Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis

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The biological antagonism between Notch and Numb controls the proliferative/differentiative balance in development and homeostasis. Although altered Notch signaling has been linked to human diseases, including cancer, evidence for a substantial involvement of Notch in human tumors has remained elusive. Here, we show that Numb-mediated control on Notch signaling is lost in ~50% of human mammary carcinomas, due to specific Numb ubiquitination and proteasomal degradation. Mechanistically, Numb operates as an oncosuppressor, as its ectopic expression in Numb-negative, but not in Numb-positive, tumor cells inhibits proliferation. Increased Notch signaling is observed in Numb-negative tumors, but reverts to basal levels after enforced expression of Numb. Conversely, Numb silencing increases Notch signaling in normal breast cells and in Numb-positive breast tumors. Finally, growth suppression of Numb-negative, but not Numb-positive, breast tumors can be achieved by pharmacological inhibition of Notch. Thus, the Numb/Notch biological antagonism is relevant to the homeostasis of the normal mammary parenchyma and its subversion contributes to human mammary carcinogenesis.

Introduction

Notch is a plasma membrane receptor involved in the control of cell fate specification and in the maintenance of the proliferative/differentiative balance in many cell lineages (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000), and alterations in Notch signaling have been implicated in tumorigenesis (Robbins et al., 1992; Gallahan et al., 1996; Capobianco et al., 1997). However, with the exception of a rare translocation in T cell malignancies (Ellisen et al., 1991), no genetic lesion of the *Notch* locus has been described in human tumors. Increased Notch signaling profoundly impairs normal mammary gland morphogenesis in mice, promoting the rapid development of poorly differentiated adenocarcinomas (Jhappan et al., 1992; Gallahan et al., 1996). This finding raises the possibility that subversion of Notch signaling might play a role in human breast tumors; yet little direct evidence for such an involvement has been provided so far (Weijzen et al., 2002). Thus, one major question is whether and how *Notch* is altered in human breast tumors, and how deregulation of its signaling might contribute to the neoplastic phenotype.

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Abbreviations used in this paper: ICD, intracellular domain; LOH, loss of heterozygosity.

During asymmetric cell division in embryogenesis, the activity of Notch is biologically antagonized by the cell fate determinant Numb (Rhyu et al., 1994; Guo et al., 1996). However, Numb is also expressed in many adult mammalian cells (Santolini et al., 2000), where it does not display asymmetrical partitioning at mitosis, suggesting additional functions. We reasoned that if the Numb/Notch antagonism is relevant to control of the proliferative/differentiative balance in the normal mammary parenchyma, then subversion of the Numb-mediated regulation of Notch might play a causative role in naturally occurring breast cancers. The present work was undertaken to test this possibility.

Results and discussion

A preliminary survey of the expression of Numb in normal and tumor tissues of different origins (unpublished data) revealed frequent alterations in breast tumors. Thus, we characterized 321 consecutive breast cancers by immunohistochemistry (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200406140/DC1). The normal breast parenchyma invariably showed intense and homogeneous Numb staining (Fig. 1 A). Conversely, tumors displayed marked heterogeneity and in many cases complete absence of Numb immunoreactivity, which allowed their classification into three classes (Fig. 1 A).

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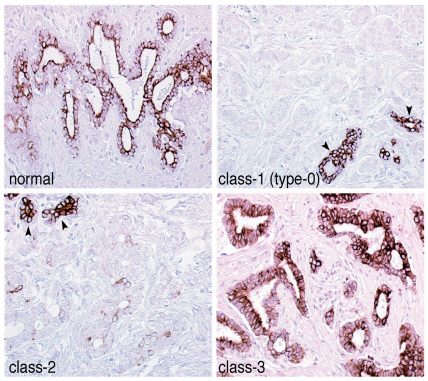
A



B

	N	NUMB class1(%)	class2(%)	class3(%)	p-value
All subjects	321	38.3	16.8	44.9	
Grade1	70	11.4	17.1	71.4	
Grade2	124	44.4	17.7	37.9	
Grade3	127	47.2	15.7	37.0	<.0001
Ki-67 < 22%	158	26.6	15.8	57.6	
Ki-67 ≥ 22%	163	49.7	17.8	32.5	<.0001
LN - Negative	231	39.4	16.9	43.7	
LN - Positive	90	35.6	16.7	47.8	0.492

