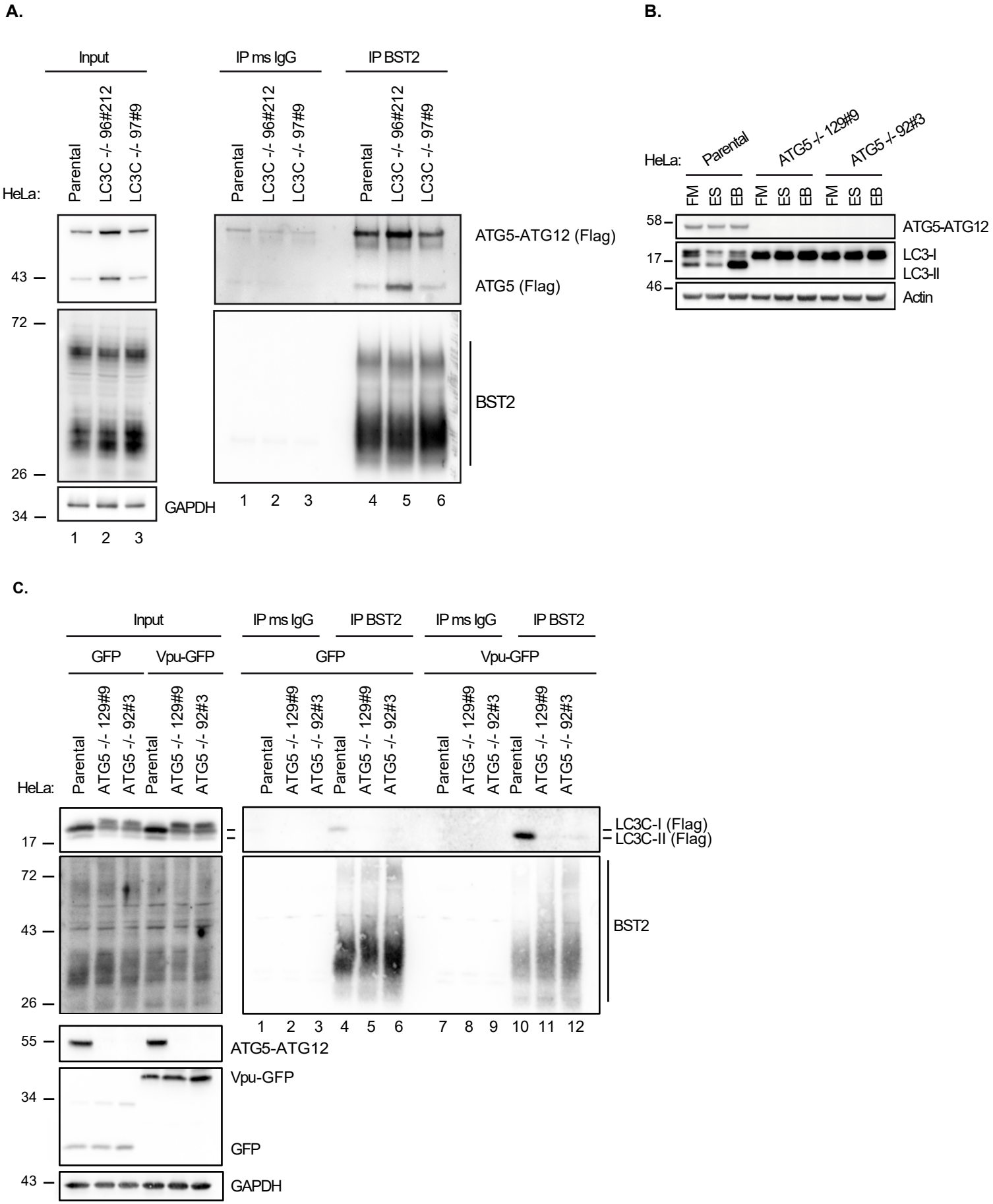
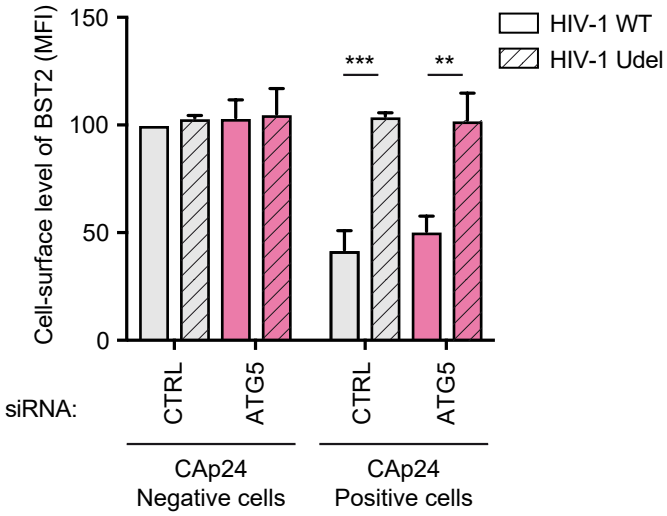

A.
Input
IP ms IgG
IP BST2
HeLa:
Parental
LC3C -/- 96#212
LC3C -/- 97#9
43
72
26
34
GAPDH
ATG5-ATG12 (Flag)
ATG5 (Flag)
BST2
1 2 3 4 5 6
B.
HeLa:
Parental
ATG5 -/- 129#9
ATG5 -/- 92#3
FM ES EB FM ES EB FM ES EB
58
17
46
ATG5-ATG12
LC3-I
LC3-II
Actin
C.
Input
IP ms IgG
IP BST2
IP ms IgG
IP BST2
GFP
Vpu-GFP
GFP
Vpu-GFP
HeLa:
Parental
ATG5 -/- 129#9
ATG5 -/- 92#3
17
72
43
26
55
34
43
LC3C-I (Flag)
LC3C-II (Flag)
BST2
1 2 3 4 5 6 7 8 9 10 11 12
ATG5-ATG12
Vpu-GFP
GFP
GAPDH

