neuralized and ligand endocytosis are required for the ability of ligands to activate signaling, but the relationship between ubiquitylation, receptor-ligand interaction and internalization of ligand is yet only partially understood. Two principally different, although not mutually exclusive, models have been presented for how endocytosis affects Notch ligand function. The ligand-maturation model suggests that endocytosis is required for producing an active form of ligand that is capable of receptor binding and activation, whereas another model proposes that endocytosis is required to remove the extracellular domain of the receptor before signaling can occur, i.e. a 'pulling-force' model (Brou, 2009; Chitnis, 2006; D'Souza et al., 2008; Le Borgne et al., 2005).

In this report, we identify a mutant form of Jagged1, Jagged1^{Ndr}, which differs from Jagged1^{WT} only by a missense mutation in the extracellular domain. Jagged1^{Ndr} has specifically lost the capacity to bind to Notch receptors and activate Notch signaling, but it retains a wild-type intracellular distribution in the absence of active Notch signalling. This notion is based on the observation that Jagged1^{Ndr}-expressing cells fail to bind and trans-endocytose NECD, and do not activate signaling in juxtaposed, Notch-receptor-expressing cells. By contrast, Jagged1^{Ndr} exhibits a similar cellular distribution as Jagged1^{WT} in the absence of Notch signaling, and can interact with MIB1. These data argue that receptor-ligand interaction is dispensable for intracellular trafficking and MIB1 interaction.

Blockade of endocytosis or MIB1 function led to loss of receptor activation, but not of receptor binding. This is compatible with a more broadly defined ligand-maturation model, in which endocytosis alters certain properties of the ligand, such that activation is achieved only after ligand endocytosis. The data however argue against more 'extreme' forms of the ligand-maturation model, where function and endocytosis of Mib1 would be considered necessary also for receptor recognition by the ligand. Our findings also provide support for the 'pulling-force' model. Previous work has demonstrated that NECD is trans-endocytosed into the ligand-expressing cell (Nichols et al., 2007; Parks et al., 2000), but the ultimate fate of NECD has not been determined. We extend the earlier reports on NECD trans-endocytosis by demonstrating that NECD is targeted for the endocytic degradation pathway, because NECD, co-transported with Jagged1, could be traced progressively over time from early endosomes through multivesicular bodies to lysosomes. The finding that Jagged1 directly interacts with Hrs, an adaptor protein in early or sorting endosomes and likely to be involved in sorting protein cargo into the degradation pathway (Raiborg et al., 2003), lends further support to the idea that Jagged1 is routed to the degradation pathway.

Our data argue that the extent of receptor-ligand interaction controls the cellular distribution of Jagged1 in vivo, because the Jagged1 protein was localized predominantly to the plasma membrane in the *Jag1*$^{Ndr/Ndr}$ mouse rather than to intracellular vesicles, as seen in *Jag1*$^{+/+}$ mice. Data on enhanced ligand cell-surface localization have recently been reported from zebrafish when Notch receptor was knocked down (Matsuda and Chitnis, 2009), and collectively this shows that bulk endocytosis is not sufficient to explain the normal cellular distribution of ligand in vivo. We observed an increased localization to the plasma membrane in the heterozygous *Jag1*$^{Ndr/+}$ mouse, which might be a result of accumulation of mutant Jagged1 protein at the plasma membrane and a longer half-life of the ligand in its non-receptor-interacting state, whereas the wild-type protein engages in functional receptor activation, and is more rapidly turned over. Jagged1^{WT} protein in *Jag1*$^{Ndr/+}$ mice most likely can engage in productive receptor interaction, because *Jag1*$^{Ndr/+}$ mice exhibit only a very mild, head-nodding, phenotype, in contrast to the embryonic lethality observed in the *Jag1*$^{Ndr/Ndr}$ mouse. The relatively mild Jagged1$^{Ndr/+}$ phenotype is also in keeping with our observation that Jagged^{Ndr}, when co-expressed with Jagged1^{WT}, was unable to exert a dominant-negative effect on ligand activation. It is of note that a non-signaling form of Delta accumulates at the cell surface in *Drosophila* (Parks et al., 2000), and in Mib1-deficient mice and zebrafish, Dll1 also accumulates at the cell surface (Itoh et al., 2003; Koo et al., 2005; Matsuda and Chitnis, 2009; Song et al., 2008). Consequently, interaction-deficient ligands, as well as ligands in cells lacking Mib1, are similarly unable to enter the endocytic route and are stranded at the cell surface.

