High levels of Notch signaling down-regulate Numb and Numblike

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Inhibition of Notch signaling by Numb is critical for many cell fate decisions. In this study, we demonstrate a more complex relationship between Notch and the two vertebrate Numb homologues Numb and Numblike. Although Numb and Numblike at low levels of Notch signaling negatively regulated Notch, high levels of Notch signaling conversely led to a reduction of Numb and Numblike protein levels in cultured cells and in the developing chick central nervous system. The Notch intracellular domain but not the canonical Notch downstream proteins Hes 1 and Hey 1 caused a reduction of Numb and Numblike. The Notch-mediated reduction of Numblike required the PEST domain in the Numblike protein and was blocked by the proteasome inhibitor MG132. Collectively, these observations reveal a reciprocal negative regulation between Notch and Numb/Numblike, which may be of relevance for stabilizing asymmetric cell fate switches and for tumor development.

Results and discussion
Numb and Numblike negatively regulate Notch signaling and reduce the level of Notch protein

A dose-dependent reduction of the levels of both a truncated membrane-tethered (Notch 1 ∆E) and an intracellular (Notch 1 ICD) form was observed in response to increasing amounts of Numb (Fig. 1, a and b). The Notch 1 ∆E protein was cleaved in the presence of Numb, although cleavage appeared to be somewhat reduced at higher Numb levels (Fig. 1, a and b).

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Furthermore, Numb and Numblike negatively regulate Notch signaling both from full-length Notch, Notch 1 ΔE, and Notch 1 ΔEΔE, as assessed by Western blotting (Fig. 1 a, b). Transfection of Numb into C2C12 cells followed by 3 d under differentiation-promoting conditions resulted in a dramatic increase in the differentiation of Numb-expressing cells at low levels of Notch signaling (Fig. 2 a). 88 and 95% of the Numb-positive cells were also myosin heavy chain (MHC) positive in C2C12/3T3 and C2C12-N1/3T3 cocultures, respectively, and 92% were positive in C2C12 cells cultured alone (Fig. 2 c). Similar values (88%) were obtained for Numblike (Fig. 2 d). The proportion of differentiated cells after Numb and Numblike transfection was considerably higher compared with the blocking of Notch signaling by the γ-secretase inhibitor DAPT, which only causes an increase in MHC-positive cells from 32 to 50% (Fig. 2 d). The addition of DAPT did not further enhance the differentiation-promoting effect of Numb and Numblike (Fig. 2 d). This argues for a more instructive role for Numb and Numblike in myogenic differentiation rather than only blocking Notch signaling. In keeping with a differentiation-promoting role, we observed that a C2C12 cell line stably expressing Numblike showed accelerated myogenic differentiation as compared with the parental cell line; 2 d after the switch to prodifferentiation conditions, 12.2% of the cells were MHC positive as compared with only 4.1% in the parental cell line (Fig. 2 e). The stable expression of Numblike was accompanied by somewhat elevated levels of the myogenic differentiation factor MyoD and a robust induction of myogenin during differentiation (Fig. 2 f). At high levels of Notch signaling (i.e., in C2C12/3T3-J1 and C2C12-N1/3T3-J1 cocultures), we found no MHC-positive cells even after the transfection of Numb (Fig. 2 c). Unexpectedly, Numb-expressing cells were also rare in C2C12/3T3-J1 and C2C12-N1/3T3-J1 cocultures (~1% as compared with C2C12/3T3 and C2C12-N1/3T3; Fig. 2 c). The combination of Numb expression and elevated Notch signaling did not increase...
cell death (Fig. S2 b, available at http://www.jcb.org/cgi/content/full/jcb.200602009/DC1). In summary, this indicates that Numb can override the differentiation-inhibiting effects of Notch at lower levels of Notch signaling and promote myogenic differentiation, but, at higher Notch levels, differentiation is blocked, and there is a strong decrease in Numb expression.

