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# Article

# NUMB enhances Notch signaling by repressing ubiquitination of NOTCH1 intracellular domain

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**The release and nuclear translocation of the intracellular domain of Notch receptor (NICD) is the prerequisite for Notch signaling-mediated transcriptional activation. NICD is subjected to various posttranslational modifications including ubiquitination. Here, we surprisingly found that NUMB proteins stabilize the intracellular domain of NOTCH1 receptor (N1ICD) by regulating the ubiquitin–proteasome machinery, which is independent of NUMB's role in modulating endocytosis. BAP1, a deubiquitinating enzyme (DUB), was further identified as a positive N1ICD regulator, and NUMB facilitates the association between N1ICD and BAP1 to stabilize N1ICD. Intriguingly, BAP1 stabilizes N1ICD independent of its DUB activity but relying on the BRCA1-inhibiting function. BAP1 strengthens Notch signaling and maintains stem-like properties of cortical neural progenitor cells. Thus, NUMB enhances Notch signaling by regulating the ubiquitinating activity of the BAP1–BRCA1 complex.**

**Keywords:** NUMB, Notch intracellular domain, BAP1, ubiquitination, neural progenitor cells

## Introduction

Diversifying cell fates during animal development requires crosstalk between neighboring cells. The Notch signaling is an evolutionarily conserved signaling pathway that regulates a plethora of developmental and oncogenic processes (Artavanis-Tsakonas et al., 1999; Pierfelice et al., 2011; Aster et al., 2017). The binding of the single-transmembrane Notch receptors with their ligands (Delta and Serrate in *Drosophila*; Delta-like and Jagged in mammals) triggers cleavage of the Notch receptor and release of the Notch intracellular domain (NICD). NICD then translocates into the nucleus to associate with the CSL/RBPJκ transcription complex and subsequently activates transcription of target genes including *HES1* and *HES5* (Kopan and Ilagan, 2009), which maintain the proliferative state (self-renewal) of neural progenitor cells (NPCs) in the developing nervous system (Ishibashi et al., 1995; Imayoshi et al., 2010).

Both Notch receptors and ligands are extensively regulated at the posttranslational level. Notch receptors are highly ubiquitinated, and several E3 ligases and deubiquitinating enzymes (DUBs) have been reported to regulate their turnover and activities (Le Bras et al., 2011; Moretti and Brou, 2013). For instance, the E3 ubiquitin ligase SCF/Sel-10/FBXW7 can ubiquitinate the PEST (rich in proline, glutamic acid, serine, and threonine residues) domain of NICD in the nucleus and promote its degradation (Guptarossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). On the other hand, a few DUBs, namely USP7, USP11, and USP15, were identified to be binding partners of NICD in the nuclei of human T cell acute lymphoblastic leukemia cells (Yatim et al., 2012). Particularly, USP12 collaborates with its activator UAF1 to deubiquitinate the full-length NOTCH1 receptor to regulate its intracellular trafficking and USP12 silencing results in an increased amount of receptor at the cell surface and a higher Notch activity. But it is unclear whether USP12 also regulates NICD's ubiquitination state. Moreover, DUBs screened in the study, including USP12, exert no effects on NICD's transcriptional activities (Moretti et al., 2012).

The cell fate determinant NUMB has been reported to antagonize Notch signaling in *Drosophila* (Guo et al., 1996; Spana and Doe, 1996; Couturier et al., 2012) and in mammalian cells probably via endocytosis- and proteasome-dependent mechanism (McGill and McGlade, 2003; McGill et al., 2009). However, NUMB could specify cell fates of *Drosophila* sensory organ precursor lineage independent of endocytosis or proteasome pathway (Tang et al., 2005). Moreover, gain- and loss-of-function studies in mice indicate that NUMB and Notch might play

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similar but not antagonizing roles in cortical neurogenesis of the brain, i.e. NUMB is also essential for maintenance of the cortical NPC pool (Petersen et al., 2002, 2004; Yang et al., 2004; Zhou et al., 2007). Although NUMB interacts with E3 ubiquitin ligase Itch/AIP4 to promote intracellular trafficking and subsequent degradation of the NOTCH1 receptor, as well as ubiquitination and degradation of NICD (McGill and McGlade, 2003; Pece et al., 2004; McGill et al., 2009), *Itch* knockout mice do not show defects of neural development as found in *Numb* or *Notch* loss (Perry et al., 1998; Fang et al., 2002). Moreover, it remains to be investigated whether NUMB can regulate Notch receptor and/or NICD posttranslationally via DUBs.

Here, we found that NUMB stabilizes NOTCH1 intracellular domain (N1ICD) rather than promotes its degradation. NUMB associates with N1ICD via NUMB very N-terminal motif, but not the region for regulating endocytosis and proteasome. In a DUB screening, BRCA1-associated protein 1 (BAP1) was identified to stabilize N1ICD and associate with both NUMB and N1ICD. Depletion of BAP1 partially reverts the stabilization of N1ICD by NUMB overexpression. Intriguingly, BAP1 stabilizes N1ICD independent of its DUB activity but by inhibiting the E3 ligase activity of BRCA1–BARD1 complex. Finally, BAP1 promotes self-renewal of embryonic neural precursors and inhibits cortical neurogenesis *in vivo*. This study depicted a novel posttranslational regulation of N1ICD mediated by NUMB and might have implication in designing strategies to treat Notch-related disorders.

## Results

*NUMB relies on its very N-terminus to associate with N1ICD*

Although studies in *Drosophila* and in mammals indicate that NUMB may antagonize Notch signaling by facilitating Notch receptor degradation or post-endocytic trafficking, there have been contradictory reports. Moreover, little is known whether NUMB has a role in the posttranslational regulation of N1ICD. To address these issues, we established HeLa cell lines that stably express FLAG-tagged N1ICD (HeLa$^{N1ICD\text{-}FLAG}$), ligands for Notch receptors including MYC-tagged DLL1 or JAG1, and HA-tagged JAG2 under constitutive CMV promoter (Supplementary Figure S1A and B). Immunoblotting assays showed that the protein level of the activated N1ICD (Val1744) in HeLa$^{N1ICD\text{-}FLAG}$ was mostly comparable to those in human cells including 293T, SH-SY5Y, and H4 (Supplementary Figure S1C). The expression of *HES1* or *HES5* in HeLa$^{N1ICD\text{-}FLAG}$ cells did not respond to DAPT, the γ-secretase inhibitor that prevents the release of NICD from the cell membrane (Supplementary Figure S1D), confirming the extracellular signaling-independent regulation by stably expressed N1ICD. Moreover, HeLa$^{N1ICD\text{-}FLAG}$ cells but not parental HeLa cells can drive the expression of 12×CSL-d1EGFP Notch reporter (Supplementary Figure S1E), and the stably expressed N1ICD could associate with *HES1* and *HES5* promoters in HeLa$^{N1ICD\text{-}FLAG}$ cells (Supplementary Figure S1F). Thus, FLAG-tagged N1ICD in HeLa$^{N1ICD\text{-}FLAG}$ cell is biochemically functional and can be regarded as endogenous N1ICD.

We validated the interaction between N1ICD and endogenous NUMB by FLAG-bound immunoprecipitation (IP) (Figure 1A). The NUMB protein contains a phosphotyrosine-binding (PTB) domain for protein scaffolding, as well as the DPF (Asp-Pro-Phe) and NPF (Asn-Pro-Phe) motifs at its carboxyl-terminus for putative roles in mediating endocytosis (Figure 1B). To define the precise region of NUMB for NUMB–N1ICD interaction, we expressed different NUMB truncations in HeLa$^{N1ICD\text{-}FLAG}$ cells. Co-IP assay showed that the NUMB–N1ICD interaction depends on NUMB very N-terminal region, also known as the ACBD3-binding (AB) motif that mediates the interaction with ACBD3 (Zhou et al., 2007) but not the PTB domain or the C-terminal region (Figure 1C). Similarly, we mapped the domain of N1ICD for N1ICD–NUMB interaction, showing that N1ICD RAM–ANK and TAD–PEST domains could pull down endogenous NUMB (Figure 1D), but the TAD domain alone could not. This result is consistent with the previous study indicating *Drosophila* NUMB association with the RAM and PEST domains of NICD (Guo et al., 1996). Nuclear localization of NUMB has been reported in breast cancer cell lines and thymocytes (Dhami et al., 2013; Martinblanco et al., 2014). Immunofluorescent staining and subcellular fractionation showed that endogenous NUMB in HeLa cells is largely localized in the cytosol with a fraction in the nucleus (Figure 1E and F). Together, NUMB associates with N1ICD via its very N-terminal region.

