NRBP1-Containing CRL2/CRL4A Regulates Amyloid β Production by Targeting BRI2 and BRI3 for Degradation

Highlights

- Homodimeric NRBP1 assembles into a Cul2- and Cul4A-containing heterodimeric CRL
- NRBP1-containing CRL2/CRL4A targets BRI2 and BRI3 for degradation
- NRBP1 CRL assembly is enhanced by TSC22D3 and TSC22D4
- RNAi-mediated depletion of NRBP1 reduces Aβ production in neuronal cells

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In Brief

Yasukawa et al. demonstrate that BRI2 and BRI3, physiological inhibitors of Aβ production and aggregation, are substrates of NRBP1-ubiquitin ligase. In the presence of TSC22D3 and TSC22D4, a dimer of the substrate receptor NRBP1 assembles into a functional Cul2- and Cul4A-containing heterodimeric CRL through overlapping BC-box and cryptic H-box motifs on NRBP1.
NRBP1-Containing CRL2/CRL4A Regulates Amyloid β Production by Targeting BRI2 and BRI3 for Degradation

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SUMMARY

Alzheimer’s disease (AD) is a progressive neurodegenerative disease caused by accumulations of Aβ peptides. Production and fibrillation of Aβ are downregulated by BRI2 and BRI3, which are physiological inhibitors of amyloid precursor protein (APP) processing and Aβ oligomerization. Here, we identify nuclear receptor binding protein 1 (NRBP1) as a substrate receptor of a Cullin-RING ubiquitin ligase (CRL) that targets BRI2 and BRI3 for degradation. Moreover, we demonstrate that (1) dimerized NRBP1 assembles into a functional Cul2- and Cul4A-containing heterodimeric CRL through its BC-box and an overlapping cryptic H-box, (2) both Cul2 and Cul4A contribute to NRBP1 CRL function, and (3) formation of the NRBP1 heterodimeric CRL is strongly enhanced by chaperone-like function of TSC22D3 and TSC22D4. NRBP1 knockdown in neuronal cells results in an increase in the abundance of BRI2 and BRI3 and significantly reduces Aβ production. Thus, disrupting interactions between NRBP1 and its substrates BRI2 and BRI3 may provide a useful therapeutic strategy for AD.

INTRODUCTION

Alzheimer’s disease (AD) is characterized by accumulations of insoluble deposits of Aβ peptides in the extracellular space of the brain parenchyma (Haass and Selkoe, 2007). Aβ is derived by sequential proteolytic processing of a type I transmembrane protein, the amyloid precursor protein (APP). Cleavage of APP by β-secretase, which is known as β-site APP-cleaving enzyme 1 (BACE1), leads to shedding of the ectodomain of APP (sAPPβ) and generation of an APP carboxy-terminal fragment (β-CTF). β-CTF is then cleaved by γ-secretase into the APP intracellular domain (AICD) and amyloidogenic Aβ peptides, including Aβ40 and Aβ42 (Haass and Selkoe, 2007; Haass et al., 2012; De Strooper et al., 2012; Vassar et al., 2014). Of the amyloidogenic peptides, Aβ42 is more prone to form pathogenic oligomers (Haass and Selkoe, 2007; Haass et al., 2012). In an alternative non-amyloidogenic proteolytic pathway, APP is first cleaved in the middle of the Aβ region by α-secretase, shedding soluble APPβ (sAPPβ) and generating a truncated APP CTF (α-CTF), which lacks the amino-terminal portion of the Aβ domain. α-CTF is then cleaved by γ-secretase into two peptides, AICD and a truncated peptide p3, which is apparently non-pathogenic (Haass et al., 2012).

Type II transmembrane proteins BRI2 and BRI3 have been reported to negatively regulate Aβ production by binding to APP and inhibiting its processing by secretases. Both proteins mask the cleavage sites of β- and γ-secretase on APP, while BRI2 also masks the γ-secretase docking site on β-CTF (Fotino-poulou et al., 2005; Matsuda et al., 2005, 2009). Of note, two mutations in the BRI2 gene have been identified as the cause of autosomal dominant familial British dementia (FBD) and familial Danish dementia (FDD), which share pathological and clinical similarities with AD (Del Campo and Teunissen, 2014). BRI2 is composed of 266 amino acids and is cleaved by furin-like proteases at its C terminus to produce mature BRI2 and a soluble 23 amino acid peptide (Bri2-23), which is capable of inhibiting Aβ aggregation (Kim et al., 2008). Disease-causing mutations at or near the stop codon result in the production of C-terminally extended, 277 amino acid mutant BRI2 proteins, which are cleaved at the normal protease processing site to generate mature BRI2 and distinct 34 amino acid amyloidogenic peptides (ABri in FBD and ADan in FDD), which accumulate in the brain of affected patients (Vidal et al., 1999, 2000). Moreover, mutations in the BRI2 gene render mutant BRI2 protein unstable, such that it is mostly degraded (Takayev et al., 2010a, 2010b).
Consequently, proteolytic processing of APP is deregulated, and formation of APP metabolites including Aβ is increased (Matsuda et al., 2011). Based on these findings, it has been suggested that lack of BRI2 function may play an important role not only in FBD and FDD development but also in AD pathogenesis (Del Campo and Teunissen, 2014; Cantlon et al., 2015).

The ubiquitin-proteasome pathway plays a vital role in the degradation of proteins. Protein ubiquitination, which targets proteins for degradation by the proteasome, is catalyzed by an enzymatic cascade involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase that determines the substrate specificity (Pickart, 2001). Cullin-RING ubiquitin ligases (CRLs) constitute a major subclass of E3-ligase complexes. They generally consist of Cullin scaffolds, RING-box (Rbx) proteins, adaptors, and substrate receptors (Petroski and Deshaies, 2005). In humans, six closely related Cullins (Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5) have been identified, and each forms a modular complex with one of the Rbx proteins Rbx1 or Rbx2. The Rbx proteins function as a docking site for the ubiquitin-charged E2 enzyme at the C terminus of the Cullin, while adaptor proteins such as SKP1, Elongin BC, and DDB1 bind to the N terminus of the Cullin and recruit distinct classes of substrate receptors that target substrates for ubiquitination (Zimmerman et al., 2010). The interfaces between the substrate receptor and the adaptor and/or Cullin N terminus have been shown to be important structural elements that determine Cullin selectivity (Cardote et al., 2017; Nguyen et al., 2015).

Nuclear receptor binding protein 1 (NRBP1) is a pseudokinase that is ubiquitously expressed and highly conserved across species (Kerr and Wilson, 2013). As NRBP1 has been shown to be capable of binding to Elongin BC and also contains a sequence similar to the canonical Cul2-box downstream of its Elongin BC-box, NRBP1 has been speculated to serve as a substrate receptor of a CRL of unknown substrate specificity (Kerr and Wilson, 2013; Mahour et al., 2008; Wilson et al., 2012). Using a combination of immunoaffinity chromatography and mass spectrometry (MS) analysis, Mahour et al. (2008) demonstrated that NRBP1 also associates with members of the TSC22 domain family (TSC22DF), TSC22D1-1, TSC22D1-2, TSC22D2, TSC22D3, and TSC22D4. However, whether these interactions are relevant to NRBP1’s putative ubiquitin ligase function remains unknown.

In this study, we searched for substrates of NRBP1-ubiquitin ligase using trypsin-resistant tandem ubiquitin-binding entity (TR-TUBE) (Yoshida et al., 2015) and identified BRI3 and BRI2 as its targets. NRBP1 dimerizes via an LiSH-motif at its C terminus and, through its BC-box and a cryptic H-box overlapping with the BC-box, forms an NRBP1-containing Cul2/Cul4A heterodimeric CRL that efficiently targets both BRI2 and BRI3 for ubiquitination in the presence of TSC22D3 and TSC22D4. Dicer-substrate small interfering RNA (siRNA)-mediated depletion of NRBP1 in neuronal cells resulted in an increase in the amounts of endogenous BRI2 and BRI3 and significantly reduced Aβ production. Taken together, our results suggest that interaction of NRBP1 with its substrates BRI2 and BRI3 could be potential therapeutic targets for AD, as well as FBD and FDD.