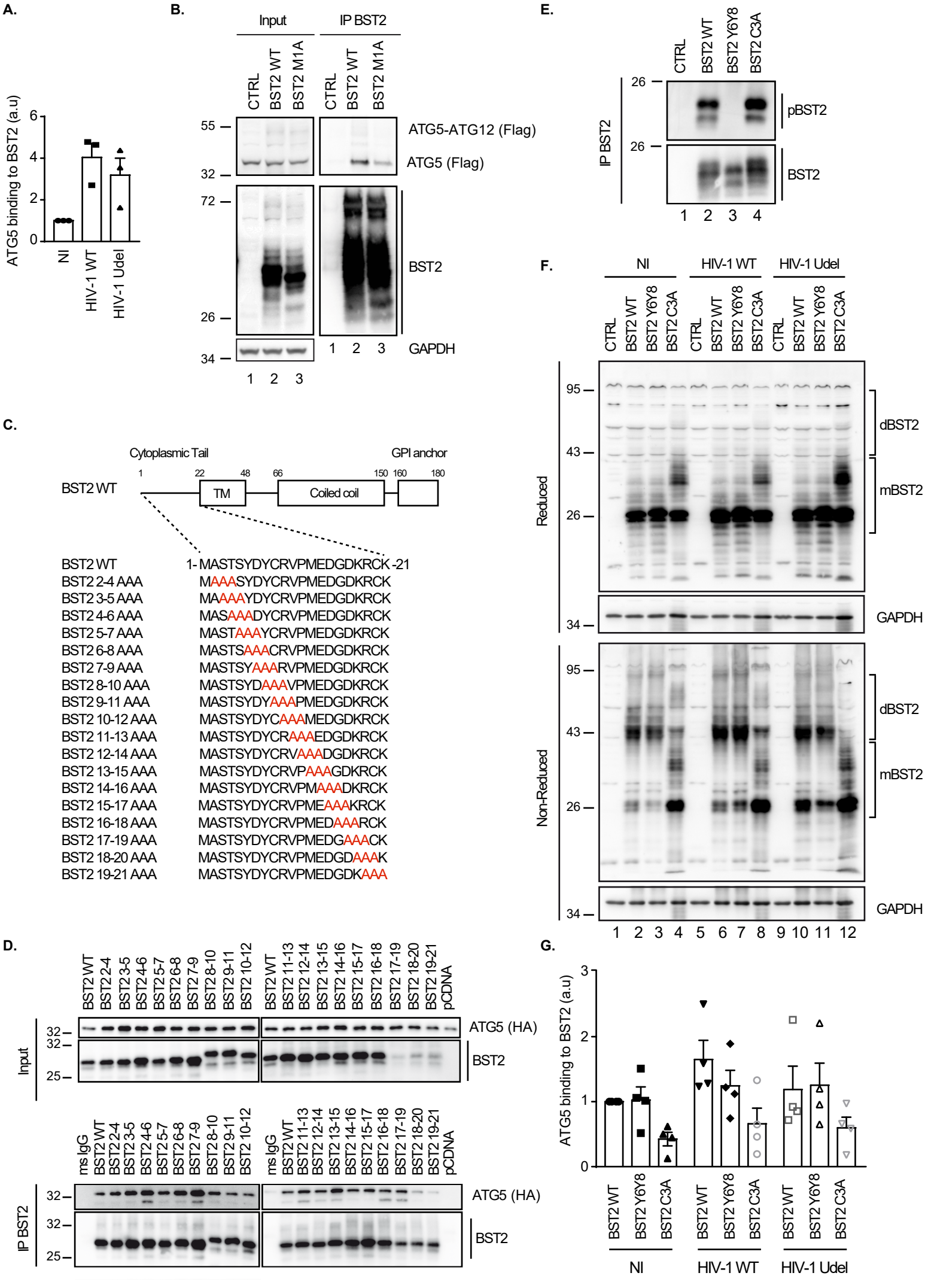
Supplemental figure 1. related to Figure 1

(A) Immunoprecipitation of endogenous BST2 in Parental (CTRL) or LC3C knockout (LC3C -/- 196#212 or 97#9) HeLa cells extracts co-transfected with p3XFlag-ATG5. Immunoprecipitated proteins were detected by western blotting using anti-BST2 and anti-Flag-HRP antibodies. **(B)** Parental (CTRL) or ATG5 knockout (ATG5 -/- 129#9 or 92#3) HeLa cells were incubated in full medium (FM) or EBSS for amino acid depletion (ES) without or with Bafilomycin A1 (EB) for 2 h before immunoblotting for ATG5, Actin, and LC3B. **(C)** Immunoprecipitation of endogenous BST2 in Parental (CTRL) or ATG5 knockout (ATG5 -/- 129#9 or 92#3) HeLa cells extracts co-transfected with vectors encoding for Flag-LC3C and GFP or Vpu-GFP. Immunoprecipitated proteins were detected by western blotting using rabbit anti-BST2 and anti-Flag-HRP antibodies. All Western blots presented are representative of at least three independent experiments. Immunoprecipitation of endogenous BST2 were done with mouse anti-BST2 antibodies in all experiments.