*NUMB stabilizes the intracellular domain of NOTCH1 receptor*

We next asked whether NUMB has a role in regulating N1ICD posttranslationally. Knockdown of NUMB in HeLa and HeLa$^{N1ICD\text{-}FLAG}$ cells significantly decreased levels of endogenous cleaved NOTCH1 and N1ICD-FLAG, respectively (Figure 2A). Overexpression of NUMB increased expressions of endogenous cleaved NOTCH1 and N1ICD-FLAG (Figure 2B; Supplementary Figure S2A), and NUMB overexpression rescued decreased N1ICD-FLAG caused by NUMB knockdown (Supplementary Figure S2B). The levels of *HES1* and *HES5*, transcriptional targets of N1ICD, were also changed accordingly upon manipulations of NUMB (Supplementary Figure S2C), and NUMB promotes N1ICD expression in a dose-dependent manner (Figure 2C). In addition, although reports suggested distinct roles among NUMB isoforms (Verdi et al., 1999; Zong et al., 2014), we found that they could indiscriminately increase N1ICD levels (Figure 2C; Supplementary Figure S2D and F).

We went on to explore whether the positive regulation of N1ICD by NUMB is conserved among different cells. The enhanced expression of endogenous and exogenous N1ICD and its target genes by NUMB overexpression was mostly conserved among a few non-neural and neural cell lines including 293T, H4, and Neuro-2a cells (Supplementary Figure S2D–G). In contrast, the expressions of Notch ligands were not altered upon NUMB overexpression (Supplementary Figure S2H), indicating the specific action of NUMB against N1ICD.

We next treated HeLa$^{N1ICD\text{-}FLAG}$ cells with 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole or cycloheximide (CHX) to inhibit processes of transcription or translation, respectively, and found that N1ICD was rapidly degraded (Supplementary Figure S3A), probably via the ubiquitin–proteasome or lysosome

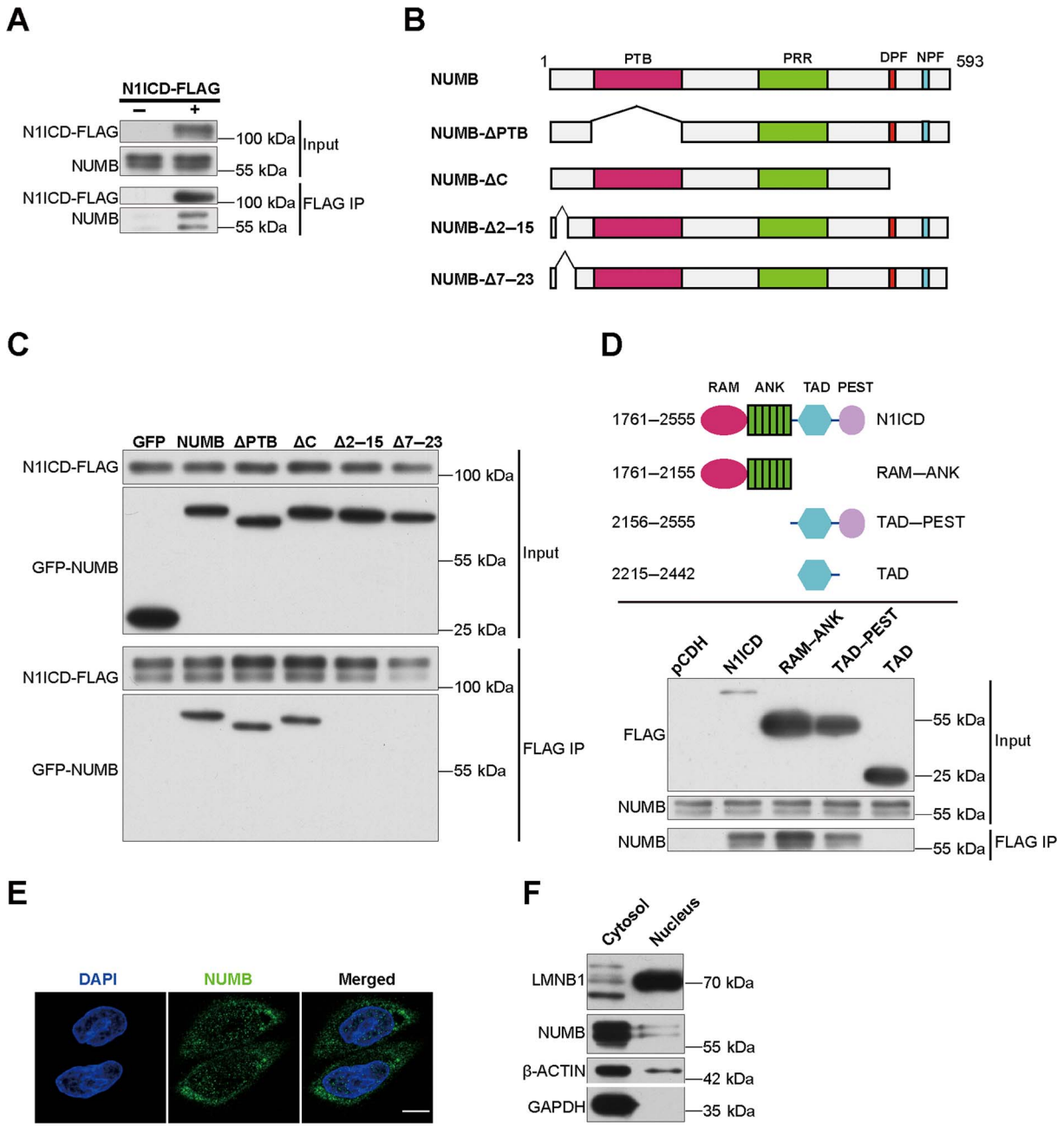


**Figure 1** N1ICD interacts with the N-terminal domain of NUMB. (**A**) Cell lysates extracted from HeLa$^{N1ICD\text{-}FLAG}$ and parental HeLa cells were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**B**) Domain organization of the NUMB protein and schemes of NUMB truncations. (**C**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with vectors expressing indicated NUMB truncations for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**D**) Domain organization of N1ICD and schemes of N1ICD truncations. 293T cells were transfected with vectors expressing FLAG-tagged N1ICD truncations for 24 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**E**) Confocal images of HeLa cells stained with the antibody against NUMB (green) and DAPI. Scale bar, 10 μm. (**F**) Subcellular fractionation of HeLa cells. Cytosol and nuclear fractionations were subjected to immunoblotting with indicated antibodies.

mechanism. To discriminate between the two possibilities, HeLa$^{N1ICD\text{-}FLAG}$ cells were treated with MG-132 or chloroquine to block the ubiquitin–proteasome or lysosome pathway, respectively. MG-132 treatment substantially increased N1ICD levels (Supplementary Figure S3B), whereas chloroquine treatment had no effect (Supplementary Figure S3C), suggesting N1ICD is largely posttranslationally regulated via the ubiquitin–proteasome pathway. As expected, overexpressing NUMB