RESULTS

Dimerization and Assembly of NRBP1 with Cul2 Are Facilitated by TSC22D3 and TSC22D4

Consistent with previous findings (Mahour et al., 2008), NRBP1 and Cul2 did not detectably interact in cells in the absence of exogenously expressed TSC22DF members; however, assembly of NRBP1 with Cul2 was markedly increased by co-expression of TSC22D3 (Figure 1A). As TSC22DF proteins possess a leucine zipper (Gluderer et al., 2010), the effect of simultaneous co-expression of each TSC22DF protein with TSC22D3 was then analyzed. Assembly of NRBP1 with Cul2 was increased most prominently when TSC22D4 was also co-expressed (Figure 1A). Moreover, as NRBP1 has been reported to be able to homodimerize in cells (Lim et al., 2002), the effect of TSC22DF proteins on NRBP1 dimerization was also analyzed by co-transfecting differentially tagged versions of NRBP1. As shown in Figure 1B, NRBP1 dimer formation was augmented most prominently when both TSC22D3 and TSC22D4 were overexpressed.

We also observed that NRBP1 expression was increased when it was co-expressed with TSC22D4, suggesting interaction with TSC22D4 could enhance the stability of NRBP1. Indeed, cycloheximide chase analysis demonstrated that although TSC22D3 had little effect on the stability of NRBP1, TSC22D4 markedly increased its stability, while the stability of Cul2 was increased when both TSC22D3 and TSC22D4 were co-expressed (Figure 1C). Together, these findings suggest that TSC22D3 and TSC22D4 appear to serve as molecular chaperones to promote NRBP1-Cul2 assembly by different mechanisms. TSC22D3 seemed to have either induced conformational changes in NRBP1 that increased the affinity for Cul2 or stabilized the interaction between NRBP1 and Cul2, whereas TSC22D4 facilitated the dimerization of NRBP1 and protected it from degradation, thus increasing the amount of NRBP1 available for Cul2 binding.

Isolation of BRI Family Proteins BRI3 and BRI2 as Substrates for NRBP1-Ubiquitin Ligase

Immediately downstream of its BC-box, NRBP1 contains sequences distantly related to the canonical Cul2-box consensus \( ^{\phi}P\text{xx}^{\phi}P\text{xx}^{\phi} \), which in other BC-box proteins has been shown to specify assembly into Cul2-based ubiquitin ligases (Nguyen et al., 2015; Figure S1A). We generated a Cul2-box point mutant NRBP1[I348A;P349A;L353A] (NRBP1-IPL), which contains alanine substitutions of predicted key Cul2-box residues. To screen for substrates of NRBP1-ubiquitin ligase, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of peptides prepared using the dual-enrichment method using TR-TUBE and anti-diGly antibody (Yoshida et al., 2015) from cells co-expressing HA-TR-TUBE, TSC22D3, TSC22D4, and FLAG-tagged wild-type (WT) NRBP1 or NRBP1-IPL (Figure 2A). We selected 16 proteins as substrate candidates because their numbers of spectral counts matched to ubiquitinated peptides (Ub-peptides) increased in WT NRBP1-expressing cells but decreased in NRBP1-IPL-expressing cells (Figure 2B; Table S1). We then performed immunoblotting to evaluate the ability of NRBP1 to ubiquitinate these candidates. Out of these 16 proteins, BRI3 (also known as ITM2C) was the only candidate whose ubiquitination was clearly
detected in cells co-expressing WT NRBP1 and TR-TUBE but not in cells co-expressing NRBP1-IPL and TR-TUBE (Figure 2C). Interaction between ectopically expressed NRBP1 and BRI3 in HEK293T cells was also examined by co-immunoprecipitation analysis. As shown in Figure 2D, substantially less BRI3 co-immunoprecipitated with WT NRBP1 than with NRBP1-IPL, as would be expected if WT NRBP1 targets BRI3 for ubiquitination and degradation by the proteasome more efficiently than NRBP1-IPL.

BRI3 is a member of a BRI gene family comprising the homologs BRI1, BRI2, and BRI3 in both mice and humans (Vidal et al., 2001). BRI2 and BRI1 share 43.7% and 38.3% amino acid sequence identity, respectively, with BRI3 (Figure S1B). We therefore checked whether BRI1 and BRI2 are also the NRBP1 substrates. Co-immunoprecipitation analysis verified that ectopically expressed BRI2, but not BRI1, interacts with NRBP1 in HEK293T cells. As with BRI3, less BRI2 co-immunoprecipitated with WT NRBP1 than with NRBP1-IPL (Figure 2E). Moreover, substantially more ubiquitinated BRI2 was detected in cells co-expressing WT NRBP1 and TR-TUBE than in cells co-expressing NRBP1-IPL and TR-TUBE (Figure 2F). As mutations in the BRI2 gene lead to the production of variant proteins FBD-BRI2 and FDD-BRI2, which are 11 amino acids longer than WT BRI2 and cause familial dementias FBD and FDD, respectively (Vidal et al., 1999, 2000), we checked whether these two mutant proteins are also NRBP1 substrates. Both FBD-BRI2 and FDD-BRI2 interacted with NRBP1-IPL as well as WT BRI2, and nearly equivalent amounts of ubiquitination of these proteins as that of WT BRI2 were observed in cells expressing WT NRBP1 and TR-TUBE (Figure S2).

We then examined whether NRBP1 affects the stability of the expressed BRI2 and BRI3 proteins in cells. Cycloheximide chase experiments revealed that the stability of both BRI2 and BRI3 was decreased in cells overexpressing WT NRBP1 compared...
with cells overexpressing NRBP1-IPL (Figure 2G). Furthermore, the stability of BRI2 and BRI3 was increased in NRBP1-depleted cells compared with mock-depleted cells (Figure 2H). Taken together, these results are consistent with the idea that an NRBP1-containing ubiquitin ligase ubiquit.ates both BRI2 and BRI3 and regulates their turnover in cells.

Both Cul2 and Cul4A Are Involved in NRBP1-Mediated Ubiquitination of BRI2 and BRI3

NRBP1 contains a sequence element similar to a Cul2-box and associates with Cul2 in the presence of TSC22D3 and TSC22D4 (Figure 1A). We therefore examined whether (1) Cul2 contributes to NRBP1-dependent BRI2 and BRI3 ubiquitination and (2) other Cullin subtypes might also do so using various dominant-negative (DN) Cullins that lack the regions responsible for E2 ubiquitin-conjugating enzyme recruitment and thus are catalytically inactive (Jin et al., 2005). In these experiments, we performed in vivo ubiquitination assays by using TR-TUBE enrichment with cells stably expressing WT-NRBP1 or NRBP1-IPL with HA-TR-TUBE, and BRI2 or BRI3. Forty-eight h after transfection, cells were lysed and immunoprecipitated with anti-HA or anti-FLAG antibody, and the resulting precipitates as well as the original cell lysates were separated using SDS-PAGE and immunoblotted with the indicated antibodies. (C) Ubiquitinated BRI3 is accumulated in the presence of WT NRBP1. (D) Interaction of NRBP1 with BRI3. (E) Interaction between NRBP1 and BRI2. (F) Ubiquitinated BRI2 is accumulated in the presence of WT NRBP1. (G and H) HEK293T cells stably expressing TSC22D3, TSC22D4, and either FLAG-tagged NRBP1 WT or IFL with HA-TR-TUBE. See also Figures S1 and S2.
ubiquitination of both BRI2 and BRI3 was dramatically reduced by co-expression of not only DN-Cul2 but also DN-Cul4A. We also asked whether overexpression of Cul2 and Cul4A affects BRI2 and BRI3 ubiquitination. The ubiquitination of BRI2 and BRI3 was indeed strongly enhanced by co-expression of either Cul2 or Cul4A (Figure 3B). Both the DN-Cul2- and DN-Cul4A-dependent reduction and Cul2- and Cul4A-dependent enhancement of these ubiquitination events were also observed in cells in which NRBP1 was not exogenously expressed (Figures 3C and 3D). These observations suggest that BC-box protein NRBP1 uses not only Cul2, which has long been known to act with BC-box-containing substrate receptors, but, surprisingly, also with Cul4A, to regulate the ubiquitination of both BRI2 and BRI3.

