Supplementary Information

A novel Rac1 GAP splice variant relays poly-Ub accumulation signals to mediate membrane-specific Rac1 inactivation

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Supplementary Information Inventory:

Supplementary text, describing Figure S1 (related to Figure 1)

Figure S1 – S8

Supplementary Methods

Supplementary References
Supplementary text (for Figure S1):

From the predicted size of the BGIN doublet produced from constructs comprising the 5’ UTR, a larger BGIN product was initiating from a codon upstream of the peptide identified (Figure 1B), while a smaller BGIN product was initiating approximately 200 nts downstream (producing an isoform ~8 kDa smaller). Two non-ATG start codons resided upstream of the BGIN N-terminal peptide identified, a Val (GTG) and Leu (CTG) codon. Mutagenesis of the Val (V13, Figure S1C) had no effect on expression of the doublet, while mutagenesis of the Leu (L1R) resulted in deletion of the larger BGIN isoform (Figure S1C, black arrow). Constructs initiating from the L1 through a L1M mutation produced a band matching the larger doublet band in wild-type BGIN (Figure S1C, black arrow).

The first ATG encountered downstream of the CTG initiation site was M73, which provided an ideal candidate downstream initiation site to produce a BGIN isoform ~8 kDa smaller in relation to the L1 initiated full-length product. To test this, we expressed a construct initiating at the first in-frame Met (M73, M73-Start) and compared its size to wild-type, and L1 initiating constructs (Figure S1E). As expected, constructs initiating at this downstream ATG corresponded to the lower BGIN doublet. Moreover, cumulative mutation of the downstream methionines in the BGIN ORF shifted the lower doublet band to progressively smaller sizes (Figures S1E, S1F), demonstrating that the initiation of this smaller doublet band is a product of ATG initiation downstream of the CTG initiator signal. This indicates that excision of the 2nd 3BP1 BAR exon recodes the first 42 aa’s within the BGIN BAR domain, and initiation of BGIN at the CTG initiator produces a full-length product of 677 aa with a predicted molecular weight of 73.59 kDa.
The smaller BGIN form which initiates from the first in-frame ATG is predicted to be 605 aa with a molecular weight of 66.19 kDa.

As mentioned in the main text, only the larger ~74 kDa isoform is observed in brain, indicating that BGIN initiation primarily utilizes the CTG (L1) codon identified.
Figure S1. BGIN translation initiates using a Leu (CTG) codon. (Supplemental data related to Figure 1) (A) Comparison of the N-terminal BAR domains from BGIN, 3BP1 and RICH1. Intronic excision of the 2nd BAR domain exon in BGIN results in a frame
shift of the 3BP1 di-Met pair into the -1 reading frame. This results in an alternate sequence coded in-frame for BGIN. Conserved residues between BGIN, 3BP1 and RICH1 are shown in gray, and 3BP1 exons 1 and 2 are shown in black and red (below).