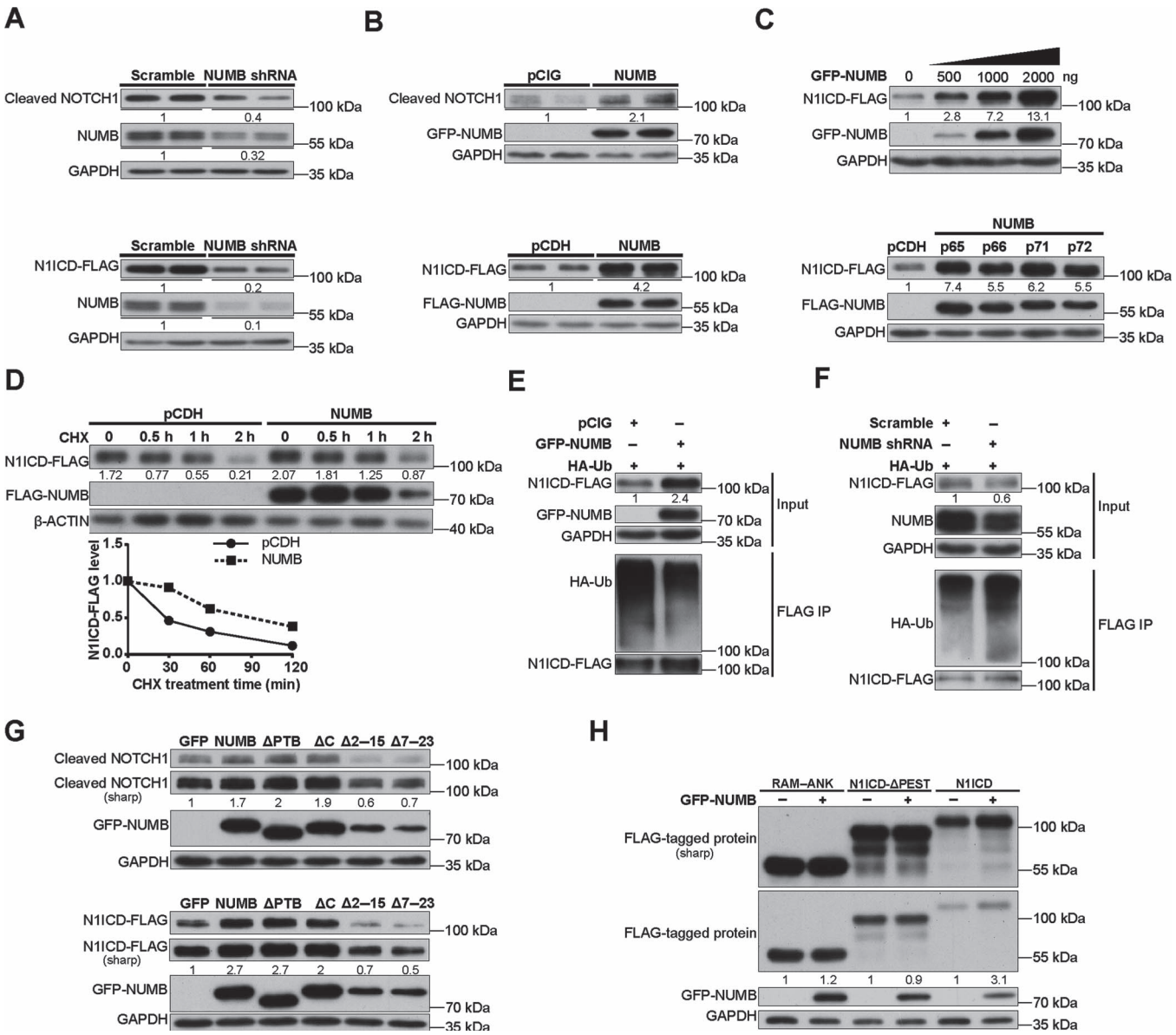


**Figure 2** NUMB inhibits the degradation of N1ICD. (**A** and **B**) HeLa (top) and HeLa$^{N1ICD\text{-}FLAG}$ (bottom) cells were infected with lentiviruses expressing indicated shRNAs for 72 h (**A**) or transfected with indicated vectors for 48 h (**B**). The expressing levels of cleaved NOTCH1 (top), N1ICD-FLAG (bottom), and NUMB were assessed by immunoblotting with GAPDH as the loading control. (**C**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated amounts of vectors (top) or transfected with indicated vectors (bottom) for 48 h. The expression levels of N1ICD and NUMB were assessed by immunoblotting with GAPDH as the loading control. (**D**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. Cells were treated with CHX (20 μg/ml) for the indicated time before harvesting. The expression levels of N1ICD and FLAG-NUMB were assessed by immunoblotting with β-ACTIN as the loading control. The band intensities of N1ICD were normalized to β-ACTIN, and the values at 0 min were set as 1. (**E** and **F**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h (**E**) or infected with lentiviruses expressing indicated shRNAs for 24 h and then tranfected with a vector expressing HA-ubiquitin for 48 h (**F**). Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**G**) HeLa (top) and HeLa$^{N1ICD\text{-}FLAG}$ (bottom) cells were transfected with indicated vectors for 48 h. The expression levels of cleaved NOTCH1 (top), N1ICD (bottom), and GFP-NUMB were assessed by immunoblotting with GAPDH as the loading control. (**H**) HeLa cells were transfected with indicated vectors for 48 h. The expression levels of RAM–ANK, N1ICD-ΔPEST, N1ICD, and GFP-NUMB were assessed by immunoblotting with GAPDH as the loading control.

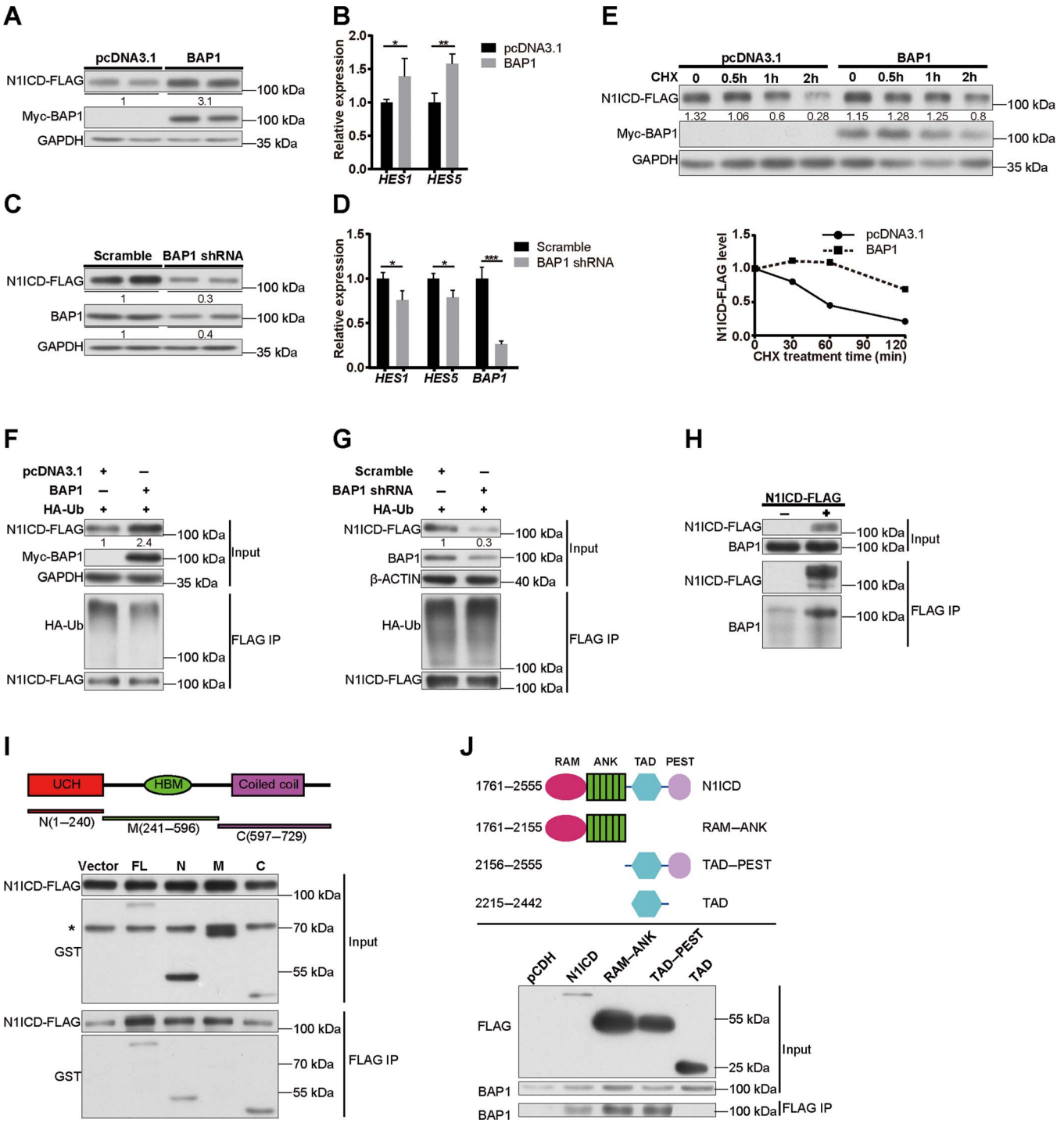


**Figure 3** BAP1 stabilizes N1ICD. (**A** and **B**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. (**A**) The expression levels of N1ICD and Myc-BAP1 were assessed by immunoblotting with GAPDH as the loading control. (**B**) *HES1* and *HES5* mRNA levels were analyzed by quantitative polymerase chain reaction (RT-qPCR). (**C** and **D**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 72 h. (**C**) The expression levels of N1ICD and BAP1 were assessed by immunoblotting with GAPDH as the loading control. (**D**) *HES1*, *HES5*, and *BAP1* mRNA levels were analyzed by RT-qPCR. (**E**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with the vector expressing Myc-tagged BAP1 or empty vector for 48 h. Cells were treated with CHX (20 μg/ml) for the indicated time before harvesting. The expression levels of N1ICD and Myc-BAP1 were assessed by immunoblotting with GAPDH as the loading control. The band intensities of N1ICD were normalized to GAPDH, and the values at 0 min were set as 1. (**F** and **G**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with the vector expressing HA-ubiquitin plus the vector expressing Myc-tagged BAP1 (**F**), shRNA against BAP1 (**G**), or control vectors for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**H**) Cell lysates extracted from HeLa$^{N1ICD\text{-}FLAG}$ and parental control HeLa cells were

delayed the degradation of N1ICD (Figure 2D) and decreased the ubiquitination of N1ICD (Figure 2E), whereas downregulating NUMB increased the ubiquitination of N1ICD (Figure 2F). Thus, NUMB stabilizes N1ICD by inhibiting its degradation via the ubiquitin–proteasome mechanism.

