Characterization of E3 ligases involved in lysosomal sorting of the HIV-1 restriction factor BST2.

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Summary Statement

We identified two regulators of BST2/Tetherin, a restriction factor of viral release: the E3-ubiquitin ligases NEDD4 and MARCH8 and provide further understanding of the mechanisms underlying BST2 turnover in cells.

Abstract

The cellular protein BST2/ Tetherin acts as a major intrinsic antiviral protein that prevents the release of enveloped viruses by trapping nascent viral particles at the surface of infected cells. Viruses have evolved specific strategies to displace BST2 from viral budding sites in order to promote virus egress. In HIV-1, the accessory protein Vpu counters BST2 antiviral activity and promotes sorting of BST2 for lysosomal degradation. Vpu increases poly-ubiquitination of BST2 through recruitment of the E3 ligase complex SCF adaptor β-TrCP, a post translation modification required for Vpu-induced BST2 down-regulation. Herein, we further investigated the role of the ubiquitination machinery in the lysosomal sorting of BST2. Using a small siRNA screen we highlighted two additional regulators of BST2 constitutive ubiquitination and sorting to the lysosomes: the E3 ubiquitin ligases NEDD4 and MARCH8. Interestingly, Vpu does not hijack the cellular machinery constitutively involved in BST2 ubiquitination to sort BST2 for degradation in the lysosomes, but instead promotes the recognition of BST2 by β-TrCP. Altogether, our results provide further understanding of the mechanisms underlying BST2 turnover in cells.
Introduction

The interferon-inducible cellular protein “Bone marrow stromal antigen 2” (BST2 also known as either Tetherin, CD317 or HM1.24) is an intrinsic antiviral factor that restricts the release of many enveloped viruses such as retroviruses, herpes viruses, filoviruses, rhabdoviruses, paramyxoviruses and arenaviruses, making it an essential component of the innate immune defence against viral dissemination (Neil, 2013, Tokarev et al., 2009).

Although up-regulated by type-I interferon and pro-inflammatory stimuli, BST2 is constitutively expressed in several cell types as a 30-36 kDa type II integral membrane glycoprotein, present both at the plasma membrane and in intracellular compartments such as the trans G0lgi network (TGN) and early/recycling endosomes. BST2 contains a short N-terminal cytoplasmic tail linked to a transmembrane domain and a large extracellular coiled-coil domain anchored to the membrane through a C-terminal glycosyl-phosphatidylinositol (GPI) moiety (Evans et al., 2010, Kupzig et al., 2003, Masuyama et al., 2009). This atypical topology is responsible of its restrictive function on viral release; BST2 assembles as parallel disulfide–bonded homo-dimers and bridges virions and cellular membranes via its GPI anchors and its transmembrane domain respectively, leading to the retention of the nascent viral particles at the surface of infected cells (Iwabu et al., 2009, Neil et al., 2008, Perez-Caballero et al., 2009, Van Damme et al., 2008, Venkatesh and Bieniasz, 2013).

To efficiently egress from cells, viruses have evolved strategies to displace BST2 from viral assembly sites and in such a way counteract its antiviral activity (Evans et al., 2010, Neil, 2013, Roy et al., 2014). Several virus-encoded proteins devoted to this function have been characterized, notably Vpu of human immunodeficiency virus type 1 (HIV-1) (Neil et al., 2008, Van Damme et al., 2008), Nef of most strains of simian immunodeficiency virus (SIV) and of HIV-1 Group O (Jia et al., 2009, Kluge et al., 2014, Sauter et al., 2009, Zhang et al., 2009), Envelope glycoproteins of HIV-2 and SIVtan, (Gupta et al., 2009, Le Tortorec and Neil, 2009) and K5 of Kaposi’s sarcoma-associated herpes virus (KSHV) (Mansouri et al., 2009).

Antagonism of BST2 by lentiviral Vpu, Env and Nef relies on an alteration in BST2 intracellular trafficking though a complex interplay with host components regulating proteins vesicular transport, leading ultimately in most cell types to an overall decrease of BST2 at the surface of infected cells (Goffinet et al., 2009, Iwabu et al., 2009, Le Tortorec and Neil, 2009, Van Damme et al., 2008). HIV-1
Vpu was notably shown to prevent the recycling of BST2 internalized from the cell surface, as well as plasma membrane (PM) access of de novo synthetized BST2, thereby decreasing the resupply of BST2 to the PM (Dube et al., 2011, Lau et al., 2011, Schmidt et al., 2011).

Vpu-induced down-regulation of cell surface BST2 is also associated with enhanced targeting of the restriction factor for lysosomal degradation (Douglas et al., 2009, Iwabu et al., 2009, Mitchell et al., 2009). Both we, and others have shown that Vpu promotes ESCRT-mediated sorting of BST2 for lysosomal degradation (Agromayor et al., 2012, Janvier et al., 2011). The ESCRT machinery regulates sorting of ubiquitinated membrane proteins to the multivesicular bodies (MVB) for their subsequent degradation in lysosomes (Raiborg and Stenmark, 2009). Interestingly, BST2 undergoes ubiquitination (Gustin et al., 2012, Pardieu et al., 2010, Tokarev et al., 2010) through a not fully characterized process, and Vpu has been reported to induce increased poly-ubiquitination of BST2 on serine (S3; S5) and threonine (T4) residues located in its cytoplasmic tail (Tokarev et al., 2010). However, numerous questions remain regarding the significance of BST2 ubiquitination on its constitutive trafficking and sorting for degradation, and there are contradictory results concerning the contribution of poly-ubiquitination of BST2 S3T4S5 residues on Vpu-induced degradation of BST2 and viral egress (Cocka and Bates, 2012, Gustin et al., 2012, Tokarev et al., 2010). Poly-ubiquitination of BST2 by Vpu is mediated by the recruitment of β-TrCP, a subunit of the Skp1-Cullin1-F-Box (SCF) ubiquitin E3 ligase via a DS52GxxS56 consensus motif located in the cytoplasmic tail of Vpu (Gustin et al., 2012, Tokarev et al., 2010). β-TrCP is required for Vpu-mediated BST2 down-regulation at the cell surface and its targeting for endo-lysosomal degradation (Douglas et al., 2009, Iwabu et al., 2009, Mangeat et al., 2009, Mitchell et al., 2009). However, the requirement of β-TrCP in Vpu antagonism of BST2 antiviral activity remains controversial (Iwabu et al., 2009, Kueck et al., 2015, Mangeat et al., 2009, Mitchell et al., 2009, Tervo et al., 2011). Furthermore, the importance of β-TrCP in the constitutive ubiquitination and turnover of BST2 has not been addressed.

