The viral restriction factor tetherin/BST2 tethers cytokinetic midbody remnants to the cell surface

Graphical abstract

Highlights

- BST2/tetherin localizes at the surface of cytokinetic midbodies in a variety of cells
- BST2 promotes retention and capture of midbody remnants
- BST2 tethers midbody remnants to the cell surface via its GPI anchor
- This work reveals new parallels between viral biology and cytokinesis

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In brief

Cytokinesis generates a midbody remnant (MBR) that is retained by a daughter cell or captured by distant cells. Presle et al. report that BST2/tetherin, a restriction factor for numerous enveloped viruses, also tethers MBRs. This work reveals a tethering role for BST2 in uninfected cells and a new parallel between cytokinesis and viral biology.
The viral restriction factor tetherin/BST2 tethers cytokinetic midbody remnants to the cell surface

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SUMMARY

The midbody at the center of the intercellular bridge connecting dividing cells recruits the machinery essential for the final steps of cytokinesis.1–5 Successive abscission on both sides of the midbody generates a free midbody remnant (MBR) that can be inherited and accumulated in many cancer, immortalized, and stem cells, both in culture and in vivo.6–12 Strikingly, this organelle was recently shown to contain information that induces cancer cell proliferation, influences cell polarity, and promotes dorso-ventral axis specification upon interaction with recipient cells.13–16 Yet the mechanisms by which the MBR is captured by either a daughter cell or a distant cell are poorly described.10,14 Here, we report that BST2/tetherin, a well-established restriction factor that blocks the release of numerous enveloped viruses from the surface of infected cells,17–20 plays an analogous role in retaining midbody remnants. We found that BST2 is enriched at the midbody during cytokinesis and localizes at the surface of MBRs in a variety of cells. Knocking out BST2 induces the detachment of MBRs from the cell surface, their accumulation in the extracellular medium, and their transfer to distant cells. Mechanistically, the localization of BST2 at the MBR membrane is both necessary and sufficient for the interaction between MBRs and the cell surface. We thus propose that BST2 tethers post-cytokinetic midbody remnants to the cell surface. This finding reveals new parallels between cytokinesis and viral biology21–26 that unexpectedly extend beyond the ESCRT-dependent abscission step.

RESULTS AND DISCUSSION

BST2 is enriched at the midbody during cytokinesis and localizes to the surface of MBRs

In most cell types, midbody remnants (MBRs) are either released into the extracellular medium or interact with the surface of a recipient cell, usually one of the two daughter cells, before being engulfed and degraded (Figure S1A).6,7,9,10,12,14,27–33 Although integrins partially account for the attachment of purified MBRs,14 understanding how MBRs are captured and retained remains a key question, because MBRs can influence cell fate and promote cell proliferation.7,14,34 Recently, we achieved the purification of intact MBRs from HeLa cells and reported the quantitative proteome of this organelle that we termed Flemmingsome.35 Looking back on the results, we were intrigued to identify BST2/CD317/tetherin as a protein 3.6-fold enriched in MBRs, as compared to the total cell fraction. BST2 is a single-pass transmembrane protein with a GPI anchor that physically tethers numerous enveloped viruses to the surface of infected cells. BST2/tetherin thereby restricts viral propagation (Figure 1A).17,18,36–43 More recently, BST2 was shown to retain exosomes at the plasma membrane,44 revealing a tethering function of BST2 in non-infected cells. We therefore reasoned that BST2 might play a role in retaining MBRs at the cell surface (Figure 1A).

To investigate whether BST2 functions in MBR anchoring, we first characterized its localization in fixed samples. In addition to BST2 being present at the cell surface, immunofluorescence in HeLa cells labeled with the midbody marker CEP55 revealed that 94% of midbodies (present at the center of cytokinetic intercellular bridges; n = 100) and 98% of MBRs (present at the surface of individual cells; n = 100) were positive for BST2 (Figures 1B and 1C). Super-resolution microscopy using structured illumination (SIM) showed that BST2 localized in a ring-like pattern at the outer rim of the MBR, wrapping around CEP55, suggesting
A cellular process is illustrated in Figure A, where a virus or exosome interacts with the cell, potentially leading to the formation of midbodies or MBRs (midbody remnants). The figure shows the localization of BST2 and CEP55 proteins in relation to midbodies and MBRs.

Figure B presents images of midbodies and MBRs stained with ac-tub and DAPI, highlighting BST2 and CEP55 localization. The percentage of BST2-positive midbodies and MBRs is shown in Figure C, indicating a higher percentage in MBRs compared to midbodies.

Figures D, E, and F display immunofluorescence images of DAPI (cell nuclei), CEP55, BST2, GFP-MKLP1, and SIR-Tubulin (microtubules) at different time points (0 min, 10 min, 50 min, 80 min, 120 min, 240 min, and 300 min) to observe the dynamic changes in protein localization.