(B) Expression of BGIN-GFP with the 5’UTR from HeLa cells results in a doublet. Expression of full-length BGIN or BAR constructs in HeLa cells tagged with a C-terminal GFP tag and the 5’ UTR region results in a doublet by immunoblot analysis of lysates. Expression of the 3BP1 ORF produces a single band. (C through E) BGIN initiates from an upstream CTG (Leu). (C) BGIN constructs comprising the 5’ UTR with C-terminal GFP tags were mutated at potential initiator codons (CTG – L1, and GTG – V13) upstream of the minimal BAR region. Mutation of an upstream CTG eliminated the higher BGIN doublet band (black arrow). Gray arrow denotes the lower band doublet initiating at a site downstream of the CTG. (D and E) The lower BGIN doublet is produced from downstream Met initiation. (D) C-terminal GFP-tagged BGIN constructs with 5’ UTR were expressed in HeLa cells and lysates were immunoblotted for size comparison with the wild-type BGIN doublet. The upper band of ~110kDa indicates initiation at the CTG in wild-type constructs, and constructs with the CTG initiator codon switched to ATG (L1M, black arrow), while “M73-Start” constructs forced initiation at the first available Met (M73) by placing M73 directly behind the CMV promoter results in a size corresponding to the lower BGIN doublet (gray arrow). “L1R” constructs are unable to initiate using the CTG codon, and therefore lack the ~110kDa upper doublet band. (E and F) Consecutive mutation of BGIN in-frame Mets shifts the initiation site the lower BGIN isoform downstream. (E) Schematic indicating downstream Met profile of the mutant constructs used. The wild-type construct is left
unchanged, while constructs listed 1-5 comprise 1 to 5 cumulative Met to Arg in-frame mutations with respect to the L1M initiator. The putative downstream Met initiators in constructs 1 through 5 are indicated in gray highlight. (F) HeLa cells were transfected with BGIN constructs complete with 5’ UTR tagged/C-terminal GFP harboring from 1 to 5 cumulative Met to Arg mutations downstream from the M73 residue, where 1 through 5 indicates the number of cumulative mutations downstream of the L1 CTG initiation codon. GFP immunoblotting (left) indicates changes in the BGIN doublet, specifically a shift to lower molecular weight forms of the lower band with cumulative mutation of initiator Met residues, which ranges from 7.9kDa in the wild-type construct to 13.6kDa with 5 cumulative Met to Arg mutations downstream of the L1 initiator. A black arrow indicates initiation from the CTG (L1) initiator, while the gray arrow indicates initiation from a putative downstream Met. The L1M construct initiates primarily from the L1 initiator through a L1M mutation, and M73 start construct lacks the 5’UTR and initiates from M73 immediately downstream of the pCDNA3 CMV promoter. (G) Schematic of antibody epitopes used to detect BGIN. The N-terminal BGIN antibody recognizes the first 1-42 recoded BGIN amino-acids resulting from exon 2 excision, while the C-terminal BGIN antibody recognizes the CIN exon 2 module in both BGIN and CIN. (H) Multiple tissue western blots (IMGENEX) were probed with an antibody recognizing recoded amino acids 1-42 in the BGIN N-terminus. (I) Immunoblots of Neuro2A and SHSY5Y cells with BGIN N-terminal and CIN exon 2 antibodies produce a band of ~73 kDa.
Figure S2. Characterizing poly-Ub/BGIN interactions. (Supplementary data supporting Figure 2). (A) BGIN/poly-Ub interactions are enhanced with proteasomal inhibition. HeLa cells were transfected with GST, GST-BGIN or GST-3BP1 constructs.
and treated with 5µM MG132 for the indicated time. GST precipitates were
immunoblotted for GST or Ub as indicated. (B, C) The CIN exon 2 module interacts
non-covalently with poly-Ub. (B) GST or GST-CIN exon 2 expressed and precipitated
from HeLa cells using glutathione sepharose was washed in increasing concentrations of
SDS as indicated and immunoblotted for GST or Ub. (C) GST, wild-type CIN exon 2 or
K594R CIN exon 2 GST constructs were expressed in HeLa cells, precipitated using
glutathione sepharose and immunoblotted for GST or Ub. (D) The CIN exon 2 domain
interacts with poly-Ub in HeLa and Neuro2A cells. GST alone or GST-CIN exon 2
expressed in HeLa (left panels) or Neuro2A (right panels) cells were precipitated and
immunoblotted for GST or Ub as indicated. (E) Increasing amounts of recombinant
purified GST or GST-CIN exon 2 immobilized on glutathione beads were incubated with
purified his₆myc-tagged poly-Ub purified from HeLa cells. Precipitates were washed and
immunoblotted for GST or myc-tagged Ub. 0.7µg and 10µg of purified poly-Ub were
included in the respective input and binding reactions. (F) Recombinant purified GST or
CIN exon 2-GST constructs were incubated with recombinant purified Ub2, Ub4, or Ub5,
precipitated with glutathione sepharose, and subjected to immunoblotting for Ub or GST.
Coomassie staining of the input components is shown adjacently. (G, H) Proteomic
analysis of poly-Ub chain conjugates in CIN exon 2 precipitates. (G) GST or GST-
tagged CIN exon 2 expressed in HeLa cells treated with 5µM MG132 for 5h were
precipitated with glutathione sepharose and eluates were subjected to inspection by
Coomassie staining or immunoblot as indicated. Eluates were dialyzed, trypsin digested
and analyzed by mass spectrometry for Ub peptides comprising a residual lysine-
conjugated di-Gly (GG) signature. (H) Three Ub K(GG) peptides denoting K48 (blue)
and K29 (red) poly-Ub chain types were identified. (I) Comparing poly-Ub interactions
in vivo between Ub binding domains. GST-constructs expressing the NEMO/ABIN
UBAN domain, MUD1 or hRAD23A UBA domains or CIN exon 2 were transfected into
HeLa cells and GST-proteins were precipitated using glutathione sepharose. Poly-Ub or
GST were detected in precipitates by immunoblot as indicated.
Figure S3. (Supplementary data supporting Figure 3) (A) Alignment of the RhoGAP family GAP catalytic domain. GAP domain sequences indicated were aligned using CLUSTALW, and the catalytic 3BP1 R312/BGIN R320 residue is indicated (arrow). (B)
MG132 attenuates Rac1-GTP levels in SH-SY5Y cells. SH-SY5Y cells were treated with 5µM MG132 for the time indicated, harvested and subjected to PBD pulldown assay as described in Supplemental Methods. Normalized Rac1-GTP levels (mean +/- SE) from 3 experiments is depicted in the graph below (**p<0.003). (C and D) Effects of MG132 on BGIN activity and stability. (C) Stably-expressing BGIN-GFP HeLa cells were treated with control or BGIN-targeting oligos and subjected to PBD-pulldown assay following MG132 treatment. Bar graphs depict mean +/- SEM from three experiments (*p<0.02). (D) MG132 treatment does not affect BGIN stability. HeLa cells stably expressing BGIN-GFP were treated with 5µM MG132 for the time indicated and lysates were immunoblotted for Ub, BGIN (GFP) or actin. (E through G) BGIN distributes to membranes through the CIN exon 2 domain. (E) GST-tagged domain constructs as shown in the schematic were transfected into HeLa cells and separated into cytosolic and membrane fractions below. (F) Membrane and cytosolic fractions were immunoblotted for the GST fragments along with a scaled amount of purified recombinant GST. Abundance of GST components in membrane/cytosol fractions were determined by plotting band densities against a linear standard as shown in the graphs below, and as described in Supplemental methods. Percentage GST distribution in membranes are displayed in (G) (mean +/- SE from 4 experiments). (H) Subcellular distribution of poly-Ub. HeLa cells were separated into cytosolic, detergent soluble and insoluble fractions as described in Supplemental Methods. Equal protein quantities were immunoblotted for Ub or EGFR/ERK markers. Band intensities from three experiments were quantified by densitometry and mean +/- SEM in comparison to steady state samples (set to 1.0) are presented in the adjacent graphs. (I) BGIN homodimerizes through the BAR domain.
HeLa cells stably expressing BGIN-GFP were transfected with GST constructs (schematic shown on the left) as indicated and precipitated with glutathione sepharose. Precipitates were immunoblotted for GFP or GST as indicated. (J) poly-Ub accumulation does not significantly affect BGIN self-interaction. HeLa cells stably expressing BGIN-GFP were transfected with GST or BGIN-GST and treated with MG132 (5µM for 5h). Glutathione sepharose precipitates were immunoblotted for GST or GFP as indicated.
Figure S4. Screening a CIN exon 2 mutant library for non-poly-Ub binding alleles.