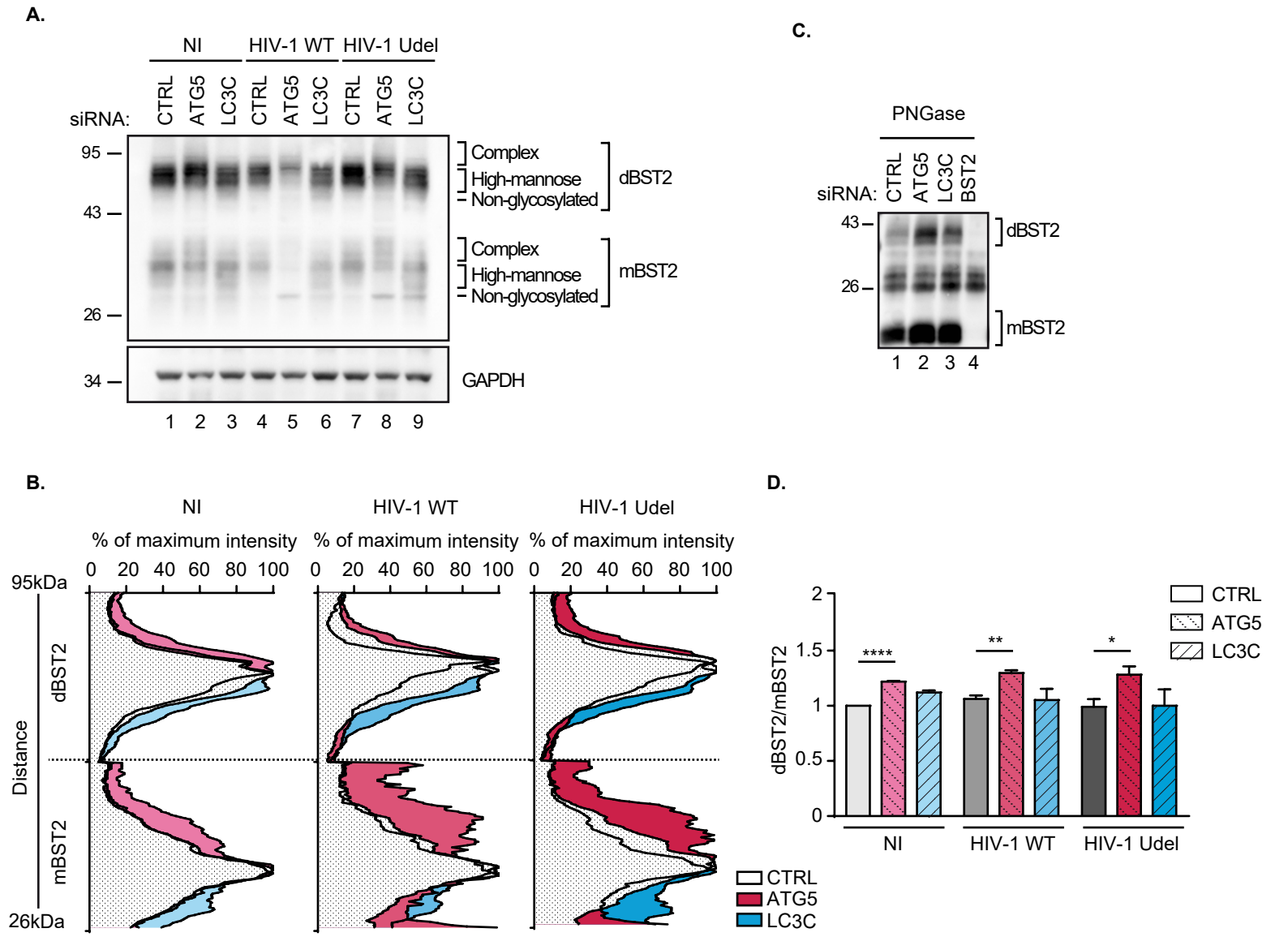
Supplemental figure 2. related to Figure 2

HeLa cells transfected with either control siRNA (CTRL) or siRNA targeting ATG5 were infected with a VSV-G pseudotyped WT or Udel NL4.3 HIV-1 at a MOI of 0.5. Twenty-four hours later, cells were stained at the cell surface with anti-BST2 antibody. Cells were then fixed, permeabilized and stained for Gag using anti-CAp24 antibody. Cells were then processed for flow cytometry analysis. Bar graphs represent cell surface level of BST2 in CAp24 negative and positive cells for each siRNA condition. Values are expressed as the Mean Fluorescence Intensity (MFI). Statistical analysis using two-way ANOVA with Tukey's multiple comparison test, mean ± SEM, n=3 experiments; ** $p \leq 0.01$, *** $p \leq 0.001$.


A.
ATG5 binding to BST2 (a.u)
NI
HIV-1 WT
HIV-1 Udel
B.
Input
IP BST2
CTRL
BST2 WT
BST2 M1A
ATG5-ATG12 (Flag)
ATG5 (Flag)
BST2
GAPDH
C.
Cytoplasmic Tail
GPI anchor
BST2 WT
TM
Coiled coil
BST2 WT 1- MASTSYDYCRVPMEDGDKRCK -21
BST2 2-4 AAA MAAASYDYCRVPMEDGDKRCK
BST2 3-5 AAA MAAAAYDYCRVPMEDGDKRCK
BST2 4-6 AAA MASAAADYCRVPMEDGDKRCK
BST2 5-7 AAA MASTAAAYCRVPMEDGDKRCK
BST2 6-8 AAA MASTSAAACRVPMEDGDKRCK
BST2 7-9 AAA MASTSYAAARVPMEDGDKRCK
BST2 8-10 AAA MASTSYDAAAVPMEDGDKRCK
BST2 9-11 AAA MASTSYDYAAAPMEDGDKRCK
BST2 10-12 AAA MASTSYDYCAAAMEDGDKRCK
BST2 11-13 AAA MASTSYDYCRAAAEDGDKRCK
BST2 12-14 AAA MASTSYDYCRVAAADGDKRCK
BST2 13-15 AAA MASTSYDYCRVPAAAGDKRCK
BST2 14-16 AAA MASTSYDYCRVPMAAADKRCK
BST2 15-17 AAA MASTSYDYCRVPMEAAAKRCK
BST2 16-18 AAA MASTSYDYCRVPMEDAAARCK
BST2 17-19 AAA MASTSYDYCRVPMEDGAAACK
BST2 18-20 AAA MASTSYDYCRVPMEDGDAAAK
BST2 19-21 AAA MASTSYDYCRVPMEDGDKAAA
D.
BST2 WT
BST2 2-4
BST2 3-5
BST2 4-6
BST2 5-7
BST2 6-8
BST2 7-9
BST2 8-10
BST2 9-11
BST2 10-12
BST2 11-13
BST2 12-14
BST2 13-15
BST2 14-16
BST2 15-17
BST2 16-18
BST2 17-19
BST2 18-20
BST2 19-21
pCDNA
ms IgG
Input
IP BST2
ATG5 (HA)
BST2
E.
CTRL
BST2 WT
BST2 Y6Y8
BST2 C3A
IP BST2
pBST2
BST2
F.
NI
HIV-1 WT
HIV-1 Udel
Reduced
Non-Reduced
dBST2
mBST2
GAPDH
G.
ATG5 binding to BST2 (a.u)
BST2 WT
BST2 Y6Y8
BST2 C3A
NI
HIV-1 WT
HIV-1 Udel