C

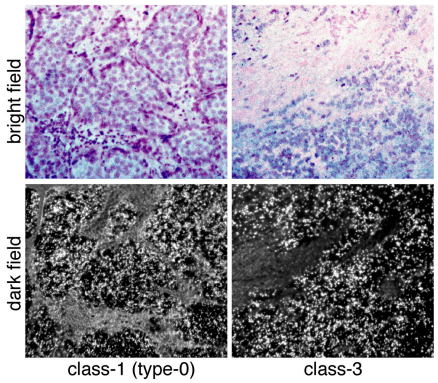


Figure 1. **Numb expression in human mammary tumors.** (A) The typical immunoreactivity for Numb in normal breast (normal) showed intense staining in the vast majority of ductal (luminal) and lobular epithelial cells, with a prominent membranous staining pattern. Examples are shown of typical class-1(type-0), class-2, and class-3 tumors. Arrowheads point to normal glands within the context of the tumors. (B) Correlation between Numb status and clinical-pathological features. Details and explanations are in Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200406140/DC1. Ki67, proliferative index; LN, lymph nodes. P value was obtained using the Mantel-Haenszel Chi square statistics. (C) In situ hybridization with an antisense probe for Numb mRNA was performed on paraffin sections. Control hybridizations with a corresponding sense probe gave no signal (not depicted). Examples of matching bright fields (top) and dark fields (bottom) of class-1 (left) and class-3 (right) tumors are shown. *Numb* transcripts appear as bright spots in the dark fields (bottom).

Class-1 (38.3% of the cases) tumors showed Numb staining in <10% of the neoplastic cells. Within this category, in fact, more than 50% of class-1 tumors displayed no detectable Numb immunoreactivity (type-0 tumors), whereas class-2 and -3 tumors (16.8% and 44.9% of investigated cases, respectively) showed Numb immunoreactivity in 10–50% and >50% of the tumor cells, respectively. Thus, more than one half of all breast tumors (classes 1 and 2 combined) had reduced levels of Numb. Remarkably, a strong inverse correlation was found between Numb expression levels and tumor grade ($P = 0.001$) and Ki67 labeling index ($P = 0.001$), which are known indicators of aggressive disease (Fig. 1 B and Table S1).

We analyzed the presence of *Numb* transcripts in human mammary tumors by in situ hybridization. Five class-3 tumors and 14 class-1(type-0) tumors were analyzed. All of the class-3 tumors (and normal glands surrounding the tumors) displayed readily detectable levels of *Numb* transcripts (Fig. 1 C). Interestingly, 12 of 14 class-1(type-0) tumors displayed levels of *Numb* mRNA expression comparable to those detected in normal tissues and class-3 tumors (Fig. 1 C). In addition, we could not detect any genetic alteration affecting the *Numb* locus by analysis of loss of heterozygosity (LOH) or by direct sequencing of *Numb* cDNAs prepared from several Numb-negative tumors (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200406140/DC1). Thus, genetic alterations at the *Numb* locus are unlikely to account for the absence of Numb protein in these tumors.

To gain insight into the molecular mechanisms responsible for loss of Numb expression, we established primary cultures from class-1(type-0) and class-3 mammary tumors and from normal breast tissues from the same patients, and analyzed them within the first two passages in vitro (Fig. S1 B). All primary cultures from normal breast and tumor cultures from class-3 patients displayed high levels of Numb expression, which were only marginally affected by treatment with the proteasome inhibitor MG132 (Fig. 2, A and B). In contrast, primary cultures from class-1(type-0) patients displayed little, if any, basal Numb expression. However, Numb levels were restored to high levels by treatment with MG132 (Fig. 2, A and B). Reduction of Numb levels in class-1 tumors did not appear to be the consequence of a generally increased proteasomal activity, as the basal levels of other cellular proteins also regulated by proteasomal degradation, such as β-catenin, were not affected under the same experimental conditions (Fig. 2 C).

It has been proposed that ubiquitination followed by proteasomal degradation represents a major mechanism for cellular regulation of Numb (Juven-Gershon et al., 1998; Susini et al., 2001; Nie et al., 2002); thus, we investigated the pattern of Numb ubiquitination in tumor cells. As shown in Fig. 2 D, the restoration of Numb levels in class-1 tumor cells by treatment with MG-132 for 6 h was accompanied by a dramatic increase in Numb polyubiquitination, whereas no effect of MG132 on Numb polyubiquitination was evident in class-3 tumors. Together, these results provide compelling evidence that enhanced ubiquitination and consequent increased proteasome-mediated degradation accounts for the loss of Numb expression in a substantial proportion of mammary tumors.

The possibility that enhanced degradation of Numb is causally involved in the progression of breast cells toward malignancy prompted us to test the consequences of restoration of Numb levels in primary tumor cells. Retrovirally mediated transient overexpression of a fusion Numb-GFP protein in primary cells from class-1 tumors resulted in a clear reduction in colony-forming ability (Fig. 3 A). Conversely, class-3 tumor cells were unaffected, despite similar levels of Numb-GFP expression upon transient infection (Fig. 3 B).