**Dimerized NRBP1 Bridges the Formation of a Cul2/Cul4A Dimeric E3-Ligase Complex**

Because our data support a role for both Cul2 and Cul4A in NRBP1-mediated ubiquitination of BRI2 and BRI3, we asked whether NRBP1 is also able to assemble with Cul4A. As shown in Figure 4A, NRBP1 and Cul4A did not detectably interact in cells in the absence of exogenously expressed TSC22DF proteins; however, assembly of NRBP1 with Cul4A and DDB1 was markedly increased by co-expression of TSC22D3 and TSC22D4. One of the substrate recognition subunits of CRL4, DDB1/Cul4-associated factor 1 (DCAF1), has been reported to dimerize through a LIS1 homology (LisH) motif, L-X2-L-X3-L-X3-L, which is present in numerous eukaryotic proteins and is frequently located N-terminal to WD40 motifs (Ahn et al., 2011). NRBP1 has also been shown to be capable of homodimerizing through amino acid residues 406–479 (Lim et al., 2002). Although NRBP1 does not possess sequences similar to WD40 domain, residues 465–476 of NRBP1 contain a putative LisH motif (Figure 4B). In order to examine whether these Leu-repeats in NRBP1 are important for its dimerization, we constructed two NRBP1 point mutants, NRBP1[L465E;L468E;L472E;L476E] (LisH-M1) and NRBP1[L464E;L465E;L466E;L468E;L472E;L476E] (LisH-M2), and three deletion mutants, NRBP1[Δ(464-476)], NRBP1[Δ(466-485)], and NRBP1[Δ(406-479)] (Figure 4B). Co-immunoprecipitation analysis of each of these mutants with V5-tagged WT NRBP1
demonstrated that all of these LisH mutants were impaired in their ability to interact with WT NRBP1 (Figure 4C; Figure S3), suggesting that LisH domain in NRBP1 and its mutants, LisH-M1 and LisH-M2, analyzed in this study. The LisH motif, L-X2-L-X3-5-L-X3-5-L, is displayed at the top. X represents any amino acid.

As NRBP1 is able to homodimerize and also capable of assembling with both Cul2 and Cul4A, it is possible that NRBP1 could unite Cul2 and Cul4A to form a Cul2/Cul4A dimeric E3-ligase complex. To test this possibility, differentially tagged versions of Cul2 and Cul4A were co-immunoprecipitated in the presence of TSC22D3 and TSC22D4 in combination with NRBP1 WT or dimerization-defective LisH-M2. As shown in Figure 4D, Cul2 and Cul4A interacted in the presence of NRBP1 WT, but not LisH-M2 mutant. HEK293T cells were transfected with vectors expressing TSC22D3, TSC22D4, and the indicated combinations of HA- or Myc-tagged Cullins, FLAG-tagged NRBP1 WT, or LisH-M2.

In (C) and (D), cells were treated with 10 μM MG132 for 12 h, then lysed and immunoprecipitated with anti-V5 (C) or anti-HA (D) antibody thirty-six h after transfection. In (A), (C), and (D), immunoprecipitates as well as the original cell lysates were separated using SDS-PAGE and immunoblotted with the indicated antibodies.

See also Figure S3.

**Figure 4. Dimerized NRBP1 Bridges the Assembly of a Cul2- and Cul4A-Containing Heterodimeric E3-Ligase Complex**

(A) NRBP1 interacts with Cul4A in the presence of TSC22D3 and TSC22D4. HeLa cells were transfected with the indicated combinations of FLAG-NRBP1, HA-Cul4A, and TSC22D4-Myc constructs. Forty-eight h after transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody.

(B) Amino acid sequences of putative LisH domain in NRBP1 and its mutants, LisH-M1 and LisH-M2, analyzed in this study. The LisH motif, L-X2-L-X3-5-L-X3-5-L, is displayed at the top. X represents any amino acid.

(C) The putative LisH motif at the C terminus contributes to dimerization of NRBP1. HEK293T cells were transfected with vectors expressing V5-NRBP1, TSC22D3, and TSC22D4 in combination with FLAG-tagged NRBP1 WT or the indicated mutants.

(D) Cul2 and Cul4A interact in the presence of NRBP1 WT, but not LisH-M2 mutant. HEK293T cells were transfected with vectors expressing TSC22D3, TSC22D4, and the indicated combinations of HA- or Myc-tagged Cullins, FLAG-tagged NRBP1 WT, or LisH-M2.

In (C) and (D), cells were treated with 10 μM MG132 for 12 h, then lysed and immunoprecipitated with anti-V5 or anti-HA antibody thirty-six h after transfection. In (A), (C), and (D), immunoprecipitates as well as the original cell lysates were separated using SDS-PAGE and immunoblotted with the indicated antibodies.

Dimer Formation and Assembly of NRBP1 with Cul2 and Cul4A Contribute to Ubiquitination of BRI2 and BRI3

In CRL4A ubiquitin ligases, WD40 motifs and a short α-helical motif called the H-box in substrate receptor DCAFs are involved in interactions with the adaptor protein DDB1, which bridges the assembly of DCAFs with Cul4A (Zimmerman et al., 2010). Although NRBP1 does not possess WD40 motifs, residues 331–343 of NRBP1, which include a canonical BC-box element, also contain potential H-box motif sequences (Figure 5A). Notably, potential H-box sequences are also present in the BC-box of another substrate receptor for CRL2, FEM1B (Figure 5A). In order to test the functional significance of these putative H-boxes in NRBP1 and FEM1B and to identify NRBP1 mutants that are selectively impaired in their ability to assemble with either Cul2 or Cul4A, we constructed three more NRBP1 mutants: NRBP1[H339E] (H-box-M1), in which histidine 339 was substituted for glutamic acid because changing the charge at position 9 of H-box motif in HBx and DCAF1 interfered with their abilities to bind to DDB1 (Gérard et al., 2014; Li et al., 2014).
2010); NRBP1[L336P;H339E] (H-box-M2), in which leucine 336 was additionally mutated to proline to completely disrupt the predicted α-helix; and H-box-M3, in which the putative H-box of NRBP1 was replaced by a sequence that is almost identical to the putative H-box of FEM1B (residues 595–607) (Figure 5A).

To prevent ectopically expressed NRBP1 with WT or mutant H-box like elements from dimerizing with endogenous NRBP1, we used monomeric NRBP1 constructs that lack residues 406–479 and hence cannot dimerize. Co-immunoprecipitation analysis of the monomeric form of each NRBP1 mutant with HA-tagged Cul2 or Cul4A revealed that NRBP1[D forty-six forty-seven] and monomeric H-box-M3 assembled with both Cul2 and Cul4A (Figure 5B). In contrast, NRBP1[D forty-six forty-seven] with the H-box-M1 mutation bound to Cul2 but exhibited reduced binding to Cul4A, while NRBP1[D forty-six forty-seven] with the H-box-M2 mutation was profoundly impaired in its ability to assemble with both Cul2 and Cul4A (Figure 5B). As expected, monomeric NRBP1-IPL was selectively impaired in its ability to assemble with Cul2 (Figure 5B). These results suggest that potential H-box sequences of NRBP1 and FEM1B are indeed responsible for assembling with Cul4A.

As NRBP1 can dimerize and assemble with both Cul2 and Cul4A in the presence of TSC22D3 and TSC22D4, it seems likely that various NRBP1-containing CRL complexes, such as monomeric CRL2 or CRL4A, homodimeric CRL2 or CRL4A, and heterodimeric CRL2/CRL4A, could be formed in cells. To investigate whether dimerization or assembly with Cul2 or Cul4A is required for NRBP1-based CRL to ubiquitinate BRI2 and BRI3, we performed in vivo ubiquitination assays using TR-TUBE with cells co-expressing TSC22D3, TSC22D4, and BRI2 or BRI3.
combination with NRBP1 WT or mutants. Although apparently increased accumulation of the ubiquitinated BRI2 and BRI3 was observed in cells co-expressing WT NRBP1, either no accumulation or substantially less accumulation of the ubiquitinated BRI2 and BRI3 was observed in cells co-expressing LisH-M2 or NRBP1-IPL, H-box-M1, H-box-M2, and H-box-M3, respectively (Figure 5C). The reason why NRBP1-IPL and H-box-M1 showed slightly more accumulation of ubiquitinated BRI2 and BRI3 than H-box-M2 and LisH-M2 is most likely that the former at least partially heterodimerized with endogenous NRBP1 in cells, where NRBP1-IPL or H-box-M1 and each dimerization partner could assemble with Cul4A or Cul2, and Cul2 or Cul4A, respectively, and exert E3-ligase activity. Taken together, these results therefore suggest that not only the dimerization but also the assembly with both Cul2 and Cul4A are important for NRBP1 and that NRBP1-mediated heterodimeric CRL selectively targets BRI2 and BRI3 for ubiquitination-dependent degradation (Figure 5D). In addition, the difference in the sequences of BC- and H-boxes between NRBP1 and FEM1B appears to make a substantial contribution to NRBP1’s E3-ligase activity, as H-box-M3 was impaired in its ability to support BRI2 and BRI3 ubiquitination, even though it can assemble with both Cul2 and Cul4A.