In this study, we investigated the mechanisms underlying BST2 ubiquitination and its role in the regulation of BST2 trafficking and turnover. To this aim, we carried out a small siRNA screening to identify the E3 ligases involved and then unravelled the role of two specific E3 ligases: the HECT E3 ligase NEDD4 and the Membrane-Associated RING-CH 8 (MARCH8) E3 ligase, in the regulation of BST2 constitutive ubiquitination and turnover. Depletion of NEDD4 and MARCH8 in cells resulted in altered ubiquitination of BST2 associated with a delayed turnover of the restriction factor. Conversely,
their overexpression caused an overall increase of BST2 ubiquitination. However, NEDD4 and MARCH8 do not contribute in Vpu–induced down-regulation of BST2. In accordance with previous studies, we showed that Vpu modulates BST2 expression through recruitment of β-TrCP that did not contribute to regulation of constitutive ubiquitination of BST2. This suggests that Vpu bypasses to some extent, the machinery involved in the constitutive turnover of BST2 to direct the restriction factor for degradation.
Results

**MARCH8, NEDD4 or β-TrCP depletions lead to increased levels of BST2**

To characterize the E3 ligase(s) involved in the constitutive ubiquitination of BST2 and its sorting for lysosomal degradation, we performed a small siRNA screen targeting a subset of well-characterized E3 ligases involved the regulation of protein trafficking in the endo-lysosomal pathway: the HECT-E3 ligases NEDD4, NEDD4-L, ITCH, WWP1 (Boase and Kumar, 2015, Ingham et al., 2004); the RING domain-containing E3 ligases c-Cbl and the transmembrane protein MARCH8 (Goh and Sorkin, 2013, Ohmura-Hoshino et al., 2006a). To this list was added a well-characterized siRNA targeting both isoforms of the SCF E3 ligase adaptors: β-TrCP1 and β-TrCP2 (referred herein as β-TrCP), that contribute to Vpu-induced sorting of BST2 for lysosomal degradation (Douglas et al., 2009, Iwabu et al., 2009, Mitchell et al., 2009). The previously described control siRNA (siCD) (Janvier et al., 2011) and an irrelevant siRNA targeting the E3 ligase RNF138 that is not expressed in HeLa cells (data not shown) were used as negative controls.

We first evaluated the outcome of their depletion on the expression level of BST2. HeLa cells were transfected with the selected siRNA, or siCD, and left for 4 days to allow significant depletion of the targeted proteins. Depletion was confirmed by western-blot (Fig. 1A) or by RT-qPCR (Fig. 1B). Steady state BST2 levels in siRNA-treated cells were then assessed by quantitative western blotting and normalized to the amount of Tubulin, used as a loading control (Fig. 1C-D). No major alteration in the amount of BST2 was observed upon knockdown of the E3 ligases NEDD4-L, ITCH, WWP1, or c-Cbl compared to control cells (Fig.1). On the contrary, depletion of NEDD4, MARCH8 and β-TrCP resulted in a marked increase in the amount of BST2 detected in cell lysates, suggesting a role for these enzymes in the regulation of BST2 levels. Quantification of BST2 transcripts in cells depleted for NEDD4, MARCH8 or β-TrCP was further assessed by quantitative RT-PCR (Fig. 1E) and showed no significant difference compared to control cells. This suggests that the augmentation of BST2 was not due to increased BST2 transcription, but most likely, to post-transcriptional stabilization of the BST2 protein.
MARCH8 and NEDD4 regulate ubiquitination of BST2

We next tested whether NEDD4, MARCH8 or β-TrCP are involved in BST2 ubiquitination (Fig. 2). To this end, siRNA-treated HeLa cells were lysed under stringent conditions (cf. Materials and Methods) and subjected to immunoprecipitation of BST2. Western-blot analysis of the immunoprecipitated proteins using an anti-ubiquitin antibody revealed a few stacked bands between 35kDa to 60 kDa that probably correspond to mono and multi-ubiquitinated forms of BST2 (these bands will be referred as such hereafter) (Fig. 2A, Lane 1). In addition, smeared higher molecular bands could also be detected to a lower intensity and might represent previously described poly-ubiquitinated BST2 (Gustin et al., 2012, Tokarev et al., 2010). Analysis of BST2 ubiquitination in cells depleted for the E3 ligases showed a significant decrease in the amount of mono/multi-ubiquitinated forms of BST2 upon depletion of NEDD4 and MARCH8 compared to control cells (siCD) (Fig. 2A-B, lanes 3-4). These results suggest that both NEDD4 and MARCH8 contribute to BST2 ubiquitination. Surprisingly, β-TrCP knockdown did not significantly alter BST2 mono/multi-ubiquitination, arguing that β-TrCP expression is not strictly required for constitutive ubiquitination of BST2 (Fig. 2A-B, lane 5).

To confirm these results, we analyzed the outcome of overexpressing WT and catalytically impaired mutant forms of the E3 ligases on the profile of BST2 ubiquitination (Fig. 2C-D). Cysteine at position 867 in NEDD4 and positions 80-83-123-126 in the RING domain of MARCH8 required for catalytic activity were mutated to serine (referred hereafter as NEDD4 C/S and MARCH8 C/S), as previously described (Anan et al., 1998, Ohmura-Hoshino et al., 2006b). The well-characterized mutant of β-TrCP devoid of its F-box domain (β-TrCPΔF) was used as catalytically dead mutant for this enzyme (Winston et al., 1999). The ubiquitination profile of BST2 was then assessed in HeLa cells transfected with GFP-tagged WT, or mutated NEDD4, MARCH8 and β-TrCP. Overexpression of NEDD4 induced an increase in the level of mono/multi-ubiquitinated BST2 compared to control cells expressing Histone2B-GFP (H2B-GFP) (Fig. 2C-D, lane 2 vs 1). This effect was abrogated upon expression of the catalytically impaired mutant NEDD4 C/S confirming the relevance of NEDD4 for BST2 ubiquitination (Lane 3). Similarly, overexpression of MARCH8 increased BST2 mono/multi-ubiquitination (Lane 4), whereas mutation of the catalytic cysteines of MARCH8 (MARCH8 C/S) abolished this effect (Lane 5), supporting as well a role of this E3 ligase in BST2 ubiquitination. In line with the siRNA-based experiments (Fig. 2A-B), overexpression of β-TrCP WT or the negative mutant β-TrCPΔF did not potently alter the ubiquitination profile of BST2 compared to control cells (Lanes 6-7 vs 1). These data
are consistent with the notion that β-TrCP is not essential for constitutive ubiquitination of BST2 and that the increased amount of BST2 observed in β-TrCP knockdown cells does not result from impaired BST2 ubiquitination.

**Analysis of NEDD4 and MARCH8 interaction with BST2**

Ubiquitination involves recognition and binding of the E3 ligases to their protein targets (Pickart, 2001). The above results suggest that expression of NEDD4 and MARCH8 is required for efficient ubiquitination of BST2. We thus determined whether this process involves binding of BST2 to these E3 ligases (Fig. 3A). HEK293T cells were transfected with expression vector for FLAG-BST2 along with vectors encoding the HA-tagged E3 ligases. Interaction of FLAG-BST2 with the HA-E3 ligases was assessed by co-immunoprecipitation using an anti-FLAG antibody, followed by western-blot analysis. No interaction was observed between FLAG-BST2 and HA-WWP1 used as a negative control (lower right panels) nor with β-TrCP (lower left panels), which is consistent with the lack of requirement of β-TrCP for BST2 ubiquitination (Fig. 2A). On the contrary, FLAG-BST2 co-immunoprecipitated with HA-NEDD4, albeit weakly (upper left panels), as well as HA-MARCH8 (upper right panels). Epitope tagging of BST2 was reported to alter some of its functions (Lv et al., 2014). Consequently, binding of these E3 ligases to exogenously expressed untagged BST2 in HEK293T cells (Fig. S1A), or endogenous BST2 in HeLa cells (Fig. S1B) was evaluated by co-immunoprecipitation using an anti-BST2 antibody. Similarly, binding of BST2 with HA-MARCH8 was detected, as well a weak but specific interaction with HA-NEDD4 (Fig. S1A-B), further confirmed for endogenous NEDD4 (Fig. S1C-D). Altogether, our results suggest that NEDD4 and MARCH8 might promote BST2 ubiquitination via binding to BST2.