Lastly, overexpressing NUMB lacking the PTB domain (NUMB-ΔPTB) or the C-terminus (NUMB-ΔC) could stabilize endogenous cleaved NOTCH1 and N1ICD-FLAG (Figure 2G). On the other hand, overexpressing NUMB lacking part of the very N-terminal AB motif (NUMB-Δ2–15 or NUMB-Δ7–23) could not enhance or even downregulate N1ICD expression levels in a dose-dependent manner (Figure 2G; Supplementary Figure S3D), suggesting their dominant-negative roles; whereas the N-terminal NUMB lacking the AB motif (NUMB-NΔ2–23) did not have such effect (Supplementary Figure S3E). These results corroborate the aforementioned findings showing that NUMB interacts with N1ICD through NUMB AB motif. Notably, NUMB overexpression did not alter levels of exogenously-expressed RAM–ANK of N1ICD or N1ICD lacking the PEST domain (N1ICD-ΔPEST) (Figure 2H), indicating that NUMB acts on the PEST domain of N1ICD to regulate N1ICD stability.

*NUMB stabilizes N1ICD via BAP1*

N1ICD is degraded via the ubiquitin–proteasome pathway and NUMB has a negative role on it, but NUMB itself has no ubiquitin ligase or DUB activity. We thus sought to identify E3 ubiquitin ligases and DUBs that modulate N1ICD stability and are regulated by NUMB. Through DUB screening, BAP1 was identified as one of the DUBs that stabilize N1ICD in HeLa$^{N1ICD\text{-}FLAG}$ cells (Supplementary Figure S4A). BAP1 was reported to target mono-ubiquitinated histone 2A lysine 119 (H2AK119) (Scheuermann et al., 2010). Previous studies in *Drosophila* and zebrafish also indicated BAP1 might positively regulate Notch signaling (Tse et al., 2009; Zhang et al., 2012). BAP1 overexpression or knockdown significantly elevated or decreased N1ICD levels, as well as the transcripts of *HES1* and *HES5* in HeLa$^{N1ICD\text{-}FLAG}$ cells (Figure 3A–D). As expected, BAP1 overexpression significantly enhanced the mRNA expression of *HES1* (*Hes1*) and *HES5* (*Hes5*) in 293T cells and Neuro-2a cells (Supplementary Figure S4B). Overexpressing BAP1 also stabilized N1ICD in CHX-treated HeLa$^{N1ICD\text{-}FLAG}$ cells (Figure 3E). Importantly, overexpressing BAP1 decreased the ubiquitination of N1ICD (Figure 3F) and downregulating BAP1 increased the ubiquitination of N1ICD (Figure 3G). Next, we asked whether BAP1 is associated with N1ICD. Exogenously expressed Myc-BAP1 and N1ICD could be found in the same protein complex in 293T cells (Supplementary Figure S4C). In a more stringent assay, the interaction between N1ICD and endogenous BAP1 was validated by FLAG-bound IP in HeLa$^{N1ICD\text{-}FLAG}$ cells (Figure 3H). Co-IP experiments further showed that the full-length, N-terminal (aa 1–240), or C-terminal (aa 597–729) but not the medial region (aa 240–596) of BAP1 could bind to N1ICD-FLAG (Figure 3I). Interestingly, we found that the RAM–ANK and PEST domains of N1ICD, regions required for NUMB association, mediate N1ICD interaction with BAP1 as well (Figure 3J), and BAP1 regulates N1ICD stability through the PEST domain (Supplementary Figure S4D).

We next examined whether BAP1 binds to NUMB and whether this association regulates N1ICD stability. First, both exogenously expressed and endogenous BAP1 could bind to endogenous NUMB (Figure 4A–B). Second, BAP1 is associated with NUMB proline-rich region (PRR; aa 183–555) but not the PTB or C-terminal domains (Figure 4C–E). Third, similar to N1ICD, NUMB could bind to BAP1 N-terminal or C-terminal part (Figure 4F). Fourth, depletion of or overexpressing BAP1 dampened or enhanced NUMB's role in stabilizing N1ICD (Figure 4G and H), without affecting NUMB levels (Supplementary Figure S5A). Finally, NUMB overexpression enhanced the interaction between BAP1 and N1ICD (Figure 4I), but co-expression of non-N1ICD-bound NUMB-Δ2–15 could not promote BAP1–N1ICD association, thus failing to boost stabilization of N1ICD (Figure 4H and I). Collectively, NUMB collaborates with BAP1 to stabilize N1ICD via physical associations.

*BAP1 stabilizes N1ICD by inhibiting BRCA1 ubiquitinating activity*

We further asked whether BAP1 prevents degradation of N1ICD by directly deubiquitinating N1ICD. Surprisingly, overexpressing wild-type or the DUB-dead (C91S) BAP1 could equally stabilize N1ICD (Figure 5A). Concordantly, the ubiquitination levels of N1ICD were equally downregulated upon overexpression of wild-type or the BAP1-C91S (Figure 5B), suggesting N1ICD is not directly deubiquitinated by BAP1. BAP1 could regulate protein stability via its associated complexes including the HCF1–OGT complex or the BRCA1–BARD1 complex (Yu et al., 2010; Ruan et al., 2012; Carbone et al., 2013; Supplementary Figure S5B). We first downregulated HCF1 or OGT1 expression in HeLa$^{N1ICD\text{-}FLAG}$ cells using shRNAs (Supplementary Figure S5C), but revealing that the N1ICD levels were not altered (Figure 5C). In contrast, silencing BRCA1, a tumor suppressor with E3 ligase activity, significantly increased N1ICD protein levels (Figure 5D), and

immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**I**) Domain organization of the BAP1 protein and schemes of BAP1 truncations. HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with vectors expressing GST-tagged BAP1 truncations for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. *Nonspecific bands. (**J**) Domain organization of N1ICD and schemes of N1ICD truncations. 293T cells were transfected with indicated vectors for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. In **B** and **D**, data are presented as mean ± SD, $n = 3$. The y-axis represents relative expression normalized to *GAPDH*, and expression levels in control groups were set to 1. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t*-test).