Figures G and H show the effect of IFNα on midbody and MBR formation. The western blot (Figure H) reveals the expression levels of BST2, B2, and GAPDH in the presence and absence of IFNα in Caco-2 and HEK 293 cells.
that BST2 resided at the MBR surface (Figure 1D). To confirm this, we purified intact, EDTA-detached MBRs from HeLa cells that stably express the midbody marker GFP-MKLP1 using flow cytometry, as described previously (GFP+ MBRs; Figure S1B) and stained them without permeabilization with anti-BST2 antibodies (Figure 1E). We found that 99% of purified MBRs (n = 100) were positive for BST2, demonstrating that BST2 indeed localizes at the plasma membrane surrounding the MBR. Finally, using time-lapse fluorescent microscopy of HeLa cells that expressed GFP-tagged BST2, we observed that BST2 accumulated at the midbody soon after furrow ingression and persisted after abscission, explaining BST2 presence on MBRs (Figure 1F; Video S1).

BST2 is constitutively expressed in many human cell types and can be strongly induced upon cytokine stimulation like interferon-α (IFN-α). BST2 was constitutively found both at the midbody and the MBR in the cancer cells that we examined, such as the Caco-2 colon cancer cells (Figure 1G), the HepG2 hepatocellular carcinoma, and the SK-MEL2 melanoma cells (Figure S1C). This was also the case in primary cells like human T lymphocytes and HUVEC endothelial cells (Figure S1C). In the HEK293 immortalized kidney cells in which BST2 was undetectable, treatment with IFN-α strongly induced BST2 expression and localization, both at the midbody and the MBRs (Figure 1H). Altogether, we conclude that BST2 localizes at the MBR in a variety of cancer and primary cells, either constitutively or upon induction.

**BST2 promotes MBR retention at the cell surface**

Because BST2 is a well-characterized tether for enveloped viruses and localizes at the surface of MBRs, we next investigated whether this protein could favor the retention of MBRs at the cell surface. Thanks to CRISPR-Cas9 technology, we established a BST2 knockout (BST2 KO) HeLa cell line that stably expressed the MBR marker GFP-MKLP1. We selected a clone in which the BST2 protein expression was abolished, as shown by western blot (Figure 2A). This resulted from two insertion/deletion events at the genomic level, leading to a translational frameshift and a premature STOP codon in the transcript (Figures S2A and S2B). As expected, BST2 signal was lost from MBRs (Figure 2B), confirming the specificity of our BST2 staining. Importantly, when comparing BST2 KO cells with control KO cells (CTRL KO), we did not observe any differences in their cell cycle (Figure S2C), in the number of cells undergoing cytokinesis (Figure S2D), or in the number of cells that successfully completed abscission (Figure S2E). Thus, BST2 KO did not change the rate of MBR production. We next compared the number of MBRs found at the cell surface in control and BST2 KO conditions. To unequivocally discriminate between internalized versus cell-surface-localized MBRs, we carried out surface staining for CD81 and CD9P1 transmembrane proteins. Indeed, our recent proteomic study showed the enrichment of several tetraspanins and associated proteins in MBRs, and we now took advantage of antibodies against the extracellular domains of CD81 or CD9P1 to reliably label non-permeabilized, purified MBRs (Figures S2F and S2G). Remarkably, surface staining of MBRs with CD81 (Figure 2C) or CD9P1 (Figure S2H) revealed a decrease in the number of MBRs at the cell surface after BST2 KO. These data suggest that, in the absence of BST2, MBRs are not properly retained at the cell surface and are likely released into the extracellular medium.

We then quantified, by flow cytometry, the amount of MBRs in the extracellular medium released from GFP-MKLP1 control KO and BST2 KO HeLa cells and observed a 2-fold increase upon BST2 KO (Figure 2D). As described for virions, we hypothesized that the release of MBRs into the medium upon BST2 KO would promote long-range transfer to distant cells within the population. To test this idea, we used co-culture experiments using control KO or BST2 KO HeLa cells expressing GFP-MKLP1 (which labels nuclei, midbodies, and MBRs) as donor cells and non-fluorescent wild-type (WT) HeLa cells as recipients (Figure 2E, left). Importantly, when compared to control co-cultures where BST2 was expressed both on donor and recipient cells, we found that BST2 KO MBRs (GFP-labeled) were twice as frequently transferred onto WT recipient cells (Figure 2E, middle and right). We therefore conclude that BST2 prevents MBR release into the extracellular medium and their long-range transfer between cells.

**GPI anchoring is required for MBR retention by BST2**

To further understand how BST2 controls MBR retention, we turned to time-lapse microscopy and followed the fate of single...
Figure 2. BST2 promotes MBR retention at the cell surface

(A) Western blot of deglycosylated lysates from CTRL KO or BST2 KO GFP-MKLP1 HeLa cells. Loading control is GAPDH. Asterisks indicate BST2 deglycosylated monomers (*) and dimers (**). See Figures S2A–S2E and Table S1 for BST2 KO cell characterization.

(B) Endogenous BST2 staining of CTRL KO and BST2 KO GFP-MKLP1 cells. Arrowheads mark MBRs. Scale bars, 10 μm.

(C) Left: CD81 surface staining of CTRL KO and BST2 KO GFP-MKLP1 cells. Arrowheads mark MBRs. Scale bars, 10 μm. Right: percentage of MBRs at the cell surface is shown (CD81-positive; mean ± SD). 4 independent experiments; n = 470–533 MBRs. Paired two-sided Student’s t test is shown. See Figures S2F–S2H for additional MBR markers.