We wondered whether the two E3 ligases cooperate with each other in binding to and ubiquitination of BST2. We thus tested the impact that depleting each ligase had on the binding of BST2 to the other enzyme in HeLa cells (Fig. 3B-C) or HEK293T cells (not shown). Binding of FLAG-BST2 to HA-NEDD4 or HA-MARCH8 in cells knocked-down for MARCH8 or NEDD4 respectively was assessed by co-immunoprecipitation. Depletion of NEDD4 did not significantly impact on binding of FLAG-BST2 with HA-MARCH8, suggesting that NEDD4 expression is not required for BST2 interaction with MARCH8 (HA-MARCH8 panel, lanes 23 versus 17). Reciprocally, MARCH8 knockdown did not impair binding of FLAG-BST2 to HA-NEDD4 (Fig. 3C, HA-NEDD4 panel, lanes 22 versus 16). Taken
together, one concludes that binding of BST2 by either NEDD4 or MARCH8 does not rely on the expression of the other ligase.

**NEDD4 AND MARCH8 regulate BST2 turnover**

Ubiquitination is involved in the regulation of many trafficking processes such as internalization and sorting for degradation (Pickart, 2001). BST2 traffics between the TGN, the plasma membrane and endosomes with a fraction targeted to lysosomes for degradation (Habermann et al., 2010, Masuyama et al., 2009, Rollason et al., 2007). NEDD4 has been shown to regulate the internalization as well as endosomal sorting for degradation of proteins such as Connexin 43 and PTEN (Leykauf et al., 2006, Wang et al., 2007). MARCH8 has also been proposed to regulate internalization and sorting for degradation of cargoes such as TfR, HLA-B7-2 and CD98 (Ablack et al., 2015, Bartee et al., 2004, Fujita et al., 2013, Ohmura-Hoshino et al., 2006a). We investigated therefore, the role of the two E3 ligases, and by extension ubiquitination, in the regulation of BST2 trafficking (Fig. 4). We first analyzed the consequences of depleting their expression on the kinetics of BST2 internalization (Fig. 4A). In control cells (siCD), almost 40% of BST2 present at the cell surface were internalized within 10 min, followed by a stabilization of the level of the pool of surface-labeled BST2, reflecting the balance between its internalization and recycling. In cells depleted for NEDD4 and MARCH8, the internalization rate of BST2 remained unchanged compared to control cells, or cells depleted for β-TrCP. This suggests that ubiquitination of BST2 by the two E3 ligases is not involved in regulation of BST2 endocytosis.

We then assessed the role of the E3 ligases in BST2 sorting for degradation (Fig. 4B-C). The turnover of BST2 was monitored in siRNA-transfected HeLa cells after incubating the cells in growth medium containing cycloheximide, followed by quantitative western-blot analysis of BST2 expression. Consistent with our previous studies (Janvier et al., 2011), almost 80% of BST2 was degraded within 4 h in cells transfected with control siRNA (siCD). By contrast, in NEDD4- or MARCH8-depleted cells, the half-life of BST2 was prolonged, with only 20% of the initial BST2 pool degraded after 4 h (siNEDD4; siMARCH8). This stabilization might account for the increased amount of BST2 at steady state (Fig. 1C-D) and suggests that ubiquitination of BST2 by NEDD4 and/or MARCH8 regulates its sorting for lysosomal degradation. Surprisingly, in cells depleted for β-TrCP, no major alteration of
BST2 turnover was observed compared to control cells, leaving unsolved the mechanism responsible for the enhanced level of BST2 in these cells (Fig. 1C).

**Interplay between NEDD4 and MARCH8 in regulating BST2 trafficking**

We have identified two E3 ligases NEDD4 and MARCH8 participating in BST2 ubiquitination and subsequent sorting for degradation. We next assessed by immunofluorescence (IF) analyses the consequences of their depletion on the intracellular distribution of BST2 along with well-characterized markers of intracellular compartments (TIR, early/recycling endosomes; HRS, endosomes/MVB; TGN46, TGN and LAMP1, lysosomes) in HeLa cells (Figs 5 and S2). In cells depleted for NEDD4 or MARCH8, BST2 staining appeared more intense compared to control cells (siCD) (Figs 5A, C and S2A, C), consistent with the enhanced amount of BST2 observed by western-blot (Fig. 1). In agreement with previous studies, in control cells BST2 is present in TIR-, HRS- and TGN46-positive compartments (Pearson’s correlation coefficient (Bolte and Cordelieres, 2006): 0.52 for TIR, 0.36 for HRS and 0.39 for TGN46; Figs 5B-D and S2B). No striking co-localization of BST2 with LAMP1 was detected (0.27 for LAMP1; Figs S2 C-D), consistent with BST2 not being stably present in lysosomes. Analysis of NEDD4-depleted cells showed no profound alteration of BST2 distribution despite enhanced BST2 levels, with a coefficient of co-localization of BST2 with TFR, HRS and TGN46 similar to that of control cells (Figs 5 and S2). In contrast, cells depleted for MARCH8 displayed a significant increase of the proportion of BST2 in clumped structures co-stained with HRS suggesting an accumulation of BST2 in this compartment (Pearson’s coefficient=0.55; Fig. 5C-D).

We further analyzed the distribution of BST2 with the aforementioned intracellular markers in cells overexpressing GFP-tagged versions of NEDD4 or MARCH8 (Fig. 6). GFP-NEDD4 displayed a diffuse localization in cells, which prevented us from drawing any conclusion on its co-localization with BST2 (Fig. 6A). Furthermore, no potent alteration in the distribution and expression level of BST2 was observed in GFP-NEDD4 expressing cells (Fig. 6A-C). On the other hand and consistent with previous studies (Bartee et al., 2004, Goto et al., 2003), membrane-spanning MARCH8 is present in perinuclear endosomal compartments co-stained with HRS and CD63, with a fraction of MARCH8 present at the cell surface (Fig. S3). Analysis of BST2 distribution showed an intense co-localization with MARCH8, mainly in HRS- and CD63-positive compartments (Fig. 6A), suggesting increased targeting of BST2 in the MVB upon MARCH8 overexpression. We also noticed in some cells that
overexpression of MARCH8 induced decreased expression of BST2, that was further confirmed by western-blot and flow cytometry analyses of BST2 overall and cell surface levels, respectively (Fig. 6B-C). In agreement with a previous report (Bartee et al., 2006), this indicates enhanced turnover of BST2 under these conditions. We next examined whether MARCH8-induced degradation of BST2 occurs in the lysosomes. HeLa cells overexpressing the E3 ligases were treated with DMSO or Concanamycin A (CMA), a vacuolar H(+) -ATPase inhibitor that blocks endosomal acidification and thus lysosomal degradation, and then analyzed by western-blot and flow cytometry analyses (Fig. 6D-E). Compared to DMSO-treated cells, MARCH8 overexpression in cells treated with CMA no longer induced down-regulation of BST2 level, which was similar to that observed in cells expressing H2B-GFP or GFP-NEDD4 or -β-TrCP, suggesting that MARCH8-induced down-regulation of BST2 is mediated by lysosomal degradation.

NEDD4 and MARCH8 are dispensable for Vpu-induced down-regulation of BST2

Vpu was shown to increase BST2 ubiquitination through recruitment of β-TrCP (Gustin et al., 2012, Tokarev et al., 2010) and target BST2 for ESCRT-mediated lysosomal degradation (Agromayor et al., 2012, Janvier et al., 2011). We analyzed therefore the involvement of NEDD4 and MARCH8 in Vpu-induced down-regulation of BST2 (Figs 7 and S4).