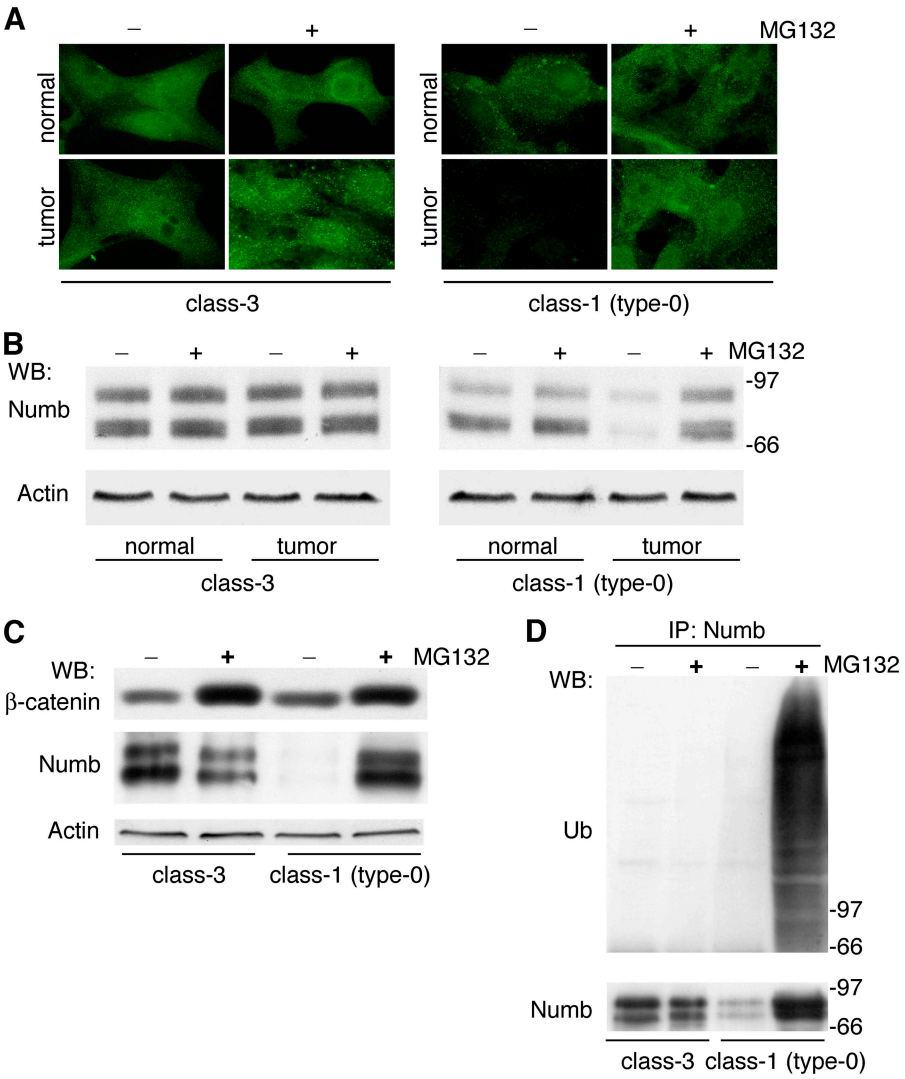


Figure 2. **Loss of Numb expression in tumors is due to enhanced ubiquitination and proteasomal degradation.** (A) Matched normal (top) and tumor (bottom) primary cells from class-1 (right) and class-3 (left) patients were treated with MG132 (+) for 12 h or mock treated (−) and stained with anti-Numb. (B) Total cellular lysates from the same cells as in A were immunoblotted with anti-Numb (top). Molecular mass is indicated in kilodaltons on the right. Typically, two Numb-specific bands (each probably corresponding to a tightly spaced doublet) are detected in human mammary cells. Equal loading was checked with anti-actin (bottom). (C) Primary tumor mammary cells were either mock treated (−) or exposed to MG132 (+) for 12 h. Lysates were immunoblotted (WB) with the indicated antibodies. (D) Tumor mammary cells from class-1 and class-3 patients were either mock treated (−) or exposed to MG132 (+) for 6 h. Lysates were immunoprecipitated (IP) with a monoclonal anti-Numb antibody and immunoblotted (WB) with the indicated antibodies. Molecular mass is indicated in kilodaltons on the right. Results in all panels are representative of three independent experiments. In addition, similar results were obtained with primary cultures from three class-1(type-0) and three class-3 patients (not depicted).

