Supporting Information

Colanzi et al. 10.1073/pnas.1222413110

SI Materials and Methods

ADP-Ribosylation Assay. Total membrane fractions (50 μg) were incubated with 1 μg His-BARS [brefeldin A (BFA) ADP-ribosylation substrate] in the absence or presence of BFA (80 μg/mL) in 50 μL ADP-ribosylation buffer [50 mM Tris·HCl (pH 7.4), 4 mM DTT, 500 μM $MgCl_2$, 30 μM total NAD^+, and 4 μCi [^{32}P]-NAD^+) at 37 °C for 1 h. At the end of the incubation, the reaction was stopped by adding SDS sample buffer, and the mixtures were analyzed by SDS/PAGE and Western blotting. The incorporated radiolabel then was evaluated using an Instant Imager (Packard Instruments).

BFA–ADP-Ribose Conjugate Synthesis. Total membranes from rat brain were incubated in the presence of BFA (80 μg/mL), NAD^+ (5 mM), and 0.01 μCi/μL [^{32}P]-NAD^+ in metabolite buffer (20 mM Tris·HCl at pH 7.0, 50 mM NaCl) at 37 °C for 2 h. The samples then were centrifuged at 40,000 × *g* at 4 °C for 45 min, and the collected supernatant was filtered using an ultrafiltration apparatus (Amicon; molecular weight cutoff, 10 kDa). Then, the flow-through was extracted twice in two volumes of MeOH/$CHCl_3$ (1:2, vol/vol), and the aqueous phase containing the metabolite of interest was lyophilized and stored at −20 °C for further purification.

Purification of Recombinant Proteins. GST-E1A (1), His-BARS (2), GST-14-3-3γ (3), and GST-PAK1 (4) were purified as previously described.

Cell Culture and Fractionation. HeLa cells were grown in DMEM supplemented with 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% (vol/vol) FBS. Total membrane fractions were prepared starting from confluent HeLa cells, which were washed three times with ice-cold PBS, and detached mechanically in 800 μL Hepes buffer (20 mM Hepes at pH 7.4, 1 mM EDTA, 250 mM sucrose). The cells were recovered and then sonicated on ice three times for 15 s; unbroken cells were removed by centrifugation at 500 × *g* for 5 min. The resultant supernatants were ultracentrifuged for 1 h at 100,000 × *g*, with the supernatants representing the cytosolic fraction and the pellets the total membrane fraction. The total membrane fractions then were resuspended in 20 mM Hepes at pH 7.4 containing 1 mM EDTA and protease inhibitors, and stored at −80 °C.

Microinjection Experiments. HeLa cells were grown on fibronectin-coated glass coverslips. Once attached, they were incubated in growth medium plus 9 μM RO-3306 for 20 h to accumulate the cells in G2 phase (5). Two hours before G2-block removal, the cells were microinjected with HPLC-purified BFA–ADP-ribose conjugate (BAC) or buffer alone (20 mM Hepes, 10 mM sucrose), previously mixed with dextran-FITC as a tracer of microinjection. The G2-block was removed by washing out RO-3306. The cells were incubated in complete growth medium for 40 min, fixed, and stained with the DNA dye Hoechst 33342.

Rat Brain Cytosol and Total Membranes. Rat brain cytosol and total membranes were prepared as described previously (6), with modifications. Rat brains were isolated, washed in buffer A (25 mM Tris·HCl at pH 7.4, 320 mM sucrose) and transferred to 12 mL ice-cold buffer B (25 mM Tris·HCl at pH 8.0, 250 mM sucrose, 500 mM KCl, 1 mM DTT, 2 mM EGTA, protease inhibitors) and homogenized (Ultra-Turrax; IKA Works). The homogenate was centrifuged (5,000 × *g*, 30 min, 4 °C), and the supernatant was ultracentrifuged (150,000 × *g*, 90 min, 4 °C). The resulting pellet was resuspended in five volumes of PBS, named total membranes, aliquoted, frozen in liquid nitrogen, and stored at −80 °C. The supernatant obtained after the ultracentrifugation (150,000 × *g*, 90 min, 4 °C) was dialyzed against buffer C (25 mM Tris·HCl at pH 8.0, 50 mM KCl, 1 mM DTT, protease inhibitors) with one change of buffer, after 2 h, for a total of 4 h (ratio of sample to dialyzed buffer C, 1:140) using dialysis membranes with a molecular weight cutoff of 2,000 Da (Spectrum Laboratories). The precipitate formed during the dialysis was removed by centrifugation for 60 min at 150,000 × g at 4 °C. The supernatant obtained, named cytosol, was divided into aliquots, frozen in liquid nitrogen, and stored at –80 °C.