We first assessed the outcome of depleting each ligase on Vpu-induced cell surface down-regulation of BST2 (Fig. 7A-B). Considering the role of β-TrCP in Vpu-induced down-regulation of BST2, siRNA targeting β-TrCP was used as a control. SiRNA-treated Hela cells were infected with HIV-1 NL4-3 WT (HIV-1 WT) or defective for Vpu (HIV-1 Udel) pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) that enables virus entry by endocytosis. Cell surface levels of BST2 were then assessed by flow cytometry analysis (Fig. 7A-B). Intracellular staining of the HIV-1 Capsid protein (CAp24) was used to distinguish infected (Fig. 7A-B, right gates and bars) from non-infected cells (Left gates and bars). Depletion of NEDD4 or MARCH8 did not significantly alter the ability of Vpu to down-regulate BST2 at the cell surface. Indeed, Vpu expression induced a ~2-fold decrease of BST2 cell surface expression, as observed in HIV-1 WT infected control cells (siCD). On the contrary and consistent with previous studies (Douglas et al., 2009, Mitchell et al., 2009), depletion of β-TrCP significantly altered the ability of Vpu to down-regulate BST2 from the cell surface compared to control cells, confirming that β-TrCP is required for this activity of Vpu.
We then analyzed the ability of Vpu to target BST2 for degradation following depletion of NEDD4, MARCH8, or β-TrCP (Fig. 7C-D). As cells depleted for NEDD4 or MARCH8 displayed low permissiveness to infection by HIV-1 (Fig. S4), these analyses were performed by IF (Fig. 7C) and flow-cytometry (Fig. 7D) analyses to allow identification of infected cells. SiRNA-treated infected cells were permeabilized and stained for BST2 and the HIV-1 Envelope protein (Env) to distinguish infected cells. Microscopy and flow cytometry analyses of control cells (siCD) showed decreased intensity of BST2 staining when infected with HIV-1, compared to the neighboring uninfected cells. A similar decrease in BST2 staining intensity was observed in HIV-1 infected cells depleted for both NEDD4 and MARCH8 suggesting that decrease in BST2 is due to Vpu recruitment of β-TrCP (Douglas et al., 2009, Mangeat et al., 2009, Mitchell et al., 2009). Consistently, cells depleted for β-TrCP and infected with HIV-1 did not show degradation of BST2. Indeed, similar levels of BST2 were observed in infected cells compared to uninfected neighboring cells (Fig. 7C-D, lower panels). These results were further confirmed by western-blot analysis (Fig. S4) that also showed a noticeable modification of BST2 profile in β-TrCP-depleted cells infected with WT virus compared to cells infected by HIV-1 Udel virus (Fig. S4, lane 5 versus 10) or control cells (siCD, lane 2). This indicates that Vpu uses β-TrCP to induce BST2 down-regulation and bypasses NEDD4 and MARCH8 that are involved in constitutive turnover of BST2.

**Vpu connects BST2 to β-TrCP without interfering with BST2 binding to NEDD4 or MARCH8**

To further decipher the molecular mechanism involved, we investigated the impact of Vpu expression on the interaction of BST2 with its E3 ligases by co-immunoprecipitation, as one might speculate that Vpu interferes with the binding of BST2 to its E3 ligases in favor of β-TrCP. No major alteration in FLAG-BST2 binding to HA-NEDD4 (Fig. 8, Lane 4) or HA-MARCH8 (Lane 8) was observed upon expression of Vpu-GFP, compared to GFP expressing control cells (Lanes 3 and 7, respectively). This argues that the viral protein does not interfere with the binding of BST2 to the two E3 ligases involved in its sorting for degradation. In cells expressing Vpu-GFP we did detect binding of FLAG-BST2 with HA-β-TrCP (Lane 13), which was not observed in cells expressing GFP alone (Lane 12), or in cells expressing Vpu mutated on the phopho-serine S52, S56 (Vpu 2.6-GFP) unable to bind β-TrCP (Lane 14) (Margottin et al., 1998), suggesting that Vpu connects BST2 to β-TrCP (Mangeat et al., 2009). To confirm this model, co-immunoprecipitation between FLAG-BST2 and HA-β-TrCP was assessed in
cells expressing Vpu mutated on the residues W22 and A14 required for its interaction with BST2 (Skasko et al., 2012, Vigan and Neil, 2010) (Fig. 8B). As expected, interaction of FLAG-BST2 with HA-β-TrCP was affected in cells expressing the mutant W22A-A14L (VpuW22A14-GFP) (Lane 16), compared to cells expressing Vpu WT (Lane14), consistent with the notion that Vpu bridges BST2 to β-TrCP. Altogether, our data suggest that Vpu connects the restriction factor BST2 to β-TrCP, inducing its down-regulation, without interfering with the binding of BST2 to the E3 ligases, NEDD4 and MARCH8, involved in its constitutive trafficking and turnover.
Discussion

Ubiquitination regulates virtually every cellular processes and therefore cellular homeostasis and fitness (Pickart, 2001). The ubiquitination pathway is commonly manipulated by pathogens to propagate and to evade the host cell immune defense (Randow and Lehner, 2009). In this study, we have highlighted the role of two E3 ligases, NEDD4 and MARCH8 in the regulation of BST2 ubiquitination, trafficking and delivery to the lysosomes for degradation. We showed that NEDD4 and MARCH8 overexpression increases BST2 ubiquitination, an effect that is abrogated upon mutation of their catalytic sites (Fig. 2C-D). Conversely, NEDD4 and MARCH8 depletion decreased BST2 ubiquitination (Fig. 2A-B) and resulted in its delayed turnover (Fig. 4B-C). Surprisingly, both E3 ligases appeared dispensable for Vpu’s ability to promote cell-surface down-regulation and degradation of BST2 (Fig. 7). This activity requires the recruitment of β-TrCP that does not potently contribute to the regulation of basal ubiquitination and turnover of BST2 (Figs 2, 4 and 7) suggesting that the viral protein does not hijack the cellular machinery involved in constitutive ubiquitination and turnover of BST2, but usurps an alternative machinery for this process.

Our results unraveled the contribution of NEDD4 and MARCH8 in the regulation of constitutive ubiquitination and trafficking of BST2 within the endocytic pathway (Figs 2, 4 and 5). Depletion of each E3 ligase independently, altered BST2 sorting for degradation (Figs 2A-B and 4B), with no alteration in the internalization rate of BST2 from the PM (Fig. 4A). This suggests that the alteration in BST2 trafficking most likely occurs at the endosome level, and by extension, that NEDD4 and MARCH8 regulate endosomal sorting of BST2. Knockdown of these E3 ligases resulted in an overall increase in BST2 levels (Fig. 1C-D). However, NEDD4 knocked-down cells displayed no major alteration of BST2 intracellular distribution (Fig. 5). Depletion of MARCH8 on the other hand induced an enhanced distribution of BST2 on an HRS-positive compartment suggesting retention of the protein in this compartment (Fig. 5). Consequently, one might propose that MARCH8 acts downstream of NEDD4 on BST2 ubiquitination and trafficking along the endosomal pathway. The delayed turnover of BST2 observed upon depletion of NEDD4 might result from enhanced and sustained recycling of BST2 from early endosomes to the cell surface. Later on the endosomal pathway, MARCH8 present at the level of the MVB (Fig. S3) might interact with BST2 and trigger its ubiquitination and sorting from this compartment. This notion is supported by the build-up of BST2 in an HRS-positive compartment upon depletion of MARCH8 (Fig. 5), suggesting inability for BST2 to recycle back to the plasma membrane,
and the redistribution of BST2 into HRS-positive endosomes and ultimately reduced levels of BST2 upon overexpression of MARCH8 (Fig. 6).