**High levels of Notch 1 activation reduce Numb protein levels**

We examined the apparent loss of Numb expression at high Notch levels in a C2C12 cell line stably expressing HA-tagged Numb (Numb-HA), which was cocultured with 3T3 or 3T3-J1 cells. The Numb protein level was markedly reduced in 3T3-J1 cocultures but not in 3T3 cocultures or in a mixed lysate of Numb-HA and 3T3 or 3T3-J1 cells cultured separately (Fig. 3 a). This effect was dependent on Notch signaling, as addition of the GSI L-685,458 to cocultures of Numb-HA and 3T3-J1 cells blocked Numb down-regulation (Fig. 3 b). In contrast, Numb mRNA levels were not reduced (Fig. S2 c). Transfection of Notch 1 ΔE led to a similar down-regulation of Numb in the cell line stably expressing Numb-HA (Fig. 3 c). To learn whether down-regulation required the activation of Hes 1 and Hey 1, we transiently expressed Notch 1 ICD, Hes 1, or Hey 1 from adenoviral vectors in the stable Numb-HA (Fig. 3 d) and Nbl-HA (Fig. 3 e) cell lines. Robust down-regulation of Numb was observed by Notch 1 ICD but to a much lesser extent by the canonical Notch downstream genes Hes 1 or Hey 1 (Fig. 3, d and e).

**Importance of the PEST domain for Notch-mediated down-regulation of Numblike**

To further investigate the Notch-mediated down-regulation of Numb and Numblike, we speculated that the PEST domain may be of importance, as PEST domains have been shown to be involved in protein turnover and have been implicated in proteasome-mediated degradation (Reverte et al., 2001). The Numb protein contains two PEST domains, and Numblike contains only one, and, as Numblike in all assays behaved similarly to Numb, we generated a Numblike construct lacking the PEST domain (amino acids 260–273; Fig. 4 a) and produced stable cell lines with HA-tagged Numblike (Fig. 3 e) or PEST-deficient Numblike (Nbl-HA and Nbl-HA–ΔPEST, respectively). Like Numb, Nbl-HA protein was reduced by high levels of Notch signaling through coculture with Jagged-1–expressing cells, whereas the mixing of Nbl-HA and Jagged-1 cells immediately
before lysis did not reduce Nbl-HA levels (Fig. 4 b). In contrast, Nbl-HA–∆PEST protein levels did not change after coculture (Fig. 4 b). As for Numb, L-685,458 blocked the 3T3-J1 coculture–induced reduction on Nbl-HA protein levels, whereas Nbl-HA–∆PEST protein levels were unaffected (Fig. 4 b). No substantial differences in the levels of Nbl-HA or Nbl-HA–∆PEST mRNA were observed (Fig. S2, d and e), nor was mouse Numb mRNA expression increased by the expression of Notch 1 ICD (Fig. S2 f). Nbl-HA–∆PEST was more efficient than Nbl-HA in negatively regulating Notch signaling as measured by 12XCSL-luc activation (Fig. S3 a, available

Figure 4. Notch-induced reduction of Numblike protein levels depends on the PEST domain. (a) A schematic representation of the mouse Numb and Numblike (Nbl) proteins (with the amino acid positions for the phosphotyrosine-binding [PTB] and proline-rich region [PRR] domains [gray] and PEST domains [black] depicted) as well as Numblike–∆PEST (Nbl∆PEST), in which the 13–amino acid–long PEST domain has been removed. (b) Cells stably expressing Numblike-HA (Nbl-HA; left) or Nbl-HA–∆PEST (right) were cocultured with 3T3 cells or with Jagged-1–expressing cells (3T3-J1) as indicated (coculture) in the presence or absence of L-685,458 (GSI) or were only mixed with 3T3 or 3T3-J1 cells just before lysis (mixed). Western blots of cell extracts were stained with an anti-HA antibody. The presence of two bands in the Western blot probably reflects the partial degradation of Numblike, and a similar doublet of bands has previously been observed for Numb (Pece et al., 2004). Loading controls (β-actin) are shown below. (c) The stable Nbl-HA and Nbl-HA–∆PEST cell lines were pulsed with [35S]methionine for 1 h at 16 h after coculture with Jagged-1–expressing cells and chased for 0 or 4 h as indicated. Note that Nbl-HA but not Nbl-HA–∆PEST levels are reduced after the 4-h chase. (d) Transfection of myc-tagged Notch 1 ICD (N1ICD) into cell lines stably expressing Nbl-HA or Nbl-HA–∆PEST in the presence or absence of the proteasome inhibitor MG132. Western blots of cell extracts were incubated with an anti-HA antibody and reprobed with an anti-myc antibody to control for Notch 1 ICD expression.
at http://www.jcb.org/cgi/content/full/jcb.200602009/DC1). This is in keeping with the increased stability of Nbl-HA–∆PEST and its resistance to degradation by Notch.