BAC Purification. BAC purification was performed using a Waters 2487 Binary Pump HPLC system equipped with a Waters 1525 Dual λ Absorbance Detector (Kontron HPLC Pump 420). The lyophilized BAC fraction was resuspended in 1 mL buffer A (10 mM KH_2PO_4 containing 2.5 mM tetrabutylammonium chloride) and loaded onto a semipreparative C18 reverse-phase column (25 × 250 mm; pore size, 10 μm; Viosfer) equilibrated in buffer A. The elution was carried out at a flow rate of 2.5 mL/min using a nonlinear gradient of buffer B (40% buffer A, 60% methanol): time (T) 0 min (100% A, 0% B); T 20 min (50% A, 50% B); T 30 min (0% A, 100% B); T 35 min (0% A, 100% buffer B); and T 40 min (100% A, 0% buffer). The collected fractions then were analyzed in ADP-ribosylation assays as described above, and those positive were pooled, supplemented with sucrose (10 mM), and lyophilized. The sample then was resuspended in 1 mL water and purified further using the same C18 reverse-phase column equilibrated with water. The elution was performed at a flow rate of 2.5 mL/min using a nonlinear gradient of buffer B (80% methanol, 20% water): T 0 min (100% A, 0% B); T 10 min (77.5% A, 22.5% B); T 13 min (50% A, 50% B); T 23 min (0% A, 100% B); and T 35 min (100% A, 0% B). With this procedure, the molecule eluted at 17 min. The fractions containing the purified metabolite were pooled, supplemented with 10 mM sucrose, and lyophilized. The sample was resuspended in 20 mM Hepes, pH 7.2 (BAC final concentration, 100 μM), and was aliquoted and stored at −20 °C.

Quantitative Measure of BAC Production in the Presence of Cyclic ADP-Ribose. Total membranes from rat brain were incubated in the presence of BFA (80 μg/mL), NAD^+ (1 mM), and 0.01 μCi/μL [^{32}P]-NAD^+ in metabolite buffer (20 mM Tris·HCl at pH 7.0, 50 mM NaCl) at 37 °C for 2 h. BAC then was purified by HPLC (as described above), and the fraction containing BAC was analyzed by liquid scintillation counting for a quantitative estimation of BAC concentration. To produce BAC in the presence of cyclic ADP-ribose (cADPR), the same procedure was followed, except NAD^+ and [^{32}P]-NAD^+ were substituted with cADPR (1 mM). The resulting product then was purified by HPLC (as described above). A chromatographic peak with the same elution time as BAC was observed. The area of the peaks was used to estimate the relative abundance of BAC produced in the presence of cADPR vs. NAD^+ (as a standard). By this approach, we estimated that 13.0 nmol of BAC was produced using NAD^+, vs. 3.2 nmol produced using cADPR.

Immunoprecipitation Experiments. HeLa cells were transiently transfected with cDNA coding for wild-type YFP-BARS or its point mutant, His304Ala, using *Trans*IT-LT1 transfection reagent (Mirus), according to the manufacturer's instructions. Twenty-

four hours after transfection, the cells were treated with BFA (80 μg/mL) or vehicle alone as a control (DMSO) in DMEM for 4 h at 37 °C, in the presence of 5 mM total NAD^+ or 1 mM cADPR. After 4 h, the cells were washed three times in ice-cold PBS and lysed using 1% Triton lysis buffer [50 mM Tris·HCl at pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, and 1% (wt/vol) Triton X-100, supplemented with protease inhibitor mixture]. Total lysates were centrifuged (15,000 × *g*, 10 min, 4 °C) and incubated with an anti-BARS antibody. After an overnight incubation, 25 μL Protein A Sepharose beads (Amersham) were added, with incubation for an additional 1 h at 4 °C. The suspensions then were centrifuged for 5 min at 500 × *g*, and the supernatants were recovered. The matrices were washed five times, and the bound proteins were eluted by boiling the samples for 10 min in 80 μL SDS sample buffer. The immunoprecipitated proteins were separated on 10% SDS/PAGE gels, transferred onto nitrocellulose, and subjected to Western blotting.

GST Pull-Down. Equimolar amounts of GST-PAK1, GST-14-3-3γ, GST-E1A, or GST were incubated with 5 μg His-BARS or His-BARS bound to BAC (see below) in GST incubation buffer (20 mM Tris at pH 8.0, 100 mM KCl, 1 mM EDTA, 0.2% Triton X-100, and protease inhibitors) for 2 h at 4 °C, with gentle agitation. After this step, 25 μL glutathione Sepharose 4B matrix (previously equilibrated in GST incubation buffer) was added to each of the samples, which then were incubated further for 1 h at 4 °C. The suspensions then were centrifuged at 500 × *g* for 5 min to sediment the matrix. Following extensive washing of the beads, the interacting proteins were eluted with 100 mM Tris at pH 8.0, 20 mM reduced glutathione, and 5 mM DTT; the eluted fractions were examined by SDS/PAGE and Western blotting.