(D) Cell culture media were collected from CTRL KO and BST2 KO GFP-MKLP1 cells. MBRs were quantified by FACS with fluorescent beads and normalized with the number of cells and volume for each condition (a.u., arbitrary units). 3 experiments; n = 667–3,783 MBRs per experiment. Paired two-sided Student’s t test is shown.

(E) Left: co-culture experimental setup scheme with non-fluorescent and GFP-MKLP1 cells. GFP-MKLP1 localizes in nuclei, midbodies, and MBRs. A GFP-positive MBR transferred onto a non-fluorescent cell is arrowed. Middle: representative image of co-culture with non-fluorescent CTRL and GFP-MKLP1 BST2 KO HeLa cells is shown, stained for BST2. A GFP-MKLP1 MBR transferred on a CTRL cell is arrowed, and an arrowhead marks a GFP-MKLP1 MBR on a GFP-MKLP1 cell. Note the loss of BST2 staining in the GFP-MKLP1 BST2 KO cells (marked by stars). Right: percentage of GFP-MKLP1 MBRs from either CTRL or BST2 KO HeLa cells transferred onto non-fluorescent CTRL HeLa cells is shown. 3 experiments; n = 1,000 MBRs per experiment. Paired two-sided Student’s t test is shown. Scale bar, 10 μm. See also Table S1 and Figure S2.
GFP-MKLP1-labeled MBRs in control and BST2 KO cells. In control cells, most of the MBRs generated after abscission were retained and roamed over the cell surface of one of the two daughter cells, before being engulfed, as previously described (Figures S1A and 3A). The GFP-MKLP1 signal at the MBR initially remained constant both at the cell surface and right after internalization (Figure 3A; time 0–570 min). Then, the GFP-MKLP1 fluorescence progressively disappeared over 2 h (Figure 3A; time 585–705 min), due to quenching in the acidic, degradative environment of late endosomes/lysosomes. In rare instances, however, we noticed a sudden and complete disappearance of the MKLP1-GFP signal between two successive time frames, corroborated by the concomitant disappearance of the dark MBR shape in phase contrast (Figure 3B); between time 750 min and 765 min), demonstrating that the MBR had been released from the cell surface into the medium. While this sudden jump occurred in 4% of control cells, the BST2 KO cell line showed a 4-fold increase in MBR release into the extracellular medium (Figure 3C). Thus, knocking out BST2 increases the number of MBR-releasing events. This finding is consistent with the observed increase of MBRs in the medium (Figure 2D) as well as the reduced rate of cells with MBRs at the cell surface upon BST2 KO (Figure 2C).

It is well established that the overexpression of the HIV-1 accessory protein VPU downregulates BST2 from the cell surface and induces virion release. Similarly, we observed that VPU overexpression downregulated BST2 both from the cell surface and the midbody. Again, this induced MBR release to levels identical as observed upon BST2 KO (Figure 3D).

Tethering of viruses to the cell surface via BST2 relies on its unique topology and requires the presence of the transmembrane domain together with the C-terminal GPI anchor. These two domains can insert into the host plasma membrane on the one hand and the virion membrane on the other hand, thus allowing BST2 to physically tether viral particles to the infected cell (Figures 1A and 3E). We thus tested whether the GPI anchor requirement also applied to retain MBRs at the cell surface. First, we stably re-expressed in the BST2 KO cell line either BST2 WT or a mutant in which the two tyrosines (tyrosines 6 and 8) in the N-terminal cytoplasmic tail were mutated to alanines (hereafter named BST2 Y2A). The latter mutant was used as a control because it fails to induce nuclear factor-kB (NF-kB) signaling but fully retains tethering activities toward viruses. As shown by fluorescence-activated cell sorting (FACS) analysis, the surface expression levels of the re-introduced BST2 WT and BST2 Y2A were comparable but lower than endogenous BST2 levels (Figure S3A). In addition, both BST2 WT and BST2 Y2A localized to MBRs (Figures 3F and S3B). Importantly, both MBR accumulation at the cell surface (fixed samples; Figure 3G) and MBR retention (video microscopy; Figure 3H) were rescued to normal levels in BST2 KO cells that re-expressed either BST2 WT or BST2 Y2A. Next, we stably expressed in the BST2 KO cell line a BST2 mutant lacking the GPI anchor (hereafter named BST2 delGPI), which is unable to tether virions. Of note, BST2 delGPI and BST2 WT were expressed at comparable levels at the MBR (Figures 3F and S3B), and BST2 delGPI expression at the cell surface was higher than the endogenous BST2 and thus was not limiting (Figure S3A). However, BST2 delGPI did not rescue the defects in MBR retention at the cell surface (Figure 3G) or the increased release, measured by time-lapse microscopy (Figure 3H), and observed in the BST2 KO cells. Thus, like for viral tethering, the GPI anchor of BST2 is essential to promote MBR retention at the cell surface.