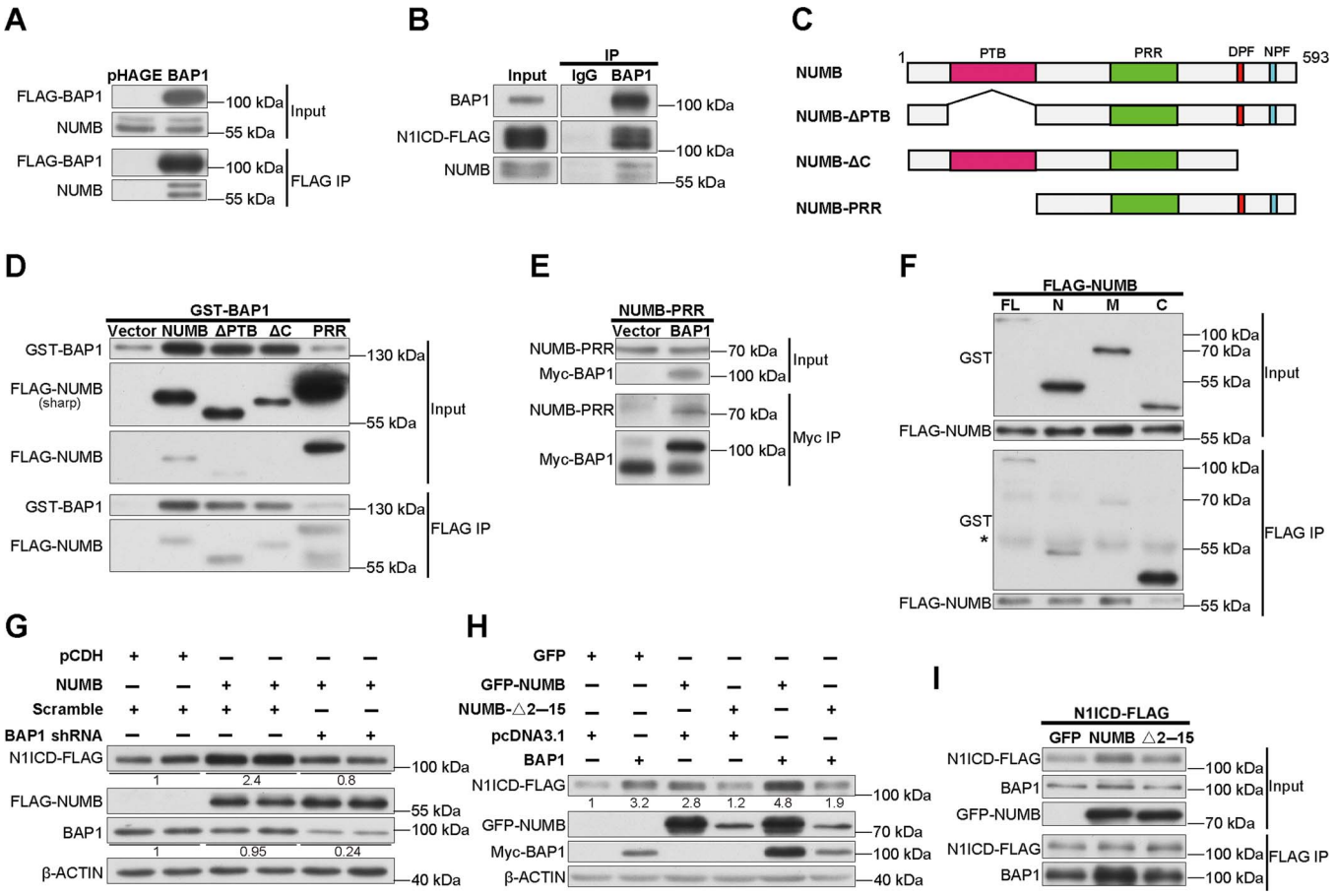


**Figure 4** NUMB interacts with BAP1 and promotes the association of N1ICD with BAP1. (**A**) 293T cells were transfected with indicated vectors for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**B**) Cell lysates extracted from HeLa$^{N1ICD\text{-}FLAG}$ cells were immunoprecipitated with anti-BAP1 or IgG antibodies, followed by immunoblotting with indicated antibodies. (**C**) Domain organization of the NUMB protein and schemes of NUMB truncations. (**D**–**F**) 293T cells were transfected with indicated combinations of vectors for 48 h. Cells lysates were immunoprecipitated with anti-FLAG resin (**D** and **F**) or anti-Myc magnetic resin (**E**) and subjected to immunoblotting with indicated antibodies. *Nonspecific bands. (**G** and **H**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated combinations of vectors for 72 h (**G**) or 48 h (**H**). The expression levels of indicated proteins were assessed by immunoblotting with β-ACTIN as the loading control. (**I**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. Cells were treated with MG-132 (10 μg/ml) for 12 h before harvesting. Cell lysates were then immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies.

BRCA1 overexpression destabilized N1ICD by enhancing N1ICD ubiquitination (Figure 5E and F). BRCA1 is largely distributed in the nucleus, and BRCA1 overexpression or knockdown has no effect on NUMB expression (Supplementary Figure S5D and E). Similar to BAP1 or NUMB, BRCA1 destabilizes N1ICD through its PEST domain (Supplementary Figure S5F). Moreover, co-expressing BAP1 reverted the decrease of N1ICD upon BRCA1 overexpression (Figure 5G) and dampened the interaction between N1ICD and BRCA1 (Supplementary Figure S5G). This suggests BAP1 regulates N1ICD stability by inhibiting BRCA1/BARD1 association with and subsequent ubiquitination of N1ICD, but independent of BAP1's DUB activity. We went on to determine whether NUMB acts as the adaptor to facilitate BAP1–BRCA1 role in stabilizing N1ICD. Co-expression of NUMB indeed reversed the decreased N1ICD and elevated N1ICD ubiquitination caused by BRCA1 overexpression in HeLa$^{N1ICD\text{-}FLAG}$ cells (Figure 5H).

### *BAP1 enhances Notch signaling in vivo*

Notch signaling promotes self-renewal and blocks differentiation of cortical NPCs (Lui et al., 2011; Engler et al., 2018). We wondered whether BAP1 regulates cell-fate choices of cortical NPCs via Notch signaling. *In situ* hybridization (ISH) revealed that *Bap1* is ubiquitously expressed in the developing mouse neocortex (Figure 6A). Parallelly, protein levels of activated NOTCH1 was found to be highly expressed in cortical NPCs (Supplementary Figure S6A), and BAP1 associates with endogenous activated N1ICD in cortical NPCs (Supplementary Figure S6B). We overexpressed BAP1 in primarily cultured E12.5 cortical NPCs

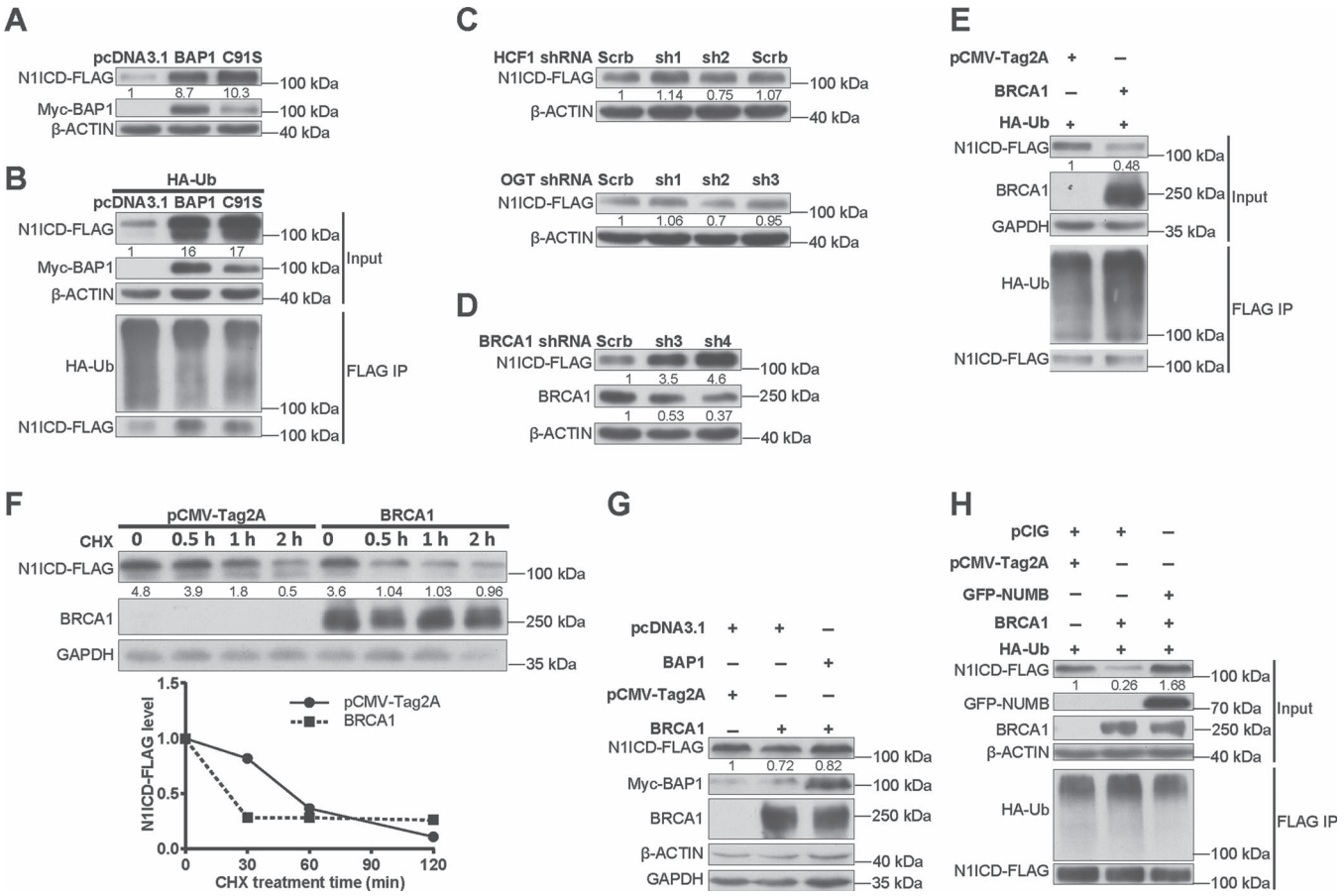


**Figure 5** BAP1 promotes N1ICD via inhibiting the ubiquitinating activity of BRCA1. (**A**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. The expression levels of N1ICD and Myc-BAP1 were assessed by immunoblotting with β-ACTIN as the loading control. (**B**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**C** and **D**) HeLa$^{N1ICD\text{-}FLAG}$ cells were infected with lentiviruses expressing indicated shRNAs for 72 h. The expression levels of N1ICD and BRCA1 were assessed by immunoblotting with β-ACTIN as the loading control. (**E**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies. (**F**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with the vector expressing BRCA1 or empty vector for 48 h. Cells were subsequently treated with CHX (20 μg/ml) for indicated time. The expression levels of N1ICD and BRCA1 were assessed by immunoblotting with GAPDH as the loading control. The band intensities of N1ICD were normalized to GAPDH, and the values at 0 min time points were set as 1. (**G**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. The expression levels of N1ICD, Myc-BAP1, and BRCA1 were assessed by immunoblotting with β-ACTIN and GAPDH as loading controls. (**H**) HeLa$^{N1ICD\text{-}FLAG}$ cells were transfected with indicated vectors for 48 h. Cell lysates were immunoprecipitated with anti-FLAG resin and subjected to immunoblotting with indicated antibodies.