His-BARS was previously incubated for 3 h at 37 °C with HPLC-purified BAC (120 μM) or with buffer alone (20 mM Tris at pH 7.4, 10 mM sucrose) to allow binding of BAC to His-BARS. The reaction mixture then was stopped on ice and used for GST pull-down assay as described above.

MALDI-TOF MS Analysis. Bands from SDS/PAGE containing recombinant BARS bound and not bound to BAC were excised manually from the gel, placed in 200-μL PCR tubes (Eppendorf), and washed to remove Coomassie blue staining. The cysteines then were reduced with 50 μL 10-mM DTT and subsequently alkylated with 50 μL 55-mM iodoacetamide. After derivatization, 20 μL of a solution of endoproteinase AspN at 10 ng/μL in 50 mM ammonium bicarbonate was added to the samples and incubated overnight at 37 °C. After adding 3 μL 10% (vol/vol) trifluoroacetic acid (TFA) to stop the digestion, the samples were ready for analysis.

Tryptic peptides were desalted and concentrated by reverse-phase extraction using ZipTip C_{18} (Millipore) according to the manufacturer's guidelines. Elution was performed with a 50% (vol/vol) acetonitrile/0.1% TFA solution of α-cyano-4-hydroxycinnamic acid at 3.6 mg/mL directly on the MTP Ground Steel 384 target (Bruker Daltonics).

MALDI-TOF MS and MS/MS spectra were acquired on an UltraFlex III TOF/TOF mass spectrometer (Bruker Daltonics) in reflectron-positive (RP) and LIFT modes, respectively. The voltages for RP acquisitions were set at 25.0 kV and 21.7 kV, for the first and second ion extraction stages, respectively; 9.0 kV for the lens; 26.3 kV for reflector 1; and 13.8 kV for reflector 2. The voltages for the LIFT acquisition were set at 8.0 kV and 7.2 kV for the first and second ion extraction stages, respectively; 3.6 kV for the lens; 29.5 kV for reflector 1; 13.8 kV for reflector 2; 19.0 kV for LIFT 1; and 3.2 for LIFT 2. The laser power attenuator value was adjusted for each acquisition to balance the differences in sample preparation and maximize resolution and intensity. Quadratic external calibration of the instrument was performed on monoisotopic masses of Bruker peptide calibration standard II, containing angiotensin II, angiotensin I, substance P, bombesin, renin substrate, Adrenocorticotropic hormone (ACTH) (1–17), ACTH (18-39), and somatostatin. FlexAnalysis 3.0 (Bruker Daltonics) and BioTools 3.0 were used to process the spectra and for the database search, respectively. The SwissProt protein database was interrogated through the MASCOT search engine, with the query restricted to *Rattus norvegicus*, using the following parameters: 50 ppm tolerance on the peptides and 0.3 Da for the fragments, one missed cleavage allowed, fixed carboamidomethylation of cysteines, and possible oxidation of methionines.

Conjugation of BFA to Hemocyanin to Raise Anti-BFA Antibodies. To produce succynylated BFA for coupling to hemocyanin, BFA was treated overnight with trimethylsilyl chloride–triethylamine in tetrahydrofuran, then silylated with *tert*-butyldimethylsilyl chloride in methylene chloride containing 2,6-lutidine, and finally treated with diluted hydrochloric acid in tetrahydrofuran before exposure to succinic anhydride and treatment with hydrofluoric acid/CH_3CN, as described previously (7). Coupling of succinylated BFA to hemocyanin was performed using 10 mM 1-ethyl-3 (3-diethylaminopropyl) carbodiimide. Keyhole limpet hemocyanin/BFA (500 μg) was dissolved in 0.1% (wt/vol) NaCl, mixed with 0.7 mL complete Freund adjuvant, and injected s.c. in rabbits. The next injections were made 2 wk after using incomplete Freund adjuvant. Two weeks later, a series of six i.v. injections were made into the peripheral vein of the ear, in two rounds of three each. The rounds were spaced at weekly intervals, and injections were daily. The inoculum was prepared by dissolving 150 μg protein in 0.3 mL of 0.1% (wt/vol) NaCl and was mixed with two volumes of aluminium potassium sulfate solution [10% $KAl(SO_4)_2 0.12H_2O$ in water]. The pH of the solution was neutralized by slowly adding 10 M NaOH until a precipitate was generated. The precipitate was incubated 4 h with continuous tumbling, then resuspended in 1 mL of 17 mM NaCl for injection. The antibody was developed in the animal laboratory of CBMSO (Centro de Biologia Molecular Severo Ochoa Universidad Autonoma de Madrid) with the license function (06/24/1993 registration number 280) of the Ministry of Agriculture, Fisheries and Food approved by Royal Decree 223/1988 (point 2 article 8).