These data might argue that a finely tuned balance between E3 ubiquitin ligase activity and receptor-ligand interaction controls the extent of ligand internalization. Such an activation-controlled regulation of endocytosis is found not only in the Notch pathway, but also in other signaling mechanisms, where endocytosis exerts a key control step. Studies of the EGF and TGF-β signaling pathways show that the level of receptor stimulation determines the intracellular route of the receptor following activation-induced endocytosis, i.e. a high degree of activation results in receptor targeting to the lysosome and subsequent degradation, whereas at lower levels of activation the receptor enters the recycling route and re-appears at the cell surface (Di Guglielmo et al., 2003; Sigismund et al., 2008). In this regard, it is of note that ubiquitylated Notch ligands are routed through discrete, lipid-raft-containing endosomal compartments, whereas non-ubiquitylated ligands are concentrated in endosomal compartments lacking lipid rafts (Heuss et al., 2008). This supports the notion that internalized Notch ligands can be routed through different endosomal compartments.

In conclusion, the discovery of an interaction-dead form of Jagged1 provides a new tool to dissect the specific importance of receptor-ligand interaction in vitro and in vivo. The data lend support to the 'pulling-force' model, but are also compatible with a broader version of the ligand-maturation model, although they rebut more radical versions of this model, in which the function and endocytosis of Mind bomb would also be considered necessary for receptor recognition.

Materials and Methods

Cell lines and cell culture

HEK293T and HEK293-Flp-In cells were cultured in Dulbecco's Minimal Essential Medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (all from Invitrogen). The isogenic HEK293-Flp-Jagged1, HEK293-Flp-Jagged1Ndr, HEK293-Flp-Jagged1Slm, HEK293-Flp-Jagged1Htu cell lines were generated by co-transfection of parental HEK293-Flp-In cells with the respective expression construct together with a plasmid encoding Flp, followed by selection according to the supplier's instructions (Invitrogen). HEK293T:Jagged1 and HEK293T:Notch1 cells have been described previously (Chapman et al., 2006). For transient transfections, cells were transfected using Fugene 6 according to the manufacturer's instructions (Roche) or Lipofectamine 2000 (Invitrogen).

Recombinant proteins and pharmacological inhibitors

Recombinant rat Notch1 extracellular domain 1-12 fused to the human Fcγ chain of IgG1 (R&D Systems) was used essentially as described (Ladi et al., 2005). In brief, Notch1-Fc was coupled to Cy3 anti-human IgG (Jackson) by incubation for 1 hour

at 4°C before addition to cells. Cy3 anti-human IgG antibody was used as a control in these experiments. Recombinant rat Notch1 EC-Fc chimera (R&D Systems) was used at 1 μg/ml. The ADAM metalloproteinase inhibitors used were GM6001 (Calbiochem) and TAP-1 (Calbiochem), both at 20 μM unless stated otherwise.

Flow cytometry

Cells labeled with recombinant Notch ECD, as described above, were carefully scraped off the plate in ice-cold PBS supplemented with 4% FBS, and fluorescence was analyzed on a FACSAria (Becton Dickinson). Percentage of fluorescently labeled cells per culture is presented.