(Supplementary data supporting Figure 4) (A) BGIN-GFP was expressed in HeLa cells and subsequently treated with DMSO or MG132 (5µM for 5h). After generating cytosolic, detergent soluble or insoluble fractions, equal protein quantities were immunoblotted with increasing concentrations of purified GFP. BGIN-GFP band intensities were quantified in comparison to the linear GFP gradient to determine the
amount of BGIN in each fraction. The percentage of BGIN-GFP was determined from
the total protein yield from each fraction, BGIN-GFP abundance was compared between
DMSO and MG132 treatments in each fraction. Mean +/- SEM of percentage
distributions from four independent experiments are presented in the adjacent bar graphs
(**p<0.002, ***p<0.0002). (B) Mutagenesis scheme for uncoupling CIN exon 2/poly-
Ubiquitin interactions. 184 CIN exon 2 clones generated from random PCR mutagenesis
were individually assayed for poly-Ub binding by GST-pulldown assay. Eight missense
alleles were identified as non-poly-Ub binding alleles as shown by GST pulldown assay
in the lower immunoblot. The number of mutations recovered in each allele is noted in
brackets. (C) CLUSTALW sequence alignment of mutations recovered in non-poly-Ub
binding alleles identified in (B), with the mutational hit frequency of each residue within
the CIN exon 2 domain presented numerically in the sequence alignments and in the bar
graph (below).
Figure S5. Distribution and activity of poly-Ub coupling and uncoupling alleles. (Supplementary data supporting Figure 5) (A) Clonal HeLa cells stably expressing wild-type or poly-Ub non-binding (mut159) BGIN constructs were assayed for total (left panel) or membrane-specific (right panel) Rac1-GTP levels by PBD pulldown assay as
described in Methods in the main text. Bar graphs depict normalized Rac1-GTP levels from four independent experiments (mean +/- SEM, **p<0.001, *p<0.05 by paired t-test). (B and C) BGIN distributes to membranes and inactivates membrane-associated Rac1 in Neuro-2A cells. (B) Wild-type or poly-Ub non-binding mut159 BGIN-HA expressed in Neuro-2A cells were subjected to biochemical fractionation, and their distribution to cytosol or membranes were determined by immunoblot. Graph depicts densitometry values of BGIN (mean +/- SE from three experiments, * p<0.003) where wild-type BGIN density was set to 1.0. (C) BGIN mediates membrane-associated Rac1 inactivation in Neuro2A cells. Neuro2A cells co-transfected with HA-BGIN and myc-Rac1 were separated into cytosolic and membrane fractions. Membrane fractions were subjected to PBD pulldown assay and immunoblotted for myc-Rac1. Ratio of normalized Rac1-GTP levels are shown below relative to the empty vector control (set to 1.0). (D) Effect of BGIN on membrane-associated Cdc42 and RhoA activity. HEK293T cells were co-transfected with HA-BGIN and myc-Cdc42 or myc-RhoA constructs as indicated, and subjected to membrane fractionation (Left panel). Membranes were resuspended and subjected to PBD or RBD pulldown assay for Cdc42 or RhoA activity respectively (Right panel). Graph depicts measurements from 3 experiments (mean +/- SE, **p<0.0006). (E through G) Recoupling poly-Ub interactions to BGIN mut159. (E) HeLa cells were transfected with vectors expressing GST alone (control), GST-CIN exon 2, or the mut159 CIN exon 2 allele with single residue reversions to wild-type residues as indicated. GST proteins were precipitated with glutathione sepharose and immunoblotted for GST or Ub. (F) Reversion of two mutational clusters, R602C/V603I and P637L/P39L in the mut159 allele were assayed for poly-Ub recoupling as described in (E). The P637/639L reversion
pair yielded a partial restoration of poly-Ub interaction. (G) Rec1 and rec2 poly-Ub recoupling alleles were integrated into full-length GST-BGIN, expressed in HeLa cells, and assayed for poly-Ub interaction by precipitation with glutathione-sepharose in the presence or absence of MG132. Precipitates were immunoblotted for GST or Ub. Rec1/rec2 alleles restored poly-Ub co-precipitation with MG132 treatment (5µM for 5h).

(H and I) Distribution of the CIN exon 2 domain to membranes is dependent on poly-Ub interactions. (H) GST-tagged wild-type, poly-Ub non-binding (mut159, mut167) or recoupling (rec1, rec2) alleles were expressed in HeLa cells and lysates were subjected to cytosol/membrane fractionation and immunoblotting. Only alleles that associated with poly-Ub (wild-type, rec1, rec2) were abundantly present in membrane fractions. (I) GST-tagged Ub binding domains and modules were expressed in HeLa cells as indicated and lysates were subjected to cytosol/membrane fractionation. Distribution of GST components were examined by immunoblot as indicated.
Figure S6. BGIN attenuates ROS generation through the Nox1 complex. (Supplementary data supporting Figure 6) (A) ROS generation in a reconstituted HEK293T system requires NoxO1, NoxA1 and Nox1 expression. HEK293T cells were transfected with myc-tagged NoxO1, NoxA1 and/or Nox1 as indicated. For all subsequent ROS measurements, a portion of the transfected cells was assayed for ROS activity and lysates from the remainder were probed for myc-NoxA1/O1 expression. Quantification of ROS generation by chemiluminescence assay is shown below with transfection of all three Nox (O1/A1/Nox1) components (last lane) set to 1.0, with all other samples expressed as a comparative ratio (mean +/- SD of triplicate samples). (B) Rac1 inhibition suppresses Nox1 ROS generation. HEK293T cells were transfected with either empty vector, or Nox1/A1 and O1 plasmids as indicated in combination with myc-tagged Rac1 T17N. ROS generation was calculated as in (A). (C) BGIN expression suppresses ROS generation. HEK293T cells were co-transfected with plasmid constructs as indicated, and ROS generation was measured. Kinetic chemiluminescence measurements are shown in the adjacent line graph. (D) BGIN GAP activity is required for BGIN-dependent attenuation of ROS generation. HEK293T cells were transfected with myc-tagged NoxO1/NoxA1/Nox1 in combination with HA-tagged BGIN constructs as indicated. (E) 2.5 x 10⁵ untransfected or SHSY5Y cells stably expressing BGIN-GFP were assayed for ROS generation by chemiluminescence. Cells were treated with the NADPH oxidase inhibitor DPI (10µM, 5h) to gauge ROS generation through the NOX complexes. Immunblots of lysates from the cells assayed are shown in the inset. (F) The BGIN C-terminal CIN exon 2 module is required for the formation of perinuclear aggregates. GFP-tagged BGIN, BGIN^AC-term (BGIN^1-573), and CIN exon 2 (BGIN^574-677)
constructs (schematic shown, middle) were expressed in HeLa cells. Three distinct localization features were observed in BGIN-expressing cells: largely cytoplasmic, loose perinuclear staining (perinuclear), and perinuclear tubule clusters. Quantification of perinuclear tubule clusters, or other features as depicted were enumerated from two experiments (mean +/- SD). (G) MG132 treatment enhances the formation of BGIN perinuclear aggregates. HeLa cells transfected with either full-length BGIN-GFP or BGIN lacking the CIN exon 2 domain (BGIN<sup>ΔC-term</sup>, BGIN<sup>1-573</sup>) were treated with DMSO or MG132 for 5h as indicated, fixed with paraformaldehyde and scored for perinuclear aggregate formation.
Figure S7