Gel Filtration. Five milligrams of rat brain cytosol was applied to a Superose 12 High-Resolution 10/30 gel filtration column (Amersham Pharmacia), as previously described (2). The eluted proteins were collected using an AKTA FPLC system (Amersham Pharmacia) and detected by monitoring absorbance at 280 nm.

1. Nardini M, et al. (2003) CtBP/BARS: A dual-function protein involved in transcription co-repression and Golgi membrane fission. *EMBO J* 22(12):3122–3130.
2. Valente C, Spanò S, Luini A, Corda D (2005) Purification and functional properties of the membrane fissioning protein CtBP3/BARS. *Methods Enzymol* 404:296–316.
3. Valente C, et al. (2012) A 14-3-3γ dimer-based scaffold bridges CtBP1-S/BARS to PI(4) KIIIβ to regulate post-Golgi carrier formation. *Nat Cell Biol* 14(4):343–354.
4. Liberali P, et al. (2008) The closure of Pak1-dependent macropinosomes requires the phosphorylation of CtBP1/BARS. *EMBO J* 27(7):970–981.
5. Vassilev LT, et al. (2006) Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc Natl Acad Sci USA* 103(28):10660–10665.
6. Spanò S, et al. (1999) Molecular cloning and functional characterization of brefeldin A-ADP-ribosylated substrate. A novel protein involved in the maintenance of the Golgi structure. *J Biol Chem* 274(25):17705–17710.
7. Paulik M, et al. (1999) Drug-antibody conjugates with anti-HIV activity. *Biochem Pharmacol* 58(11):1781–1790.