We also observed increased level of BST2 in cells depleted for β-TrCP, without alteration of BST2 turnover and ubiquitination (Figs 1, 2 and 4). β-TrCP regulates proteasome-associated quality control of proteins in the biosynthetic pathway as well as endo/lysosomal degradation of proteins (Fuchs et al., 2004). One might propose that depletion of β-TrCP induces ubiquitin-dependent alteration of a yet-to-be determined regulator of BST2 in the ER and/or at the endo/lysosomal pathway at a cryptic level, leading ultimately to an increased of BST2 levels during the course of our experiments.

Substrate recognition by NEDD4 relies on the interaction of its WW domain with a proline rich PPxY (PY) motif within its substrates (Boase and Kumar, 2015). BST2 does not harbor a typical PY motif suggesting the potential involvement of an adaptor protein for its interaction with NEDD4. Examples of adaptors for NEDD4 binding have been described such as Ndfip (Boase and Kumar, 2015), or members of the Arrestin family (Becuwe et al., 2012). WW domains can also interact with phosphoserine/threonine residues in substrates (Lu et al., 1999, Sudol et al., 1995) and it’s noteworthy that BST2 contains two serines and one threonine (S3T4S5) in its cytoplasmic tail. Moreover, albeit controversial (Gustin et al., 2012), these residues were proposed to be the targets of Vpu-induced ubiquitination of BST2 (Tokarev et al., 2010). Their contribution on BST2 binding to NEDD4 therefore would be worth testing. To date, no specific determinants for MARCH8 recognition have been characterized. Of note, interaction of Myc-BST2 with MARCH8-GFP had been previously addressed by pulling down MARCH8, but did not reveal any interaction between both proteins (Fujita et al., 2013). This discrepancy suggests that BST2 might not be one of the main partner of MARCH8 and/or alternatively that the interaction between both proteins is very transient.

MARCH8 (initially called c-MIR) was initially identified as the cellular functional homolog of the KSHV membrane-bound ubiquitin ligases K3 and K5 (also called MIR1 and MIR2 for modulator of immune response) (Goto et al., 2003). Antagonism of BST2 antiviral activity by K5 relies on ubiquitination of BST2 on lysine K18 located in its cytoplasmic tail and subsequent sorting of the restriction factor for ESCRT-dependent lysosomal degradation (Bartee et al., 2006, Pardieu et al., 2010). We previously showed that BST2 undergoes ESCRT-mediated targeting to the lysosome (Janvier et al., 2011) and have demonstrated herein the role of MARCH8 in BST2 ubiquitination and delivery to lysosomal
degradation. Altogether, this supports the view that K5 mimics the endogenous cellular activity of MARCH8 to counteract BST2 restriction activity on KSHV infection. Further characterization of the role of the lysine residue of BST2 in mediating its sensitivity to MARCH8–induced ubiquitination will be required to confirm the mechanistic resemblance between both cellular and viral proteins on BST2 regulation.

Vpu favors ESCRT-mediated targeting of BST2 to the lysosome (Agromayor et al., 2012, Janvier et al., 2011). This process was shown to be ubiquitin dependant (Douglas et al., 2009, Goffinet et al., 2010, Mitchell et al., 2009, Tokarev et al., 2010), but does not involve hijacking of the machinery involved in the regulation of constitutive ubiquitination and sorting of BST2 for degradation (Fig. 7). Indeed, depletion of NEDD4 or MARCH8 had no impact on the ability of Vpu to modulate the expression of BST2, an activity that relies on the recruitment of β-TrCP by Vpu (Figs 7-8), as previously reported (Mangeat et al., 2009, Mitchell et al., 2009, Tokarev et al., 2010). Vpu has been proposed to promote poly-ubiquitination of BST2 on its serine and threonine residues (Tokarev et al., 2010). Our data suggest that NEDD4 and MARCH8 promote mono/multi-ubiquitination of BST2. This might explain the irrelevance of both E3 ligases on Vpu-induced sorting of BST2 for degradation.

Vpu-induced down-regulation of BST2 has been suggested to be dispensable in counteracting BST2 antiviral activity, which mainly requires active removal of BST2 from the HIV-1 budding sites by the viral protein (Chu et al., 2012, Goffinet et al., 2010, Lewinski et al., 2015). The requirement of β-TrCP and consequent ubiquitination of BST2 in Vpu-induced antagonism of BST2 antiviral activity remains controversial (Iwabu et al., 2009, Kueck et al., 2015, Mangeat et al., 2009, Mitchell et al., 2009). We did not observe any potent effect of β-TrCP depletion on the amount of HIV-1 released into cell supernatants (Fig. S4). However, these cells displayed an increased expression of viral components, notably Env and Vpu, suggesting either increased sensitivity for HIV infection, or enhanced transcription/translation of viral genome that precludes drawing any conclusion on the involvement of β-TrCP on Vpu-mediated viral release. In the same line of thoughts, measurement of virus production in cells treated with siRNA against NEDD4 or MARCH8 did not allow us to draw any conclusions due to the low permissiveness of these cells to HIV-1 infection (Fig. S4). Interestingly, a recent paper revealed the importance of MARCH8 in the regulation of HIV-1 Env glycoproteins trafficking and incorporation into budding viruses (Tada et al., 2015). Countering BST2 antiviral activity by most strains of HIV-2 and a subset of SIV strains (SIV\text{agm}Tan and SIV\text{mac}239\Delta\text{nef} isolates) is performed by
Env glycoproteins (Gupta et al., 2009, Jia et al., 2009, Le Tortorec and Neil, 2009, Serra-Moreno et al., 2011). Future work will explore in depth the role of MARCH8 in HIV-2 Env-induced antagonism of BST2.

In summary, this study has highlighted two additional regulators of BST2, namely, NEDD4 and MARCH8 that together provide greater understanding on the mechanisms underlying BST2 turnover in cells under basal conditions. Furthermore, our data showed that Vpu bypasses the machinery constitutively involved in BST2 ubiquitination and sorting for degradation and favors recognition of the restriction factor by β-TrCP to trigger its lysosomal targeting. Future studies will decipher the molecular and cellular mechanisms underlying regulation of BST2 expression and trafficking by Vpu.
Materials and Methods

Cell culture
HeLa (NIH; AIDS Reagent Program), and HEK293T (ATCC) cells were grown in DMEM plus glutamine, antibiotics and 10% decomplemented-FBS (foetal bovine serum) (Gibco®, Life Technologies).

Recombinant DNA and transfection
The cDNAs for NEDD4 WT, catalytically inactive NEDD4 C\textsubscript{867}S mutant (gifts from Dr Snyder), MARCH8 WT and catalytically inactive C\textsubscript{83/86/123/126}S mutant (referred herein MARCH8 C/S) (gifts from Drs Kelly and Jannhe) were cloned into pEGFP-C2 vector (Clontech, France). Expression vectors for BST2, wild-type and mutated NEDD4 and MARCH8 fused to the HA or the FLAG affinity tags, were obtained by cloning the cDNAs into pAS1B vector (Selig et al., 1999) or p3XFLAG vector (Janvier et al., 2011) respectively, that enable N-terminal tagging of the proteins. Expression vectors for GFP or HA-tagged β-TrCP WT and delta F-box deletion mutant (β-TrCPΔF) were obtained from Dr Margottin-Goguet (Margottin et al., 1998). The NL4-3 Vpu mutants S\textsubscript{52/56}N, and A\textsubscript{14}L-W\textsubscript{22}A were made by PCR mutagenesis using the QuikChange II site directed mutagenesis kit (Stratagene, France). WT and mutants cDNAs of NL4-3 Vpu were cloned into pEGFP-N1 vector (Clontech, France). Transfections of expression vectors in HeLa and HEK293T cells were performed using Lipofectamine LTX (Life Technologies) following the manufacturer’s instructions.

siRNA transfection
Cells were transfected with 5 to 30 nM siRNA using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s instructions and analysed four days post transfection. The 21-nucleotides RNA duplex designed to target both β-TrCP1 and β-TrCP2 (5’-GUGGAAAUUUGUGGAACAUCAUCdTdT-3’ at positions 448-465 and 265-285 respectively) was previously described (Jin et al., 2003). The On-target plus SMART-pool siRNAs targeting BST2 (#L-011817-00), NEDD4 (#L-007178-00), NEDD4-L (#L-007187-00), MARCH8 (#L-007161-00), CBL (#L-003003-00), ITCH (#L-007196-00), WWP1 (#L-004251-00) were purchased from Dharmacon. The On-target plus non-targeting siRNA 1 (#D-001810-01 from Dharmacon) was used as control.
**SDS-PAGE and western blot analyses**

SDS-PAGE and Western blotting were performed as described previously (Caillet et al., 2011). Antibodies used are listed in Table S1.