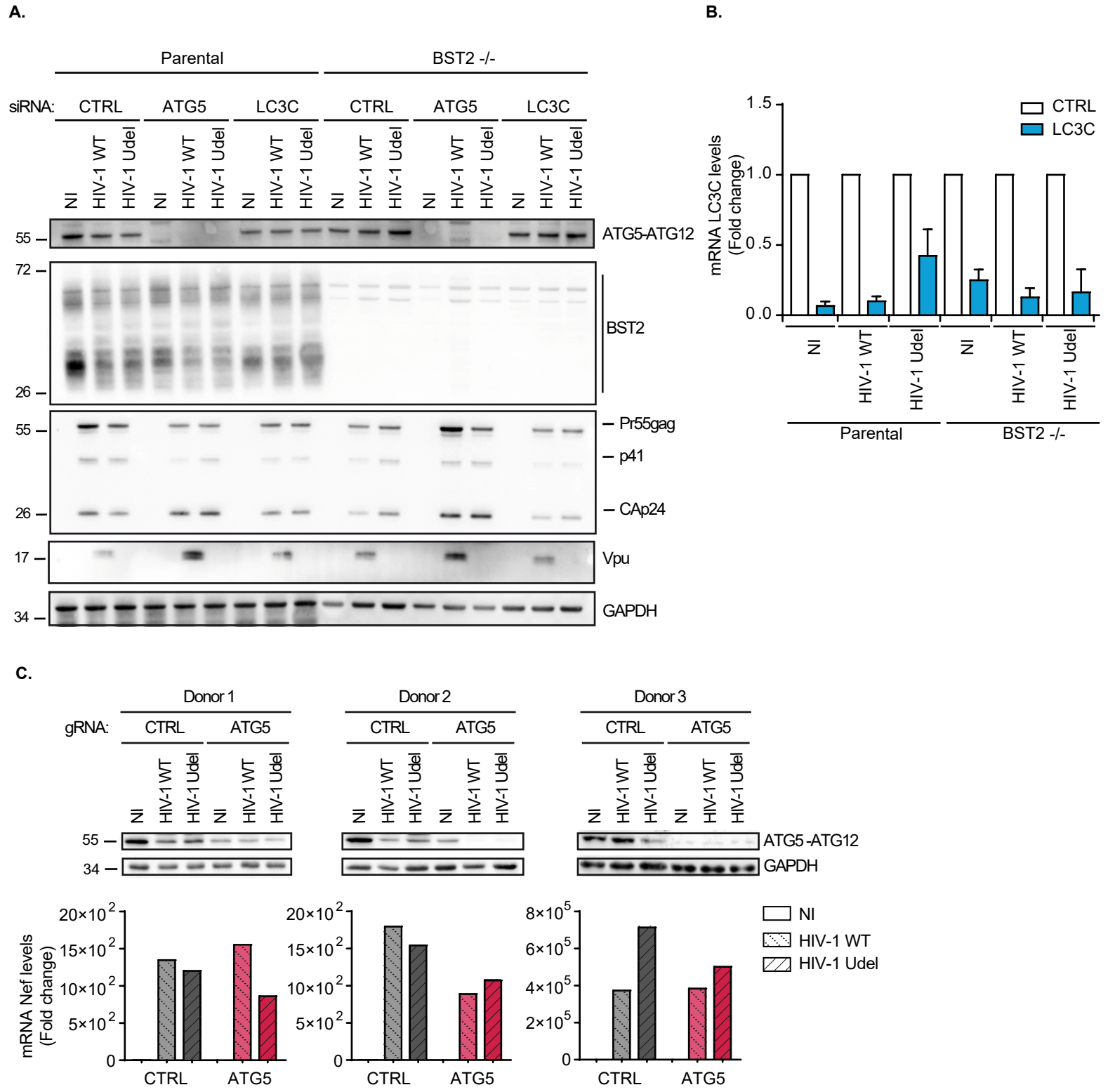
Supplemental figure 3. related to Figures 3 and 4

(A) Quantification of the relative amount of ATG5 immunoprecipitated with BST2 WT upon infection from Figure 3A. Quantification presented are representative of at least three independent experiments. **(B)** HEK293T cells were transfected with p3XFlag-ATG5 and either pcDNA, pcDNA-BST2 WT, or pcDNA-BST2 M1A encoding for the short isoform of BST2. BST2 was immunoprecipitated with mouse anti-BST2 antibodies. Immunoprecipitated proteins were detected by western blotting using anti-flag-HRP and rabbit anti-BST2 antibodies. **(C)** Schematic representation of alanine mutagenesis in the cytoplasmic domain of BST2. **(D)** HEK293T cells were transfected with pCMV-HA-ATG5 and either pcDNA, pcDNA-BST2 WT, or plasmids encoding for mutated BST2 as described in (B). BST2 was immunoprecipitated with mouse anti-BST2 antibodies. Immunoprecipitated proteins were detected by western blotting using anti-HA-HRP and rabbit anti-BST2 antibodies. **(E-F)** HEK293T cells were co-transfected with either pcDNA-BST2 WT or BST2 Y_6Y_8 (double tyrosine phosphorylation defective mutant) or BST2 C3A (triple cysteine dimerization defective mutant) and the WT or Udel NL4.3 HIV-1 provirus. BST2 dimerization and phosphorylation were analyzed. For the detection of BST2 phosphorylation, BST2 was immunoprecipitated with a mouse anti-BST2 antibody and deglycosylated precipitates were analyzed by western blot using a rabbit antibody specific of phosphorylated tyrosine 6 and 8 of BST2 and a mouse anti-BST2 antibody (E). For BST2 dimerization, cell lysates were prepared under reducing or non-reducing conditions. dBST2: dimer of BST2; mBST2: monomer of BST2 (F). All Western blots presented are representative of at least three independent experiments. **(G)** Quantification of the relative amount of ATG5 immunoprecipitated with BST2 WT; BST2 Y_6Y_8 or BST2 C3A upon infection in four experiments from Figure 4C.


A.
NI
HIV-1 WT
HIV-1 Udel
siRNA:
CTRL
ATG5
LC3C
95
43
26
34
Complex
High-mannose
Non-glycosylated
dBST2
mBST2
GAPDH
1 2 3 4 5 6 7 8 9
C.
PNGase
BST2
43
26
1 2 3 4
B.
% of maximum intensity
0 20 40 60 80 100
95kDa
26kDa
Distance
D.
dBST2/mBST2
****
**
*

Supplemental figure 4. related to Figure 5

(A) HeLa cells transfected with either control siRNA (CTRL) or siRNA targeting ATG5 or LC3C were infected with a VSV-G pseudotyped WT or Udel NL4.3 HIV-1 at a MOI of 0.5 for 24 hrs. Cells were treated for 20min with formaldehyde 1% before preparing cell extracts under reducing conditions. Western blot analysis of BST2 and GAPDH in non-infected and infected siRNA-treated cells. **(B)** Line-scan profiles of BST2 intensity obtained on western blot of extracts of siRNA-treated HeLa cells infected with a VSV-G pseudotyped HIV-1 NL4.3 WT or Udel at a MOI of 0.5 for 24hrs and formaldehyde crosslinked. Analysis of BST2 patterns was determined by western blot and line-scan profiles of BST2 intensity were plotted across 95 to 26 kDa**.** The x-axis represents the % of maximum intensity for the monomeric and dimeric forms of BST2 and the y-axis, the distance between molecular weights. **(C)** HeLa cells transfected with either control siRNA (CTRL) or siRNA targeting ATG5, LC3C or BST2 were infected with a VSV-G pseudotyped WT or Udel NL4.3 HIV-1 at a MOI of 0.5 for 24hrs. Endogenous BST2 was immunoprecipitated with a mouse anti-BST2 antibody and deglycosylated precipitates were analyzed by western blot using a rabbit anti-BST2 antibody. dBST2: dimer of BST2; mBST2: monomer of BST2. All Western blots presented are representative of at least three independent experiments. **(D)** Quantification of the relative amount of the dimeric form of BST2 (dBST2) after normalization to the monomeric form (mBST2) from Figure 5A. Statistical analysis using two-tailed unpaired Student's *t* test, mean $\pm$ SEM, n=3 experiments, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. dBST2: dimer of BST2; mBST2: monomer of BST2.