Overall, the sum of our results points to a direct link between increased degradation of Numb and uncontrolled cell proliferation in a subset of human mammary gland tumors. The well established biological antagonism between Numb and Notch allowed us to test the hypothesis that aberrant regulation of Notch signaling in human breast tumors is due to a deficiency of Numb activity. Lack of Numb in tumor cells would be expected to be reflected in Notch activity. Notch is activated through a series of proteolytic cleavages ultimately leading to the release of its soluble intracellular domain (ICD) from the plasma membrane (Schroeter et al., 1998; Struhl and Adachi, 2000). The ICD is translocated to the nucleus, where it regulates gene transcription by interacting with the DNA binding-protein RBP-*J*k/CBF-1 (Jarriault et al., 1998). The biochemical mechanisms through which Numb antagonizes Notch are not yet clear; however, one leading hypothesis is that direct binding of Numb to Notch prevents nuclear translocation and hence transcriptional activity of the ICD (Frise et al., 1996; Guo et al., 1996; Zhong et al., 1996).

Therefore, we used primary tumor cells to monitor the subcellular distribution and activation state of Notch. In class-1(type-0), Notch immunostaining appeared lower than in class-3 tumors or normal cells (Fig. 4 A). We reasoned that this finding might be consistent with increased processing of Notch at the plasma membrane followed by increased nuclear translocation of the ICD into the nucleus, whereby it is promptly degraded by the nuclear proteasomal machinery. Indeed, even brief (1 h) MG132 treatment revealed nuclear accumulation of Notch (ICD) in all class-1(type-0) cells, but not in class-3 tumors or normal counterparts (Fig. 4 A). We measured Notch function by following luciferase activity driven from a Notch-dependent CBF1-responsive reporter (6x-RBP-*J*k-luc) transfected into primary cells. Luciferase activity was similar among class-3 and normal cultures but clearly increased in all class-1 cultures (Fig. 4 B). Furthermore, by using quantitative RT-PCR, we found that endogenous expression of the *HES-1* mRNA, a known target gene for Notch transcriptional activity (Sasai et al., 1992; Jarriault et al., 1998), was significantly higher in class-1 tumor cells than in class-3 cells or their normal counterparts (Fig. 4 C).

These results prompted us to assess directly a possible functional link between Numb levels and Notch activity in tumor cells. RNAi-mediated silencing of Numb in primary normal breast cells resulted in a significant increase in *HES-1* mRNA transcripts in comparison to cells transfected with a