Quantitative western blots for BST2 and Tubulin were performed by incubation of the membranes with rabbit anti-BST2 (NIH) and mouse anti-Tubulin (Sigma-Aldrich) antibodies followed by incubation with Dylight™-800 conjugated anti-Rabbit IgG and Dylight™-680 conjugated anti-Mouse IgG (KPL-Thermo Scientific). The corresponding signals were then acquired using the Odyssey® Infrared Imaging System (Li-Cor) for further quantification using ImageJ software.

**Ubiquitination assay**

HeLa cells transfected with siRNA or GFP-tagged constructs were lysed 30 min at 4°C using lysis buffer (50 mM Tris pH 7.6, 300 mM NaCl, 2 mM EDTA, 1% (v/v) NP-40, 0.1% (v/v) sodium deoxycholate and 0.2% SDS), supplemented with 20 mM N-ethylmaleimide (Calbiochem) and complete protease inhibitor Cocktail (Roche Diagnostics). Cell extracts were immunoprecipitated using BST2 antibody (2E6, Abnova) coupled to Dynabeads® Protein G (Life Technologies). Immunoprecipitates were resolved on SDS-PAGE before immunoblotting using antibodies to Ubiquitin (HRP-linked FK2 antibody, Enzo Life Sciences) and BST2 (NIH).

**Cycloheximide treatment**

BST2 turnover was assessed by incubating the cells with cycloheximide (Calbiochem), as previously described (Janvier et al., 2011), followed by quantitative western-blot analyses using the Odyssey® Infrared Imaging System (Li-Cor).

**Co-immunoprecipitation experiments**

Cells were harvested 24 h after transfection and lysed 30 min at 4°C in lysis buffer (50 mM Tris-HCl pH 7.6, 2 mM EDTA, 150 mM NaCl, 0.5% NP-40) complemented with protease inhibitor cocktail. The cell lysates were immunoprecipitated with anti-FLAG M2 affinity Gel (Sigma-Aldrich) or anti-BST2 (2E6) antibody or the corresponding IgG isotype (Biolegend) coupled to Dynabeads® Protein G for 4 h at 4°C. Purified proteins were resolved by SDS-PAGE followed by western blotting using anti-HA-
peroxidase (HRP) (Roche© Diagnostics), anti-FLAG®M2-peroxidase (Sigma-Aldrich) anti-GFP-HRP (Genetex), anti-BST2 (NIH) or anti-NEDD4 (Millipore) antibodies.

**Immunofluorescence microscopy**

Immunofluorescence analyses in cells transfected with siRNA or GFP-tagged constructions and/or infected were performed as described previously (Caillet et al., 2011). Antibodies used are listed in Table S2. Coverslips were mounted in Fluoromount-G (SouthernBiotech).

Cells were analysed with a Leica DMI6000B Microscope Spinning Disk. Series of 0.2 µm optical sections were recorded and images were processed using ImageJ software. Quantitative colocalization analysis was done with JACoP tool (ImageJ software) using calculation of Pearson’s correlation coefficient (Bolte and Cordelieres, 2006). The Pearson’s coefficient was calculated for eight images per condition, with about 3 to 5 cells per image.

**Flow cytometry**

Cells transfected with plasmids encoding GFP-tagged constructs were stained 24 h after transfection with Alexa-647 conjugated anti-BST2 antibody (clone RS38E, Biolegend) or control IgG1 (Biolegend) in PBS/1% BSA for 1 h at 4°C. Cells were washed and fixed in PBS/1% BSA/1% paraformaldehyde (PFA, Electron Microscopy Science) before analysis using the Cytomics FC500 Flow Cytometer (Beckman Coulter) or the Accur™ C6 Flow cytometer (BD Biosciences). Gates for GFP were set using non-transfected cells. All the data were analysed using the CXP software or the C6 cytometer software.

Surface staining of BST2 in HIV-1 infected cells infected was performed as described above. Cells were then fixed, permeabilized and stained with a FITC-conjugated anti-CAp24 antibody (Beckman Coulter) prior analysis.

Intracellular staining of infected cells was performed in permeabilized fixed cells using antibodies against BST2 (2E6, Abnova) and HIV-1 Env (2G12, NIH), followed by staining with the appropriate secondary antibodies (Table S2) prior analysis.
Internalization assay
siRNA transfected cells were surface-stained with anti-BST2 antibody (RS38E, Biolegend) or IgG1 isotype control for 1 h at 4°C in growth medium (DMEM+FBS 10%). Cells were washed 3 times in cold medium, an aliquot representing t=0 was kept at 4°C. The remaining cells were transferred at 37°C for different periods of time to allow internalization of BST2. At each time point, cells were transferred in cold medium then stained with Alexa647-conjugated anti-mouse secondary antibody for 30 min at 4°C, washed 3 times and fixed in PBS/1% BSA/1% PFA before analysis by flow cytometry. BST2 internalization rate was calculated as the amount of BST2 present at the cell surface at each time point compared to the amount present at time zero.

RNA extraction and RT-qPCR
RNA extractions and RT-qPCR analyses were performed as described previously (Madjo et al., 2016). RNAs extracted using the QIAGEN RNeasy Mini kit and treated with DNase (Qiagen, RNAse-free DNase set) were reverse transcribed using the High Capacity Reverse transcription kit (Applied Biosystem) and quantified by real-time PCR using the Roche LightCycler 480 SYBR Green 1 Master kit (Roche Diagnostics) and specific primers. Each point was performed in technical triplicate. The relative abundance of each mRNA tested was normalized to that of GAPDH mRNA level. The primers used are: MARCH8 (5’-ACAGGAAGCCTCCACTTCG-3’, 5’-GAGTCCTGTAGGTTGCAG-3’), βTrCP1 (5’-CCAACATGGGCACATAAACTCG-3’, 5’-CCTACGGTTTAGTATACGACG-3’), βTrCP2 (5’-ACGAATGGTACGACGCACTGATGATCC-3’, 5’-CCTACACTTGTGCCCACTTCA -3’) and GAPDH (5’-GCATGGACTGTGGTACGAG-3’, 5’-TGCACCACCAACTGCTTAGC-3’).

Viral stocks and HIV-1 production assay
Stocks of HIV-1 NL4-3 WT (NIH AIDS Reagent Program) and HIV-1 NL4-3 Udel (from Dr. Strebel), pseudotyped with VSV G glycoprotein were prepared as previously described (Janvier et al., 2011). HIV-1 production assays were performed as described (Janvier et al., 2011). Pelleted viruses and cell lysates were analyzed by western blotting using anti-CAP24 (ARP366), anti-SUgp120 (110H), anti-TMgp41 (41A), anti-Vpu, anti-BST2 and anti-Tubulin antibodies (see Table S1).
Statistical analysis
Statistical significance was analyzed by paired two-tailed Student’s \( t \)-test and the one way Anova test, and expressed as a \( P \) value. Each experiment was performed independently at least three times to ensure minimal robustness of the statistical analyses.