To address whether the down-regulation of Nbl-HA was a result of increased protein turnover or reduced synthesis, we performed pulse-chase experiments in the stable cell lines. After a 1-h [35S]methionine pulse 16 h after coculture with Jagged-1–expressing cells, there was no difference in the synthesis rate between Nbl-HA and Nbl-HA–∆PEST chase, but, after 4 h of chase, considerably less Nbl-HA was observed as compared with Nbl-HA–∆PEST (Fig. 4 c). This suggests that degradation rather than the synthesis rate is affected in the Notch-mediated down-regulation of Nbl-HA. Addition of the proteasome inhibitor MG132 abrogated the Notch 1 ICD–mediated down-regulation of Nbl-HA and, in fact, increased levels to more than what was observed in the absence of Notch signaling (Fig. 4 d, compare the first and third lanes). No effect of MG132 was observed with Nbl-HA–∆PEST (Fig. 4 d).

In conclusion, these experiments argue that the PEST domain is important for the Notch-mediated reduction of Numblike protein levels and that Numblike protein degradation is proteasome dependent.

The negative regulation of Numb and Numblike by Notch demonstrated in this study may play a role in stabilizing the cell fate switch by an asymmetric cell division, which generates two distinct cells: one daughter cell that receives high levels of Numb and, therefore, down-regulates Notch signaling and a second daughter cell with no or low Numb and continued Notch signaling (for review see Cayouette and Raff, 2002). In Fig. 5 a, we propose that in the latter cell, down-regulation of Numb/Numblike by Notch may assure that Numb levels are kept low and, thus, safeguard the outcome of the cell fate switch resulting from Numb segregation. Such a mechanism would reduce the requirements on the asymmetric segregation machinery to perfectly distribute Numb to only one daughter cell, as small amounts of Numb segregating to the Notch-signaling cell would be eliminated. It may also be particularly important to reduce Numblike protein levels in this cell, as Numblike appears not to be asymmetrically localized (Zhong et al., 1997).

To test the model, we wanted to learn whether the level of Notch signaling was inversely correlated with Numb levels in a tumor cell line because a correlation between reduced Numb protein levels and elevated Notch expression is frequently found in breast tumors (Pece et al., 2004; Stylianou et al., 2006). The human ovarian carcinoma cell line SKOV-3 was selected for analysis because it contains detectable levels of both Notch 1 ICD and Numb (Fig. 5 b). Treatment with L-685,458 resulted in reduced levels of Notch 1 ICD and, importantly, elevated levels of endogenous Numb (Fig. 5 b), suggesting that a reduction of Notch activity leads to enhanced Numb levels. Treatment with L-685,458 also reduced the proliferative rate in the SKOV-3 cells (Fig. 5 c). To test the converse situation (i.e., the experimental elevation of Notch), we introduced Notch 1 ICD by in ovo electroporation into the developing chick central nervous system. Areas of Notch 1 ICD overexpression showed reduced levels of Numb protein (Fig. 5 d, arrowhead), whereas areas of low Notch ICD expression contained higher levels of Numb (Fig. 5 d, arrow). In conclusion, these data support the idea that Notch-mediated down-regulation of Numb can be observed in vivo.