A. Workflow outline

Tissue culture cells:
Observation - Overexpressed BGN/GFP co-localizes to Ub-enriched aggregates (~10% cells):
Dependent on poly-Ub interactions (CIN exon 2 domain)
Enhanced with MG132

Brain tissue Ub aggregate pathology:
Determine potential BGN/Ub interaction in AD, PD and HD by BGN/Ub co-localization in Ub-enriched aggregates.

Biochemical tissue characterization
Determine whether BGN/Ub co-aggregate is perturbed in neuropathological settings

Proteotoxic neuronal cell model
Determine whether BGN perturbation can influence Rac1 signaling in an appropriate proteotoxic model as determined by BGN/Ub colocalization

B. Human AD

C. re-synuclein

E. Cytosol:
AD PD HD control
Erk1/2
CIN exon 2
BGN

G. membrane BGN

F. Detergent soluble membranes:

BGN
CIN
Na+/K+ ATPase

Erk1/2
actin

G. normalized membrane Rac1-GTP

H. normalized membrane p-cofilin

AD control
AD control
Figure S7. Localization and distribution of BGIN in neurodegenerative brain tissue.
(Supplementary data supporting Figure 7) (A) Experimental strategy outline. The formation of Ub-enriched BGIN-GFP inclusion aggregates in tissue culture cells suggests that neuropathological aggregates (plaques, tangles, Lewy bodies, etc.) possibly also accumulate BGIN. Ub/BGIN co-localization will give good indication that Ub may influence BGIN distribution and possibly function in response to proteotoxic stressors within a specific neurodegenerative context. This will direct subsequent investigation into whether BGIN distribution and downstream Rac1 signaling may be influenced in certain neurodegenerative tissues or by a particular proteotoxic species. (B) BGIN and Ub partially co-localize in neurofibrillary tangles. Human AD brain sections were co-stained with antibodies recognizing BGIN or Ub and visualized by fluorescence microscopy. Merged images shown in the right panels. (C) Human PD brain sections were co-stained with antibodies detecting α-synuclein and BGIN, and visualized by fluorescence microscopy following incubation with fluorescent secondary antibodies. Merged panel is shown on the right. (D through F) Surveying neurodegenerative brain tissue for aberrations in BGIN/Rac1 signalling. (D) Human brain tissue from three AD, PD, HD and control patient samples were extracted for total protein as described in Supplemental Methods and subjected to immunoblotting with the antibodies indicated. Brain extracts were separated into cytosol, membrane and detergent insoluble fractions and immunoblotted for ERK1/2, Na+/K+ ATPase and actin to determine separation fidelity. (E and F) Cytosolic (E) and detergent-soluble membrane (F) brain fractions were immunoblotted for the signaling components indicated. In all analyses (E, F), band intensity of immunoblots obtained from the three of each of the AD, PD, HD or control
samples were grouped and expressed as an average +/- SEM (bar graphs)(*p<0.002).

(G) Individual densitometry scans of membrane BGIN and normalized Rac1-GTP and phosphocofilin levels in (Figure 7E) from AD (black bars) and control patients (gray bars) are depicted in bar graphs. All values are expressed as a ratio compared to the control group mean (set to 1.0); quantified blots represented from (Figure 7E) are shown in the graph insets.
Figure S8. Investigating BGIN/Rac1 signaling in an AD neuroblastoma cell model.

(A) Aβ₁₋₄₂ detection in untransfected and stably-expressing APP SH-SY5Y cells. Media from normal (WT) and APP-expressing (+APP) SH-SY5Y cells were collected and subjected to Aβ₁₋₄₂ detection by ELISA as described previously (Kounnas et al., 2010). Left graph depicts Aβ₁₋₄₂ / 25µls media, right graph shows fold Aβ₁₋₄₂ production in APP cells in comparison to non-expressing cells (set to 1.0).

(B) GST, GST-BGIN or the
non-polyUb binding allele GST-BGIN mut159 was expressed in SH-SY5Y cells with or without stable APP expression as indicated, and GST precipitates were generated from lysates and immunoblotted for GST or Ub. The graph below indicates the poly-Ub smear intensity normalized against GST-BGIN wild-type band intensity. (C) Stable APP expression attenuates ROS generation. Non-expressing or APP transgenic SH-SY5Y cells were seeded at equal density and left untreated or treated with the NOX inhibitor DPI (10µM) for 5h prior to chemiluminescence detection for ROS generation (see Supplemental Methods). Upper line graph depicts kinetic ROS values, and the bar graph below shows normalized ROS values from four experiments measured in triplicate. (**p<9.7 x 10^-8). (D) Short term BGIN siRNA treatment has no effect on Rac1-GTP levels. Normal or APP-expressing SH-SY5Y cells transfected with 20nM siRNA for 48h were harvested and subjected to PBD pulldown assay. (E) SH-SY5Y cells transfected with 20nM siRNA for 48h were subjected to Rac1 activity assay by PBD pulldown method. Bar graphs depict mean BGIN and Rac1-GTP values from 3 experiments (mean +/- SE). Lower graph shows fold-change in Rac1-GTP levels in BGIN-treated cells compared to control siRNA-transfected samples (set to 1.0). (F) Cells were transfected as described in (E) and subjected to chemiluminescence assay for ROS output. Kinetic ROS output values are shown in the upper line graph, relative ROS values from 3 experiments measured in triplicate are depicted in the lower bar graph (**p<0.008). (G) Long-term siRNA results in APP-expressing SH-SY5Y cells results in elevated Rac1 activity. APP-expressing SH-SY5Y cells were transfected with control or CIN1 (BGIN non-targeting) or CIN4 (BGIN-targeting) siRNA oligos for 1 round over 48h (short-term
RNAi) or 2 rounds over 4 days (long-term RNAi). Cells were then harvested and subjected to Rac1-GTP detection by PBD pulldown assay.