A.
Parental
BST2 -/-
siRNA:
CTRL
ATG5
LC3C
NI
HIV-1 WT
HIV-1 Udel
55
72
26
17
34
ATG5-ATG12
BST2
Pr55gag
p41
CAp24
Vpu
GAPDH
B.
mRNA LC3C levels (Fold change)
1.5
1.0
0.5
0.0
CTRL
LC3C
Parental
BST2 -/-
C.
Donor 1
Donor 2
Donor 3
gRNA:
ATG5 -ATG12
GAPDH
mRNA Nef levels (Fold change)
20×10^2
15×10^2
10×10^2
5×10^2
0
8×10^5
6×10^5
4×10^5
2×10^5

Supplemental figure 5. related to Figure 5

(A) Western blot analysis of ATG5, HIV-1 Gag and CAp24 products, BST2, Vpu and GAPDH in parental and BST2 -/- HeLa cells transfected with either control siRNA (CTRL) or siRNA targeting ATG5 (ATG5) or LC3C and transfected with the WT or Udel NL4.3 HIV-1 provirus for 28hrs. The western blot is representative of three independent experiments. **(B)** Total RNA profile for LC3C mRNA levels relative to KDSR by qRT-PCR of HeLa BST2 WT and HeLa BST2 -/- cells transfected with either control siRNA (CTRL) or siRNA targeting LC3C and transfected with the provirus HIV-1 NL4.3 WT or Udel for 28hrs. n= 3 experiments. **(C)** Purified CD4+T cells from three independent donors were nucleofected with the indicated gRNA and then infected with a VSV-G pseudotyped HIV-1 NL4.3 WT or Udel at a MOI of 0.5 for 48 hrs. Total RNA was analyzed for Nef mRNA levels relative to FKBP4 by qRT-PCR. Western blot analysis of ATG5 and GAPDH in purified CD4+T cells from three independent donors nucleofected with the indicated gRNA and then infected with a VSV-G pseudotyped HIV-1 NL4.3 WT or Udel at a MOI of 0.5 for 48 hrs.

Supplemental Materials and Methods

Cell lines

HeLa and HEK293T cells were grown in DMEM (Dulbecco's modified Eagle's medium) with GlutaMAX and 10% decomplemented-FCS (fetal calf serum) (Gibco, Life Technologies). HeLa WT and HeLa BST2 KO cells were a kind gift of Stéphane Fremont (Institut Pasteur, France).

Primary CD4+ T cells

CD4+ T cells were obtained from HIV-seronegative human buffy coats. Peripheral blood mononuclear cells (PBMCs) were purified by density centrifugation using Ficoll-Paque Plus (density, 1.077 g/mL; GE Healthcare) at 400 x g for 30 minutes at 20°C. CD4+ T cells were negatively selected from PBMCs using magnetic-activated cell sorting (MACS) CD4+ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's recommendations. CD4+ T cells were then activated and expanded using T Cell TransAct reagent (Miltenyi Biotec) according to the manufacturer's recommendations. After 2 days of stimulation, T Cell TransAct reagent was removed and CD4+ T cells were cultured in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 10% decomplemented fetal calf serum (FCS), 1% antibiotic-antimycotic cocktail and 30 IU/mL Human IL-2 (Miltenyi Biotec). Three days after initial stimulation, the purity and the activation of CD4+ T cells were assessed by flow cytometry analysis of the following cell surface markers: CD3, CD4, CD8, CD25 and CD69.

CRISPR-Cas9 knockout in HeLa cells

Expression plasmids for single guide RNA (sgRNA) (pMLM3636 vector, a gift from Keith Joung; Addgene plasmid #43860) targeting an exon within ATG5 genes were transiently transfected into HeLa cells using Lipofectamine LTX with PLUS Reagent (Life technologies), along with a plasmid expressing Cas9 fused with GFP (pCas9 GFP, gift from Kiran Musunuru: Addgene plasmid #44719). The following target sequences were used: 5'-AACTTGTTTCACGCTATATC-3' (ATG5, exon 1; guide 129) and 5'-AAGATGTGCTTCGAGATGTG-3' (ATG5, exon 1; guide 92). 48 hours after transfection, GFP expressing HeLa cells were sorted with BD FACS Aria III *Cell Sorter*, cultivated for 7 days in complete medium, cloned by limiting dilution in 96-well flat-bottomed culture plates and expanded for 15 days. The clones were then screened by PCR amplification of the targeted region of the genome. The PCR products were cloned into pCR-Blunt II TOPO (Life technologies) and up to 20 independent clones were

sequences in each case. The primer pair used for PCR amplification of the sgRNA target sites was: ATG5fw 5'-TCCAAAATAAGCATGAATTAGCTGT-3' and ATG5rev 5'-TGGGCTTGAAAGACTGATGCA-3' (ATG5 target site). ATG5 knockout was checked by western blot. CRISPR LC3C cell lines were previously described (1).

Nucleofection of primary CD4+ T cells

Six days after initial activation, CD4+ T cells were electropored with CRISPR-Cas9 ribonucleoproteins (crRNP) using P3 Primary Cell 4D X Kit (Lonza). Gene-specific CRISPR RNAs (crRNA) were obtained from Integrated DNA technologies (IDT) and selected to target ATG5: 5'-/AltR1/rArArC rUrUrG rUrUrU rCrArC rGrCrU rArUrA rUrCrG rUrUrU rUrArG rArGrC rUrArU rGrCrU/AltR2/-3' or the negative control Alt-R® CRISPR-Cas9 Negative Control crRNA (catalog number 1072544; IDT).