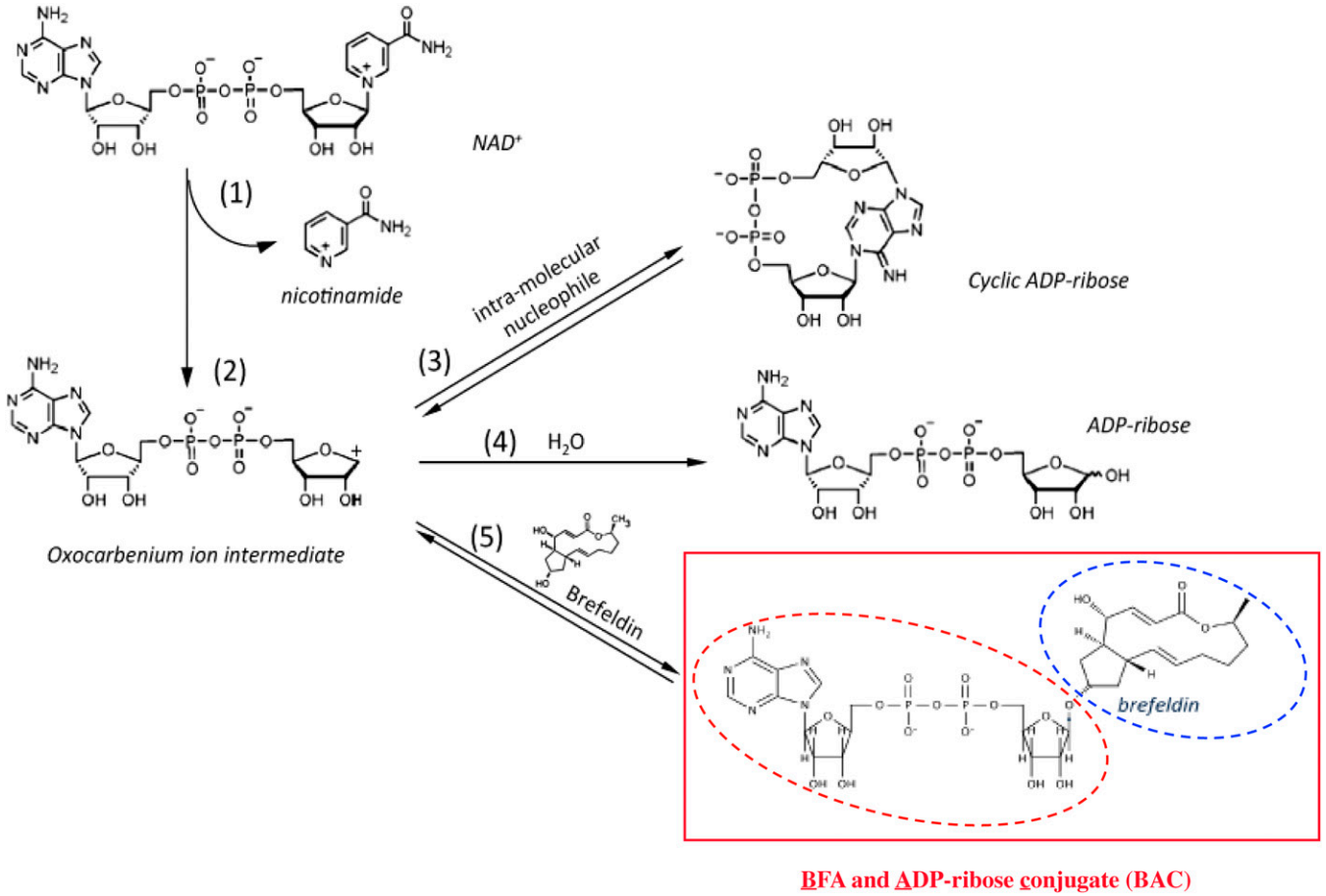

Fig. S1. Model of the molecular mechanism of BAC synthesis. The ADP-ribosyl cyclases convert NAD^+ to cADPR and/or ADPR through cleavage of the nicotinamide–ribose bond (1) and formation of an enzyme-stabilized ADP-ribosyl–oxocarbenium ion intermediate (2). This intermediate is a good nucleophile acceptor that can react with the intramolecular adenine (3) or water (4) to give cADPR or ADPR, respectively. As BFA has two hydroxyl groups, it may act as a nucleophile and form a conjugate with the oxocarbenium ion intermediate (5), leading to the formation of the BFA and ADPR conjugate (BAC).

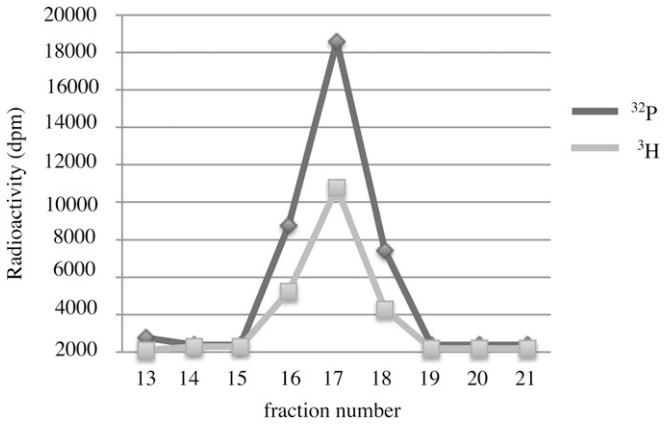

Fig. S2. The active derivative is a BAC. Total membranes from rat brain (400 μg/mL) were incubated for 2 h at 37 °C in the presence of BFA (80 μg/mL) and NAD^+ (1 mM). [^{3}H]-BFA and [^{32}P]-NAD^+ were added as tracers (300 and 600 dpm/pmol specific activity, respectively). The samples then were purified through the two-step HPLC procedure (*SI Material and Methods*). Fractions were collected at 1-min intervals and analyzed by liquid scintillation counting.

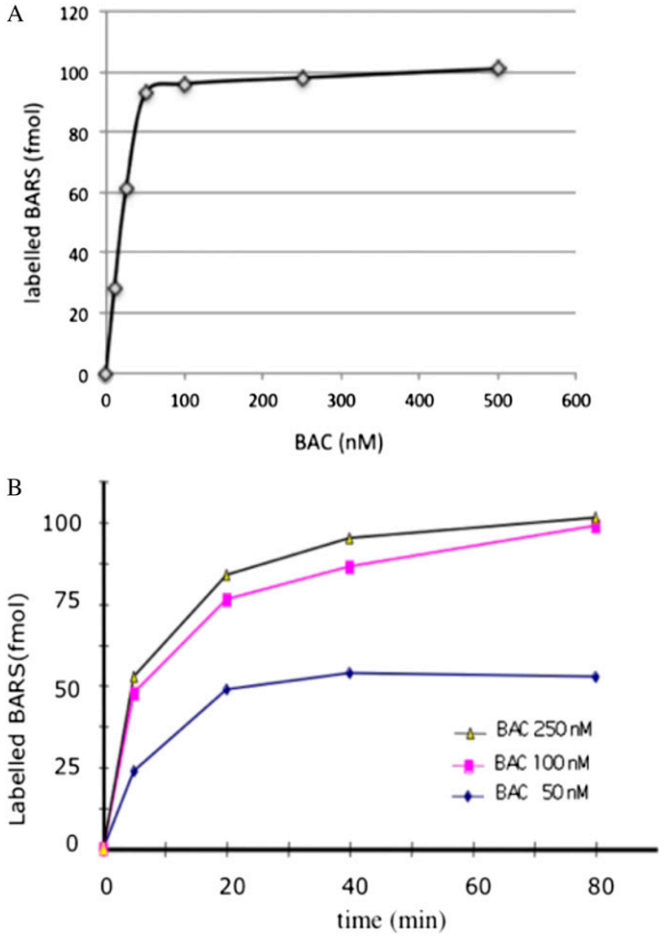

Fig. S3. Dose–response and time course of BAC binding to cytosolic BARS. (*A*) Rat brain cytosol was incubated for 2 h at 37 °C with the indicated concentrations of [^{32}P]-labeled BAC. (*B*) Rat brain cytosol was incubated for various times at 37 °C with the indicated concentrations of [^{32}P]-labeled BAC. (*A* and *B*) The samples were analyzed by SDS/PAGE. The amounts of modified BARS were estimated using an autoradiography reader (Instant Imager) to measure the radioactivity associated with BARS.