DNA constructs

A DNA fragment containing the entire coding region of mouse *Jag1* cloned into pBluescript was used as template to generate Jagged1^{Ndr}, Jagged1^{Slm} and Jagged1^{Htu} by site-directed mutagenesis using QuikChange (Stratagene). The wild-type and mutated forms of *Jag1* were subcloned by PCR to the pcDNA5FRT/TO vector (Invitrogen) to generate pcDNA5-Jagged1, pcDNA5-Jagged1^{Ndr}, pcDNA5-Jagged1^{Slm} and pcDNA5-Jagged1^{Htu}. pcDNA3-HAMib1 was a kind gift from Young-Yun Kong (Pohang University of Science and Technology, Pohang, South Korea). The antimorphic *Mib1* construct Mib1(M989R), which corresponds to the ta52b (white tail) mutation in zebrafish Mind bomb (Zhang et al., 2007), was generated by mutagenesis of coding position 989 (from methionine to arginine) starting from the pcDNA3-HAMib1 construct. Expression constructs for HA-Notch1 and EGFP-DynaminIIK44A were generously supplied by Pier Pablo Di Fiore (IFOM, Milan, Italy). The EGFP-2xFYVE, EGFP-EEA1 and Myc-Hrs constructs have been described previously (Gillooly et al., 2000; Gillooly et al., 2003). 2xFYVE acts as an endosomal marker through its interaction with the endosomal lipid, phosphatidylinositol 3-phosphate (Gillooly et al., 2000). EGFP-Ub was generously provided by Nico Dantuma (Karolinska Institutet, Stockholm, Sweden). 12XCSL-luc was a kind gift from Tasuko Honjo (Kyoto University, Kyoto, Japan), and CMV-βgal was obtained from Thomas Perlmann (Ludwig Institute for Cancer Research, Stockholm, Sweden). EGFP-F is commercially available (BDBiosciences, Clontech).

RNA interference

Specific knockdown of human *MIB1* was achieved by transfecting cells using Lipofectamine 2000 (Invitrogen) with the Stealth siRNA duplex HSS166394 specific for the *MIB1* coding region (Invitrogen). As a control, cells were transfected with a scrambled siRNA duplex with similar GC content recommended by the manufacturer (Invitrogen). 12 hours after the initial transfection, cells were retransfected with siRNA and DNA plasmid as indicated using Lipofectamine 2000. Lipofectamine 2000 was used according to protocols provided by the manufacturer.

Antibodies and fluorescent compounds

The following primary and secondary antibodies were used in the study: rabbit anti-Jagged1 (Santa Cruz), goat anti-Jagged1 (Santa Cruz), rabbit anti-pan Cadherin (BD Biosciences), mouse anti-HA (Nordic Biosite and Covance), rabbit anti-GFP (Abcam), rat anti-PECAM (BD Biosciences), rabbit anti-nicastrin (Sigma), anti-mouse Alexa Fluor 488 (Molecular Probes/Invitrogen) anti-mouse Cy3 (Jackson), anti-mouse Cy5 (Jackson), anti-rabbit Alexa Fluor 488 (Molecular Probes/Invitrogen), anti-goat Alexa Fluor 488 (Molecular probes/Invitrogen), anti-goat Cy3 (Jackson) anti-human Cy3 (Jackson), anti-human Cy5 (Jackson), anti-rat HRP (Vector Laboratories), anti-goat HRP (DAKO), anti-mouse HRP (DAKO), and anti-rabbit HRP (DAKO). Transferrin-633 (Molecular Probes/Invitrogen) was used according to the manufacturer's instructions.

Cell-surface biotinylation

Cell-surface proteins were captured as described in a previously published protocol (Hansson et al., 2005). Briefly, cell surface proteins were biotinylated using the EZ-link NHS-LC-Biotin substrate (Pierce), at a concentration of 1 mg/ml for 30 minutes at 4°C. Residual activity of the biotinylation reagent was quenched by incubation with serum-free DMEM for 10 minutes. Cells were then washed and lysed, and biotinylated proteins were precipitated with Streptavidin-agarose beads (Pierce).

RNA extraction, cDNA synthesis and quantitative PCR

RNeasy (Qiagen) was used according to the manufacturer's instruction for RNA extraction. Reverse transcription was performed on 1 μg total RNA using oligo-dT12-18 and Superscript II reverse transcriptase (Invitrogen). A mastermix containing nucleotides, Taq polymerase, SYBRGreen and buffer (Applied Biosystems) were mixed with primers and cDNA. Real-time PCR was performed using a LightCycler rapid thermal cycler system (Applied Biosystems) in accordance with the manufacturer's instructions. *MIB1* expression levels were normalized against the internal control *GAPDH*. Primer sequences are available on request.

