Chapman et al., http://www.jcb.org/cgi/content/full/jcb.200602009/DC1

DNA constructs

Mouse Numb and Numblike (gifts from W. Zhong, Yale University, New Haven, CT) were PCR amplified and cloned into pCMXHA using the Gateway system, forming CMV–Numb-HA and CMV–Nbl-HA. CMV–Nbl-ΔPHA lacking amino acids 260–273 was generated by QuikChange mutagenesis using the primer sequence 5′-ACCTGCCCAGCCTGGGGGA GAGGCGGGCACCC-3′ according to the manufacturer's instructions (Stratagene). Numb-HA, Nbl-HA, Nbl-ΔPHA, and FLN1-myc (a gift from J. Nye, Pfizer Inc., Kalamazoo, MI) were Gateway cloned into pCAG-IRES-Puro (a gift from S. Wood, Mental Health Research Institute, Parkville, Victoria, Australia). Myc-tagged N1IC was removed from pGADN1IC and Gateway cloned into pCMX. pCS2-N1ΔEFmyc, which includes the OPA and PEST domains and a myc tag, was generated by cloning a BglII–SpeI fragment from pcDNA-FLN1myc into pCS2-N1ΔE6myc (Kopan et al., 1996).

Cell culture, transfection, and reporter gene analysis

Luciferase assay transfections in 24-well trays contained 50 ng CMV-lacZ plasmid, 200 ng of reporter plasmid 12XCSL-luc (p6xTP1-luc; Kato et al., 1997), and 100 ng of each expression plasmid or pCMX plasmid to a total of 450 ng. Cultures were harvested 36 h after transfection in 150 μl Galacto-Lite lysis buffer (Tropix). Luciferase activity was assayed using GeneGlow (Biothema) and measured in an Anthos Luminoscan Lucy 1. β-galactosidase activity was determined using the Galacto-Lite kit (Tropix). Luciferase counts were normalized against β-galactosidase activity to account for differences in transfection efficiency.

Generation of antibodies and immunocytochemistry

Rabbit antisera were raised against the peptide NGVDNGGLASGNRHAE found near the C terminus of mouse Numb (Agrisera). Peptide-reactive antibodies were affinity purified according to the manufacturer's instructions (PSL GmbH). Purchased primary antibodies used in this study were rabbit anti-HA (CLONTECH Laboratories, Inc.), mouse anti-HA antibody (Covance), mouse 9E10 anti-myc antibody (BD Biosciences), and anti–act-N1 (Cell Signaling). Mouse antimyosin heavy chain developed by D. Fischman (Weill Medical College of Cornell University, New York, NY) was obtained from the Developmental Studies Hybridoma Bank (The University of Iowa). Immunofluorescence was performed as described previously (Dahlqvist et al., 2003). Immunoreactivity was visualized by fluorescence (Eclipse E800; Nikon) or confocal microscopy (LSM510 Meta; Carl Zeiss MicroImaging, Inc.). Fluorescent images