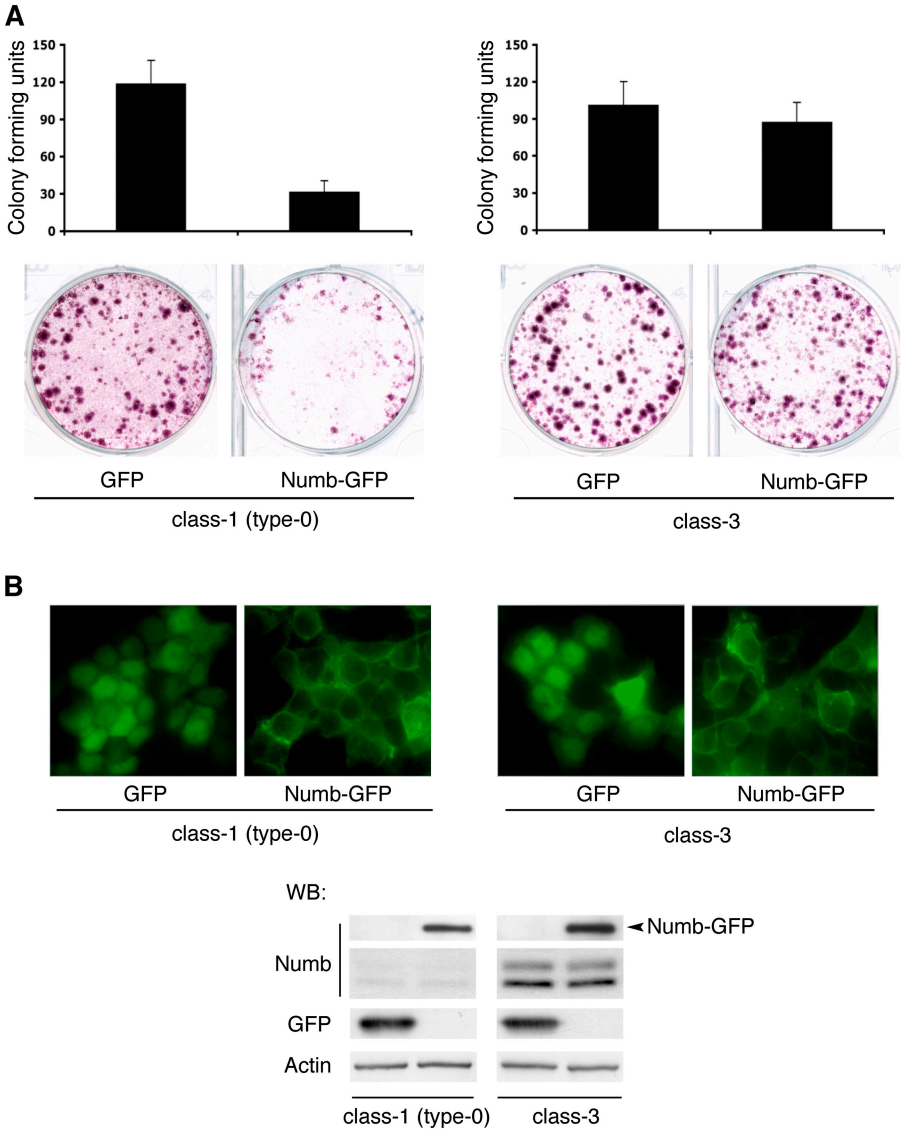


Figure 3. **Re-expression of Numb selectively suppresses growth in Numb-negative tumors.** (A) Primary tumor cells from class-1(type-0) (left) and class-3 (right) patients were transduced with retroviruses encoding GFP or Numb-GFP. After 3 wk, plates were fixed and stained (bottom). The histograms show the average number of colonies (colony-forming units ± SD) from three plates. Similar results were obtained with primary cultures from three independent class-1(type-0) and three independent class-3 patients (not depicted). (B) The expression of GFP and Numb-GFP, as detected by epifluorescence (top) or immunoblot (bottom), upon transient retroviral delivery is shown to demonstrate equal efficiency of infection/expression.

control siRNA (Fig. 5 A). As expected, a similar increase in Notch-dependent transcriptional activity was observed in class-3 tumor cells, but not in class-1 tumor cells (Fig. 5 A). Accordingly, retrovirally mediated overexpression of Numb caused a significant decrease in basal Notch activity in class-1, but not in class-3, tumor cells (Fig. 5 B) or in normal cells from the same patients (not depicted). As overexpression of Numb in class-1 tumor cells also caused a significant growth-suppression effect (Fig. 3), we directly tested the possibility that deregulated Notch activity arising from loss of Numb might be responsible for uncontrolled cell proliferation in class-1 tumors. We took advantage of the small molecule peptidomimetic presenilin inhibitor DFP-AA, which blocks Notch signaling and effectively suppresses the growth of Notch1-transformed lymphoid cell lines in vitro (Weng et al., 2003). DFP-AA treatment of class-1 tumor cells was sufficient to cause a dramatic suppression of their growth potential (Fig. 5 C), which was accompanied by a marked decrease in Notch activity, as assessed by *HES-1* mRNA levels (Fig. 5 D). In contrast, no significant effect was observed in class-3 tumor cells (Fig. 5, C and D).

Using breast tumor specimens and primary cells derived from them, this work has established that deregulation of Numb-mediated control on Notch signaling is a frequent occurrence in human breast cancer. Indeed, loss of Numb expression leads to activation of Notch, which in turn is responsible for increased proliferation of tumor cells. Accordingly, restoration of physiological Numb levels or inhibition of Notch activity attenuated the hyperproliferative state of Numb-negative tumors. Importantly, Numb-positive tumors were unaffected by these manipulations. Thus, *Numb* and *Notch* fulfill the operational definitions of oncosuppressor and oncogene, respectively, in human breast cancers.

Loss of Numb expression in breast cancers is due to its increased ubiquitination and resultant proteasomal degradation, raising the issue of the genetic lesion responsible for excessive Numb ubiquitination. Consistent with findings that Numb levels are regulated by E3-ligases, such as LNX, Siah-1, and Mdm2 (Juven-Gershon et al., 1998; Susini et al., 2001; Nie et al., 2002), an obvious possibility is that there are increased levels or activity of an E3-type ubiquitin ligase. In this context, it would be interesting to test in our ex vivo model the effects of a new class of