and revealed that BAP1 promotes Notch signaling, as evidenced by elevated protein levels of activated NOTCH1 and HES1, as well as increased expression of *Hes1*, *Hes5*, and *Myc*. Expression levels of proliferating markers such as *Ccnd1* and *Pcna* were also increased (Figure 6B). Consistently, *Pax6* (a marker for self-renewing NPCs) expression was increased whereas *Tuj1* (a neuronal marker) was decreased upon BAP1 overexpression, indicating the blockade of NPC differentiation (Supplementary Figure S6C). In addition, BAP1 overexpression leads to enhanced self-renewal of cultured cortical NPCs (Figure 6C). Similar to wild-type BAP1, BAP1-C91S overexpression promotes Notch signaling (Figure 6B) and self-renewal of cortical NPCs (Figure 6C; Supplementary Figure S6C). To investigate the direct impact of BAP1 on Notch signaling *in vivo*, plasmids expressing BAP1 were electroporated in the E13.5 mouse cortex (Supplementary Figure S6D), together with a Notch activity reporter (12×CSL-H2B-Citrine) (Sprinzak et al., 2010). Brains were collected 48 h later for phenotypic analysis. Immunofluorescent staining of control cortices with PAX6, TBR2, and NEUROD2 showed that most Citrine$^{+}$ cells co-localize with PAX6 and only a few Citrine$^{+}$ cells express TBR2 or NEUROD2, markers for committed NPCs and cortical projection neurons, respectively (Supplementary Figure S6E–G), indicating that cells with high Notch activity are largely self-renewing NPCs. BAP1 overexpression leads to significant more mCherry$^{+}$ electroporated cells (73.7% ± 1.9% compared to 67.4% ± 0.2% in control) in the

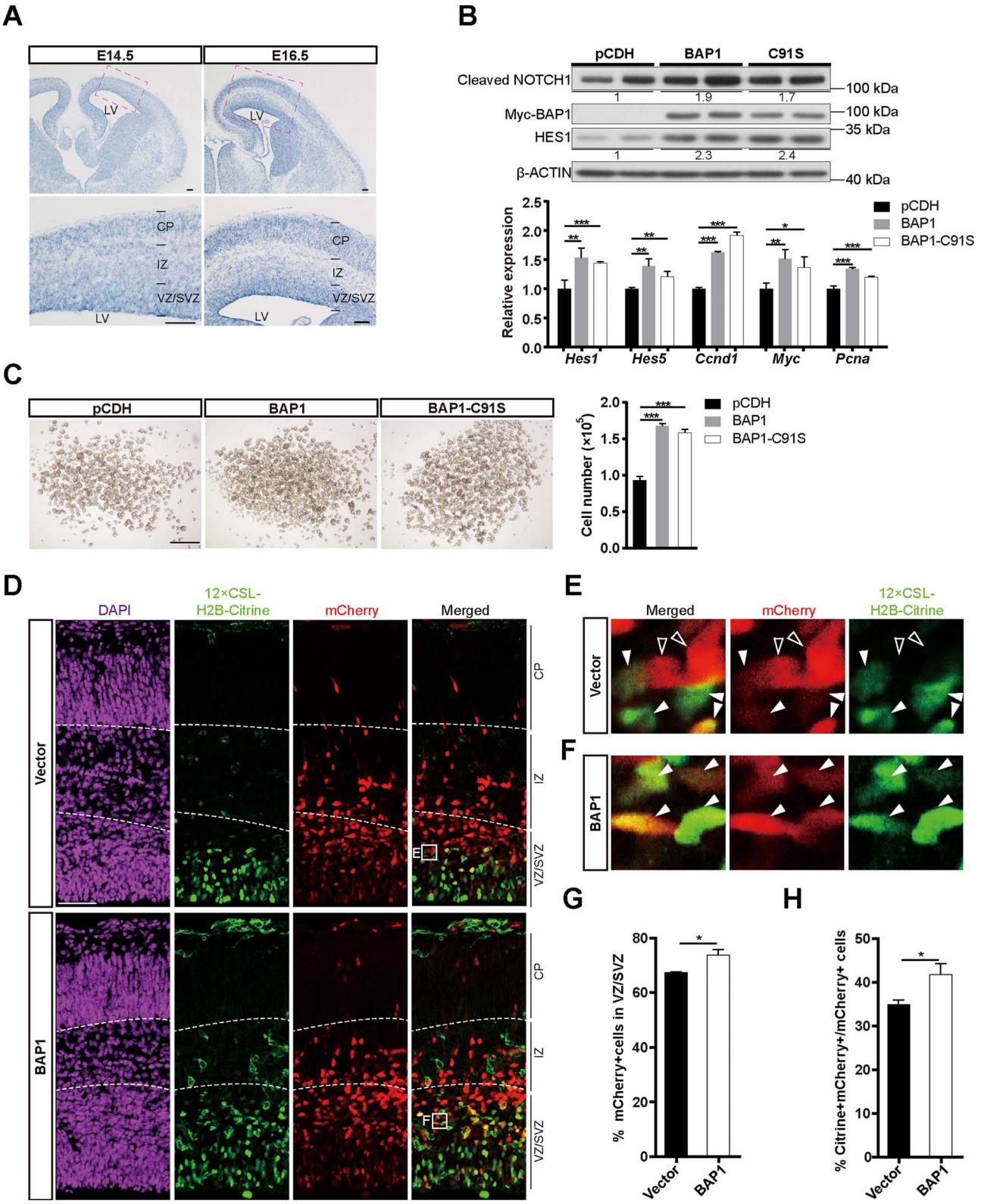


**Figure 6** BAP1 promotes Notch signaling *in vivo*. (**A**) ISH of *Bap1* on coronal sections of E14.5 (left) and E16.5 (right) CD-1 mouse dorsal forebrains. Boxed regions are enlarged in the bottom. Scale bar, 100 μm. (**B**) E12.5 cortical NPCs were infected with indicated lentiviruses for 96 h. Top: the expression levels of cleaved NOTCH1, HES1, and Myc-BAP1 were assessed by immunoblotting with β-ACTIN as the loading control. Bottom: *Hes1*, *Hes5*, *Ccnd1*, *Myc*, and *Pcna* mRNA levels were analyzed by RT-qPCR. The y-axis represents relative expression normalized to *Gapdh*, and expression levels in control groups were set to 1. (**C**) Representative images showing cortical NPCs maintained

ventricular/subventricular zone (VZ/SVZ; the location where most NPCs reside) (Figure 6D–G), suggesting enhanced self-renewal and partial blocking of NPC differentiation. Moreover, significant more BAP1-expressing cells showed high Notch activity (Citrine$^+$; 41.8% ± 2.4% compared to 35% ± 0.9% in control) (Figure 6D–F and H). Together, BAP1 enhances Notch signaling *in vivo*.