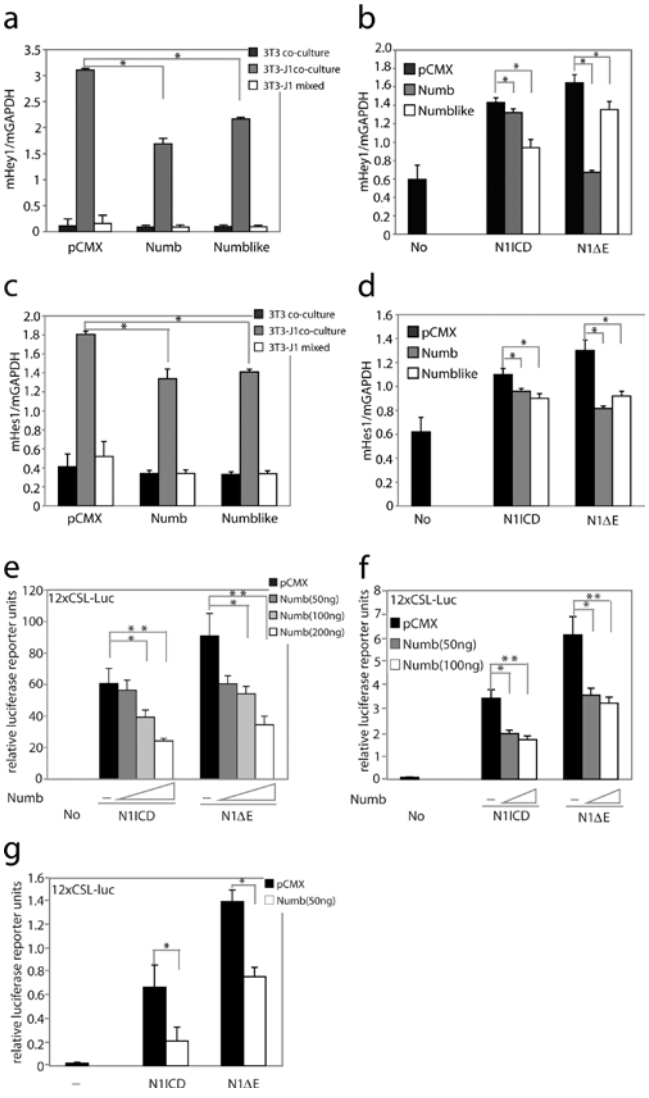

Figure S1. **Numb and Numblike down-regulate Notch signaling.** (a–d) Real-time PCR for mouse *Hey 1* (a) or *Hes 1* (c) mRNA levels after the transfection of Numb, Numblike, or vector only and after the activation of endogenous Notch signaling in C2C12 cells co-cultured with 3T3-J1 or 3T3 cells or when C2C12 cells are mixed with Jagged-1–expressing cells just before lysis (3T3-J1 mixed). Real-time PCR for mouse *Hey 1* (b) or *Hes 1* (d) mRNA levels after Notch activation by the transfection of Notch 1 ICD (N1ICD) or Notch 1 ΔE (N1 ΔE) and transfection with Numb, Numblike, or vector only (pCMX). Bars represent *Hey 1* or *Hes 1* mRNA expression relative to the expression of *mGAPDH*. (e) The level of activation of the Notch reporter 12XCSL-luc in C2C12 cells after the transfection of 50 ng Notch 1 ICD or Notch 1 ΔE together with various amounts of Numb (as indicated). (f) A similar experiment as in panel e but with 25 ng Notch 1 ICD or Notch 1 ΔE transfected. (g) The level of activation of the Notch reporter 12XCSL-luc in 293T cells after the transfection of 50 ng Notch 1 ICD or Notch 1 ΔE together with 50 ng Numb or empty pCMX vector. Values are significant at **, $P < 0.01$ and *, $P < 0.05$. Data represent the mean ± SEM (error bars) of three independent experiments performed in triplicate.

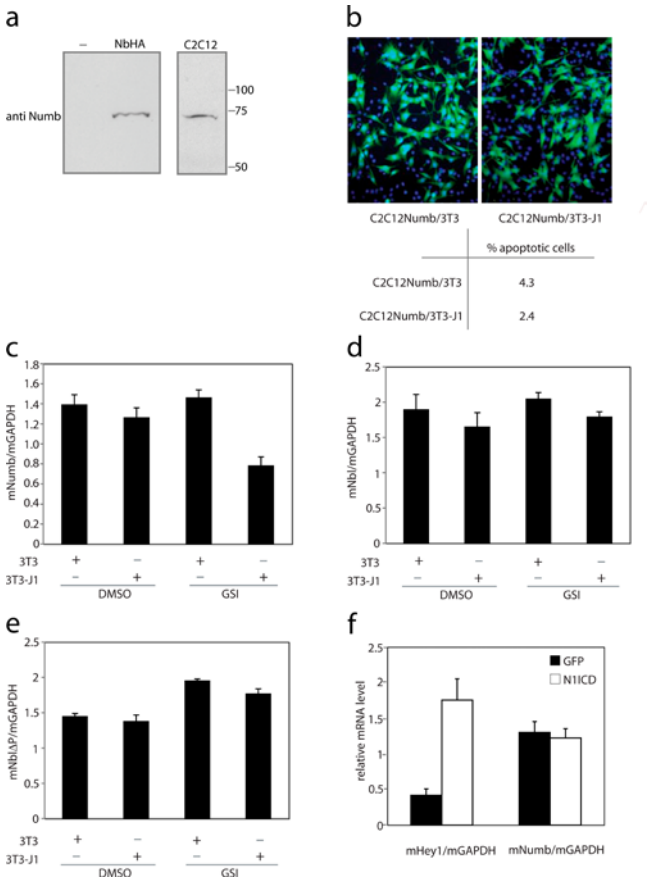

Figure S2. **Detection of the Numb protein and the role of Notch and Numb for apoptosis.** (a) Detection of Numb protein by the new anti-Numb antiserum in Western blots of cell extracts from C2C12 (endogenous Numb) and 293T transfected with Nb-HA. No immunoreactivity was observed in an extract from untransfected 293T cells (−). This antiserum was used for immunocytochemistry in Fig. 2 c. (b) The combination of Notch signaling and Numb expression does not increase the level of apoptosis. Before coculturing, equal numbers of C2C12 cells stably expressing Numb were labeled with CytoTracker green (Invitrogen) according to the manufacturer's instructions. Labeled cells were cocultured with 3T3-Jagged (3T3-J1) or 3T3 cells. Cell death after 48 h of coculture was assessed by flow cytometry of fixed, propidium iodide–stained cells. Cell death was specifically measured in the C2C12-Numb cells expressing CtyoTracker green. The combination of Notch signaling and Numb expression did not increase cell loss. (c–f) Numb and Numblike mRNA levels are not altered by the activation of Notch. Real-time PCR for mouse *Numb-HA* (c), *Numblike-HA* (*Nbl-HA;* d), or *Numblike-HA–ΔPEST* (*Nbl-HA-ΔPEST;* e) mRNA levels after the various coculture experiments as compared with GAPDH mRNA expression. (f) Expression of Notch ICD (6 h after infection with a Notch 1 ICD adenovirus) in C2C12 cells increased *Hey 1* mRNA expression but not the expression of *Numb* (EGFP expression from adenovirus as a control). Data represent the mean ± SEM (error bars) of three independent experiments performed in triplicate.