Each Alt-crRNA and the trans-activating CRISPR RNA (tracrRNA) (catalog number 1072534; IDT) were reconstituted to 100µM with Nuclease-free Duplex Buffer (IDT). Oligos were mixed at equimolar concentrations in a PCR tube (e.g., 1.5 µL Alt-R crRNA and 1.5 µL Alt-R tracrRNA). Oligos were then annealed by heating at 95°C for 5 min in PCR thermocycler and the mix was slowly cooled to room temperature. Then, one crRNA-tracrRNA duplexes were incubated with 10 µg of TrueCut cas9 Protein v2 (catalog number A36499; ThermoFisher Scientific) at room temperature for at least 10 min allowing the formation of RNP complex.

For the nucleofection, CD4+ T cells were washed once prior to be resuspended in primary cell nucleofection solution and then quickly mixed with RNP complex. The mix was transferred to Nucleofection cuvette strips (4D-Nucleofector X kit S; Lonza). Cells were electroporated using a 4D nucleofector (4D-Nucleofector Core Unit: Lonza, AAF-1002B; 4D-Nucleofector X Unit: AAF-1002X; Lonza) with the pulse program EO-115. After nucleofection, prewarmed T cell media was added to cells and the strips were incubated 15 min at 37°C. Cells were then transferred in 24-well plates and cultured at 2 x 106 cells/mL in T cell media supplemented with IL-2 (30 IU/mL). Targets deficiency were checked by immunoblot.

Small interfering RNA and transfection

Cells were transfected with relevant small interfering RNA (siRNA) oligonucleotide using Lipofectamine RNAiMAX (Life Technologies), according to the reverse transfection procedure described in the

manufacturer's recommendations. The final concentration of siRNA oligonucleotides was 7.5 to 30 nM. 21-nucleotide RNA duplexes with 2-nucleotide 3-(2-deoxy) thymidine overhangs were used. Specific siRNA sequence targeting ATG5 was used: ATG5 (5'-GGATGCAATTGAAGCTCATdTdT-3', positions 647-665 for variant 1, 574-592 for variant 2, 519-537 for variant 3, 647-665 for variant 4, 412-430 for variant 6 and 284-302 for variant 7). The siRNA targeting LC3C (5'-GCTTGGCAATCAGACAAGAGGAAGT-3', position 143-167) was previously described (1, 2). The On-Target plus SMART pool siRNA targeting BST2 (L-011817-00) was purchased from Dharmacon. The siRNA (D-001810-01 from Dharmacon) was used as negative control (referred as siRNA Control).

Mammalian expression vectors and transfection

NL4-3 HIV-1 proviral DNA were obtained, respectively from NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID). NL4-3 Udel HIV-1 proviral DNA and wild type NL4-3 (MA/YFP) HIV-1 was, respectively, a gift from Dr. K. Strebel (3) and Dr. P. Bienasz (4). HIV-1 NL4-3 Vpu ORF was cloned into pEGFP-N1 (Clontech, France). The ORF of human ATG5 was cloned in frame with HA tag into pAS1B vector (pAS1B-HA-ATG5). The ORFs of human LC3C, human ATG5 and human Beclin 1 were cloned in frame with FLAG or HA affinity tag into the p3XFlag vector (p3XFlag-LC3C, p3XFlag-ATG5 and p3XFlag-Beclin 1) or pCMV-HA (pCMV-HA-LC3C). The ORFs of human ATG5 was cloned in frame with GFP tag into the pEGFP-C1 vector. The cDNA encoded the WT and short isoforms of human BST2 was cloned in pcDNA3.1 vector (pcDNA-BST2 WT; pcDNA-BST2 M1A) and in frame with FLAG tag in p3XFlag vector (p3XFlag-BST2 WT). The mutated forms of BST2 (BST2 2-4-AAA, BST2 3-5-AAA, BST2 4-6-AAA, BST2 5-7-AAA, BST2 6-8-AAA, BST2 7-9-AAA, BST2 8-10-AAA, BST2 9-11-AAA, BST2 10-12-AAA, BST2 11-13-AAA, BST2 12-14-AAA, BST2 13-15-AAA, BST2 14-16-AAA, BST2 15-17-AAA, BST2 16-18-AAA, BST2 17-19-AAA, BST2 18-20-AAA, BST2 19-21-AAA, BST2 Y_6Y_8-AA, BST2 C3A, BST2 M1A) and ATG5 (ATG5-K130R) were made by PCR mutagenesis using the QuikChange II XL site directed mutagenesis kit (Stratagene, France). The cDNA encoded ATG5-K130R was cloned in frame with GFP tag into the pEGFP-C1 vector. Similarly, the deleted form of BST2 (BST2 Delta CT) was generated by site directed mutagenesis by introducing a stop codon in position 21 of BST2 in p3XFlag-BST2 WT. Mutagenesis and subclonings were verified by DNA sequencing.

Transfections of HeLa cells or HEK293T cells with mammalian expression vectors were performed using Lipofectamine LTX with PLUS Reagent (Life technologies), following the manufacturer's instructions.

Viral stocks

Stocks of VSV-G pseudotyped wild type (WT) NL4-3 HIV-1, NL4-3 Udel HIV-1, WT NL4-3 (MA/YFP) HIV-1 and Udel NL4-3 (MA/YFP) HIV-1 were obtained by transfection of HEK293T cells with HIV-1 proviral DNA along with a VSV-G expression vector (pMD.G) and polyethylenimine (PEI) (Polysciences). Twenty-four hours after transfection, cells media were removed and cells were cultured for additional 24hrs in fresh media. Supernatants were then collected and filtered (0.45 µm). Viral titers were determined by infection of HeLa cells with serial dilutions of the viral stocks for 24hrs, followed by flow cytometry analysis of CAp24 antigen expression on fixed and permeabilized cells labelled with KC57-fluorescein isothiocyanate (FITC) (Beckman Coulter).

Antibodies

The following antibodies were used for immunoblotting and/or immunofluorescence: Mouse monoclonal anti-CAp24 HIV-1 (National Institute for Biological Standards and Control Centralized Facility for AIDS Reagents (NIBCS); ARP366), human monoclonal anti-SUgp120 (National Institute for Biological Standards and Control Centralized Facility for AIDS Reagents (NIBCS); 2G12), mouse monoclonal anti-BST2 (Abnova; H00000684-M15), mouse monoclonal anti-GAPDH (Santa Cruz; sc-47724), mouse monoclonal anti-α-Tubulin (Sigma-Aldrich; T9026), mouse monoclonal anti-β-Actin (Sigma-Aldrich; A2228), mouse monoclonal anti-Flag (M2)-HRP (Sigma-Aldrich; A8592), rabbit polyclonal anti-ATG5 (Cell Signaling Technology; 12994S), rabbit polyclonal anti-LC3B (Novus Biologicals; NB600-1384), rabbit monoclonal anti-Syk (Cell Signaling; #12358), rabbit monoclonal anti-phosphoSyk (Cell Signaling; #2715), rabbit polyclonal anti-BST2 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; 11721) and rabbit polyclonal anti-Vpu (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH; 969), rat monoclonal anti-HA (3F10)-HRP (Roche; 12013819001), goat polyclonal anti-GFP-HRP (GeneTex; GTX26663).

