NUMB enhances Notch signaling by repressing ubiquitination of NOTCH1 intracellular domain

Zhiyuan Luo†, Lili Mu†, Yue Zheng, Wenchen Shen, Jiali Li, Lichao Xu, Bo Zhong, Ying Liu*, and Yan Zhou*

College of Life Sciences, Renmin Hospital of Wuhan University, Medical Research Institute at School of Medicine, Wuhan University, Wuhan 430072, China
† These authors contributed equally to this work.
* Correspondence to: Ying Liu, E-mail: y.liu@whu.edu.cn; Yan Zhou, E-mail: yan.zhou@whu.edu.cn

Edited by Zhen-Ge Luo

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 nucleus 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; Couttier 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...
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 ICH/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). ICH 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 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.

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-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-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-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-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.

To discriminate between the two possibilities, HeLa^{N1ICD-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 HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-FLAG cells were transfected with indicated vectors for 48 h (E) or infected with lentiviruses expressing indicated shRNAs for 24 h and then transfected 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 HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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 HeLaN1ICD-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-N-Δ2–23) 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 HeLaN1ICD-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 HeLaN1ICD-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 HeLaN1ICD-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 HeLaN1ICD-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 HeLaN1ICD-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. HeLaN1ICD-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).
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 HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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) HeLaN1ICD-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 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 Pcnal (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 deletion 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 ubiquitinactivating 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 (Berndnik 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−mCherry+/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 N1ICD 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/BARD1'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 *Brae1* 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

---

*Supplementary materials available at https://academic.oup.com/jmcb/article/12/5/345/5558388.*

---
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**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.

**Acknowledgements**

We thank Drs Weimin Zhong, HUDAN Liu, Ceshi Chen, Duanqing Pei, Xiaodong Zhang, Jingyi Hui, Feng Li, and Xiaozhong Peng for sharing reagents and technical support.

**Funding**

This work was supported by grants from the National Key R&D Program of China (2018YFA0800700), the National Natural Science Foundation of China (31671418, 31970676, 31970770, and 31471361), Hubei Natural Science Foundation (2018CFA016), Fundamental Research Funds for the Central Universities (2042017KFO242), Medical Science Advance-ment Program (Basic Medical Sciences) of Wuhan University (TFJC2018005), and Wuhan University Experiment Technology Project Funding (WHU-2018-SYS-01).

**Conflicts of interest:** none declared.

**References**

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science 284, 770–776.

Aster, J.C., Pear, W.S., and Blacklow, S.C. (2017). The varied roles of Notch in cancer. Annu. Rev. Pathol. 12, 245–275.

Berndt, D., Torok, T., GonzalezGaitan, M., et al. (2002). The endocytic protein α-adaptin is required for Numb-mediated asymmetric cell division in Drosophila. Dev. Cell 3, 221–231.

Blain, J., Bedard, L., Thompson, M., et al. (2017). C-terminal deletion of NOTCH1 intracellular domain N1ICD increases its stability but does not amplify and recapitulate N1ICD-dependent signalling. Sci. Rep. 7, 5034.

Carbone, M., Yang, H., Pass, H.I., et al. (2013). BAP1 and cancer. Nat. Rev. Cancer 13, 153–159.

Couturier, L., Vedovaro, N., and Schweiguth, F. (2012). Endocytosis by Numb breaks Notch symmetry at cytokinesis. Nat. Cell Biol. 14, 131–139.

Dhami, G.K., Liu, H., Galka, M., et al. (2013). Dynamic methylation of Numb by SET9 regulates its binding to p53 and apoptosis. Mol. Cell 50, 565–576.

Engler, A., Zhang, R., and Taylor, V. (2018). Notch and neurogenesis. In: Bortgrefe, T. and Giaimo, B.D. (eds). Molecular Mechanisms of Notch Signaling. Cham: Springer International Publishing, 223–234.

Fang, D.Y., Elly, C., Gao, B.X., et al. (2002). Dysregulation of T lymphocyte function in itchy mice: a role for itch in T42 differentiation. Nat. Immuno. 3, 281–287.

Guo, M., Jan, L.Y., and Jan, Y.N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron 17, 27–41.

Guptarossi, N., Bail, O.L., Gonen, H., et al. (2001). Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. J. Biol. Chem. 276, 34371–34378.

Imayoshi, I., Sakamoto, M., Yamaguchi, M., et al. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. J. Neurosci. 30, 3489–3498.

Ishibashi, M., Ang, S., Shiota, K., et al. (1995). Targeted disruption of mammalian hairy and enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. 9, 3136–3148.

Jensen, D.E., Proctor, M.L., Marquis, S.T., et al. (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene 16, 1097–1112.

Jory, A., Le Roux, I., Gayraud-Morel, B., et al. (2009). Numb promotes an increase in skeletal muscle progenitor cells in the embryonic somite. Stem Cells 27, 2769–2780.

Ke, H., Zhao, L., Zhang, H., et al. (2018). Loss of TDP43 inhibits progression of triple-negative breast cancer in coordination with SRSF3. Proc. Natl Acad. Sci. USA 115, e3426–e3435.

Kopan, R., and Ilagan, M.X.G. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137, 216–233.

Kwon, C., Cheng, P., King, L.N., et al. (2011). Notch post-translationally regulates β-catenin protein in stem and progenitor cells. Nat. Cell Biol. 13, 1244–1251.

Le Bras, S., Loyer, N., and Le Borgne, R. (2011). The multiple facets of ubiquitination in the regulation of Notch signaling pathway. Traffic 12, 149–161.

Lui, J.H., Hansen, D.V., and Kriegstein, A.R. (2011). Development and evolution of the human neocortex. Cell 146, 18–36.

Martinblanco, N.M., Checquolo, S., Gaudio, F.D., et al. (2014). Numb-dependent integration of pre-TCR and p53 function in T-cell precursor development. Cell Death Dis. 5, e1472.

Maser, R.S., Choudhury, B., Campbell, P.J., et al. (2007). Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. Nature 447, 966–971.

McGill, M.A., Dho, S.E., Weinmaster, G., et al. (2009). Numb regulates post-endocytic trafficking and degradation of Notch1. J. Biol. Chem. 284, 26427–26438.

McGill, M.A., and McGlade, C.J. (2003). Mammalian Numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. J. Biol. Chem. 278, 23196–23203.

Mizutani, K., Yoon, K., Kang, D.T., et al. (2007). Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature 449, 351–355.

Moretti, J., and Brou, C. (2013). Ubiquitinations in the Notch signaling pathway. Int. J. Mol. Sci. 14, 6359–6381.

Moretti, J., Chastagner, P., Liang, C.C., et al. (2012). The ubiquitin-specific protease 12 (USP12) is a negative regulator of Notch signaling acting on Notch receptor trafficking toward degradation. J. Biol. Chem. 287, 29429–29441.

Mukherjee, A., Veraksa, A., Bauer, A., et al. (2005). Regulation of Notch signalling by non-visual β-arrestin. Nat. Cell Biol. 7, 1191–1201.

Nishikawa, H., Wu, W., Koike, A., et al. (2009). BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. Cancer Res. 69, 111–119.

Oberg, C., Li, J., Pauley, A.M., et al. (2003). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. J. Biol. Chem. 278, 35847–35853.