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Competing interests
The authors declare no competing interests

Authors contributions
\textbf{N.R.} and \textbf{G.P.} conducted the experiments, analysed the data and contributed to the writing of the manuscript. \textbf{C.B-T.} analysed the data and edited the manuscript. \textbf{K.J.} conceived, designed, performed the experiments, analysed the data, supervised the study and wrote the manuscript.

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References


Fig. 1. Silencing of NEDD4, MARCH8 or β-TrCP induces enhanced levels of BST2. (A-B) Analysis of E3-ubiquitin ligase depletion. HeLa cells transfected with indicated siRNA or siRNA control (siCD), were lysed and proteins depletion was confirmed (A) by western-blot analysis or (B) by RT-qPCR. (C-D) Impact of E3-ligase depletion on the cellular level of BST2. 20 µg of proteins of
each sample were loaded and BST2 levels were assessed by quantitative western-blotting. Tubulin is the loading control (C). BST2 relative levels were measured using ImageJ software and normalized on Tubulin levels (D). Values were normalized to those obtained for the control cells set as 100%. Data are represented as mean +/- SD from 3 independent experiments (n=3). P values were calculated using Student’s t-test. Significant results (*, p<0.05) are indicated. (E) Impact of NEDD4, MARCH8 and β-TrCP depletion on BST2 transcription level. BST2 mRNA level was quantified in siRNA-treated cells by RT-qPCR using specific primers. Values calculated were normalized to that obtained for control cells set as 100%. Bars represent the mean +/- SD (n=3).
Fig. 2. Contribution of NEDD4, MARCH8 and β-TrCP in BST2 ubiquitination. (A-B) SiRNA-transfected HeLa cells or (C-D) HeLa cells transfected with plasmid encoding for wild-type (Lanes 2, 4, 6) or catalytically inactive mutants (Lanes 3, 5, 7) of NEDD4, MARCH8 or β-TrCP fused to GFP, were
lysed in stringent buffer and BST2 was immunoprecipitated with anti-BST2 antibody. Ubiquitination of BST2 was analyzed by western-blot using anti-Ubiquitin antibody. In panels C-D, plasmids encoding for GFP-tagged Histone H2B (H2B-GFP) or WT Vpu (Vpu-GFP) or Vpu mutated on residues S52 and S56 (Vpu 2.6-GFP) were used as controls. Asterisks (*) in panel C indicate the bands corresponding to GFP-fusion proteins. (B, D) Signals obtained for Ubiquitin staining were normalized to those obtained for BST2. Values obtained for each condition were normalized to those obtained for control cells (siCD and H2B-GFP, respectively) set as 100%. Bars represent the mean ± SEM (n=4), ****p<0.0001, **p<0.01, *p<0.05.
Fig. 3. Analysis of BST2 interaction with NEDD4, MARCH8 and β-TrCP. (A) HEK293T cells were transfected with plasmid encoding FLAG-BST2 or p3xFLAG vector (used as negative control) along with expression vectors for HA-NEDD4, HA-MARCH8, HA-β-TrCP or HA-WWP1 used as negative control. Binding of HA-E3 ubiquitin ligases and FLAG-BST2 was assessed by immunoprecipitation of FLAG-BST2 followed by western-blot analyses. Left panels represent the input and right panels represent bound proteins. Data representative of 4 independent experiments. (B-C) NEDD4 or MARCH8 interacts with BST2 independently of each other. HeLa cells transfected with the indicated siRNA were transfected with both plasmids encoding FLAG-BST2 and HA-tagged E3 ligases followed by FLAG-BST2 pull-down. Binding of HA-E3 ubiquitin ligases and FLAG-BST2 was analyzed by western-blot. Left panels represent the input and right panels represent bound proteins. Data representative of 3 independent experiments.
Fig. 4. Role of NEDD4 and MARCH8 in BST2 trafficking. (A) NEDD4, MARCH8 and β-TrCP are not involved in the regulation of BST2 internalization. Internalization of BST2 in siRNA-transfected HeLa cells was monitored by immunofluorescence staining and flow cytometry analyses. The kinetics of BST2 internalization were expressed as the amount of BST2 remaining at the cell surface at each time point compared to the amount of BST2 present at t=0 (set as 100%). Error bars represent the mean +/- SD (n=3). (B) NEDD4 and MARCH8 sort BST2 for degradation. SiRNA treated HeLa cells were incubated with cycloheximide for the times indicated above each lane. Equivalent amounts of each sample (20 µg of proteins) were analyzed by quantitative western-blot using antibodies against...
BST2 and Tubulin as a loading control. For each sample, BST2 relative amount was measured using ImageJ software and normalized on Tubulin levels. Values at time 0 were set to 100% in the graph shown on the right. Error bars represent the mean +/- SD (n=3).
Fig. 5. Effects of NEDD4 or MARCH8 silencing on BST2 subcellular localization. SiRNA-transfected HeLa cells were permeabilized before fixation and immuno-stained with antibodies against BST2 (green) and transferrin receptor (TIR, recycling endosomes) (A) or HRS (endosomes/MVBs) (C) (red) along with Dapi (blue) to visualize the nucleus of the cells. Cells were observed with a confocal
microscope. Scale bars = 10µm. (B, D) Colocalization between BST2 and TIR or BST2 and HRS, was assessed by calculating the Pearson’s correlation coefficient on 8 images per conditions. Each dot represents the Pearson's correlation coefficient of one image featuring at least 3 cells. Errors bars represent the mean -/+ SEM from each image (n=3), ***p<0.001.
Fig. 6. BST2 distribution in cells overexpressing NEDD4 or MARCH8. (A) HeLa cells transfected with plasmids encoding for GFP-NEDD4 or GFP-MARCH8 were permeabilized and co-stained with antibodies against BST2 (green) and EEA1, HRS or CD63 (red) along with Dapi (blue). Cells were then analyzed by confocal microscopy. Scale bars = 10µm. Areas indicated in square are magnified in lower panels. (B) HeLa cells transfected with the indicated expression vectors were lysed and BST2 levels were assessed by western-blot analyses. Tubulin is the loading control. Asterisks (*) indicate the bands corresponding to GFP-fusion proteins. These data are representative of 3 independent experiments. (C) HeLa cells transfected with the indicated GFP-fusion constructs were surface-stained with BST2 antibody or IgG1 isotype control and processed for flow cytometry analysis. The level of cell surface expression of BST2 was calculated as the mean fluorescence intensity (MFI) values obtained for BST2 staining minus MFI values of the isotype control. Values for each condition were normalized to that of control H2B-GFP expressing cells set as 100%. Bars represent the mean +/- SD (n=4) **p<0.01, *p<0.05. (D-E) HeLa cells expressing the indicated GFP-fusion constructs for 12 hours were further cultured for 16 hours in presence of either DMSO or 50nM of Concanamycin A, an inhibitor endosomal/lysosomal acidification and processed for western-blot and flow-cytometry analyses as described in (B-C).
Fig 7. NEDD4 and MARCH8 are not required for Vpu-induced BST2 down-regulation. (A-B) Effects of NEDD4, MARCH8 or β-TrCP silencing on Vpu-induced cell surface down-regulation of BST2. HeLa cells transfected with the indicated siRNA were infected with VSV-G pseudotyped HIV-1 NL4-3 WT. Twenty-four hours later, cells were surface-stained for BST2 or an isotype IgG control. The cells were then fixed, permeabilized and stained for Gag using a FITC-conjugated monoclonal anti-CAp24. The cells were then processed for flow cytometry analysis. (A) Dot plot. Vertical lines indicate the gates set using non-infected cells stained as indicated. Left gate: non-infected cells; right gate: infected-cells. (B) Bar graph representation of cell surface level of BST2 in CAp24 negative cells (Left bars) and CAp24 positive cells (Right bars) for each siRNA condition. Values are expressed as the MFI for BST2 staining minus those of the isotype control, normalized to those of non-infected cells set as 100%. Bars represent the mean +/- SD (n=3); **p<0.01, *p<0.05. (C-D) β-TrCP is required for Vpu-induced BST2 degradation. (C) Infected siRNA-treated HeLa cells were permeabilized before fixation, and intracellular BST2 (green) and HIV-Env (red) were labelled with specific antibodies. Nuclei were stained with DAPI. Cells were imaged by confocal microscopy. Env staining discriminates infected cells (arrows) and non-infected cells. Scale bar: 10 µm. Images are representative of 3 independent experiments. (D) Infected siRNA treated cells were fixed, permeabilized and stained for intracellular BST2 and HIV-1 Env using specific antibodies prior analysis by flow cytometry analysis. BST2 expression levels on infected and non-infected cells respectively were expressed as in B. Bars represent the mean +/- SD (n=3), **p<0.01.
**Fig 8. Vpu connects BST2 to β-TrCP. (A)** HEK293T cells were transfected with plasmid encoding FLAG-BST2 or p3X-FLAG vector (control) along with expression vectors for HA-NEDD4, HA-MARCH8 or HA-βTrCP, and either Vpu WT-GFP or Vpu 2.6-GFP impaired for β-TrCP binding, or GFP (as control). Binding of FLAG-BST2 with HA-E3 ubiquitin ligases and Vpu-GFP proteins was assessed by immunoprecipitation of FLAG-BST2 followed by western-blot analyses. Upper panels represent bound proteins and lower panels represent the input. Data representative of 4 independent experiments. **(B)** Interaction between FLAG-BST2 and HA-β-TrCP in presence of Vpu WT-GFP or Vpu 2.6-GFP or mutated Vpu A14L-W22A (Vpu A14W22-GFP) impaired for BST2 binding was assessed in as described in (A). Left panels represent the input and right panels represent bound proteins. Data are representative of 2 independent experiments.
Supplementary Figures