We next examined whether dimerization of NRBP1 affects substrate recognition. To prevent ubiquitination and degradation of ectopically expressed BRI2 and BRI3, cells were treated with MG132. Co-immunoprecipitation analysis of NRBP1 WT with or without LisH mutation with either BRI2 or BRI3 was performed. As shown in Figure S4, NRBP1 WT and LisH-M2 showed comparable levels of binding to BRI2 and BRI3, suggesting that NRBP1 dimerization has little effect on its affinity for BRI2 and BRI3.

The C Terminus of NRBP1 and Central Regions of BRI2 and BRI3 Are Important for Their Interaction

To investigate the NRBP1 regions required for interaction with its substrates, a series of Flag-tagged NRBP1 deletion mutants were generated and examined for their abilities to associate with VSV-G-tagged BRI2 or BRI3. A C-terminal deletion mutant Δ(328–535) failed to bind to BRI3, and N-terminal deletion mutants Δ(2–72), Δ(2–327), and Δ(2–405) were all capable of binding to BRI3, indicating that the N terminus of NRBP1 is not required and that residues 406–535 are sufficient for the interaction. However, an NRBP1 mutant Δ(406–535) is still capable of binding to BRI3, suggesting that at least two regions in residues 328–405 and 406–535 contribute to the interaction (Figure 6A). Similar results were also obtained with BRI2 (Figure S5). We further examined the interaction between a series of BRI2 and BRI3 deletion mutants with VSV-G-tag and Flag-tagged NRBP1. BRI3 internal deletion mutants such as Δ(91–120), Δ(121–150), and Δ(181–210) failed to bind to NRBP1. Further analysis using a series of mutants with smaller deletions demonstrated that two central portions of BRI3, residues 121–140 and 191–210, are important for the interaction (Figure 6B). In addition, those truncated deletion mutants composed of residues 61–135 and 81–210 or residues 61–136 and 77–210 of BRI3 or BRI2, respectively, retained their abilities to bind to NRBP1 (Figure 6C), suggesting that the luminal portion of each protein is important and that the C-terminal peptide portion, which is secreted after cleavage by furin protease, is not required for the interaction.

NRBP1 Controls APP Processing by Targeting BRI2 and BRI3 for Degradation

As the overexpression of NRBP1 led to ubiquitination of BRI2 and BRI3 in the presence of TSC22D3 and TSC22D4 and the proteins related to NRBP1 CRL were detectably expressed in lysates prepared from the primary culture of mouse cortical neurons (Figure 7A), we next examined whether the knockdown of endogenous NRBP1 affects APP processing and the production of Aβ from F11 neuronal cells. F11 cells, rat primary cultured neurons immortalized by fusion with mouse neuroblastoma cell line N18TG2, are considered to be an excellent model for primary cultured neurons (Patlaka et al., 1985). F11 cells were transfected with control DsiRNA or an NRBP1 DsiRNA that could target both rat and mouse isoforms. Forty-eight h after DsiRNA transfection, culture media were replaced and cells were further incubated for 24 h, and cell lysates and conditioned media were prepared. DsiRNA-mediated depletion of NRBP1 resulted in an increase in the amounts of endogenous BRI2 and BRI3 (Figure 7B). The levels of secreted Aβ40, Aβ42, sAPPα, sAPPβ, and sAPPγ were significantly decreased, and the levels of α-CTF and β-CTF in the lysates were apparently decreased upon depletion of NRBP1, while the levels of APP were unaltered (Figures 7C and 7D). These observations suggest that in neuronal cells, APP cleavage by β- and γ-secretase, and perhaps cleavage of β-CTF by γ-secretase as well, is fine-tuned by endogenous levels of NRBP1. To exclude the possibility of off-target effects of the DsiRNA, we performed rescue experiments by overexpressing an NRBP1 DsiRNA-resistant form of human NRBP1 cDNA (NRBP1Dsi-R), the nucleotide sequence of which differs in 6 of 21 target nucleotides from rat and mouse isoforms. The NRBP1 knockdown-dependent increase of endogenous BRI2 and BRI3 and decrease in the levels of Aβ40, Aβ42, sAPPα, and sAPPγ in the media and α-CTF in the lysates were rescued by NRBP1Dsi-R overexpression (Figures 7B–7D). Taken together, our results suggest that NRBP1 controls Aβ production by regulating turnover of BRI2 and BRI3 in neuronal cells.

DISCUSSION

NRBP1 is an evolutionarily conserved adaptor protein that plays important roles in cellular homeostasis. NRBP1 carries a putative binding domain for Src homology 2 (SH2)-containing proteins, as well as binding sites for the myeloid leukemia factor 1 (MLF1), Elongin BC, and TSC22DF (Gluderer et al., 2010; Kerr and Wilson, 2013; Lim et al., 2002). Recently, NRBP1 has been reported to play a tumor-suppressive or tumor-promoting role in cancer depending on tumor types, but its precise functions and underlying mechanisms remain largely unknown (Ruiz et al., 2012; Wilson et al., 2012). Here, we have shown that NRBP1 homodimerizes and assembles with not only Cul2 but also Cul4A and supports ubiquitination of BRI2 and BRI3. Our findings demonstrate that a functional heterodimeric CRL complex can be formed through homodimerization of a substrate receptor. The BC-box, an ~12 amino acid degenerate sequence motif with consensus x(T,S,P)Lxxx(C,A,S)xxxx, is shared by the
Figure 6. Interaction between NRBP1 and BRI2 and BRI3

(A) Left: schematic diagram of 3 x FLAG-tagged NRBP1 deletion mutants. Right: cell lysates of Sf9 cells expressing VSV-G-tagged BRI3 WT and 3 x FLAG-tagged NRBP1 WT or deletion mutants were immunoprecipitated with anti-VSV-G antibody.

(B) Left: schematic diagram of VSV-G-tagged BRI3 internal deletion mutants.

(C) Left: diagram of VSV-G-tagged BRI3 and BRI2 N- and C-terminal deletion mutants. (B and C) Right, cell lysates of Sf9 cells expressing 3 x FLAG-tagged NRBP1 WT and VSV-G-tagged WT or deletion mutant forms of BRI3 or BRI2 were immunoprecipitated with anti-FLAG antibody. (A–C) Immunoprecipitates as well as the original cell lysates were separated using SDS-PAGE and immunoblotted with the indicated antibodies. See also Figure S5.
substrate receptors of CRL2 and CRL5 ubiquitin ligases (Mahrou et al., 2008). Analysis of the crystal structures of the ternary complexes of BC-box proteins with Elongin BC has revealed that binding of Elongin BC to BC-boxes, which adopt a helical structure, is governed by interaction of an invariant leucine at the N terminus of the BC-box with a hydrophobic pocket created by residues in the C-terminal half of Elongin C (Bullock et al., 2006; Stanley et al., 2008; Stebbins et al., 1999). The H-box is a poorly conserved 13 amino acid motif with consensus sequence \( x_f x_f x_f x_f x_f x_f (R, H, Q)x_f (V, L, I)x_f \) (Gérard et al., 2014). The crystal structure of DDB1 in complex with peptides corresponding to the H-box sequences of the hepatitis B virus X protein (HBX), woodchuck hepatitis virus X protein (WHX), or the DCAF9 has revealed that all these peptides adopt the same helical conformation and insert themselves into a deep pocket created by the BPA and BPC \( \beta \)-propeller domains of DDB1 (Li et al., 2010). The identification of H-box motifs overlapping the BC-boxes of NRBP1 and FEM1B raises the possibility of the existence of unrecognized H-boxes in CRL substrate receptors other than those found in well characterized CRL4s. Indeed, we have noticed that potential H-box sequences overlap the BC-boxes of a number of CRL2 and CRL5 substrate receptors (Figure S6). Therefore, we hypothesized that these BC-boxes could bind DDB1. To test this hypothesis, structural models of the NRBP1 BC-box in complex with either Elongin C or DDB1 were generated using the crystal structures of the VHL-Elongin BC and DCAF6-DDB1 complexes, respectively. The models suggest that the NRBP1 BC-box can form proper hydrophobic interactions not only with Elongin C (Figure S7A) but also with DDB1 (Figure S7B), without changing its conformation (Figure S7C). Moreover, superposition of the BC-box models of selected CRL2/CRL5 substrate receptors including FEM1B (15 proteins listed in Figure S6) suggests that all of them...