Anti-phosphoBST2 antiserum was elicited in rabbits by using a modified peptide of BST2 (amino acids 1 to 21) phosphorylated on tyrosines in position 6 and 8, generating a polyclonal antibody against the tyrosine-phosphorylated intracellular portion of BST2 (Proteogenix).

Secondary antibodies against the mouse, rabbit, goat or human immunoglobulin G coupled to Alexafluor-594, Alexafluor-488 or Alexafluor-647 (purchased from Invitrogen) were used for

immunofluorescence. Secondary antibodies against the mouse and the rabbit immunoglobulin G coupled to HRP (Dako) were used for immunoblotting experiments.

The antibodies used for flow cytometry are Alexa Fluor 647 mouse anti-human BST2 (Biolegend; 348404) Alexa Fluor 647 mouse anti-human CD3 (BD Biosciences; 557706), PE-Cy7 mouse anti-human CD4 (BD Biosciences; 348809), FITC mouse anti-human CD8 (BD Biosciences; 555366), APC-Cy7 mouse anti-human CD25 (Biolegend; 356122), PE mouse anti-human CD69 (BD Biosciences; 555531) and HIV-1 core antigen-FITC (KC57, Beckman Coulter; 6604665).

Immunoprecipitation assay

HEK293T or HeLa cells were co-transfected with mammalian expression vectors and HIV-1 proviral DNA NL4-3 (WT or Udel) as described above. Twenty-four hours after transfection, immunoprecipitation of the relevant protein was performed.

HeLa cells were treated with siRNA (7.5-30 nM) as described above. Forty-eight hours after transfection, siRNA-treated cells were infected with VSV-G pseudotyped NL4-3 (WT or Udel) HIV-1 for 2h30 at a multiplicity of infection (M.O.I.) of 0.5. Twenty-four hours after transfection, immunoprecipitation of the relevant protein was performed. To observed pBST2 after immunoprecipitation, cells were incubated for 5 min in DMEM containing 100µM of pervanadate before lysis.

Cells were lysed in ice-cold lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 200 µM sodium orthovanadate) with complete protease inhibitor cocktail (Roche). The protein concentrations were determined using a Bradford protein assay (Bio-Rad), and equal amounts of protein for each sample were used for the following steps. Immunoprecipitations were performed by incubating indicated whole cell extracts overnight at 4°C with monoclonal mouse anti-Flag or monoclonal mouse anti-BST2 antibody, or mouse IgG CTRL coupled to Dynabeads protein G (Life Technologies). The beads were washed 4 times with lysis buffer, and proteins were eluted in 2X Laemmli (Sigma-Aldrich) or deglycosylated by an 1h treatment with PNGase F (NEB) then eluted in 2X Laemmli as previously described.

GFP-tagged proteins were immunoprecipitated using GFP-TRAP beads (Chromotek) using TNTE buffer (20mM Tris-HCL pH 7.4, 150mM NaCl, 5mM EDTA, 0.5% Triton X-100) with complete protease inhibitor cocktail. Immunoprecipitations were performed by incubating indicated whole cell extracts for 1h30 at 4°C with GFP-TRAP beads. The beads were washed and proteins were eluted as previously described.

Western Blotting

Cell lysates were lysed in ice-cold DOC buffer (10mM Tris, pH 8, 150mM NaCl, 1mM EDTA, 1% Triton X-100 and 0.1% DOC 10%) containing complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation for 15 min at 13,000 rpm. The protein concentrations were determined using a Bradford protein assay (Bio-Rad), and equal amounts of protein for each sample were used for the following steps.

Cell lysates and immunoprecipitated proteins were subjected to SDS-PAGE gels. Laemmli 2x concentrate (Sigma) has been used as a sample buffer for reducing and loading protein samples in SDS-PAGE, whereas NuPAGE LDS sample buffer 4x concentrate (ThermoFisher Scientific) has been used for non-reducing conditions. Proteins were then transferred onto hydrophobic polyvinylidene difluoride membranes (PVDF, 0.45 µm, Millipore), followed by blocking in milk buffer (Tris-buffered saline [TBS] [0.5 M Tris pH 8.4, 9% {wt/vol} NaCl], 5% [wt/vol] nonfat dry milk, 0.05% [vol/vol] Tween 20) for 1h at room temperature (RT). Membranes were incubated overnight at 4°C with the appropriated primary antibodies in milk buffer or BSA buffer (Tris-buffered saline [TBS] [0.5 M Tris pH 8.4, 9% {wt/vol} NaCl], 3% [wt/vol] BSA, 0.05% [vol/vol] Tween 20). Blots were washed with TBS containing 0.05% (vol/vol) Tween 20 and incubated with appropriate HRP-conjugated secondary antibodies in milk or BSA buffer for 1h at RT. After washing, protein bands were detected by using Amersham ECL Select Western blotting detection reagent (GE Healthcare).

Formaldehyde crosslinking of BST2

To stabilize non-covalent oligomers of BST2 for SDS-PAGE analysis and immunoblotting, cells were harvested using PBS-EDTA 1mM, pelleted, washed once with PBS then resuspended in 1ml of serum-free DMEM containing 1% formaldehyde. Cells were incubated at 37°C for 20 min under rotation. The reaction was stopped by adding 100µl of 1.25M glycine in PBS and cells were incubated 5 min at RT under rotation. Cells were then pelleted and lysed in DOC buffer. Lines were drawn on each BST2 signal and resulting line plots of the chemiluminescence intensity were used to confirm the enrichment of N-linked glycosylated form of BST2.