**Supplemental Methods**

**Plasmid constructs**

pGEX4T3 vectors comprising NEMO and Abin1 UBAN motifs or the hRad23A UBA1 domain were a generous gift from Dr. Ivan Dikic (Goethe University Frankfurt). pGEX6-MUD1 was generously received from Dr. David Komander (MRC, UK). Fragments comprising these Ub-binding components were also cloned in-frame and 3’ of the GST tag for expression in mammalian cells in the pRK5mGST vector.

**Plasmid constructs, and generating stable cell lines.**

The original AK126873 cDNA clone (clone ID no. BRAMY3014613) was purchased from the National Institute of Technology and Evaluation (NITE, Japan), and the SH3BP1 (3BP1) clone was obtained from the Sanger Institute (UK). All BGIN PCR-based site-directed mutagenesis and PCR-generated BGIN fragments required 5% DMSO, and all PCR fragments produced for subcloning were generated using Pfu Turbo polymerase (Stratagene, Santa Clara CA).

BGIN constructs were initially generated with C-terminal GST or GFP tags in-frame with the BGIN ORF in pCDNA3, which includes the intact 5’ BGIN UTR. In order to identify the 5’ translation start site in BGIN, the BGIN N-terminal BAR domain (5’UTR comprising 211 nt’s upstream of the BGIN1-262 region) was cloned in-frame with a C-terminal GST tag (pCDNA3 5’UTR/BAR-GST), purification of this BAR fragment yielded N-terminal BGIN sequence information described in Results.
pTRChis$_6$ Ub2-Ub5 and pGEX4T3 Ub – Ub7 constructs were cloned by serially inserting Ub monomer units with BamH1/SalI ends 5’ upstream of an anchoring Ub unit (containing a stop codon) using BamH1/Xho1 sites generated using PCR oligos.

**Antibody purification**

Antibodies specifically recognizing the BGIN$^{574-677}$ region were affinity purified from rabbit antisera from rabbits immunized with the full-length CIN antigen: recombinant GST-BGIN$^{574-677}$ was immobilized on CNBr-Sepharose and immunoreactive antibodies were bound and eluted as described previously (Gohla et al., 2005). Antibodies recognizing the unique BGIN$^{1-42}$ N-terminal region were affinity purified from rabbits immunized with his$_6$-tagged recombinant BGIN$^{1-42}$, and antibodies specifically recognizing this unique N-terminal epitope were collected by binding the rabbit antisera to GST-tagged BGIN$^{1-42}$ crosslinked to glutathione sepharose (GE Healthcare, Piscataway NJ). Antibodies were eluted with 0.1M Glycine pH 2.5, and dialyzed in 1x PBS/5% glycerol.

**Protein purification**

Purification of the BAR-GST fragment used for sequence determination of the BGIN N-terminus was performed by glutathione-sepharose precipitation from HeLa cell lysates in 1%NP40/Hepes buffer 1 (20mM Hepes pH 7.5, 150mM NaCl, 10mM MgCl$_2$, 5% glycerol) expressing 5’UTR/BAR-GST using the pCDNA3 construct described above. Beads were washed in Hepes buffer 1 and eluted in 30mM reduced glutathione in buffer A (20mM Hepes/0.5M NaCl), and dialyzed in Hepes buffer 1. Proteins were then TCA
precipitated in 100% TCA, washed in acetone, dried and submitted for analysis by mass spectrometry. A portion of the precipitated sample was retained for silver staining.

For purification of BGIN-HAhis$_6$ complexes for mass spectrometry analysis, ten 150mm dishes of HeLa cells were transfected with pCDNA BGIN-HAhis$_6$ (complete with 5’ UTR), and cells were lysed in 11mls of Hepes buffer 1 (20mM Hepes pH 7.5, 150mM NaCl, 10mM MgCl$_2$, 5% glycerol) with 1% NP40 were collected ~16h following transfection. Imidazole was added to 10mM and His$_6$-tagged proteins were precipitated using 2.5mls of Ni-NTA agarose (50% slurry). Beads were then washed twice with 25mls of lysis buffer with 1% NP40/25mM imidazole, and twice with 25mls of buffer without detergent. Proteins were eluted with 300mM imidazole in Buffer A (20mM Hepes pH 8, 0.5M NaCl), dialyzed in detergent-free lysis buffer and concentrated 10-fold using an Amicon-15 filter.

Recombinant his$_6$-GST-tagged CIN exon2 expressed using pTRC (in BL21 E. coli) was solubilized from inclusion bodies by sonication in urea buffer (8M urea, 20mM Hepes pH 8.0, 100mM NaCl), and bound to Ni-NTA beads in 10mM imidazole. After two washes in urea buffer/and two washes in 1x PBS each in 20mM imidazole, bound proteins were renatured in 0.3M NDSB-201/1x PBS at 4°C for 0.5h, washed twice in 1x PBS and eluted in 0.3M imidazole/1x PBS. Proteins were dialyzed in 1x TBS/5% glycerol with 0.3mM DTT. Similarly, his$_6$ linear poly-Ub chains (Ub2 to Ub7) expressed using the pTRC vector was purified from BL21 E. coli lysed in native buffer (50mM Tris pH 7.5, 100mM NaCl, 5mM MgCl$_2$, 1mM EDTA, 1mM DTT) and bound to Ni-NTA agarose in 10mM imidazole. Beads were washed in 20mM imidazole in buffer A, and
eluted with 0.3M imidazole buffer A. Proteins were dialyzed in 1x TBS/5% glycerol/0.3mM DTT.

Recombinant GST, GST-RBD and GST-PBD were expressed from pGEX vectors in BL21, induced for 5h at 37°C (GST-RBD was induced at 30°C) and lysed in native buffer. GST proteins were bound to glutathione-sepharose, washed several times with buffer A and eluted in 30mM reduced glutathione in 0.3M Hepes pH 8.0. GST-PBD/GST-RBD was dialyzed in 25mM Tris pH 7.5, 1mM EDTA, 5mM MgCl₂, 50mM NaCl, 5% glycerol, 0.5mM DTT, and GST was dialyzed in 1x TBS/5% glycerol/0.3mM DTT.