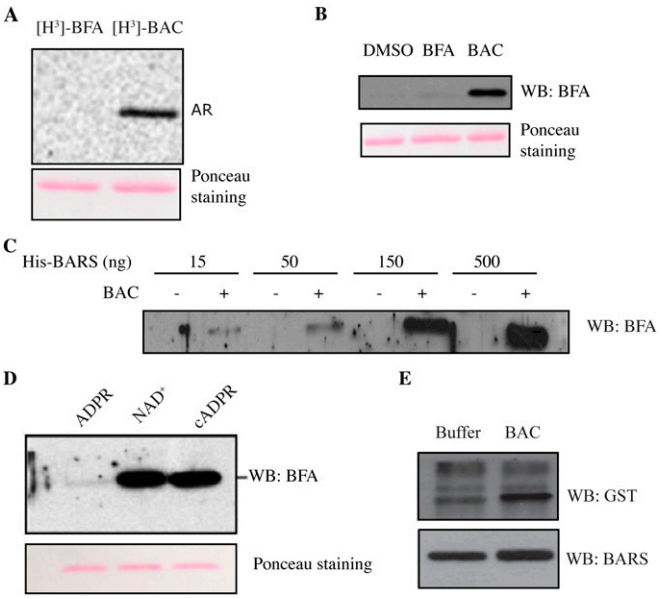

Fig. S4. The entire BAC molecule binds covalently with BARS. (*A*) BAC was produced using [^{3}H]-radiolabeled BFA. The HPLC-purified [^{3}H]-BFA-BAC or [^{3}H]-BFA was incubated for 2 h at 37 °C with recombinant BARS. The samples were separated by SDS/PAGE and examined by digital autoradiography (AR) using a Fugifilm imager. (*B*) Recombinant BARS was incubated for 2 h with BFA or BAC or vehicle alone (DMSO), then analyzed by immunoblotting with an anti-BFA antibody. (*C*) Different amounts of BARS, as indicated, were incubated with BAC for 2 h at 37 °C. The samples were separated by SDS/PAGE and examined by immunoblotting with an anti-BFA antibody. (*D*) Total rat brain membranes were incubated with BFA (80 μg/mL) at 37 °C in the presence of 100 μM ADPR, NAD^+, or cADPR. After 2 h, the membranes were removed by centrifugation and the supernatants were recovered and incubated with recombinant His-BARS for 2 h at 37 °C. The samples were analyzed by SDS/PAGE and transferred onto nitrocellulose. The modified BARS was analyzed using an anti-BFA antibody. (*A*, *B*, and *D*; *Lower*) Total levels of BARS are shown. (*E*) Recombinant BARS was incubated for 1 h with BAC or buffer alone. The samples were separated by SDS/PAGE and transferred onto nitrocellulose. The blot was incubated with GST-tagged macrodomain (200 ng/mL) for 2 h at room temperature and revealed using a GST antibody. (*E*, *Lower*) Total levels of BARS are shown.