Figure 7. NRBP1 Controls APP Processing by Targeting BRI2 and BRI3 for Degradation

(A) Both the components and substrates of NRBP1 CRL are detectably expressed in neurons. Cell lysates prepared from the primary culture of mouse cortical neurons were separated using SDS-PAGE and immunoblotted with the indicated antibodies.

(B–D) Knockdown of NRBP1 increases the amounts of endogenous BRI2 and BRI3 and blocks both amyloidogenic and non-amyloidogenic pathways in neuronal cells. F11 cells were transfected with control DsiRNA, NRBP1 DsiRNA, or NRBP1 DsiRNA plus NRBP1

\(^{Δ\text{N}}\) construct. Forty-eight h after DsiRNA transfection, culture media was replaced. Cells were grown an additional 24 h, and cell lysates and conditioned media were prepared. As controls, F11 cells were treated with 1 \( \mu \)M DAPT (C) or 2 \( \mu \)M BACE1 inhibitor C3 (D) for 24 h, and cell lysates and conditioned media were prepared.

(B) NRBP1, BRI2, and BRI3 in the lysates were analyzed using immunoblotting.

(C) NRBP1, APP, and APP-CTFs in the lysates were analyzed using immunoblotting, and secreted A\( β_{40} \) and A\( β_{42} \) in the media were measured using ELISA. To analyze the levels of APP-CTFs, cell lysates were separated using Tricine-SDS-PAGE. For APP-CTFs, a longer exposure image is shown. The asterisk indicates a non-specific band.

(D) NRBP1 and APP in the lysates and total sAPP (sAPP\( α + sAPPβ \)), detected by 22C11) in the media were analyzed using immunoblotting, and secreted sAPP\( α \) and sAPP\( β \) in the media were measured using ELISA. Raw values obtained from ELISA were normalized to protein concentrations of total lysates from transfected cells.

In (C) and (D), data represent the average of four biological replicates (mean ± SEM; \( * p < 0.005 \) and \( ** p < 0.001 \)). In (A–D), \( β \)-tubulin was used as a loading control.
would adopt a similar helical structure and would interact with DDB1 in exactly the same manner as does DCAF6 (Figure S7D). Taken together, these findings suggest that additional CRL2 or CRL5 substrate receptors besides NRBP1 may be able to dimerize and, through their BC-boxes, form Cul2/Cul4- or Cul5/Cul4-containing heterodimeric CRL complexes to regulate the turnover of their respective targets.

How, then, might dimerization and assembly of NRBP1 with both Cul2 and Cul4A help activate its E3-ligase function? A growing body of evidence indicates that a number of CRLs form functional dimers via their substrate receptors. In addition to neddylation, dimerization is a prerequisite for the full ubiquitination function of at least some CRLs (Bosu and Kipreos, 2008; Merlet et al., 2009). CRL dimerization may enhance activity by increasing the local concentration of reactants needed for ubiquitination, including substrate and ubiquitin-activated E2, or by optimally positioning a substrate-binding site and the two docking sites of activated E2. Our findings (1) that NRBP1 dimerization does not increase the affinity for substrates BR12 and BR13 and (2) that only the NRBP1-based Cul2/Cul4A-containing CRL was able to efficiently ubiquitinate BR12 and BR13, even though NRBP1 also forms a CRL complex containing Cul2/Cul2 or Cul4A/Cul4A, suggest that NRBP1-ubiquitin ligase activity is predominantly controlled by the latter mechanism. Indeed, the crystal structures of various Cullins have shown that there are flexible hinges in Cullin N-terminal domains and that the degrees of flexibility and ranges of E2-substrate distance are distinct among Cullin types (Cardote et al., 2017; Liu and Nussinov, 2011). Moreover, while a small binary Elongin BC complex serves as an adaptor for CRL2, CRL4A uses as its adaptor the 127 kDa DDB1, which consists of three WD40 β-propeller domains (BPA, BPB, and BPC) and a helical C-terminal domain (Zimmerman et al., 2010). The crystal structure of CRL4A has revealed that the interface between the substrate receptor binding module (BPA-BPC) and the Cul4A binding domain (BPB) is quite flexible, with a range of rotation of at least 150° (Scrima et al., 2008; Zimmerman et al., 2010). The different degrees of rotational and torsional flexibility between CRL2 and CRL4A could help the NRBP1-ubiquitin ligase to accommodate different distances between the two ubiquitin-activated E2 and acceptor lysines in both substrate and the elongating ubiquitin chain, thereby enabling it to target a large area centered around the captured substrate and increasing the rate of the ubiquitination reaction. It is therefore tempting to speculate that one of the CRLs, perhaps CRL2, might be responsible for adding monoubiquitin or the first few proximal ubiquitins of the growing polyubiquitin chain to substrate, whereas more distal ubiquitination events may be driven by the other CRL (Figure 5D).

TSC22DF proteins are putative transcription factors characterized by a carboxy-terminal leucine zipper and an adjacent TSC-box (Gluderer et al., 2010). TSC22DF proteins are ubiquitously expressed and control multiple biological processes, including cell proliferation, differentiation, senescence, apoptosis, and embryonic development (Ayroldi and Riccardi, 2009; Gluderer et al., 2010; Hönig-Hölzel et al., 2011; Kester et al., 1999). Little is known about molecular functions of TSC22DF proteins. BunA, a Drosophila TSC22D1.1 homolog, has been shown to be capable of increasing the localization of Madm, an NRBP1 homolog, in the Golgi apparatus in Drosophila cells (Gluderer et al., 2010). Here, we have demonstrated that the TSC22DF proteins TSC22D3 and TSC22D4 seem to serve a chaperone-like role in promoting the assembly of NRBP1-mediated Cul2/Cul4A dimeric CRL complex. Regulation of CRL assembly by chaperone-like proteins is not unprecedented; for example, cofactor CBFβ acts as a molecular chaperone to help HIV-1 Vif to fold into proper conformation, allowing it to interact with Cul5 (Han et al., 2014).

BR12 and BR13 are members of a BRI gene family with single transmembrane domains, extracellular BRICHOS domains, and furin-like protease cleavage sites near their C termini (Vidal et al., 2001). Besides their roles in APP processing, BR12 is capable of not only reducing cellular BACE1 levels (Tsachaki et al., 2013) but also increasing the secretion of IDE (Kilger et al., 2011), which is able to degrade Aβ and monomeric Ab3 and ADan peptides (Morelli et al., 2005). Furthermore, the 23 amino acid C-terminal peptide, BR12-23, and the BR12 BRICHOS domain, which are released upon BR12 processing, are both able to inhibit Aβ aggregation, leading to delayed Aβ fibrillation and subsequent formation of Aβ plaques (Kim et al., 2008; Willander et al., 2012). In addition, though aggregation of islet amyloid polypeptide (IAPP) into amyloid fibrils in islets of Langerhans is the pathological hallmark of type 2 diabetes mellitus and is also believed to be related to the pathogenesis of AD in diabetics, the BR12 BRICHOS domain has recently been reported to be able to effectively inhibit IAPP oligomerization and toxicity in pancreatic β cells (Oskarsson et al., 2018; Zhang and Song, 2017). In this study, DsirRNA-mediated depletion of NRBP1 in neuronal cells resulted in an increase in the amounts of endogenous BR12 and BR13 and successfully reduced the production of Aβ. Although there is no direct evidence that NRBP1-ubiquitin ligase activity is elevated in AD, it is noteworthy that extracellular BR12 deposits associated with Aβ plaques are increased in early stages of AD and in the hippocampus of AD patients, the amounts of BR12 associated with APP are decreased, which could lead to impaired BR12 functionality thereby promoting AD pathology (Del Campo et al., 2014). Thus, key interfaces between NRBP1 and its substrates BR12 and BR13 could be promising therapeutic targets, and elucidation of the structure of these interaction surfaces at the molecular level should contribute to the future pharmacological intervention against AD.