Because knocking out BST2 does not lead to the release of all MBRs (Figures 2 and 3), additional factors that promote MBR tethering to the cell surface must be involved. Recently, using the peptide cilengitide (RGDfV), which preferentially inhibits αV chain containing integrins, the αV/33 and/or αV/55 integrins have been proposed to promote the attachment and capture of purified MBRs to the cell’s surface. As expected for a peptide inhibiting integrins, RGDfV (but not the inactive peptide RADfV) impaired cell re-adhesion (Figure S4A) and induced progressive cell rounding of adherent cells (Figure S4B). Despite inhibiting integrins efficiently, RGDfV did not increase the release of endogenous MBRs already present at the cell surface during the treatment, neither from control KO nor from BST2 KO cells, as assessed by video microscopy (Figure 4A). This suggests that RGDfV-sensitive integrins do not cooperate with BST2 to retain endogenous MBRs. Using αV integrin KO cells generated by CRISPR-Cas9, we confirmed that αV deletion did not increase the release of MBRs either (Figure S4C). Interestingly, treating cells with the Ca²⁺/Mg²⁺ chelator EDTA and following MBRs release by video microscopy revealed a synergy between

Figure 3. GPI anchoring is required for MBR retention by BST2
(A and B) GFP-MKLP1 CTRL KO HeLa cells were imaged every 15 min, and the midbody followed from anaphase until its degradation (A; gradual loss of the GFP signal, insets) or release in the culture medium (B; sudden loss of the GFP signal, insets). Brackets and arrowheads mark the intercellular bridge and the MBR, respectively. Scale bars, 10 μm.

(G) Percentage of MBRs released in the media determined by time-lapse fluorescent microscopy (as in B), for either CTRL KO or BST2 KO cells (mean ± SD). 3 independent experiments; n = 50–100 MBRs per experiment. Paired two-sided Student’s t test is shown.

(D) Left: endogenous labeling of BST2 at the MBR (arrowhead) in CTRL KO and BST2 KO GFP-MKLP1 HeLa cells transiently transfected with plasmids encoding mCherry alone (“empty”) or VPU + mCherry. Scale bars, 10 μm. Right: percentage of MBRs released in the media determined by time-lapse fluorescent microscopy for CTRL KO and BST2 KO cells with or without VPU is shown (mean ± SD). 3 independent experiments; n = 36–50 MBRs per experiment. Paired two-sided Student’s t test is shown.

(F) Surface staining of BST2 (green, gray insets) in CTRL KO and BST2 KO GFP-MKLP1 HeLa cells, stably re-expressing BST2 WT, BST2 Y2A, or BST2 delGPI. Arrowheads mark MBRs. Scale bars, 10 μm. See Figures S3A and S3B for quantifications of BST2 levels at the MBR and cell surface.

(G) Proportion of surface (CD81-positive) versus total (MKLP1-positive) MBRs for each cell population indicated (mean ± SD). 3 independent experiments; n = 415–461 MBRs. Paired two-sided Student’s t tests are shown.

(H) Percentage of MBRs released in the media determined by time-lapse fluorescent microscopy for the indicated cell population (mean ± SD). 3 independent experiments; n = 50 MBRs per experiment. Paired two-sided Student’s t tests are shown. See also Table S1.
EDTA-sensitive factors and BST2 for tethering MBRs to the cell surface (Figure 4B). Despite the short recording due to cell detachment (3 h), approximately 50% of the GFP-MKLP1-positive MBRs were released upon BST2 KO when combined with EDTA treatment. Note that intracellular (internalized) GFP-MBRs cannot be released but are still fluorescent before reaching degradative compartments.9,11 Thus, the percentage of MBRs present at the cell surface and that were released is actually underestimated in this assay. Altogether, our results indicate that BST2 is a critical tether for MBRs that cooperates with yet to be discovered, EDTA-sensitive factors.

BST2 localization at the MBR is required for promoting MBR attachment

We next observed that a homodimerization mutant of BST2 lacking the three specific cysteines that stabilize the coiled-coil domain (BST2 C3A)37-39 failed to localize at the MBR (Figures 4C, S3C, and S3D). Remarkably, although this mutant was expressed at the cell surface as much as BST2 WT (Figure S3C), BST2 C3A also failed to retain MBRs at the cell surface (Figure 4D), as observed for virions.37 This shows the requirement of BST2 homodimerization for both its recruitment to the midbody and its tethering activity.

Experiments using BST2 KO cells (Figures 2 and 3) could not discriminate whether BST2 was required at the cell surface and/or at the MBRs to retain them, because it was depleted from both locations. To answer this question, we purified, using flow cytometry as described in Addi et al.,35 MBRs from donor GFP-MKLP1 HeLa cells expressing BST2 (CTRL KO) or not (BST2 KO). After the incubation step, we monitored the retention of purified MBRs at the surface of non-fluorescent, recipient HeLa cells that expressed or not BST2 (Figures 4E and 4F). In each combination, the same number of MBRs was incubated with the same number of recipient cells. We then quantified the release of these newly attached, GFP-labeled MBRs from the cell surface of recipient cells by time-lapse microscopy (Figure 4F). Interestingly, deposited BST2-positive MBRs were retained as efficiently by recipient cells, whether the cells expressed or not BST2 at their surface. In contrast, MBRs lacking BST2 were more often released when interacting with BST2 KO cells, consistent with the data described above (Figures 2 and 3). Importantly, this increased release was not affected by BST2 expression on the recipient cell (recipient CTRL KO; Figure 4F). We thus conclude that the localization of BST2 at the MBR membrane is necessary and sufficient to promote MBR attachment to the cell surface.