**Materials and methods**

**Cell culture, transfection, and reporter gene analysis**

3T3, 293T, and C2C12 cells were grown in DME containing 10% FCS. Transfections were performed using LipofectAMINE Plus reagent (Invitrogen) or FuGene 6 reagent (Roche) according to the manufacturer’s instructions.
The luciferase assay is described in the supplemental material (available at http://www.jcb.org/cgi/content/full/jcb.200602009/DC1). High-titer stocks of adenoviruses for Notch1 ICD, Hes1, and Hey1 were used for infection of Numb-HA or Nbl-HA stable C2C12 cells, and, in all cases, at least 70% of the cells expressed the protein 24 h after infection with minimal cell toxicity.

**Coculture and C2C12 cell differentiation**
C2C12 cells grown on 10-cm plates were transfected with 4 μg DNA using LipofectAMINE Plus reagent. For coculture experiments, transfected C2C12 cells were seeded with approximately the same number of 3T3 or 3T3-J1 cells and grown for 24–48 h in DME containing 10% FCS. Equivalent amounts of 3T3 or 3T3-J1 cells were added several hours later. Culture medium was changed the following day to DME containing 2% horse serum. Cultures were fixed 3 d later in 4% PFA and subjected to immunocytochemistry.

**Western blotting and immunoprecipitation**
Cultures were washed in PBS, harvested, resuspended in 50–200 μl whole cell extraction buffer (20 mM Heps, pH 7.8, 420 mM NaCl, 0.5% NP-40, 25% glycerol, 0.2 mM EDTA, 1.5 mM MgCl2, 1 mM DTT, 1 mM PMSF, and complete protease inhibitors [Roche]), and incubated on an end-to-end rotator for 30 min at 4°C. The lysate was centrifuged for 30 min at 12,000 rpm, and protein concentration in the supernatant was determined by Bradford analysis (Bio-Rad Laboratories). Western blotting was performed as described in the supplemental material.

**RNA isolation and quantitative RT-PCR**
RNA was isolated from cultured cells using the RNeasy Mini Kit (QiAGEN). Reverse transcription was performed on 2.5 μg of total RNA using oligo- dT12–18 and SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was performed in accordance with the manufacturer’s instructions using a rapid thermal cycler system (LightCycler; Applied Biosystems). A masternix containing nucleotides, Taq polymerase, SYBR green, and buffer was mixed with primers and cDNA.

**Pulse chase**
C2C12 cells stably expressing Nbl-HA or Nbl-HA–ΔPEST were cocultured with 3T3-J1 or 3T3 cells. At 16 h of coculture, the growth medium was replaced by serum-free DME without methionine and cysteine containing 30 μCi [35S]methionine. After a 1-h pulse, the cells were either harvested (0 h) or washed, and the medium was replaced with normal growth medium for a 4-h chase. Cells were lysed in radiomunoprecipitation buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0, supplemented with protease inhibitors [Complete; Roche]). Cell lysates were centrifuged 14,000 g for 20 min at 4°C for the removal of insoluble material followed by preclearing with Sepharose G beads. HA-tagged Nbl or Nbl–ΔPEST were immunoprecipitated using a monoclonal HA antibody (HA11; BioSite) and captured by Sepharose G beads. The immunoprecipitated proteins were eluted by Laemmli sample buffer and separated by SDS-polyacrylamide gel electrophoresis.

**Online supplemental material**
Fig. S1 shows that Numb and Numblike down-regulate Notch signaling. Fig. S2 shows the detection of Numb protein and the role of Notch localizations of a mammalian numb homolog during mouse cortical neurogenesis. (Neuron) modulates neuronal differentiation by binding to NOTCH-1. (Nature) requires notch signaling to maintain the undifferentiated cell state. (Dev. Cell) 9:617–628.

**References**


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