Quantitative RT-PCR

Total cellular RNA was extracted using the Reliaprep RNA Cell Miniprep System kit (Promega; Z6012) following the manufacturer's instructions. For each sample, 500ng to 2µg of total RNA were subjected to DNase I treatment (TURBO DNase; ThermoFisher; cat AM2239) and cDNA synthesis was performed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems; 4368814). The different mRNA levels were assayed using SYBR Green Supermix (Bio-Rad; 1725275) in a real-Time PCR detection system (LightCycler® 480). The PCR conditions and cycles were as follows: an initial DNA denaturation at 95°C for 5 min, followed by 45 cycles of amplification (denaturation: 95°C for 10 sec, annealing: 63°C for 10 sec and extension :72°C for 10 sec), followed by a melting-curve analysis cycle. Each point was performed in technical duplicate. The relative abundance of TNFα mRNA (sense: 5'-TCCTTCAGACACCCTCAACC-3' ; antisense: 5'-AGGCCCCAGTTTGAATTCTT-3'), CXCL10 mRNA (sense : 5'-TCTGAGACATTCCTCAATTGCT-3' ; antisense : 5'-AGAGGTACTCCTTGAATGCCA-3'), IFNβ mRNA (sense: 5'-AAACTCATGAGCAGTCTGCA-3' ; antisense: 5'-AGGAGATCTTCAGTTTCGGAGG-3') and Nef mRNA (sense: 5'-AGGGGCGGCGACTGGAAGA-3'; antisense: 5'- GATTGGGAGGTGGGTTGCTTTG-3' were calculated by the comparative ΔΔCt method normalizing to the housekeeping product KDSR mRNA (sense : 5'-AGATGAGTTGGACCCATTGC -3' ; antisense : 5'-AAGCCATGAGTTTCCACCAG -3') in HeLa cells or FKBP4 mRNA (sense : 5'-AAGGCGTGCTGAAGGTCAT-3' ; antisense: 5'-CCAGCCAGTGTAGTGGACAA-3') in LTCD4+ cells.

Fluorescence microscopy

Cells were grown on coverslips, transfected with siRNA, infected, and then fixed with 4% paraformaldehyde in PBS for 20 min. For staining of pBST2, cells were incubated for 5 min in DMEM containing 100µM of pervanadate before fixation with 4% paraformaldehyde. Cells were then washed three times using PBS containing 50mM NH_4Cl. Cells were permeabilized with 0.2% Triton X-100 for 10 min. Coverslips were blocked with 5% BSA for 1h, incubated with primary antibody in 1% BSA for 1h, washed with PBS, and incubated with secondary antibody in 1% BSA for 30 min, before final washing with PBS and MilliQ water.

For extracellular staining of BST2, living cells were incubated for 1h at 4°C with rabbit polyclonal anti-BST2 (NIH) together with mouse anti-Env SUgp120 (110H, Hybridolab). Then, cells were fixed with 4% PFA in PBS for 20 min and labelled with appropriate fluorophore-conjugated secondary antibodies.

For the antibody-feeding assay, living cells were incubated for 30 min at 37°C with rabbit polyclonal anti-BST2 (NIH) together with mouse anti-Env SUgp120 (110H, Hybridolab). Cells were washed, incubated 30 min in complete media then fixed with 4% PFA in PBS for 20 min and labelled with appropriate fluorophore-conjugated secondary antibodies.

Cells were mounted in DAPI Fluoromount-G (SouthernBiotech). Microscope IXplore spinning disk Olympus was used for confocal analysis. We used the 60X plan-apochromat objective, with a numerical aperture 1.42. Images were processed using ImageJ software. The experiments were repeated as indicated in the figure legends, and representative images are shown. In all experiments, images shown in individual panels were acquired using identical exposure times or scan settings and adjusted identically for brightness and contrast using Photoshop CS5 (Adobe).

Electron Microscopy

The method used is essentially those described previously (5, 6). Briefly, ultrathin cryosections (50 nm) were stained with mouse antibodies against HIV-1 p24/p55 (EVA365 and EVA366, NIBSC), rabbit anti-mouse bridging antibody (Rockland Immunochemicals Inc. Limerick, PA), and 10 nm PAG (Protein A gold reagents were obtained from the EM Lab, Utrecht University, Utrecht, The Netherlands). Sections were fixed in 1% (v/v) glutaraldehyde for 10 min, embedded in uranyl acetate in methylcellulose, as described previously, and examined with a Technai G2 Spirit transmission electron microscope (FEI Company UK. Ltd., Cambridge, UK).

Flow cytometry

Twenty-four hours post-infection, siRNA transfected HeLa cells were washed twice in PBS and collected using PBS EDTA 1mM. Cells were first stained with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermofisher) according to the manufacturer's recommendations. Then, cells were washed in cold PBS/2% (w/v) FBS 1mM EDTA and stained for 1 hour at 4°C with AlexaFluor647-conjugated anti-BST2 (Biolegend) or control isotype (Biolegend). The cells were washed three times in cold PBS/2% (w/v) FBS 1mM EDTA, then fixed in 4% paraformaldehyde (PFA) and permeabilized in PBS/1% BSA/0.1% saponin before staining with a FITC-conjugated anti-Cap24 (KC57-FITC, Beckman Coulter, France) for 1h at room temperature. Cells were washed and analysed using the BD LSRFortessa^{TM} cell analyzer.

HIV-1 production assay

For a HIV-1 production assay, HeLa BST2 WT and HeLa BST2 KO cells were treated with siRNA (7.5-30 nM) as described above and transfected with HIV-1 proviral DNA NL4-3 (WT or Udel). Twenty-eight hours after transfection, cell lysates were analyzed by qRT-PCR and western blotting.

In a single round of infection, HeLa cells were treated with siRNA (7.5-30 nM) as described above. Forty-eight hours after, siRNA-treated Hela cells were infected with VSV-G pseudotyped NL4-3 (WT or Udel) HIV-1 for 2h30 at a multiplicity of infection (M.O.I.) of 0.5. Thirty-two hours after infection, media was removed and replaced with fresh media for additional 16h. Supernatants were then collected, 0.45 µm-filtered and used for HIV-1 CAp24 quantification by ELISA (released CAp24) (Perkin Elmer). Cell lysates were analyzed by western blotting.

For CD4+ T cells infection, CRISPR cells were activated for three days as described previously, then infected by spinoculation with VSV-G pseudotyped NL4-3 (WT or Udel) HIV-1 for 2h at a multiplicity of infection (M.O.I.) of 0.5. Forty-eight hours after infection, cell lysates were analyzed by qRT-PCR and western blotting.

Statistical analysis

The statistical details of all experiments are reported in the figure including statistical analysis performed, error bars, statistical significance, and exact n numbers. Statistics were performed using GraphPad Prism 6 software, as detailed in the figure legends.

Supplemental References

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