**In vitro pulldown assays for CIN exon 2/poly-Ub interactions**

15µgs of recombinant purified GST control, GST-CIN exon 2 or Ub or linear poly-Ub chains were bound to glutathione sepharose and washed in Hepes buffer 1 with 1%NP40. 15µgs his₆-tagged binding substrate (linear Ub chains, or CIN exon 2-GFP) were incubated with the gluthione beads in Hepes buffer 1/1%NP40, incubated for 1h at 4°C with rotation and washed three times with Hepes buffer 1/1%NP40. GST-Ub/poly-Ub/his₆-CIN exon 2-GFP interactions were washed in Hepes buffer 1/1% NP40 with 0.5M NaCl to reduce background binding.

15µgs of purified proteins were visualized on 10% SDS-PAGE gels by Coomassie blue staining for quality control of the input components. Comparison of linear Ub binding between CIN exon 2, NEMO/Abin1 UBAN and hRad23A UBA1 was performed as above with the exception of using 20µgs of all proteins.
Proteomic analysis of CIN exon 2/poly-Ub chains

Four 10cm dishes of HeLa cells were transfected with the pRK5mGST empty vector, or pRK5mGST-CIN exon 2 plasmid construct and treated with 5µM MG132 for 5h, 16h post-transfection. Lysates were generated in Hepes buffer 1/1% NP40, and GST proteins were precipitated with 0.3mls glutathione sepharose slurry. Beads were washed 3 times with 15mls Hepes buffer 1/1%NP40 and eluted in 3.5mls 30mM reduced glutathione in 0.3M Hepes pH 8.0. Samples were dialyzed in 0.1M Tris-HCl pH 8.5 overnight and concentrated with an Amicon filter (10kDa cutoff) to a final protein concentration ranging from 3-5mg/ml. Samples were digested with trypsin and subjected to analysis by mass spectrometry as described below. Ubiquitin peptides comprising an additional lysine-conjugated di-glycine (GG) signature indicating poly-Ub chain conjugation were identified and mapped to specific lysines on the Ub peptide. Methods for mass spectrometric and bioinformatic analysis is described below.

Mass spectrometric analysis

Proteins were reduced with 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (Sigma-Aldrich, St. Louis, MO, product C4706) and alkylated with 10 mM 2-chloroacetamide (Sigma-Aldrich, St. Louis, MO). Proteins were digested for 18 hr at 37 °C in 2 M urea 100 mM Tris pH 8.5, 1 mM CaCl2 with 2 ug trypsin (Promega, Madison, WI, product V5111) and digest reactions were terminated with formic acid (5% final concentration). Debris was removed by centrifugation, 30 min 18000 x g.

MudPIT microcolumn assembly, equilibration and experimental data acquisition was performed as described previously (Washburn et al., 2001; Wolters et al., 2001).
Protein and peptide identification was extracted from the raw spectral data as described previously (McDonald et al., 2004). To identify poly-Ub chains in CIN exon 2 precipitates, a decoy database containing the reversed sequences of all the proteins appended to the target database was employed to accurately estimate peptide probabilities and false discovery rates (Peng et al., 2003). Tandem mass spectra were matched to sequences using the ProLuCID algorithm with 50 ppm peptide mass tolerance. The search space included all fully tryptic peptide candidates that fell within the mass tolerance window with no miscleavage constraint. Carbamidomethylation (+57.02146 Da) of cysteine was considered as a static modification. Ubiquitination (+114.042927) of lysine was considered as a differential modification.

**siRNA transfection of SHSY5Y cultures**

SH-SY5Y cells transfected with siRNA and processed for ROS generation were seeded at 1 x 10⁵ cells in a 6-well plate format, premixing 200µls of Opti-MEM containing 2µls of RNAi Max and siRNA oligos to a final culture concentration of 20nM in an equal volume of Opti-MEM prior to transfection. Transfected cells were incubated for 48h prior to harvest and processing for chemiluminescence assay for ROS generation (described below).

Serial siRNA treatment of SH-SY5Y cultures required initial seeding of 10⁶ cells in 60mm dishes. Cells were transfected with 20nM siRNA oligos/4µls of RNAi Max into a shallow volume of serum-free media (1.6mls), where the total volume was added to 4mls after transfection. Cells were replated onto 60mm dishes for PBD pulldown assay (0.8 to 10⁶ cells) or consequent transfection (10⁶ cells) of siRNA (2x RNAi) 48 to 72h
post-transfection depending on cell growth. This was repeated for a final round (3x RNAi) of transfection, where cell growth often became extremely slow after 3 rounds of siRNA transfection. PBD assay conditions for SH-SY5Y cells are described below.

**PBD pulldown assays for Rac1/Cdc42 and RhoA-GTP measurements**

Rac1-GTP levels were measured by PBD pulldown as described previously (Benard et al., 1999; Stofega et al., 2006) with some modification. For transient transfection assays from total cell lysates, transfected HEK293T cells were co-transfected with BGIN and myc-Rac1 or myc-Cdc42 plasmids (in pRK5 vectors). Cells were lysed in lysis/assay buffer (20mM Hepes, 150mM NaCl, 10mM MgCl₂, 5% glycerol, 1%NP40) and a portion of the lysates were retained for immunoblotting. Lysates were incubated with recombinant purified GST-PBD and glutathione sepharose for 1h at 4°C, and washed three times in lysis/assay buffer and boiled in Laemelli buffer. Co-precipitated myc-Rac1 or Cdc42 were visualized by immunoblotting and expressed as a ratio of normalized to Rac1/Cdc42 expressed in total lysate immunoblots.