Here, we have identified the transmembrane protein BST2/tetherin as a key factor that contributes to retain post-cytokinetic MBRs at the plasma membrane. In BST2 KO cells, the frequency of MBR detachment from the cell surface increases and MBRs accumulate in the extracellular medium and are more often transferred to distant cells (Figures 2 and 3). Strikingly, this mirrors what happens for virions, when enveloped viruses are able to counteract BST2 restriction,17-20 and we thus propose that BST2 also acts as a protein that tethers midbody remnants to the plasma membrane. Recently, BST2 was reported to tether exosomes to the cell surface after inhibition of the V-ATPase.44 To our knowledge, our data provide the first functional evidence of a BST2 tethering activity in unperturbed and uninfected cells. How does BST2 tether MBRs to the cell surface? For virions, a few molecules of BST2 can physically tether these relatively small (50–100 nm) particles by spanning the virion-cell scission site (Figure 1A).19 We cannot exclude that BST2 at the cell surface also participates in the tethering of MBRs, but testing this possibility is technically challenging, as this would require to selectively inhibit the localization of BST2 at the MBR, but not at the cell surface. However, tethering between free MBRs and the plasma membrane was observed and requires that BST2 localizes at the MBR side, but not at the interacting cell side (Figures 4E and 4F). As for viral and exosomal tethering,44 BST2 tethering absolutely depends on BST2’s terminal GPI anchor because BST2 delGPI (re-expressed at the MBR as much as BST2 WT; Figure S3B) failed to rescue MBR tethering (Figures 3G and 3H). We thus propose that the GPI moiety, initially inserted in the outer leaflet of the MBR’s membrane, can flip and insert into the plasma membrane of the facing recipient cell once both membranes are close enough (Figure 4G, left). Such transfer of a GPI moiety between membranes has been
observed for a variety of GPI-anchored proteins, both in vivo and in vitro.57,58 At the surface of HIV-1-infected cells, BST2 is incorporated into HIV-1 particles as a parallel homodimer with its GPI anchor preferentially inserted into the virion. However, in order to tether several virions together, the transmembrane domain can also be inserted in the virions.59 Although the final topology is similar for virion and MBR tethering (with the transmembrane domain and GPI anchor in opposite membranes: Figure 3E), the mechanism proposed for MBR tethering by BST2 is original. This unusual tethering might reflect the fact that, contrary to vi- rions, MBRs are, first, large particles (typically 2,000 × 1,500 nm) and, second, highly enriched for BST2 molecules (Figure 1). Besides this direct MBR tethering activity via BST2, it is possible that BST2, at the MBR and/or at the cell surface, interacts with partners or locally organizes membrane microdomains (see below),59 which also contribute to the MBR-cell attachment.

The tethering activity of BST2 raises the question of how it is localized to the MBR. BST2 localization at or close to the viral budding site depends on its GPI anchor where it interacts with cholesterol and PtdIns(4,5)P2-rich lipid domains.54,60 We observed that the GPI moiety is also an important determinant for BST2 localization at the MBR: although expressed more than BST2 WT at the cell surface, BST2 delGPI localized proportionally less than BST2 WT to the midbody/MBR (Figures S3A and S3B). Interestingly, quantitative lipidomics previously demonstrated that midbodies have a very peculiar, raft-like composition rich in cholesterol, PtdIns(4,5)P2, and sphingoli- pids.61 This suggests that the determinants of BST2 localization at viral budding sites and at MBRs are highly dependent on the local lipid composition of the membrane.

BST2 is the first protein whose depletion impairs MBR retention at the cell surface. However, not all MBRs are released in the absence of BST2. This is also true for exosomes: a fraction of exosomes is still retained at the cell surface upon BST2 KO.54 Thus, multiple, redundant molecular machineries appear to tether MBRs to the cell surface. At the molecular level, the complete release of an MBR implies the simultaneous detachment of all the links between this organelle and the plasma membrane. Interestingly, our data highlight that BST2 synergizes with EDTA-sensitive factors to retain MBRs at the cell surface (Figures 4B and 4G, right). Identifying the Ca2+/Mg2+-dependent factors (lipids or proteins) will be an important next step.

MBRs contain information that promotes cell proliferation once captured and internalized in cultured cells.14 We noticed that knocking out BST2 was not sufficient to significantly decrease progression in the cell cycle (Figure S2C), expression of the proliferation marker Ki67 (Figure S4E), or long-term proliferation in clonogenic assays (Figure S4F). This could be due to the fact that not all MBRs are released upon BST2 KO (Figure 2). Of note, we observed that MBRs tend to accumulate intracel- lularly once internalized in BST2 KO cells (Figure S4G), indicating a role for BST2 in the turnover/degradation of MBRs beyond tethering. Interestingly, the intracellular MBRs were more often present in Lamp1-negative (Lamp1−) compartments (Figure S4G, right), where they have been recently proposed to signal for prolif- eration.14 This could also explain why, despite the increase in MBR release from cells (Figures 2C and 2D), BST2 KO does not impact on overall cell proliferation. The physiological relevance of MBR tethering by BST2 thus remains to be explored, in particular in vivo. Because BST2 KO mice are viable,52 we suspect that putative signaling functions of MBRs in mammals would become apparent when BST2 and the Ca2+/Mg2+-dependent factors are simultaneously eliminated. Alternatively, the function of BST2-dependent MBR tethering might be important only in specific cell types (such as during the development of the brain, where MBRs accumulate at specific stages)5 or in pathological conditions (such as in tumor cells, where BST2 is frequently found upregulated).52,62

Altogether, we identified a striking common function of BST2 in retaining virions and MBRs at the plasma membrane. In both cases, BST2/tetherin limits their release into the extracellular medium and their spread to distant cells. Thus, beyond the ESCRT-dependent membrane scission events, our work reveals a novel and unexpected parallel between viral biology and cytokinesis.