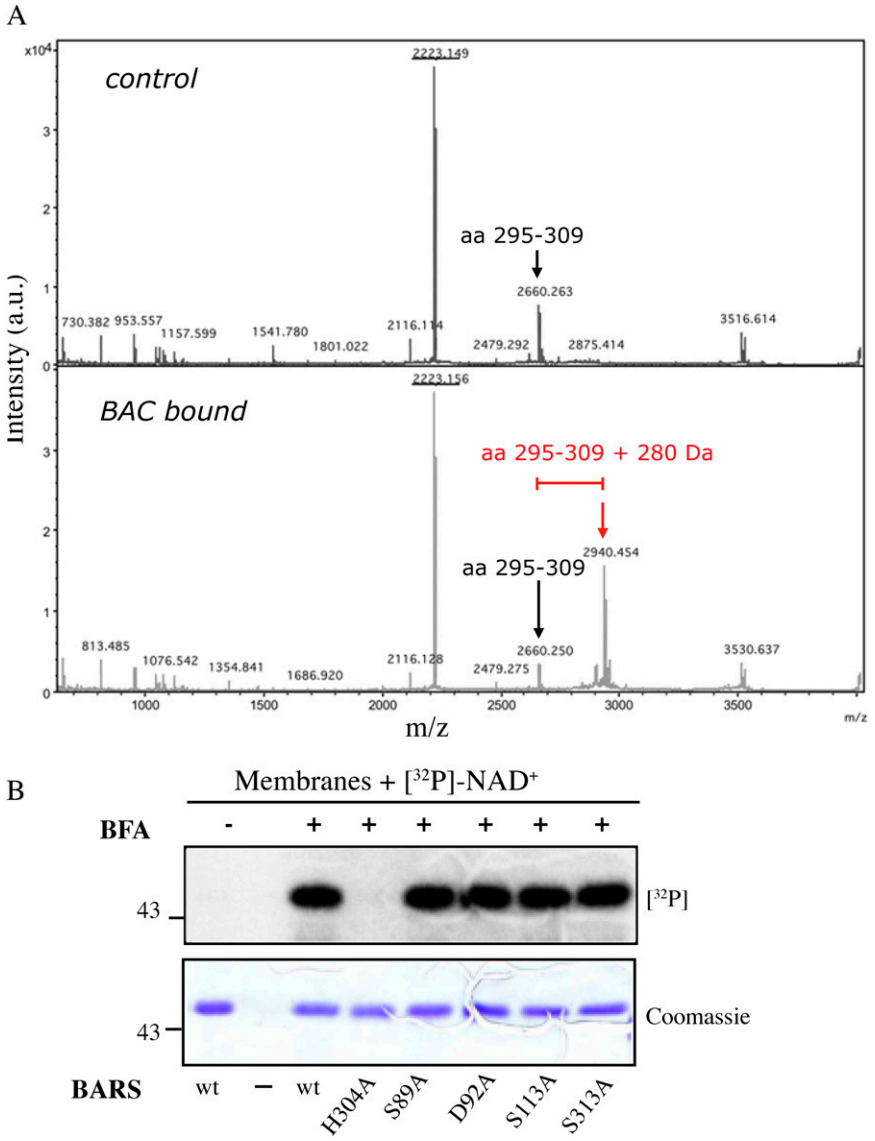

Fig. S5. Identification of the amino acid residue involved in the covalent bond to BAC. (*A*) Recombinant BARS was incubated with BAC (BAC bound) or control buffer (control) for 4 h at 37 °C. The samples then were subjected to SDS/PAGE separation, proteolytic digestion, and MALDI-TOF and LC-MS analysis. (*B*) To confirm the involvement of His304 in the formation of the covalent bond between BAC and BARS, different recombinant point mutants of the BARS protein were generated, produced in bacteria, and tested in in vitro ADP-ribosylation assays, using total brain membranes to produce BAC. The samples were separated by SDS/PAGE and examined by autoradiography for [^{32}P]. Total BARS levels are shown by Coomassie blue staining.

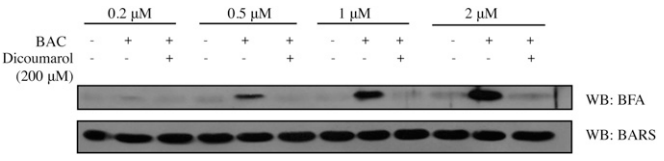

Fig. S6. Dicoumarol competes with BAC for binding to recombinant BARS. Recombinant His-BARS was incubated for 1 h at 37 °C with different concentrations of BAC in the absence and in the presence of dicoumarol (200 μM). The samples were analyzed by SDS/PAGE and Western blotting. The modified BARS was revealed using an anti–BFA-specific antibody. (*Bottom*) Total levels of BARS were monitored using an anti-BARS antibody and are shown.

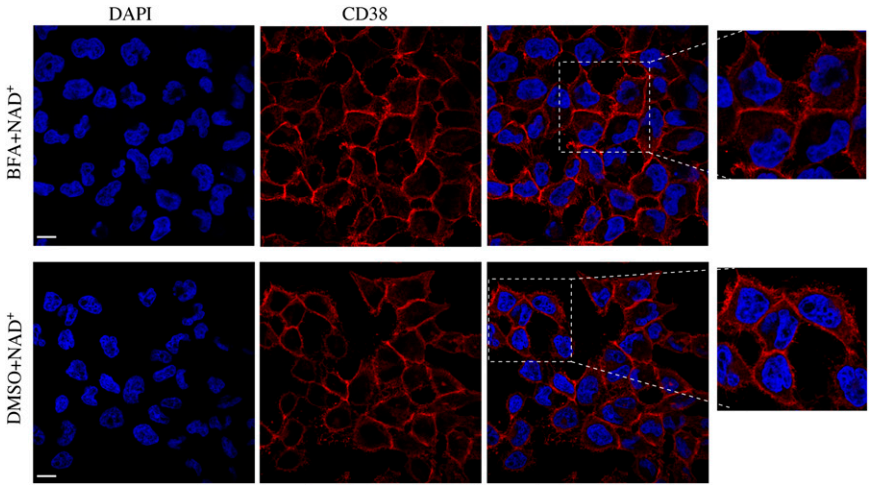

Fig. S7. Subcellular localization of CD38. CD38$^+$ HeLa cells were incubated with BFA or vehicle alone (DMSO) in the presence of 5 mM extracellular NAD$^+$. After 1 h of incubation, the cells were fixed and labeled with the DNA-specific dye Hoechst and an anti-CD38 antibody. Scale bar, 15 μM. Representative images are shown.