For Rac1, Cdc42 or RhoA-GTP measurements from membrane fractions by transient transfection, HEK293T cells were transfected as above and harvested in detergent-free lysis/assay buffer. Cells were sonicated and lysates were cleared by centrifugation at 12K x g for 10 minutes. Lysates were then separated into cytosol and detergent soluble fractions by ultracentrifugation at 55K rpm for 30mins. Membrane pellets were gently washed once in lysis/assay buffer and resuspended in lysis/assay buffer containing 1% NP40 by bath sonication. Equal protein quantities from membrane fractions (at least 500µg) was subjected to PBD assay as described above, or with
recombinant GST-RBD (Rhothekin RhoA-binding domain)(Ren and Schwartz, 2000). All precipitated GTPases detected by immunoblot were normalized to GTPase levels detected in membrane fractions.

Rac1-GTP measurements from endogenous Rac1 in HeLa cells or stably BGIN-expressing HeLa cell lines were performed as described above, where Rac1-GTP or total Rac1 was measured by immunoblotting with the 23A8 Rac1 antibody. Rac1-GTP measurements from HeLa membrane fractions required generation of a minimum of 1mg of detergent-soluble membranes from two 150mm tissue culture dishes for PBD measurements. Rac1-GTP measurements from SH-SY5Y cells were performed as described above, but required a higher GST-PBD concentration (120 to 150µg of GST-PBD, compared to ~ 30 to 50µg used for assay in HeLa/HEK293T cells).

For all membrane Rac1-GTP, Cdc42-GTP or RhoA measurements, a portion of cytosol and detergent solubilized membranes were immunoblotted for cytosolic/membrane markers and Rac1/myc-Rac1.

**Cell fractionation.**

Cell fractionation was performed as previously described with some modification (DerMardirossian et al., 2006). To separate cytosolic, detergent-soluble membranes, and high-molecular weight detergent-insoluble compounds from cultured cells, cells were scraped in fractionation buffer (100mM Pipes, pH 7.3, 100mM KCl, 3.5mM MgCl₂, 3mM NaCl), sonicated briefly and cleared at 5K x g for 10 minutes. Lysates were then centrifuged at 55K rpm for 1h at 4°C, and cytosolic fractions were removed. Pellets were washed in fractionation buffer and resuspended in fractionation buffer with 1%
deoxycholate and Triton X-100, by bath sonication. Samples were subjected to ultracentrifugation (45K rpm, 1h) and detergent-soluble and insoluble fractions were obtained. For separation of cytosolic/membrane fractions only, cells were processed as described above (with the exception of the clearing lysates at 12K x g), membrane pellets were washed once after ultracentrifugation and resuspended by pipetting and bath sonication in fractionation buffer containing 1% NP40. Human brain tissue was subjected to fractionation and analysis in a similar manner, and described in detail in below.

Quality of separation between membranes and cytosolic components was determined by immunoblotting for ERK1/2 (cytosolic) and EGFR or Na+/K+ ATPase (membrane) markers.

Estimating distribution of BGIN-GST domains or BGIN-GFP in cytosol/membrane fractions.

For estimating the abundance of GST constructs expressing full-length BGIN or BGIN BAR, GAP or CIN exon 2 domains (Figure 4E through 4G), constructs were transfected into HeLa cells and lysates were separated into cytosol and membrane fractions by ultracentrifugation. 30µgs of cytosol/membrane from each sample was immunoblotted for GST alongside a standard curve comprising increasing amounts of purified recombinant GST. The band intensity of the recombinant GST was used to form a linear correlation, and ng quantities of the BGIN-GST fragments were estimated by plotting relative band densities along a y = mx + b linear gradient. The ng GST protein quantities were adjusted for differences in molecular weight relative to GST, and the %
of GST protein was calculated from the total protein cytosol/membrane yield. The percentage of protein in membrane fractions were then calculated as a ratio of GST in membrane fractions divided by the total protein expressed in cytosol + membrane in combination.

Estimation of BGIN-GFP abundance in cytosolic, detergent-soluble membranes and detergent-insoluble fractions (Figure S4A) was performed similarly by transfecting HeLa cells with BGIN-GFP constructs with or without MG132 treatment (5µM for 5h), where biochemical cytosol, membrane and insoluble fractions were generated as described above. 10µg from each fraction was immunoblotted with a linear gradient of recombinant GFP, and the relative percentage of BGIN segregating to each fraction was calculated according to the total protein yield of each fraction.

**Mutant CIN exon 2 library screen for non-poly-Ub binding alleles.**

A random mutant library comprising the BGIN C-terminal CIN exon 2 domain (BGIN575-677) was generated using the Diversity (Clontech, Mountain View CA) error-prone PCR system. Reaction conditions producing maximum mutagenic yield (640µM MnSO₄/200µM dGTP) were used to generate a range between 1 to 15 mutations within the BGIN C-terminal domain. BamH1/Xho1 overhangs from these fragments were cloned into BamH1/Xho1 sites in the pRK5mycGST vector. In order to reintegrate mutant alleles into full-length BGIN constructs, G575 and S576 residues were converted into a unique BamH1 (5’ GGATCC 3’) site, and mutant allele cassettes were inserted using the recombinant BamH1 site.
184 mutant allele pRK5mycGST constructs were individually transfected into HeLa cells, and GST-CIN exon 2 was precipitated using glutathione sepharose and assayed for poly-Ub coprecipitation by immunoblotting. 45 alleles were identified to uncouple CIN exon 2/poly-Ub interactions, and upon sequence analysis, 37 of these clones comprised frameshift or non-sense mutations, leaving 8 allelic CIN exon 2 variants comprised exclusively of missense mutations (Figure S4B and S4C).

Cell spreading on fibronectin

HeLa cells stably expressing BGIN or the mut159 BGIN allele, or cells transiently transfected with GFP or GFP-tagged Q61L Rac1 were suspended and dropped onto glass coverslips coated with 0.0001% fibronectin for 45 mins. Cells were then fixed with paraformaldehyde and co-stained with paxillin antibodies (BD Biosciences) or phalloidin. Cell area was measured in GFP-positive cells using Metamorph morphometric analysis software in X cells from 3 experiments.