Western blot, immunoprecipitation and luciferase assays

For western blotting, cells were lysed in whole-cell extract buffer (20 mM HEPES, pH 7.5, 0.42 M NaCl, 0.5% Nonidet P-40, 25% glycerol, 0.2% EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with protease-inhibitor cocktail (Roche). For immunoprecipitations, cells from 10 cm plates were lysed in 0.5 ml IP buffer [150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM $CaCl_2$, 1% Triton X-100, 5% glycerol, 10 mM NaF, 10 mM sodium orthovanadate with protease-inhibitor cocktail (Roche) and 10 mM NEM (Sigma)]. After incubation at 100°C for 5 minutes, the samples were incubated with the appropriate primary antibody for four hours at +4°C with end-over-end rotation. Precipitated proteins were captured with Protein-A- or G-Sepharose (Amersham Biosciences). Proteins were resolved on Nu-PAGE 10% or 4-12% Bis-Tris gels (Invitrogen) and transferred to Protran nitrocellulose membrane (Schleicher and Schuell). Antibody incubations were carried out in 3% milk in TBS-T, and proteins were visualized using ECL or ECL Plus (Amersham Biosciences) and exposure on Hyperfilm ECL (Amersham Biosciences).

For luciferase assays of co-cultures, receptor-expressing cells were co-transfected with 12XCSL-luc (5.5 μg/10 cm plate) and CMV-βgal (0.5 μg/10 cm plate) using FUGENE6 (Roche) according to protocols from the supplier. 12 hours later, the cells were scraped off the plate, pipetted up and down to break cell-cell contacts, and reseeded in a 24-well plate. 12 hours after reseeding, ligand-expressing cells were added to the cultures. The ratio of ligand-expressing cells to receptor-expressing cells was 2:1 or 3:1. The following day cells were lysed in lysis buffer (Tropix/Applied Biosystems) and analyzed for luciferase activity after addition of luciferin and ATP (BioThema) and βgal activity using Galacto-light (Tropix/Applied Biosystems) in an Anthos Lucy2 luminometer. Luciferase values are proportional to the level of Notch signaling in the cell, and are normalized to βgal to control for differences in transfection efficiency. Arbitrary βgal-normalized luciferase units are presented as fold induction compared with the control.

Immunocytochemistry

Cells were fixed 36-48 hours after transfection in 3.7% formaldehyde in PBS (Histolab). After several rounds of washing in PBS, cells were incubated in blocking buffer (5% BSA, 0.3% Triton X-100 in PBS) at room temperature for 45-60 minutes, followed by incubation with primary antibodies diluted in blocking buffer in a humid chamber at 4°C over night. Cells were rinsed at least three times in PBS the following day and subsequently stained with secondary antibodies diluted in blocking buffer in darkness at room temperature for 45 minutes. Cells were rinsed thoroughly in PBS, mounted in Vectashield containing DAPI (Vector Laboratories), and visualized in a Zeiss LSM510 META confocal unit (Carl Zeiss), equipped with a 63× lens (1.4 oil objective). In some cases a Zeiss LSM510 confocal unit without META hardware was used. Images were cropped and in some cases adjusted for brightness and contrast in Photoshop, and assembled in Illustrator (both Adobe).

Cell-culture quantifications

For quantification of dsRed$^+$ and Notch$^+$ cells (Fig. 4C), 50-100 Notch$^+$ cells were assessed for dsRed positivity per condition in each experiment. This experiment was repeated three times in duplicate, the graph represents the mean of three experiments and ANOVA statistical analyses were performed by GraphPad Prism Software. For Jagged1 and EEA1 quantification (Fig. 5B), 1 μm optical slice images were acquired on a Zeiss LSM confocal microscope. 10 cells per condition were classified as falling into one of three categories 0-33%, 34-66%, or 67-100% Jagged1$^+$ EEA1$^+$. In general, cells contained circa 20-50 EEA1$^+$ vesicles in such an image. The graph represents the mean of three experiments.