STARS METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.02.039.

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DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit polyclonal anti-BST2 | Proteintech | Cat# 13560-1-AP; RRID: AB_2067220 |
| Rabbit polyclonal anti-integrin alpha V | Proteintech | Cat# 27096-1-AP; RRID: AB_2880753 |
| Mouse monoclonal anti-Cep55 | Santa Cruz Biotechnology | Cat# sc-374051; RRID: AB_10917564 |
| Mouse monoclonal anti-CRIK | BD Biosciences | Cat# 611376; RRID: AB_398898 |
| Mouse monoclonal anti-CD81 | Kind gift from Dr. E. Rubinstein | N/A |
| Mouse monoclonal anti-CD9P1 | Kind gift from Dr. E. Rubinstein | N/A |
| Rabbit polyclonal anti-Lamp1 | Abcam | Cat# ab24170; RRID: AB_775978 |
| Chicken polyclonal anti-GFP | Abcam | Cat# ab13970; RRID: AB_300798 |
| Human monoclonal anti-acetylated tubulin | Institut Curie, Paris France | Cat# A-R-H#39 |
| Mouse monoclonal anti-GAPDH | Proteintech | Cat# 60004-1-lg; RRID: AB_2107436 |
| APC mouse monoclonal isotype control | BioLegend | Cat# 400121; RRID: AB_326443 |
| APC human monoclonal anti-BST2 | BioLegend | Cat# 348410; RRID: AB_2067121 |
| Donkey anti-mouse IgG CY3 | Jackson ImmunoResearch | Cat#715-165-151; RRID: AB_2315777 |
| Donkey anti-mouse IgG Alexa647 | Jackson ImmunoResearch | Cat#715-605-151; RRID: AB_2340863 |
| Donkey anti-rabbit IgG CY3 | Jackson ImmunoResearch | Cat#711-165-152; RRID: AB_2307443 |
| Donkey anti-rabbit IgG Alexa 647 | Jackson ImmunoResearch | Cat#711-605-152; RRID: AB_2492288 |
| Bacterial and virus strains |
| E. coli DH5α | GIBCO | Cat# 18265017 |
| Chemicals, peptides, and recombinant proteins |
| Human TNFα | Thermofisher | Cat# PHC3015 |
| 123count ebeads | invitrogen | Cat# 15526296 |
| RADfV control peptide | Enzo life Sciences | Cat# BML-AM101 |
| RGDfV | Enzo life Sciences | Cat# BML-AM100 |
| SiR-tubulin | Tebu-bio | Cat# SC002 |
| DAPI | Serva | Cat# 18860.01 |
| Mowiol | Calbiochem | Cat# 475904 |
| X-tremGENE 9 | Sigma | Cat# 06365809001 |
| Lipofectamine | Invitrogen | Cat# 18324012 |
| Puromycin | GIBCO | Cat# 711138-03 |
| Zeocin | ThermoFisher | Cat# R25001 |
| PFA | Electron microscopy sciences | Cat# 15714 |
| BSA | Sigma | Cat# A7030 |
| NP-40 / Igepal | Sigma | Cat# CA-630 |
| HEPES | GIBCO | Cat# 15630-056 |
| Triton X-100 | Merck | Cat# 108643 |
| Tween-20 | Sigma | Cat# P1379 |
| Critical commercial assays |
| PNGase F | New England Biolabs | Cat# 0704S |
| ExcelLenti LTX Lentivirus Packaging Mix | Oxford genetics | Cat# EXL10 |
| TOPO 2.1 TA cloning kit | Thermofisher | Cat# 45-0641 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Arnaud Echard (arnaud.echard@pasteur.fr).

Materials availability
Plasmids and cell lines generated in this study can be obtained through the Lead Contact.

Data and code availability
This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and cell culture
GFP-MKLP1 HeLa and parental HeLa (female), HEK293 (gender not provided by ATCC), HEK293FT (female), SK-MEL2 Dynamin-GFP (male) and HepG2 (male) cell lines were grown in Dulbecco’s Modified Eagle Medium (DMEM) GlutaMax (#31966; GIBCO, Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (Pan biotech) and 1X Penicillin-Streptomycin (GIBCO). Caco-2 cells were supplemented with 1X Non-essential amino acids mix (GIBCO). GFP-MKLP1 cells were kept under G418 (40 μg/mL, GIBCO) selection and GFP-MKLP1 BST2 KO expressing one of the different BST2 constructs were kept under G418 and zeocin (50 μg/mL, Invitrogen) selection. Human TNFα (Thermofisher, #PHC3015) was diluted at 20 μg/mL in the cell culture medium for 30 min. GFP-MKLP1 HeLa ITGαV KO cells were cultured on collagen-coated dishes (Corning, #354236). All cell lines