*BAP1 maintains stem-like properties of cortical NPCs*

Next, we asked whether BAP1 maintains self-renewal of cortical NPCs *in vivo*. E13.5 embryonic cortices were electroporated with siRNAs against *Bap1 or* scramble control siRNAs, and brains were collected at E16.5 for phenotypic analysis. There were significantly fewer EGFP$^+$ electroporated cells (31.3% ± 1.5% compared to 38.5% ± 2% in control) in the VZ/SVZ and more EGFP$^+$ cells (44.8% ± 0.9% compared to 38.4% ± 2% in control) in the intermediate zone (IZ) compared to negative controls, indicating enhanced neuronal differentiation of NPCs upon BAP1 depletion (Figure 7A; Supplementary Figure S7A). In line with this, fewer *Bap1* siRNA-transduced cells (52.7% ± 0.3% compared to 59.5% ± 1.6% in control) co-localized with PAX6$^+$ NPCs in the VZ/SVZ (Figure 7B). Next, E16.5 electroporated cortices were immunostained with TBR2, NEUROD2, and SATB2, a marker for cortical projection neurons. Result showed fewer *Bap1* siRNA-transduced cells co-labeled with TBR2 (30.1% ± 0.8% compared to 34.8% ± 1.3% in control) in the VZ/SVZ (Figure 7C). In contrast, significantly more *Bap1* siRNA-transduced cells expressed NEUROD2 (83.3% ± 0.9% compared to 78.1% ± 1% in control) (Figure 7D; Supplementary Figure S7D) or SATB2 (67.4% ± 1% compared to 59.4% ± 2% in control) (Figure 7E; Supplementary Figure S7E). Collectively, silencing *Bap1* lead to enhanced differentiation of NPCs, which is similar to the phenotype of *Notch* loss (Mizutani et al., 2007). Moreover, knockdown of *Bap1* in cortical NPCs also resulted in decreased levels of activated NOTCH1 and HES1, as well as reduced expression of *Hes1*, *Hes5*, *Myc*, *Ccnd1*, and *Pcna* (Figure 7F). Consistently, depletion of *Bap1* significantly compromised the self-renewal of cultured cortical NPCs (Figure 7G) with decreased expression of *Pax6* and increased level of *Tuj1* (Supplementary Figure S7B). Lastly, the reduced self-renewal of cortical NPCs and compromised Notch signaling caused by *Bap1* silencing could be partially rescued by depletion of *Brca1* (Supplementary Figure S7C and F). All these data support that BAP1 maintains self-renewal of cortical NPCs by enhancing Notch signaling.

## Discussion

Here, we surprisingly discovered that NUMB positively regulates the Notch signaling through stabilizing the N1ICD, which is achieved by regulating the ubiquitinating activity of BRCA1. We also identified BAP1, a DUB and a negative regulator of BRCA1, physically associates with N1ICD, and the association is strengthened by NUMB (Figure 7H).

NUMB regulates cell-fate determination through Notch-dependent and independent mechanisms, reliant on development stages and cellular context (Wu and Li, 2015). Although our findings are seemingly at odds with the common notion that NUMB antagonizes Notch signaling, it supports studies showing that NUMB and Notch could play similar roles in cell fate specifications. For example, NUMB family proteins, including NUMB and its mammalian homologue NUMBL, are intrinsic factors crucial for the renewal of neural and cardiac progenitor cells (Petersen et al., 2002, 2004; Shenje et al., 2014) and might function synergistically with membrane-associated NOTCH1 (Kwon et al., 2011). Interestingly, although Numb is thought to promote differentiation of skeletal muscle by antagonizing Notch signaling, Numb-transgenic mouse embryos have expanded stem/progenitor pool size with Notch targets unaltered or significantly increased (Jory et al., 2009).

Endocytosis could both positively and negatively regulate Notch signaling. In *Drosophila*, receptor-mediated endocytosis plays critical roles in modulating Notch signaling, in particular its activation (Seugnet et al., 1997; Vaccari et al., 2008). Studies in *Drosophila* and in mammalian cells support roles of NUMB in mediating endocytosis, post-endocytic trafficking, and subsequent degradation of membrane-bound NOTCH1 receptor. NUMB suppresses Notch signaling by reducing Notch receptor level at the cell surface through receptor-mediated endocytosis and/or proteasome-mediated degradation (Berdnik et al., 2002; Smith et al., 2004; McGill et al., 2009).

N1ICD is largely localized in the nucleus and is short-lived (Blain et al., 2017), with half-life ∼30 min in the current study. Moreover, N1ICD is degraded via the ubiquitin–proteasome but not the lysosome pathway and is the target of E3 ubiquitin ligases MDM2 and FBXW7 (Moretti and Brou, 2013). Interestingly, our findings pointed out the positive regulation of N1ICD protein

under neurosphere conditions for 6 days after transducing with indicated lentiviruses, with quantification of cell numbers. Scale bar, 500 μm. (**D**) E13.5 mouse cortices were electroporated with the Notch activity reporter (12×CSL-H2B-Citrine) together with empty vector or BAP1-expressing plasmid. Electroporated cells were labeled with nuclear mCherry. Embryos were sacrificed at E15.5 for immunofluorescent analysis. Boxed regions are enlarged in **E** and **F**. Scale bar, 100 μm. (**E** and **F**) Enlarged images of boxed regions shown in **D**. Electroporated Cells with high Notch activity are Citrine$^+$ and mCherry$^+$ (solid arrowheads), whereas electroporated cells with low Notch activity are Citrine$^-$ and mCherry$^+$ (empty arrowheads). (**G**) Quantification of mCherry$^+$ cells localized in the VZ/SVZ. (**H**) Quantification of Citrine$^+$mCherrry$^+$/mCherry$^+$ cells. In **B**, data are presented as mean ± SD, $n = 3$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test). In **C**, data are presented as mean ± SEM, $n = 3$. **$P < 0.01$ (Student's *t*-test). In **G** and **H**, data are presented as mean ± SEM, $n = 5$ brains for vector and $n = 5$ brains for BAP1 overexpression. *$P < 0.05$ (Student's *t*-test).