Numerous inhibitors targeting either γ-secretase or β-secretase have been developed to reduce the production and accumulation of Aβ in the brain (Briggs et al., 2016). However, both secretases are involved in the processing of a number of endogenous substrates important for physiological functions other than APP, and strong inhibition of either of the substrates causes serious adverse effects. Long-term use of γ-secretase inhibitor causes gastrointestinal toxicity and skin cancer, probably due to blockade of Notch signaling (De Strooper et al., 2012). In addition, completely inhibiting of β-secretase disrupts muscle spindles functions in adults, resulting in impairment of coordinated movement due to blockade of Neuregulin-1 (NRG1) signaling (Vassar et al., 2014). In contrast, BR12 and BR13 are capable of inhibiting APP processing, decreasing aggregation, and increasing clearance of Aβ, without interfering with the cleavage of other substrates by these secretases (Fotinopoulou et al., 2005; Kilger et al., 2011; Kim et al., 2013).
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AUTHOR CONTRIBUTIONS

T.Y., R.C.C., J.W.C., and T.A. designed the study. T.Y., A.T., and T.A. performed experiments and analyzed the data. C.T.-S. and S.S. participated in the study design. A.S., M.P.W., and L.F. performed mass spectrometry data collection and processing. T.T. and K.S. performed modeling and analysis of the complex structures. J.W.C., R.C.C., and T.A. wrote the manuscript with contributions from T.Y., A.S., and T.T. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-FLAG (M2) monoclonal antibody | Sigma | Cat# F1804; RRID: AB_262044 |
| anti-Myc (9B11) monoclonal antibody | Cell Signaling | Cat# 2276; RRID: AB_331783 |
| anti-HA (12CA5) monoclonal antibody | Roche | Cat# 1166606001; RRID: AB_514506 |
| anti-HA (3F10) monoclonal antibody | Roche | Cat# 11867431001; RRID: AB_390919 |
| anti-V5 (6F5) monoclonal antibody | Wako | Cat# 011-23596 |
| anti-NSV-G polyclonal antibody | Bethyl | Cat# A190-131A; RRID: AB_155862 |
| anti-NRBP1 (4D2) monoclonal antibody | Abnova | Cat# H00029959-M01; RRID: AB_464241 |
| anti-GILZ (G-5) monoclonal antibody | Santa Cruz | Cat# sc-133215; RRID: AB_2287737 |
| anti-TSC22D4 antibody | Aviva Systems Biology | Cat# ARP30107_T100; RRID: AB_841770 |
| anti-Cul2 (C-4) monoclonal antibody | Santa Cruz | Cat# sc-166506; RRID: AB_2230072 |
| anti-Cul4A antibody | GeneTex | Cat# GTX129459 |
| anti-Elong2 B antibody | Santa Cruz | Cat# sc-11447; RRID: AB_641309 |
| anti-ITM2B (C-8) monoclonal antibody | Santa Cruz | Cat# sc-374362; RRID: AB_10988049 |
| anti-ITM2C polyclonal antibody | abcam | Cat# ab101389; RRID: AB_2036745 |
| anti-APP (22C11) monoclonal antibody | Millipore | Cat# MAB348; RRID: AB_94882 |
| anti-APP C-Terminal antibody | Sigma | Cat# A8717; RRID: AB_258409 |
| anti-β-tubulin antibody | Sigma | Cat# T4026; RRID: AB_477577 |
| anti-FLAG M2 Affinity Gel | Sigma | Cat# A2220; RRID: AB_10063035 |
| anti-HA Affinity Matrix; (clone 3F10) antibody | Roche | Cat# 1815016001; RRID: AB_390914 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Hygromycin B | invitrogen | Cat# 10687-010 |
| Puromycin | Sigma | Cat# P8833 |
| MG132 | Boston Biochem | Cat# I-130 |
| cycloheximide | Sigma | Cat# C1988 |
| Complete Protease Inhibitor Cocktail | Roche | Cat# 11697498001 |
| Complete EDTA-free Protease Inhibitor Cocktail | Roche | Cat# 11873580001 |
| N-ethylmaleimide | Sigma | Cat# E3876 |
| PR-619 | LifeSensors | Cat# 59568 |
| HA peptide | Roche | Cat# 1666975 |
| Tris [2-carboxy-ethyl] phosphate hydrochloride (TCEP) | Thermo Fisher | Cat# 77720 |
| methylmethanethiosulfonate (MMTS) | Thermo Fisher | Cat# 23011 |
| Trypsin | Promega | Cat# V5280 |
| Papain | Wako | Cat# 164-00172 |
| DNase I | Roche | Cat# 776785 |
| N2 Supplement | Wako | Cat# 141-08941 |
| Cytosine-1-β-D-arabinofuranoside | Wako | Cat# 030-11951 |
| N-[25-(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethylethyl ester-glycine (DAPT) | Cayman | Cat# 13197 |
| BACE1 inhibitor C3 (β-Secretase Inhibitor IV) | Sigma | Cat# 565788 |
| **Critical Commercial Assays** | | |
| PTMScan Ubiquitin Remnant Motif (K-ε-GG) Kit | Cell Signaling | Cat# 5562 |
| QuickChange Lightning Site-Directed Mutagenesis Kit | Agilent Technologies | Cat# 210519 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Gibson Assembly Master Mix | New England BioLabs | Cat# E2611 |
| Human/Rat β Amyloid(40) ELISA Kit II | Wako | Cat# 294-64701 |
| Human/Rat β Amyloid(42) ELISA Kit | Wako | Cat# 290-62601 |
| Mouse sAPPβ-w Assay Kit | IBL | Cat# 27416 |
| Mouse/Rat sAPPα (highly sensitive) Assay Kit | IBL | Cat# 27419 |

Deposited Data

| MudPIT data | This paper | Table S1; MassIVE repository (ftp://massive.ucsd.edu/MSV000084419/) ProteomeXchange accession PXD015705 |
| Original mass spectrometry data | This paper | Stowers Original Data Repository (https://www.stowers.org/research/publications/libpb-1369) |

Experimental Models: Cell Lines

| HEK293T | ATCC | CRL-3216 |
| HeLa | ATCC | CCL-2 |
| F11 | Laboratory of Dr. Takako Niikura, ECACC | Cat# 08062601 |
| S19 | invitrogen | Cat# 11496-015 |

Experimental Models: Organisms/Strains

| Mouse: ICR | Japan SLC | SLC: ICR |

Oligonucleotides

| Sequences of NRBP1 DsRNA (RNA bases are upper case, DNA bases are lower case) | N/A |
| NRBP1 DsRNA sense strand 5' - GAAG AACAGAAGAACCACUACUUtt-3' | This paper N/A |
| NRBP1 DsRNA antisense strand 5' - AAAAGUGUAGGUUCUUCGUU CUUCCC-3' | This paper N/A |
| DS NC1 (negative control DsiRNA) | Integrated DNA Technologies | Cat# 51-01-14 |
| NRBP1 DsRNA target sequence mutagenesis: Forward Primer 5'- GTGAAGACTTGTCGAGA GGAGCAAAAAATCTACACTTCTTTGC-3' | This paper N/A |
| NRBP1 DsRNA target sequence mutagenesis: Reverse Primer 5'- GCAAAGAAGTGTAGATT TTTTTGCTCCTCTCGACAAGTCTTTCAC-3' | This paper N/A |

Recombinant DNA

| pcDNA3.1-BR13 | This paper | N/A |
| pcDNA3.1-BR12 | This paper | N/A |
| pcDNA3-HA-TR-TUBE | Yoshida et al., 2015 | N/A |
| pcDNA3-DN-CuI2-FLAG | This paper | N/A |
| pcDNA3-DN-CuI5-FLAG | This paper | N/A |
| pcDNA3-DN-hCuI3-FLAG | Jin et al., 2005 | Addgene Plasmid #15820 |
| pcDNA3-DN-hCuI4A-FLAG | Jin et al., 2005 | Addgene Plasmid #15821 |
| pcDNA3-DN-hCuI4B-FLAG | Jin et al., 2005 | Addgene Plasmid #15822 |
| pcDNA3-Myc-CuI2 | Laboratory of Dr. Yue Xiong | N/A |
| pcDNA3-Myc-CuI4A | Laboratory of Dr. Yue Xiong | N/A |
| pCl-neo-Myc-CuI5 | This paper | N/A |
| pCl-neo-HA-CuI2 | Laboratory of Dr. Takumi Kamura | N/A |
| pcDNA3-HA-CuI4A | Laboratory of Dr. Takumi Kamura | N/A |