Reagent or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Experimental models: cell lines | | |
| Human: HeLa cells | ATCC | CCL-2 |
| Human: HeLa cells | Kyoto, Kind gift from Dr. M.Piel | N/A |
| Human: GFP-MKLP1 HeLa cells | 45 | N/A |
| Human: HEK293 | ATCC | CRL-1573 |
| Human: HEK293FT | Thermofisher | Cat# R70007 |
| Human: SK-MEL2 Dynamin-GFP | Kind gift from Dr. N. Sauvonnet | 64 N/A |
| Human: HepG2 | 65 | N/A |
| Human: Caco-2 | Kind gift from Dr. M. Lecuit | HTB-37 |
| Human: HUVEC | Kind gift from Dr. E. Lemichez | N/A |
| Human: primary T lymphocytes | Kind gift from Dr. N. Casartelli and Dr. O. Schwartz | N/A |

Oligonucleotides

| N/A | See Table S1 for oligonucleotides | N/A |

Recombinant DNA

| BST2 WT pcDNA3.1 | This paper | N/A |
| BST2 GFP | This paper | N/A |
| pLenti4to pDEST V5 Nter | Thermofisher | Cat# V49810 |
| pSpCas9(BB)-2A-Puro (PX459) V2.0 | Addgene Plasmid # 62988 |

Software and algorithms

| Fiji (ImageJ) | ImageJ | https://imagej.nih.gov/ij/ |
| Icy | Icy | http://icy.bioimageanalysis.org/ |
| Graphpad Prism | Graphpad | https://www.graphpad.com/scientific-software/prism/ |
| Metamorph | Molecular Devices | https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy |
| Flowjo | Flowjo | https://www.flowjo.com/ |
were grown in 5% CO₂ / 37°C, and routinely tested for mycoplasma, maintained at sub-confluent densities and in low passage number. Primary HUVEC (female), primary T lymphocytes (gender not provided) and Caco-2 (male) were fixed and processed for immunofluorescence.

**METHOD DETAILS**

**Plasmids, cloning and CRISPR/Cas9 KO clone testing**

The human BST2 coding sequence was cloned into a pcDNA3 plasmid. The kozak sequence of the plasmid was used to express BST2. BST2-GFP was generated as described in Kupzig et al. Briefly, the GFP coding sequence was amplified with PstI restriction sites at 5’ and 3’ and ligated into the PstI site of BST2.

The plasmid pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene) was a gift from Feng Zhang; [http://addgene.org/62988](http://addgene.org/62988); RRID: Addgene_62988. In which we inserted the CTRL (5’-AAGATGAAAAGGAAGCGTT-3’), BST2 (5’- GCCGGACGGCTTCCGGCA-3’) or Integrin ITGαV (5’-CACCGGTGACTGGTCTTCTACCCGC-3’) KO guide using the BbsI restriction sites. The primer pair 5’-GCCCGTAGAAGATTCCAGCA-3’ and 5’-TGTTCAAGCGAAAAGCCAG-3’ primers were used to amplify the RT product, which was migrated on agarose gel and extracted for sequencing.

**BST2 lentiviral constructions for transduction**

The lentiviral expression plasmids encoding for the different BST2 mutants were made as follows: all point mutations have been generated from the pcDNA3 BST2 plasmid using NEBaseChanger (NEB) and appropriate primers (Table S1), including BST2 C3A (C53A, C63A, C91A), BST2 Y2A (Y6A, Y8A), BST2 delGPI (S161STOP). BST2 mutants were amplified by PCR and introduced into pENTR gateway vectors, then recombined into the pLenti/V5 destination vector (ThermoFisher scientific). V5 tag is not expressed as the BST2 STOP codon was conserved.

**Stable cell lines**

**BST2 KO cell line using CRISPR/Cas9 technology**

CTRL KO and BST2 KO HeLa cells expressing GFP-MKL1 and non-fluorescent HeLa Kyoto cells were created using the plasmid pSpCas9(BB)-2A-Puro with the appropriate guides as described above. Cells were selected with 1.5 μg/mL puromycin and clones were isolated and tested for KO using genomic PCR and RT-PCR, followed by sequencing.

**ITGαV KO cell lines using CRISPR/Cas9 technology**

Either CTRL KO or BST2 KO HeLa cells expressing GFP-MKL1 (see above) were edited using the plasmid pSpCas9(BB)-2A-Puro with either CTRL or ITGαV guides. Cells were selected with 1.5 μg/mL puromycin for 4 days, and sorted by FACS after surface immunostaining with a rabbit anti-ITGαV antibody (1:100; Proteintech Group 27096-1-AP) coupled with a fluorescent secondary antibody.

**BST2 mutant cell lines using lentiviral transduction**

Lentiviral particles were produced in the HEK293 FT packaging cells using standard methods. Briefly, cells were co-transfected with the different pLenti-BST2 constructs and the Excelenti LTX Lentivirus Packaging mix (Oxford Genetics) using lipofectamine as per manufacturer’s protocol. After 48 h, the HEK293 FT culture supernatants were added to BST2 KO GFP-MKL1 HeLa cells for 24 h. Cells were selected for stable expression with 100 μg/mL zeocin and BST2-positive cells were then sorted by FACS after immunostaining with the APC-coupled anti-BST2 antibody (#BLE388410, OZYME).