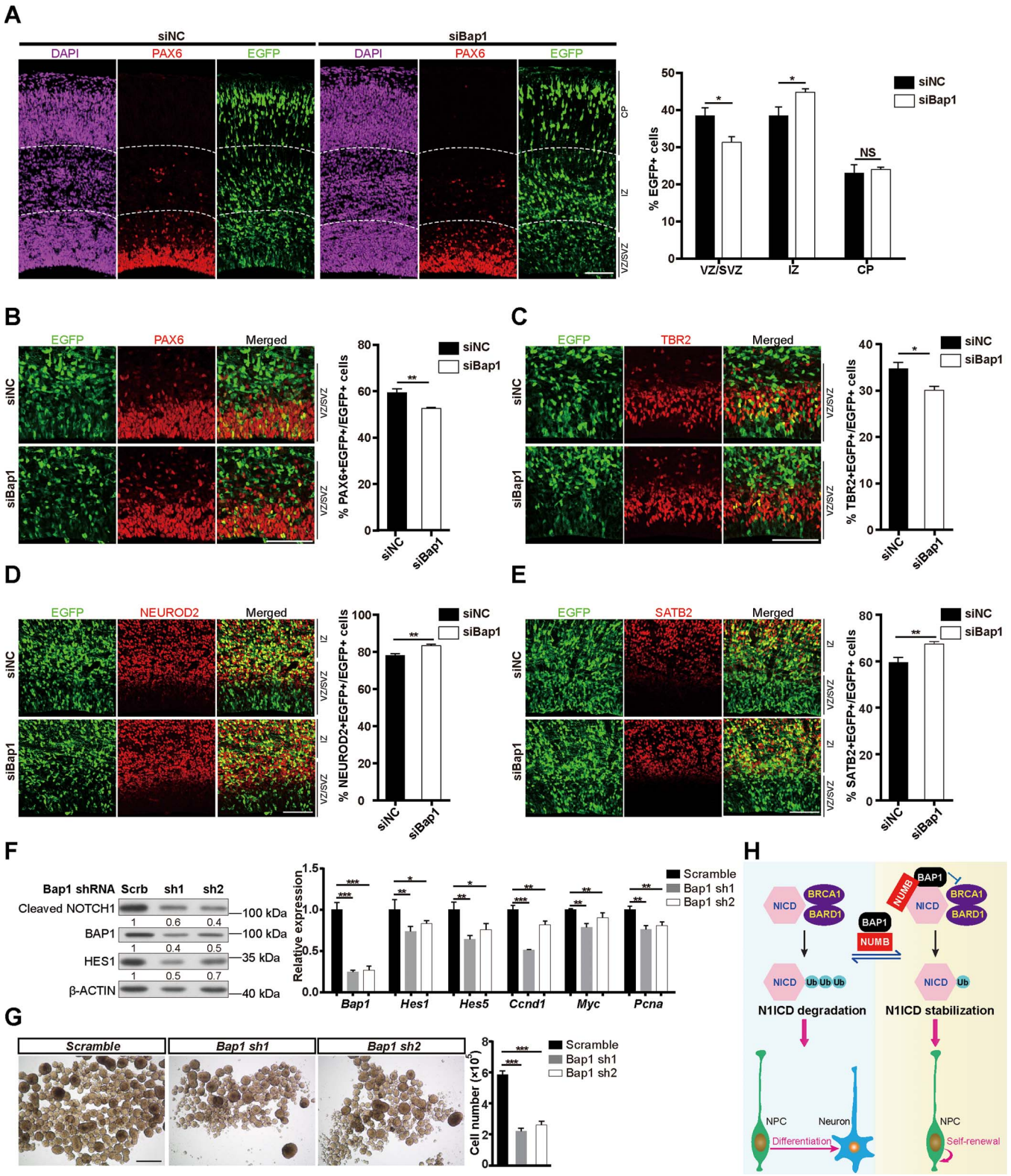


**Figure 7** BAP1 maintains self-renewal of cortical NPCs. (**A**) E13.5 mouse cortices were electroporated with indicated siRNAs, with transduced cells labeled with EGFP. Embryos were sacrificed at E16.5 for immunofluorescent analysis. The relative location of EGFP$^+$ cells was quantified. Scale bar, 100 μm. (**B**–**E**) Representative images of control or *Bap1* siRNA-electroporated sections immunostained for PAX6 (**B**), TBR2 (**C**), NEUROD2 (**D**), and SATB2 (**E**), with quantification of marker-positive transduced cells. Scale bar, 100 μm. (**F**) E12.5 cortical NPCs were infected with indicated lentiviruses for 96 h. Left: the expression levels of cleaved NOTCH1, BAP1, and HES1 were

by NUMB through inhibiting N1ICD ubiquitination. Moreover, NUMB fulfills the functions independent of its role in regulating endocytosis, as NUMB utilizes the very N-terminal AB domain but not the PTB or DPF/NPF motif to stabilize N1ICD. Thus, it is not surprising all four NUMB isoforms are equally capable of stabilizing N1ICD because they bear the same N-terminal AB motif. In certain cellular scenarios, e.g. in H4 glioblastoma cells or in neural precursor cells (Petersen et al., 2004, 2006), NUMB might preferentially stabilize N1ICD rather than facilitate the degradation of the Notch receptor. This is analogous to seemingly contradictory roles of *Drosophila* Deltex, an E3 ubiquitin ligase, in Notch receptor trafficking and activation: Deltex-mediated Notch trafficking can result in either production of NICD and subsequent signal activation or degradation of Notch receptors and signal repression (Mukherjee et al., 2005; Wilkin et al., 2008). Actually, Deltex regulates Notch signaling in a context-dependent manner, determined by its interaction with other regulatory factors (Moretti and Brou, 2013; Sancho et al., 2015).

Through DUB screening and biochemical studies, BAP1 was found to be the key effector of NUMB and the positive regulator of N1ICD. Moreover, genetic studies in *Drosophila* and in zebrafish also supported the role of *BAP1* orthologs in enhancing Notch signaling (Tse et al., 2009; Zhang et al., 2012). BAP1 associates with NUMB PRR region and N1ICD RAM–ANK and PEST domains, where NUMB also interacts with. Moreover, the presence of full-length NUMB, but not the AB-deleted NUMB, significantly enhances the interaction between BAP1 and N1ICD. BAP1 is a nuclear-localized, a member of the ubiquitin COOH-terminal hydrolase subfamily of DUBs, which was initially identified as a protein that interacts with the RING finger domain of BRCA1 to enhance BRCA1-mediated cell growth inhibition (Jensen et al., 1998). Being a component of a multiprotein complex, BAP1 can regulate gene transcription, histone modification and protein stability (Scheuermann et al., 2010; Yu et al., 2010). We revealed that BAP1 stabilizes N1ICD independent of its DUB activity but via its role in repressing BRCA1/BARD's ubiquitinating activity toward N1ICD. In fact, BAP1 interferes with the BRCA1–BARD1 association independent of its DUB activity, which results in inhibition of the E3 ligase activity of BRCA1 (Nishikawa et al., 2009). Both BAP1 and BRCA1 are highly expressed in the developing nervous system, and ablation of *Brca1* of cortical NPCs leads to smaller cortices, which could be due to massive apoptosis of NPCs and early born cortical neurons (Pulvers and Huttner, 2009). This phenotype is reminiscent of N1ICD or NUMB gain-of-function in cortical NPCs (Yang et al., 2004; Zhou et al., 2007). Consistently, our studies showed that BAP1 activates Notch signaling *in vivo*, and depletion of BAP1 in cortical NPCs results in decreased Notch signaling, compromised self-renewal, and premature differentiation of cortical NPCs.

We noticed that both NUMB and BAP1 are able to interact with N1ICD PEST domain, the region essential for the targeting by E3 ubiquitin ligase FBXW7, suggesting ubiquitination states of N1ICD could be counterbalanced by multiple modifiers. Deletion in the PEST domains of NOTCH1 or mutations in the *FBXW7* gene lead to aberrant activation of the Notch pathway and are among the most frequent mutations in T-cell acute lymphoblastic leukemia (Weng et al., 2004; Maser et al., 2007; O'Neil et al., 2007), highlighting the significance of this regulatory event. Furthermore, NUMB might regulate multiple facets of Notch signaling: although current study did not show NUMB's regulatory roles for Notch ligands DLL1, JAG1, and JAG2, NUMB functions as a sorting switch to control the post-endocytic trafficking of DLL4 in vascular endothelial cells (Shao et al., 2017). Finally, *BAP1* mutations and *NUMB* gain were reported to be associated with a number of malignant tumors (Carbone et al., 2013; Qin et al., 2015; Ke et al., 2018; Tominaga et al., 2018), and it remains to be explored whether they act on Notch signaling in these scenarios.

## Materials and methods

### *Cloning and primers*

DNA constructs used in this study were listed in Supplementary Table S1. Sequences for shRNAs and siRNAs were listed in Supplementary Table S2. Sequences for qPCR primers were listed in Supplementary Table S3.

### *Cell culture*

HeLa, HEK-293T, and SH-SY5Y cells were gifts from Drs Xiangdong Fu, Hong-Bing Shu, and Ling Zheng, respectively. U87MG and H4 cells were gifts from Dr Xiaozhong Peng at Peking Union Medical College. Neuro-2a and NE-4C cells were purchased from the Cell Bank of Chinese Academy of Sciences. Cells were maintained in appropriate culture media (Dulbecco's Modified Eagle Medium or Modified Eagle Medium) containing 10% fetal bovine serum.

### *Immunoprecipitation*

Cells were lysed with ice-cold lysis buffer (50 mM Tris–HCl, 300 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, 1 mM EDTA, pH 7.4) containing protease inhibitor cocktail (Biotool). Cell extracts were pre-cleared and centrifuged at 14000 *g* for

assessed by immunoblotting with β-ACTIN as the loading control. Right: *Bap1*, *Hes1*, *Hes5*, *Ccnd1*, *Myc*, and *Pcna* mRNA levels were analyzed by RT-qPCR. The y-axis represents relative expression normalized to *Gapdh*, and expression levels in control groups were set to 1. (**G**) Representative images showing cortical NPCs maintained in neurosphere conditions for 6 days after transducing with indicated lentiviruses, with quantification of cell numbers. Scale bar, 500 μm. (**H**) A model showing that NUMB stabilizes N1ICD by associating with BAP1 to repress the ubiquitinating activity of BRCA1/BARD1 in cortical NPCs. In **A**–**E**, data are presented as mean ± SEM, $n = 3$ brains for siNC and $n = 4$ brains for siBap1. *$P < 0.05$; **$P < 0.01$ (Student's *t*-test). In **F**, data are presented as mean ± SD, $n = 3$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test). In **G**, data are presented as mean ± SEM, $n = 3$. ***$P < 0.001$ (Student's *t*-test).

30 min at 4°C, and protein was immunoprecipitated with specific antibodies and Protein G beads (GE Healthcare) at 4°C overnight or at room temperature for 90 min. The beads were washed six times with lysis buffer and then heated at 100°C in loading buffer for immunoblotting.

*In vivo ubiquitination assay*

HeLa$^{N1ICD\text{-}FLAG}$ cells were plated into 6-well plate and transfected with indicated plasmids. After 48 h, cells were lysed with 1% SDS lysis buffer (200 µl/well) and heated at 100°C for 15 min. The lysates were then diluted with 800 µl dilution buffer (50 mM Tris–HCl, 150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, 1 mM EDTA, pH 7.4) and centrifuged at 12000 *g* for 10 min at 4°C. N1ICD was immunoprecipitated with anti-FLAG resin (Bimake; B23102) at 4°C overnight. The beads were washed six times with RIPA buffer and then heated at 100°C in loading buffer for immunoblotting.

More detailed methods are described in Supplementary material.

## Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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