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### LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Teijiro Aso (asot@kochi-u.ac.jp). Plasmids generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

#### Mice

Male and female ICR mice (12-15 weeks old) were purchased from Japan SLC. All mice were maintained on a 12 h light/dark cycle in a temperature-controlled pathogen-free animal facility at Kochi University with free access to food and water. All animal experiments

### Table: REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pQCXIP-TSC22D1-1-Myc | This paper | N/A |
| pQCXIP-TSC22D2-Myc | This paper | N/A |
| pQCXIP-TSC22D3-Myc | This paper | N/A |
| pQCXIP-TSC22D4-Myc | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1 | Mahrou et al., 2008 | N/A |
| pcDNA5/FRT-FLAG-NRBP1 truncations | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-IPL | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-LisH-M1 | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-LisH-M2 | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-H-box-M1 | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-H-box-M2 | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-H-box-M3 | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-IPL/Δ(406-479) | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-LisH-M1/Δ(406-479) | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-LisH-M2/Δ(406-479) | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-H-box-M1/Δ(406-479) | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-H-box-M2/Δ(406-479) | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1-H-box-M3/Δ(406-479) | This paper | N/A |
| pcDNA5/FRT-FLAG-NRBP1\(\text{Din}^{\text{R}}\) | This paper | N/A |
| pBacPAK-His1-NRBP1-3 × FLAG | This paper | N/A |
| pBacPAK-His1-NRBP1 truncations-3 × FLAG | This paper | N/A |
| pBacPAK-His1-BRI2-VSV-G | This paper | N/A |
| pBacPAK-His1-BRI2 truncations-VSV-G | This paper | N/A |
| pBacPAK-His1-BRI3-VSV-G | This paper | N/A |
| pBacPAK-His1-BRI3 truncations-VSV-G | This paper | N/A |

**Software and Algorithms**

- **ProLuCID**
  - Xu et al., 2015
  - [http://fields.scripps.edu/yates/wp/?page_id=17](http://fields.scripps.edu/yates/wp/?page_id=17)

- **DTASelect**
  - Tabb et al., 2002
  - [http://fields.scripps.edu/yates/wp/?page_id=17](http://fields.scripps.edu/yates/wp/?page_id=17)

- **Modeler 9.13**
  - Sali and Blundell, 1993
  - [https://salilab.org/modeler/](https://salilab.org/modeler/)

- **UCSF Chimera**
  - Pettersen et al., 2004
  - [https://www.cgl.ucsf.edu/chimera/](https://www.cgl.ucsf.edu/chimera/)

**Other**

- **GL-Tip SDB**
  - GL Sciences
  - Cat# 7820-11200

- **GL-Tip GC**
  - GL Sciences
  - Cat# 7820-11201

- **Protein A/G PLUS-Agarose**
  - Santa Cruz
  - Cat# sc-2003

- **FuGENE HD**
  - Promega
  - Cat# E2311

- **DharmaFECT1**
  - Dharmacon
  - Cat# T-2001
were performed in accordance with the Regulations for Animal Experiments at Kochi University and were approved by the Kochi University Animal Care and Use Committee.

Primary cortical neurons were prepared from cerebral cortices of embryonic day 17 (E17) ICR mice. The stage of mouse embryos was determined by taking the morning when the copulation plug was seen as E0.5. Seventeen-day pregnant ICR mice were sacrificed by CO2 inhalation, and 24 fetuses were removed and chilled to 4°C. Brain cortices were isolated from the fetuses, and were dissected and incubated with 0.25% papain (Wako), 0.01% DNase I (Roche) in Hank’s balanced salt solution (HBSS; Wako). Dissociated cells were then cultured on poly-L-lysine-coated 24-well plates in DMEM (Wako) containing N2 supplement (Wako) and 5 μM Cytosine-1-β-D-arabinofuranoside (AraC; Wako) as described previously (Sudo et al., 2000). By this procedure, neurons represent more than 98% of the cells present in cultures.

**Cell culture**

HeLa and HEK293T cells were maintained in DMEM (Wako) containing 10% FBS and were transfected with indicated plasmids using FuGENE HD (Promega) according to the manufacturer’s instructions. F11 cells, rat dorsal root ganglia neurons immortalized by fusion with mouse neuroblastoma cell line N18TG2 (Platika et al., 1985), were maintained in Ham’s F-12 (Wako) containing 2 mM Glutamine and 18% FBS. Cells were collected and lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol, and the Complete Protease Inhibitor Cocktail (Roche).

For *in vivo* ubiquitination assay using TR-TUBE, cells were lysed in the presence of 1 mM N-ethylmaleimide (Sigma) and 20 μM PR-619 (LifeSensors) to inhibit deubiquitinase activity. Lysates were centrifuged at 10,000 × g for 20 min at 4°C.

To inhibit cellular proteasome activity, cells were treated with 10 μM MG132 (Boston Biochem) 12 hr before harvesting or 60 μM MG132 6 hr before harvesting. For cycloheximide chase assay, cells were cultured in the presence of 25 or 40 μg/ml cycloheximide (Sigma) for the indicated period of time. To inhibit γ-secretase or β-secretase activity, cells were treated with 1 μM N-[2S-(3,5-difluorophenyl)acetyl]-L-allyl-2-phenyl-1,1-dimethylethyl ester-glycine (DAPT; Cayman) or 2 μM BACE1 inhibitor C3 (Sigma), respectively, 24 hr before harvesting.

SF9 cells were cultured at 27°C in SF-900 II SFM (Invitrogen) with 10% FBS and kanamycin (100 μg/ml) and infected with the recombinant baculoviruses indicated in the figures. Seventy-two hours after infection, cells were collected and lysed in ice-cold buffer containing 40 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol, and the Complete Protease Inhibitor Cocktail, and centrifuged at 10,000 g for 20 min at 4°C.

**METHOD DETAILS**

**Plasmids**

N-terminal or C-terminal V5-tagged, and untagged human BRI3, BRI2, and BRI1, and N-terminal V5-tagged human NRBP1 were subcloned into pcDNA3.1/Hygro (+) (Invitrogen). C-terminal FLAG-tagged DN-Cul2 and DN-Cul5 were subcloned into pcDNA3. N-terminal or C-terminal V5-tagged, and untagged human BRI3, BRI2, and BRI1, and N-terminal V5-tagged human NRBP1 were subcloned into pQCXIP (Clontech). Mammalian expression vectors for mutant NRBP1-IPL, LisH-M1, LisH-M2, H-box-M1, H-box-M2, H-box-M3, TSC22D4, containing Myc tag at their C-termini were subcloned into pCI-neo (Promega). Mouse TSC22D1-1, human TSC22D2, mouse TSC22D3, and human TSC22D4-Myc and pQCXIP-TSC22D4-Myc and puromycin selection.

Generation of cell lines stably expressing WT or mutant NRBP1 with TSC22D3/D4

HEK293T cells stably expressing FLAG-tagged NRBP1 WT or IPL with TSC22D3/D4 were generated by transfection of pcDNA5/FRT—FLAG-NRBP1 WT or pcDNA5/FRT—FLAG-NRBP1 IPL and hygromycin selection, and subsequently transfections of pQCXIP-TSC22D3-Myc and pQCXIP-TSC22D4-Myc and puromycin selection.

**Immunoprecipitation and western blotting**

Lysates of HeLa, HEK293T cells or baculovirus-infected SF9 cells were incubated with the antibodies in the figures for 1 hr at 4°C and then with protein A/G PLUS-agarose (Santa Cruz) for 1 hr at 4°C, or incubated with anti-FLAG M2-conjugated agarose (Sigma) or anti-HA (3F10)-conjugated agarose (Roche) for 2–12 hr at 4°C. Beads were washed five times in buffer containing 40 mM HEPES-NaOH (pH 7.9), 150–1000 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 10% glycerol.
Immunoprecipitated proteins, cell lysates, or conditioned media were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For detection of APP-CTFs, lysates were separated by Tricine-SDS-PAGE. Proteins were transferred to a polyvinylidenefluoride membrane (Millipore), and analyzed by immunoblotting with the antibodies indicated in the figures. The antibodies used in this study were listed in the Key Resources Table. Immunoblots were visualized either with Western Lightning Plus-ECL (Perkin Elmer), Immobilon Western (Millipore), or SuperSignal West Femto chemiluminescent reagent (Pierce) according to the manufacturer’s instructions.