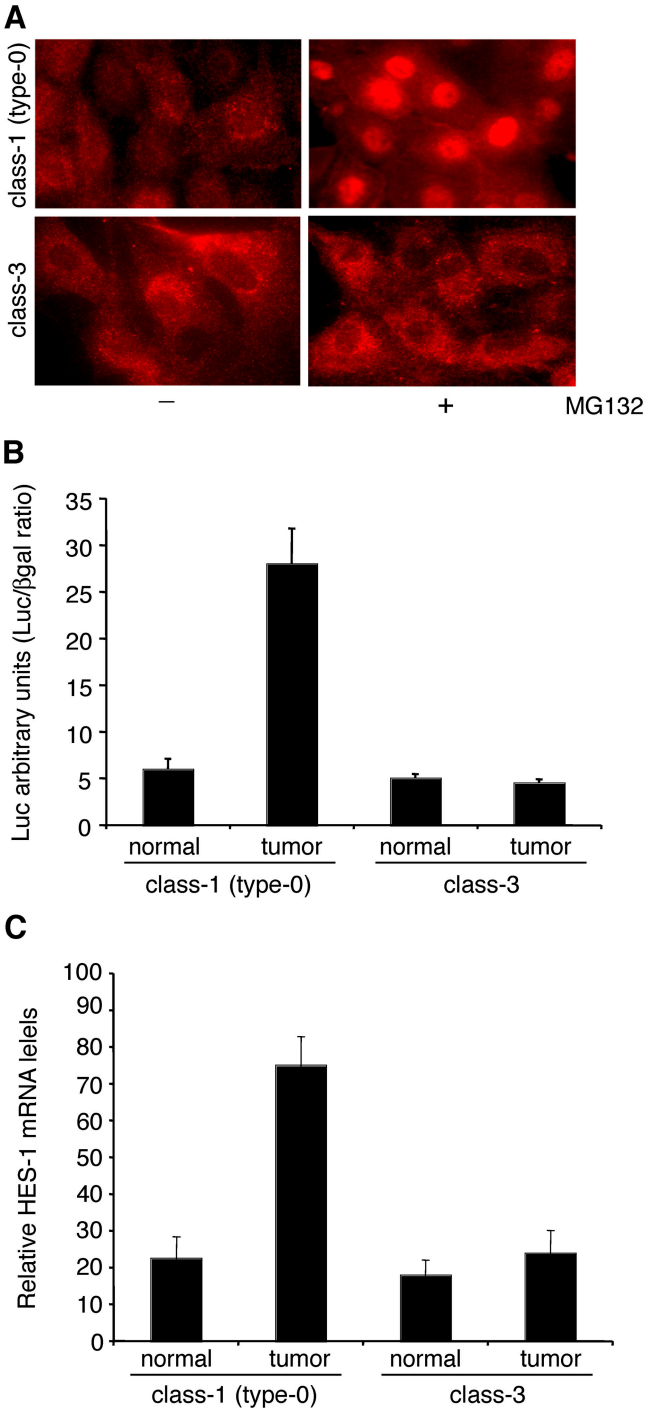


Figure 4. **Increased Notch signaling in Numb-negative tumors.** (A) Primary tumor cells from class-1(type-0) (top) and class-3 (bottom) patients were treated with MG132 (+) or mock treated (−) for 1 h and stained with anti-Notch. Note the lower basal levels of Notch expression in class-1 MG132-untreated cells and the presence of nuclear Notch in the same class upon MG132 treatment. (B) CBF1-responsive reporter gene activity was evaluated in normal and tumor cells from class-1(type-0) and class-3 patients. (C) *HES-1* mRNA expression in total RNAs from normal and tumor cells from class-1(type-0) and class-3 patients. In B and C, the mean fold induction (± SD) from two independent experiments performed in triplicate is shown. In all panels, results are representative of those obtained with primary cultures from three class-1(type-0) and three class-3 patients (not depicted).

cytotoxic agents with antiproteasomal activity, exemplified by the PS-341 compound, currently under clinical evaluation for advanced cancers (Adams et al., 1999). Alternatively, increased Numb phosphorylation might cause its ubiquitination and degradation, as a strong correlation between these two posttranslational modifications has been shown for other proteins (Pickart, 2001). Of note, in *Drosophila melanogaster*, a serine/threonine kinase, NAK, physically interacts with Numb and causes loss-of-Numb-function phenotypes upon overexpression (Chien et al., 1998). Under this scenario, the primary lesion would affect a serine/threonine kinase (Numb is serine/threonine phosphorylated), rather than an E3-ligase. Whatever the case, restoration of Numb function might in principle be obtained by pharmacological inhibition of the enzyme(s) responsible for its degradation: a therapeutic possibility with obvious appeal.