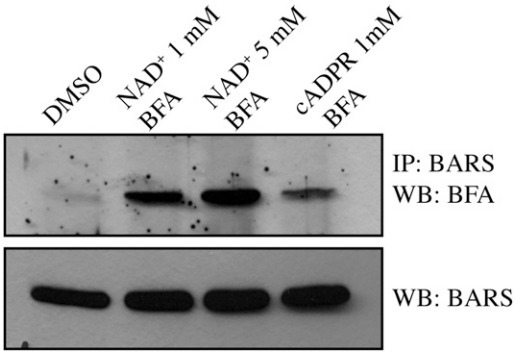

Fig. S8. cADPR also may induce BAC binding to intracellular BARS. CD38$^+$ HeLa cells were transfected with YFP-BARS. After 24 h, the cells were treated with 80 μg/mL BFA for 4 h at 37 °C, in the presence of extracellular NAD$^+$ (1 mM or 5 mM) or cADPR (1 mM). YFP-BARS was immunoprecipitated from the total lysates using an anti-BARS antibody, and the ADP-ribosylated protein was revealed using an anti-BFA antibody. (*Lower*) Total BARS levels are shown.

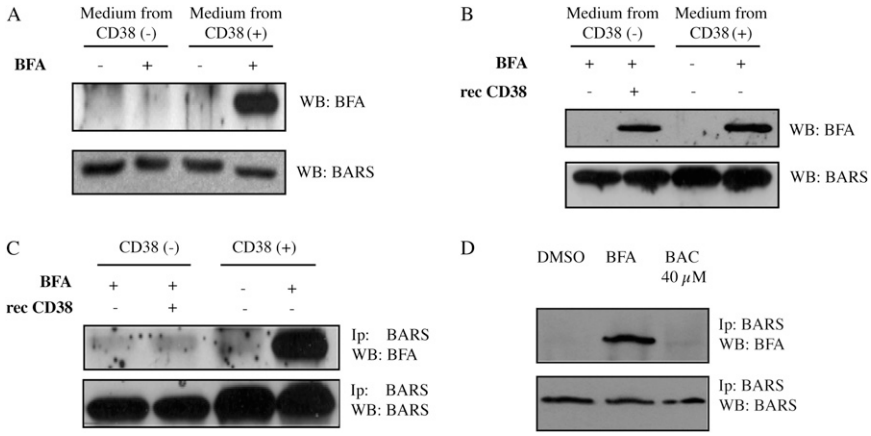

Fig. S9. BAC production and internalization require CD38. (*A*) Control ($CD38^-$) and $CD38^+$ HeLa cells were treated with BFA (80 μg/mL) at 37 °C. After 4 h of treatment, the different media were recovered and incubated with recombinant His-BARS for 2 h at 37 °C. Samples were analyzed by SDS/PAGE and transferred onto nitrocellulose. The modified BARS was analyzed using an anti–BFA-specific antibody. (*A*, *Lower*) Total levels of BARS are shown. (*B* and *C*) Control ($CD38^-$) and $CD38^+$ HeLa cells were transiently transfected with YFP-BARS. After 24 h of overexpression, the cells were treated for 4 h with BFA (80 μg/mL) and 5 mM NAD^+ at 37 °C. To compensate for the absence of CD38, a recombinant CD38 (rec CD38) enzyme was added to the extracellular medium for the time of treatment. At the end of the treatment, the different media were recovered. (*B*) Recombinant His-BARS was incubated with the recovered media for 2 h at 37 °C to induce BAC binding in vitro. An anti–BFA-specific antibody was used to check for modified BARS. (*B*, *Lower*) Total levels of BARS are shown. (*C*) At the end of the incubation, the cells were lysed and BARS was immunoprecipitated. The samples were resolved on SDS/PAGE and transferred on nitrocellulose. Modified BARS was analyzed using an anti–BFA-specific antibody. (*C*, *Lower*) Total levels of BARS are shown. (*D*) $CD38^+$ HeLa cells were transiently transfected with YFP-BARS. After 24 h of overexpression, the cells were treated with BFA (80 μg/mL) or HPLC-purified BAC for 2 h at 37 °C. At the end of the incubation, the cells were lysed and BARS was immunoprecipitated. The samples were resolved on SDS/PAGE and transferred onto nitrocellulose. Modified BARS was analyzed using an anti-BFA antibody. (*D*, *Lower*) Total levels of BARS are shown.