**Immunoprecipitation of diGly-containing peptide**

Ubiquitinated peptides were enriched using the PTMScan Ubiquitin Remnant Motif (K-GG) Kit (Cell Signaling). Briefly, eluted peptides were dried by vacuum centrifugation, dissolved in 0.6 mL of IAP buffer [50 mM Mops (pH 7.2), 10 mM Na۲HPO۴, 50 mM NaCl], adjusted to pH 7 with 1 M Tris, and incubated for 2 hr at 4°C with 30 μL of ubiquitin branch motif immunoaffinity beads. Then, the beads were washed twice with 750 μL of IAP buffer and three times with 750 μL of distilled water, and peptides were eluted with 3 × 60 μL of 0.15% TFA. The eluted peptides were desalted using GL-Tip SDB and GL-Tip GC (both from GL Sciences).

**Multidimensional protein identification technology (MudPIT)**

Enriched peptide pellets were dissolved in 100 μL of buffer A, pressure loaded on split-triple-phase fused-silica micro-capillary columns (McDonald et al., 2002) and analyzed by nanoflow liquid chromatography using an ultimate 3000 RSLC nano system coupled to a Q Exactive plus mass spectrometer equipped with a nanospray Flex Ion source (Thermo Fisher Scientific). Fully automated 10-step MudPIT separations were performed with salt pulses of 5 μL of 0%, 7%, 15%, 25%, 35%, 45%, 60%, 80%, 90%, 100% of 800 mM ammonium acetate (in 5% acetonitrile and 0.1% formic acid) prior to starting the reverse phase (RP) gradient. For reverse-phase chromatography the mobile phases were 5% acetonitrile and 0.1% formic acid (buffer A) and 80% acetonitrile and 0.1% formic acid (buffer B).

In the 1st-MudPIT step, peptides were eluted from the reverse phase onto the strong cation exchanger using a 70 min RP-gradient (wash with 2% buffer B for 5 min, 2%–10% in 5 min, 10%–45% in 40 min, 45%–98% in 10 min, hold at 98% buffer B for 5 min, wash in 2% buffer B for 5 min). For 2nd–10th MudPIT-steps, peptides were bumped with salt-pulses from the strong cation exchanger onto the reverse phase and separated using a 120 min RP-gradient (wash with 2% buffer B for 16 min, 2%–12% in 3 min, 12%–45% in 81 min, 45%–98% in 10 min, hold at 98% buffer B for 6 min, wash in 2% buffer B for 4 min). The column flow rate was maintained at 300 nl/min.

The Q Exactive plus mass spectrometer was operated in the data-dependent MS/MS mode, using Xcalibur software (Thermo Fisher). Spray voltage was set at 2.5 kV and heated capillary temperature ware set at 250°C. Peptides were fragmented by higher-energy collisional dissociation (HCD) in positive polarity mode with normalized collision energy of 27. The survey scans were acquired at a resolution of 70,000 at m/z 200 and the mass range was set to m/z 350-1700. Top ten most abundant ions were acquired at a resolution of 70,000 at m/z 200 and the mass range was set to m/z 350-1700. Top ten most abundant ions were acquired at a resolution of 30,000 at m/z 200 and the mass range was set to m/z 350-1700. Top ten most abundant ions were acquired at a resolution of 17,500, isolation window was set to 1.5 m/z, dynamic exclusion was set for 15 s. The maximum ion injection times for the survey and MS/MS scans were 50 ms and 100 ms respectively, and the ion target values were set to 1 × 10⁶ and 1 × 10⁵ for the survey and MS/MS scans, respectively.

**Protein identification from MS data**

The MS/MS datasets were searched using ProLuCID (Xu et al., 2015). The samples were searched against a database of 146186 sequences, consisting of 72956 H. sapiens non-redundant proteins (downloaded from NCBI on 2014-03-25), 193 usual contaminants (such as human keratins, IgGs, and proteolytic enzymes), and the NRBP1-muant protein sequences. To estimate false discovery rates (FDR), each protein sequence was randomized leading to a total search space of 73091 sequences. The precursor and fragment mass tolerances were set to 10 ppm and 100 ppm, respectively. Diglycine modification of Lys side chains, Ser/Thr/Tyr phosphorylation, pyroglutamate formation, acetylation, methionine oxidation and cysteine methyliothio were set as variable modifications.

Peptide/spectrum matches were sorted, selected and compared using DTASelect (Tabb et al., 2002) together with in-house software swallow and sandmartin. Proteins had to be detected by at least 2 spectra and average FDRs at the protein and spectral levels
were set to < 4%. To estimate relative protein levels, Normalized Spectral Abundance Factors (dNSAFs) were calculated for each detected protein, as described in Zhang et al. (2010). NSAF7 was used to extract total and modified label-free features for each amino acid within and calculate modification levels based on local spectral counts and generate the modification results.

RNA interference
An NRBP1 DsiRNA duplex that could target both rat and mouse isoforms was used to knockdown endogenous NRBP1 expression in F11 cells. A DS NC1 duplex (Integrated DNA Technologies) that does not target any part of the human, mouse, or rat transcriptomes was used as a control. Transfection of DsiRNAs into cells was accomplished using DharmaFECT1 (Dharmacon) at a final concentration of 10 nM in six-well dishes. For the rescue experiment, NRBP1_Dsi-R plasmid was transfected using Fugene HD (Promega) after 7 h of the NRBP1 DsiRNA transfection.

mCAT-HeLa cells stably expressing NRBP1 shRNA or EGFP short hairpin RNAs were generated pMX–puro Il-U6/siRNA as described (Yasukawa et al., 2008). The target sequence for NRBP1 was 5'-GCCGGTTGACTTCTCTGCTAG-3'.

Measurement of Aβ and sAPP by ELISA
F11 cells were either transfected with DsiRNA or DsiRNA plus NRBP1_Dsi-R construct. Forty-eight hours after transfection, culture media were replaced and cells were further incubated for 24 hr. As controls, F11 cells were treated with 1 μM DAPT or 2 μM BACE1 inhibitor C3 for 24 hr. The conditioned media were collected and cleared by spinning at 20,000 × g for 10 min. Aβ40 and Aβ42 in the media were measured using the Human/Rat β Amyloid ELISA Kits (Wako). For Aβ40, samples were diluted 1:5, whereas for Aβ42 were used undiluted. sAPPβ and sAPPα were measured with the Mouse sAPPβ-w Assay Kit (IBL) or Mouse/Rat sAPPα (h.s.) Assay Kit (IBL), respectively. For sAPPβ or sAPPα, samples were diluted 1:2 or 1:50, respectively. Aβ and sAPP concentrations (in pg/ml) were calculated based on the linear phase of the standard curve and the resulting values normalized for the amount of total protein of each cell extract and expressed as pg of Aβ/ml of medium/μg of protein.

Modeling of the complexes between various BC-boxes and Elongin BC or DDB1
Each BC-box sequence of the selected CRL2/CRL5 substrate receptors listed in Figure S6 was aligned to residues 156-168 of VHL (see Figure S1) and to residues 9-21 of DCAF6 (see Figure S6). Structural models of the BC-box sequence in complex with Elongin BC and that in complex with DDB1 were generated using the crystal structures of VHL-Elongin BC complex (PDB ID: 1VCB) (Stebbins et al., 1999) and DCAF6-DDB1 complex (PDB ID: 3I70) (Li et al., 2010) as templates. Ten models were generated by Modeler 9.13 (Sali and Blundell, 1993) for each complex and the model with the lowest DOPE score (Shen and Sali, 2006) was selected as the final model.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was performed using the two-tailed Student’s t test provided in Microsoft Excel software. A p value of less than 0.05 was considered to indicate a statistically significant difference between the two groups. Data are presented as mean ± SEM.

DATA AND CODE AVAILABILITY
The mass spectrometry dataset can be obtained from the MassIVE database at MSV000084419 from the ProteomeXchange accession PXD015705, and from the Stowers Original Data Repository at LIBPB-1369.