Materials and methods

Tissue samples and primary cells

Mammary tissue specimens were obtained from breast cancer patients undergoing surgery. Pure populations of epithelial cells were obtained as described previously (Hammond et al., 1984; Speirs et al., 1998). The epithelial origin of the cultures was confirmed by immunofluorescence with an anti-Pan cytokeratin antibody (Sigma-Aldrich). Second-passage cells, practically free of nonepithelial cells, were used for all described experiments (Fig. S1 B). MG132 was used at 10 μM. 1 μM DFP-AA (compound E; Calbiochem) was added in fresh medium every 24 h for 10 d. Mock-treated controls were exposed to equivalent concentrations of carrier (0.05% dimethyl sulfoxide).

Expression vectors and biological assays

A Numb-GFP expression vector was obtained by recombinant PCR and subcloning in a retroviral (Pinco) vector, followed by sequence verification. Primary cells (50,000 per well) were infected in 6-well plates with supernatants from ΦNX (Phoenix cells) transfected with Pinco-Numb-GFP or with Pinco-GFP every 3 d for 3 wk.

A luciferase reporter plasmid (6x-RBP-Jk-luc, provided by U. Lendahl, Karolinska Institute, Stockholm, Sweden) containing six copies of the CBF1 binding consensus sequence was used to evaluate Notch signaling. Primary mammary cells were transfected in 6-well plates with 800 ng of 6x-RBP-Jk-luc and 200 ng of CMV-β-galactosidase expression plasmids. Luciferase activity was evaluated 48 h after transfection and normalized for transfection efficiency by β-galactosidase expression.

Protein studies and siRNA

An affinity-purified anti-Numb peptide antibody was used for immunoblotting, immunofluorescence, and immunocytochemistry (Santolini et al., 2000), according to standard procedures.

To assess its ubiquitination state in tumors in vivo, Numb was immunoprecipitated with a mouse mAb raised against exon-10 of human Numb and not cross-reactive with the *Numb*-related gene Numbl, followed by immunoblot with the anti-Ub antibody FK1 (Affiniti Research Product). Other antibodies used were as follows: anti-Notch1 (c-20; Santa Cruz Biotechnology. Inc.), HRP-conjugated secondary antibodies (Amersham Biosciences), and Cy3- and FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). For siRNA experiments, delivery of siRNA oligos was achieved using Oligofectamine. The targeted sequences were as follows: Numb siRNA, CAGCCACUGAACAAGCAGA; scrambled siRNA, AGACGAACAAGUCACCGAC.

In situ hybridization

Numb expression was assessed by in situ hybridization using ^{35}S-UTP–labeled sense and antisense riboprobes. After overnight hybridization at 50°C, tissue sections were washed in 50% formamide, 2× SSC, and 20 mM 2-mercaptoethanol at 60°C and coated with Kodak NTB-2 liquid emulsion to reveal radiolabeling. Sequences of the probes are available upon request.

Quantitative RT-PCR

Quantitative RT-PCR analysis was performed on the Prism 7700 Sequence Detection System (Applied Biosystems). Each cDNA sample was tested in triplicate. For quantification of gene expression changes, the ΔΔCt