Chemiluminescence measurement of ROS generation

Measurement of ROS by the Nox1 (NADPH oxidase 1) complex in a reconstituted HEK293T cell system was assayed as described previously with some modification (Gianni et al., 2008; Kim et al., 2007). HEK293T cells were grown to 80% confluency in 6-well plates, and transfected with pRK5myc-Nox1, NoxA1 and Nox01 plasmids (0.6µg/construct/well) in addition to BGIN or Rac1 constructs. Constructs were expressed overnight, washed in 1x PBS, trypsinized and resuspended in 1ml of DMEM media and pelleted at 1K x rpm for 5 mins. Cells were washed in 1ml 1x HBSS, pelleted
and resuspended in 700µls of 1x HBSS. 60µls of the cell suspension was mixed with 60µls of 1x HBSS in a 96-well plate (Berthold Technologies, Oak Ridge TN), and mixed with 250µM luminol (final concentration) and 1 U of HRP to a final volume of 200µls prior to luminometer reading. The remainder of the cell suspension was pelleted, lysed in buffer with 1% NP40 (20mM Hepes pH 7.5, 150mM NaCl, 10mM MgCl₂, 5% glycerol) and subjected to immunoblotting. Kinetic measurements were taken for the time indicated at room temperature, and data output was generated in an Excel spreadsheet for analysis.

Endogenous ROS measurement of SHSY5Y cells was essentially undertaken as above, with some modification. Briefly, SHSY5Y cells in 6-well plates were incubated in 0.5mls of trypsin/EDTA for 5 mins. at 37°C, and harvested into 2ml tubes containing 1ml of 1 x DMEM media (complete with 8% FBS). Cells were then processed for chemiluminescence assay as described above. Stably-expressing SHSY5Y cells (APP or BGIN) were seeded at a density of 2.5 x 10⁵ cells in 6-well plates one day prior to assay.

**Human Brain Samples.**

We also thank the patients and their families who provided tissue samples to the Alzheimer’s Disease Research Center (UCSD) used in this study. Postmortem human brain samples (frontal cortex) were provided by the Alzheimer’s Disease Research Center at University of California San Diego (UCSD) from patients diagnosed with AD, PD and HD. All cases were clinically characterized during life (within 12 months before death) and histopathologically characterized postmortem. The diagnosis of AD was based on the initial presentation with dementia and presence amyloid plaques and Tau positive...
tangles. Detailed fractionation methods and Rac1 activity assay of human brain samples
are described below.

**Fractionation and analysis of human brain tissue**

Frozen human brain tissue (250mg) from the frontal cortex was resuspended in
850µls modified fractionation buffer (100mM Pipes, pH 7.3, 100mM KCl, 10mM MgCl₂,
3mM NaCl) and sonicated for 35 seconds. Debris was cleared by centrifugation (12K x g,
10 mins. at 4°C) and a portion of the lysates were subjected to PBD pulldown assay
(500µg) or immunoblotting (30µg). Total lysates were pelleted by ultracentrifugation at
50K x rpm for 50 mins. at 4°C, cytosolic extracts were obtained (and subjected to PBD
pulldown assay/immunoblotting) and the pellet was washed once in fractionation buffer.
High-speed pellets were resuspended in fractionation buffer containing 1% triton X-
100/1% deoxycholate by bath sonication, and subjected to ultracentrifugation at 50K x
rpm for 50mins. at 4°C. Detergent soluble membranes were obtained, and samples were
subjected to PBD pulldown assay (250µg protein) or immunoblotting. Pellets were
washed, and detergent insoluble fractions were resuspended in fractionation buffer in
1%NP40 by bath sonication.

**Transgenic APP mouse strains**

APP transgenic mice expressing mutated (Swedish K670M/N671L, London V717I)
human(h) APP751 under the control of the mThy-1 promoter (mThy1-hAPP751) (line
41) (Rockenstein et al., 2001) were used. We have previously shown that these mice
display loss of synaptic contacts, high levels of Aβ₁-42 production, early amyloid
deposition, Tau hyperphosphorylation and behavioral deficits (Rockenstein et al., 2003; Rockenstein et al., 2007a; Rockenstein et al., 2007b).

**Fluorescence imaging of HeLa cells.**

HeLa cells were seeded on glass coverslips and transfected with plasmid constructs as described. Coverslips were then subjected to fixation in 4% paraformaldehyde at room temperature for 45 min, followed by permeabilization with 1x PBS/0.5% Triton X-100 for 5mins. Coverslips were then washed in PBS, blocked with 3% BSA, and stained with various primary antibodies as described. Samples were visualized by fluorescence microscopy using an Olympus IX70 microscope (40x oil objective) after subsequent staining with Alexa568-coupled secondary antibodies/DAPI and mounting on glass slides in anti-fade reagent (Prolong Gold, Invitrogen).

**Immunohistochemical staining and fluorescent dual-labeling of human and transgenic mouse brain.**

For immunohistological labeling of BGIN, vibratome sections (40 µm thick) were incubated at 4°C overnight with affinity-purified antibodies against BGIN (the unique 1-42 N-terminal BGIN epitope), followed by detection using a biotin-coujugated secondary antibody (1:75, Vector Laboratories, Burlingame, CA), and labeling with Avidin D-horseradish peroxidase (HRP, ABC Elite, Vector). Samples were visualized by diaminobenzidine (DAB, 0.2 mg/ml) in 50 mM Tris (pH 7.4) with 0.001% H2O2, as previously described (Rockenstein et al., 2003; Rockenstein et al., 2006). Immunostained sections were imaged with a digital Olympus BX51 microscope and assessment of levels
of immunoreactivity was performed utilizing the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD).

To determine the co-localization between BGIN or Rac 1 and neurofibrillary tangles, double-labeling experiments were performed, as previously described (Spencer et al., 2009). Vibratome sections were immunolabeled with the antibodies against Rac1 or BGIN and the mouse monoclonal against pTau PHF1 (courtesy of Dr. Peter Davis, Albert Einstein College of Medicine). The PHF1 immunoreactive structures were detected with the Tyramide Signal Amplification™-Direct (Red) system (1:100, NEN Life Sciences, Boston, MA) while BGIN and Rac1 was detected with FITC tagged antibodies (Vector, 1:75). All sections were processed simultaneously under the same conditions and experiments were performed in triplicate in order to assess the reproducibility of results. Sections were imaged with a Zeiss 63X (N.A. 1.4) objective on an Axiovert 35 microscope (Zeiss) with an attached MRC1024 LSCM (laser scanning confocal microscope) system (BioRad).

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