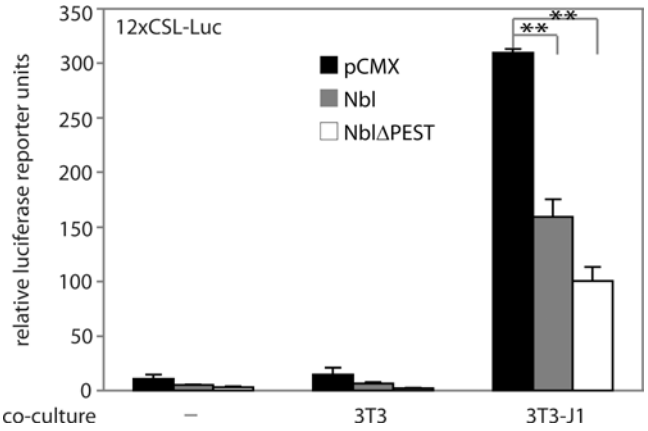

Figure S3. **Numblike-HA–ΔPEST down-regulates Notch-induced reporter gene activation more efficiently than Numblike-HA.** (a) The level of activation of the Notch reporter 12XCSL-luc in C2C12 cells after coculture with 3T3-J1 or 3T3 cells together with Numblike-HA, Numblike-HA–ΔPEST, or pCMX (as indicated). Values are significant at **, $P < 0.01$. Data represent the mean ± SEM (error bars) of three independent experiments performed in triplicate.

(AlexaFluor488 [Invitrogen] and Cy3 conjugates [Jackson ImmunoResearch Laboratories]; EGFP) were collected using a laser-scanning confocal microscope (LSM 510 META; Carl Zeiss MicroImaging, Inc.) configured on an inverted stand (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with a plan-Apochromat 63× NA 1.4 oil differential interference contrast objective. A plan-Apochromat 20× NA 0.75 objective was used for lower magnification images. Images are single z sections (<1.6 μm thick for 63× and <4.0 μm thick for 20×) generated using LSM 3.0 software (Carl Zeiss MicroImaging, Inc.). Images were processed by Photoshop (size adjustments), and Illustrator (Adobe) was used for figure layout.

Western blot and immunoprecipitation

Western blotting was performed as follows: 50 μg of protein lysates were electrophoresed on SDS-PAGE gels (Invitrogen), blotted to nitrocellulose membranes, and blocked in 5% skim milk powder in TBS (150 mM NaCl and 10 mM Tris-HCl, pH 7.4) for a minimum of 30 min. Primary antibodies were applied to Western blots for a minimum of 1 h. Membranes were then washed four times in TBS containing 0.05% Tween-20 for 10 min each before the addition of HRP-conjugated secondary antibodies. Membranes were washed four times in TBS with 0.05% Tween-20 for 10 min, and proteins were detected by ECL or ECL Plus (GE Healthcare).

Explanation of Fig. S1

The data in Fig. S1 show that the Notch-induced up-regulation of *Hey 1* mRNA, as a result of ligand stimulation of a full-length Notch 1 receptor (Fig. S1 a) or the use of a membrane-tethered ligand-independent form of Notch (Notch 1 ΔE) or the Notch ICD, was abrogated by both Numb and Numblike (Fig. S1 b). A similar, although less pronounced, down-regulation by Numb and Numblike was also observed for *Hes 1* mRNA (Fig. S1, c and d). Numb and Numblike in a dose-dependent manner down-regulated Notch 1 ICD and ΔE activation of a synthetic, highly specific Notch response element containing multimerized CSL-binding sites (12XCSL-luc, also referred to as p6XTP1-luc; Fig. S1 e), and the Numb- or Numblike-mediated down-regulation was even stronger when a smaller amount of Notch ICD (25 ng plasmid) was tested (Fig. S1 f). Furthermore, a more pro-

nounced down-regulation was seen when the experiments were conducted in 293T cells (Fig. S1 g), which may be a consequence of higher transfection efficiency in 293T cells as compared with C2C12 cells. In summary, these data suggest that Numb and Numblike negatively affect Notch signaling from both membrane-tethered and intracellular forms of Notch.

As Numb is localized to vesicular structures, presumably endosomes (see Fig. 1 c), and Notch ICD most likely is exclusively cytoplasmic or nuclear and, thus, is not associated with endosomes, we tested whether Notch ICD and Numb interacted. We could not detect a direct protein–protein interaction between Notch ICD and Numb (unpublished data), which is in contrast to a previous study (Frise et al., 1996) but is in keeping with data by Yaich et al. (1998). The lack of a direct interaction combined with the observed down-regulation of Notch ICD signaling and protein levels by Numb may provide support to a model in which Numb indirectly affects Notch ICD (for example, via the E3 ubiquitin ligase Itch; McGill and McGlade, 2003).

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