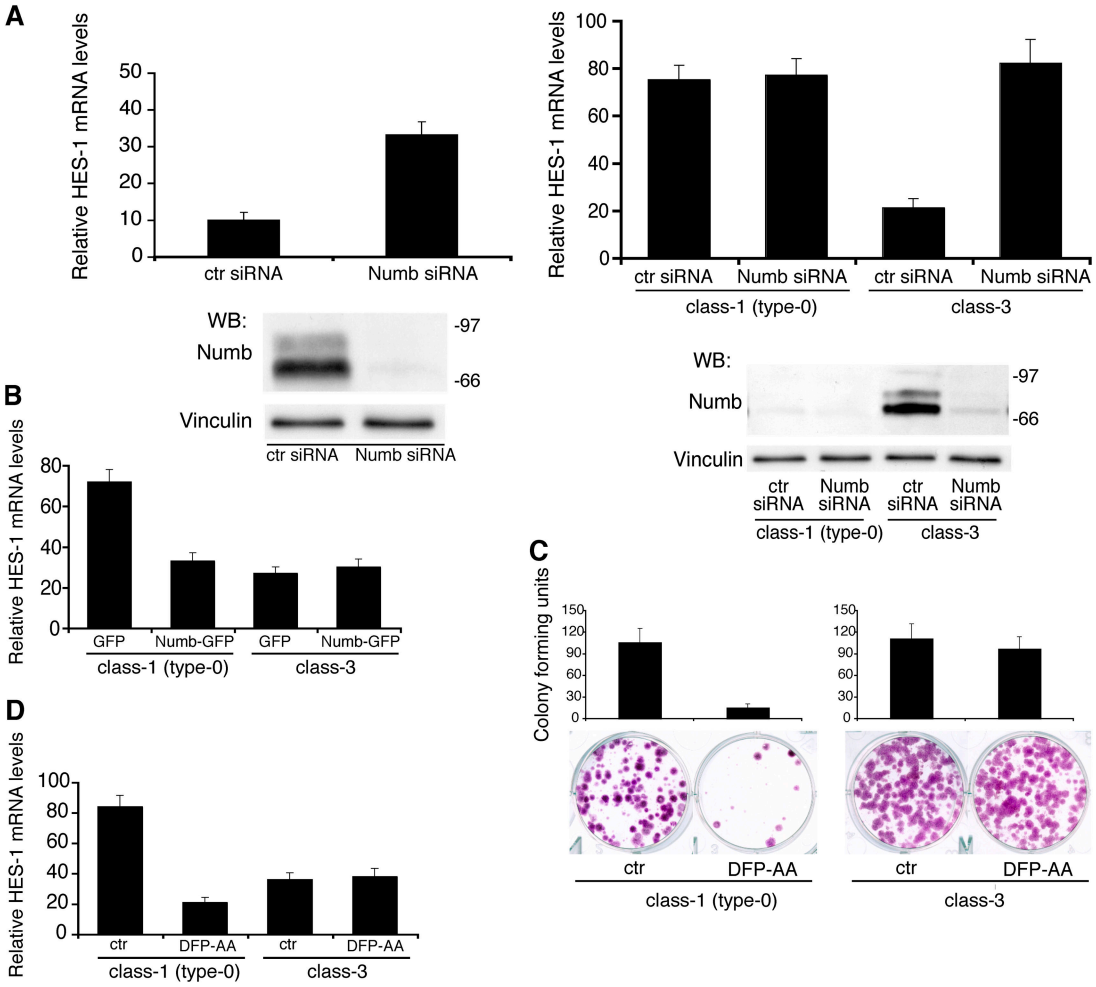


Figure 5. **Effects of perturbation of Numb or Notch activity in mammary tumor primary cells.** (A) Primary normal (left) or tumor (right) cells were transfected with siRNA oligos for Numb or control (ctr) oligos for 72 h and assayed for *HES-1* mRNA levels (histograms) or immunoblotted with the indicated antibody (WB). (B) Primary tumor cells from class-1(type-0) and class-3 patients were transduced with GFP or Numb-GFP and assayed for *HES-1* mRNA levels 72 h after infection (protein expression controls are as from Fig. 3 B, not depicted here). Normal primary cells from the same patients behaved as class-3 tumors (not depicted). In A and B, the mean fold induction (± SD) from two independent experiments performed in triplicate is shown. (C) Primary tumor cells from class-1(type-0) (left) and class-3 (right) patients were treated with DFP-AA for 10 d or mock treated (ctr), followed by staining (bottom) to count colonies. The histograms show the average colonies (colony-forming units ± SD) in triplicate plates. Results are representative of three independent experiments. (D) *HES-1* mRNA expression from cells treated as in C, the mean fold induction (± SD) from two independent experiments performed in triplicate is shown. In all panels, results are representative of those obtained with primary cultures from three class-1(type-0) and three class-3 patients (not depicted).

method was used to calculate relative fold changes normalized against the *glyceraldehyde 3-phosphate dehydrogenase* gene, as described in the manufacturer's protocol (Applied Biosystems).

Microscopic analysis and image acquisition

Immunofluorescence specimens, mounted in 90% glycerol with paraphenylenediamine as an antibleaching agent, were examined at RT using an epifluorescence microscope (model AX70 Provis; Olympus) with a 60×/1.4 NA PlanAPO oil immersion lens or a 40×/0.85 UplanAPO objective. Images were obtained with a digital camera (model C5985 B/W CCD; Hamamatsu) using an acquisition software (Hamamatsu C5985 Plugin for Adobe Photoshop). Tissue sections were observed with a DMLB microscope (Leica) equipped with a digital color camera (model DXC-950P 3CCD; Sony; TRIBVN ICS software for Windows), using a 10×/0.40 NA or a 20×/0.70 NA plan-APO objective. Digital images were computer processed using Adobe Photoshop 7.

Online supplemental material

Fig. S1 describes in detail procedures used for LOH analysis (A) and characterization of primary cultures (B). The clinical-pathological features of patients and details of immunohistochemical analysis are described in Table S1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200406140/DC1.

This work is dedicated to the beloved memory of Elisa Santolini.

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