Animal maintenance

Nodder mice were identified through an ENU screen. C3HeB/FeJ mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Mutagenesis was achieved by treating male mice three times with 100 mg N-ethyl-N-nitrosurea (ENU) per kg in a weekly interval and crossing them to wild-type C3HeB/FeJ females. The F3 progeny of the ENU-treated mice was analysed for neurological and behavioural abnormalities in a series of tests according to a modified SHIRPA (SmithKline Beecham, Harwell, Imperial College School of Medicine, Royal London Hospital, Phenotype, Assessment) protocol. Nodder mice were identified by nodding behavior and balance defects in motor-coordination assays. Breeding and phenotyping of the Nodder mice progeny revealed a dominant autosomal inheritance. For chromosomal mapping and positional cloning, C57BL/6J mice were purchased from the Jackson Laboratory and used for generating outcross progenies. SNP and microsatellite analysis procedures for positional cloning of the responsible mutated gene were performed as previously described (Paffenholz et al., 2004). All histological analysis was carried out at the animal facility at the Department of Cell and Molecular Biology, Karolinska Institute, in accordance with the ethic regulation for mouse work in Sweden. Nodder mice (kept on a pure C3H background) were genotyped by PCR amplification of genomic DNA using the primers 5′-GTGTGTTGTAAC-TATGTGCA-3′ (sense) and 5′-GAGTCCCACAGTAATTCAGA-3′ (antisense). The obtained PCR fragments were gel-purified and sequenced by the Karolinska Institute or by Eurofins/MWG (Germany) sequencing facility using the primer 5′-GGTCATACCTTTGTCACAGA-3′. To determine the age of embryos, noon of the day of the vaginal plug was set as embryonic day (E) 0.5.

Immunohistochemistry

For whole-mount immunohistochemistry, embryos were dissected in PBS and fixed in 4% paraformaldehyde overnight. The following day, embryos were rinsed three times in PBS, dehydrated in series of increasing ethanol concentration and kept at –20°C before analysis. Embryos were then bleached by incubation in 5% H_2O_2 in methanol for 5 hours at room temperature, where the solution was changed every

hour. Bleaching was stopped by three 15 minute washes in 100% methanol, and the embryos were rehydrated in series of PBS. Following rehydration, embryos were incubated in PBSMT (PBS with 3% non-fat dry milk and 0.1% Triton X-100) for twice for 1 hour at room temperature before incubation with primary antibody in PBSMT at 4°C overnight. The following day, embryos were subjected to five 1 hour washes in PBSMT and incubated with biotinylated secondary antibody in PBSMT at 4°C overnight. The following day, embryos were washed five times in PBSMT before incubation with ABC reagent (Vector Laboratories) in PBSMT at 4°C overnight. Embryos were then washed five times in PBSMT, once in PBT (PBS with 0.2% BSA and 0.1% Triton X-100) and staining was achieved by incubation in DAB staining solution (Vector Laboratories). Following washes in PBT and PBS, the embryos were post-fixed in 0.1% glutaraldehyde, 2% paraformaldehyde in PBS and transferred to 70% glycerol.

For sections, embryos were fixed in 4% PFA for 4 hours, washed three times in PBS and then incubated in 30% sucrose overnight. Subsequently, embryos were embedded in Optimum Cutting Temperature compound (TissueTek) and sectioned onto Superfrost slides (Thermo Scientific) at 14 μm on a cryostat. Slides were rehydrated in PBS and blocked in a blocking solution consisting of 5% donkey serum (Sigma) in PBS with 0.1% Triton X-100 (Roche) for 1 hour at room temperature. Primary antibodies were applied in blocking solution overnight at 4°C. Goat anti-Jagged1 (Santa Cruz) was used at 1:1000. Slides were then washed three times for 15 minutes and then incubated with the appropriate secondary antibody at 1:500 for 1 hour at room temperature. After three more PBS washes, slides were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/17/2931/DC1

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