Supplementary Figure 1 related to Figure 3

A

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C

HEK 293T cells +pCDNA-BST2

D

HeLa cells

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Fig. S1 (related to Fig. 3). (A-B) Analysis of BST2 interaction with HA-NEDD4 and HA-MARCH8. (A) HEK293T cells were transfected with plasmids encoding BST2 (pCDNA-BST2) along with expression vectors for HA-NEDD4 or HA-MARCH8. Binding of HA-E3 ubiquitin ligases and BST2 was assessed by immunoprecipitation using monoclonal anti-BST2 antibody or IgG1 isotype as a negative control, followed by western-blots using antibodies against the HA tag and BST2. Left panels represent the input and right panels represent bound proteins. Data representative of 3 independent experiments. (B) HeLa cells were transfected with expression vectors for HA-NEDD4 or HA-MARCH8. Binding of the HA-ligase with endogenous BST2 was assessed as described in (A). Data representative of 3 independent experiments. (C-D) Analysis of BST2 interaction with endogenous NEDD4. (C) HEK293T cells were transfected with plasmid encoding untagged BST2. Interaction of BST2 with endogenous NEDD4 was addressed by immunoprecipitation using monoclonal anti-BST2 antibody or IgG1 isotype as a negative control. Samples were resolved on a 4-12% gradient acrylamide gel followed by western blotting using antibodies against BST2 and NEDD4. Data representative of 3 independent experiments. (D) Binding of endogenous BST2 with NEDD4 was assessed in HeLa cells by co-immunoprecipitation assay as described in (C). Data representative of 2 independent experiments. (C-D) Western-blotting of samples resolved on gradient acrylamide gel with anti-NEDD4 antibody, directed against the WW2 domain of NEDD4, enables detection of 3 major species (*) that might correspond to isoforms of NEDD4.
Supplementary Figure 2 related to Figure 5

(A) BST2 and TGN46 localization in cells treated with different siRNAs.

(B) Scatter plot showing colocalization between BST2 and TGN46 in cells treated with different siRNAs.

(C) BST2 and LAMP1 localization in cells treated with different siRNAs.

(D) Scatter plot showing colocalization between BST2 and LAMP1 in cells treated with different siRNAs.

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Fig. S2 (related to Fig. 5). Effects of NEDD4, or MARCH8 silencing on BST2 subcellular localization. (A and C) HeLa cells transfected with siRNA targeting NEDD4, MARCH8 or control siRNA, were permeabilized before fixation and immuno-stained for BST2 (green) along with the TGN marker TGN46 (A) or the lysosomal membrane protein LAMP1 (lysosomes) (C) (red) along with Dapi (blue) to visualize the nucleus of the cells, followed by analysis by confocal microscopy. Scale bars = 10μm. (B, D) BST2-TGN46 and BST2-LAMP1 colocalizations. Colocalization was assessed by calculating the Pearson’s correlation coefficient on 8 images per conditions, using the JACoP plugin on ImageJ. Each dot represents the Pearson’s correlation coefficient of one image featuring at least 3 cells. Bars represent the mean +/- SEM from each image. The data is representative of 3 independent experiments.
Fig. S3 (related to Fig. 6). MARCH8 is localized at the plasma membrane and in endosomal compartments. (A) HeLa cells were transfected with plasmid encoding GFP-tagged MARCH8 (GFP-MARCH8) (green). Twenty-four hours later, cells were permeabilized before fixation and staining with antibodies directed against specific markers of cellular compartments such as HRS (endosomes/MVBs), TfR (early/recycling endosomes), TGN46 (TGN), LAMP1 (lysosomes) or CD63 (late endosomes) (red). Cells were then analyzed by confocal microscopy. Scale bars = 10µm. Colocalizations between GFP-MARCH8 and subcellular compartment markers were assessed by calculating the Pearson’s correlation coefficient on 7 images featuring at least 3 cells per conditions. Bars represent the mean -/+ SEM from each image. The data is representative of 2 independent experiments.
Fig. S4 (related to Fig. 7). Consequences of NEDD4, MARCH8 and βTrCP depletion on HIV-1 production. HeLa cells transfected with either control siRNA (siCD) or siRNA targeting BST2, NEDD4, MARCH8 or β-TrCP were infected with VSV-G pseudotyped NL4-3 HIV-1 (NL4-3 WT) or VSV-G pseudotyped Vpu-defective NL4-3 (NL4-3 Udel) at a MOI of ~0.5. (A) Western blot analyses of infected siRNA treated cells (upper panels) and pelleted virus (lower panels) with antibodies against BST2, Gag; Env, Vpu and Tubulin as a loading control. Asterisks (*) indicate non-specific bands. (B) CAp24 present within the cells (Cell-associated CAp24) and released in the supernatant of the infected cells (Released CAp24) was measured by ELISA. The values were normalized to those obtained for control cells (siCD) infected with WT viruses set to 100%. Bars represent the mean +/- SD from 5 independent experiments.
**Table S1:** Primary and secondary antibodies used for western blot analyses

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**Table S2:** Primary and secondary antibodies used for Immunofluorescence staining followed by Microscopy or Flow cytometry analyses

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