**Flow cytometry samples**

**MBR purification**

MBRs were purified as previously described for GFP-MKL2 HeLa cells in Addi et al. Briefly, MBRs were detached from HeLa GFP-MKL1 cells with a 2 mM EDTA-treatment. The supernatant from the 70 g centrifugation was collected and sorting of MBRs was performed on a BD Biosciences FACs ARIA III. The Threshold based on FSC was set at 250, and Neutral Density filter 1.0 has been used to detect small particles. MBRs were gated on a pseudo-color plot looking at GFP versus SSC-A parameters, both in log scales (Figure S1B).

**BST2 level measurements**

GFP-MKL1 HeLa cells grown to sub-confluency were stained on ice with an APC-coupled isotype control (1:200; BioLegend #BLE400121) or the anti-BST2 (1:100; BioLegend#BLE348410) antibody for 20 min. Then cells and MBRs were detached with 2 mM EDTA-treatment and the sample was processed on a BD Biosciences FACs ARIA III. Cells were acquired using Neutral Density filter 2.0 and the MBRs acquired using Neutral Density filter 1.0.

**Quantification of MBRs in cell culture media**

Culture media from CTRL KO and BST2 KO GFP-MKL1 cells grown for three days was harvested and a fraction of the sample was mixed with a known quantity of fluorescent beads (123count ebeads, Invitrogen #15526296). We acquired simultaneously fluorescent beads, calibrated at a known concentration, and MBRs from the same sample media and then extrapolated the total number of MBRs in the whole culture medium. Each condition was normalized to the number of cells.
**Cell cycle experiment and Ki67 labeling**

Non-confluent GFP-MKLP1 HeLa cells were EDTA-detached, washed twice with ice-cold PBS and fixed 30 min with ice-cold 80% Ethanol. 10^6 cells were washed with PBS and permeabilized in 0.2% Triton. Fixed cells were then washed and saturated for 5 min with 1% FCS. Cells were incubated with 1 μg/mL DAPI (Serva) and anti-Ki67 coupled with APC (or matched APC isotype) in 1% FCS/ PBS for 30 min. Cell cycle and Ki67 fluorescence were acquired with the CytExpert 2.4.0.028 on a CytoFLEX S (Beckman Coulter, France). DAPI signal was measured using a 405-450/45nm filter. FSC/SSC parameters were used for cell size and doublets excluded from the analysis. 10,000 singlets were recorded for each condition and analyzed with FlowJo.

**Spinoculation of MBRs**

**On glass coverslips**

Purified GFP+ MBRs isolated by flow cytometry were spinoculated at 1200 g for 45 min onto a poly-lysine coated coverslip and then processed for immunofluorescence.

**On cells**

Purified GFP+ MBRs isolated by flow cytometry were spinoculated at 1200 g for 15 min onto cells in DMEM + HEPES 25mM (GIBCO) at 37°C/ 5% CO₂. The medium was washed with fresh medium to remove MBRs that did not attach properly, and cells were then acquired with time-lapse imaging.

**Co-culture experiments**

WT, non-fluorescent HeLa cells were incubated with either CTRL KO or BST2 KO GFP-MKLP1 HeLa cells, in a 1:1 ratio. Cells were grown for 48 hours, then fixed and processed for IF. The percentage of transfected MBRs (number of GFP-MKLP1 MBRs transferred onto non-fluorescent HeLa cells divided by the total number of GFP-MKLP1 MBRs) was quantified.

**Immunofluorescence and image acquisition**

Cells were grown on coverslips and then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 3 min, blocked with 0.2% BSA/PBS for 20 min and successively incubated for 1 h at room temperature with primary and secondary antibodies diluted in PBS containing 0.2% BSA.

For surface staining, live cells were saturated with 0.2% BSA/PBS for 10 min on ice, then incubated with primary antibodies (anti-CD81 or anti-CD9P1) diluted in 0.2% BSA/PBS for 30 min on ice. Cells were fixed with 4% PFA for 10 min at room temperature, and either directly incubated with secondary antibodies diluted in PBS containing 0.2% BSA, or permeabilized with 0.1% Triton X-100 for 3 min, saturated with 0.2% BSA/PBS for 5 min and successively incubated for 1 h at room temperature with primary (anti-GFP and/or anti-Lamp1) and secondary antibodies diluted in PBS with 0.2% BSA.

**Cell adhesion assay**

Briefly, detached cells were incubated 30 min on ice with 40 μM RADfV or RGDfV peptides, and then seeded on fibronectin-coated coverslips for 15 min to allow them to adhere. The cells were then fixed with 4% PFA, permeabilized with 0.1% Triton X-100 for 3 min, stained with DAPI and mounted in Mowiol.

**Time-lapse microscopy**

For the MBR release experiments, HeLa cells were plated on glass bottom 12-well plates (MatTek) or on collagen-coated 96-well plates for the ITGα7KO experiment (Greiner), and put in an open chamber (Life Imaging) equilibrated to 5% CO₂ and maintained at 37°C. For the long-term imaging (Figures 3A–3C, 3G, 3H, 4D–4F, and 4C), time-lapse sequences were recorded every 15 min for 48 h using a Nikon Eclipse TiE inverted microscope with a x20 objective lens 0.45 NA Plan Fluor ELWD controlled by Metamorph software (Universal Imaging). The midbody of a mitotic cell was followed from its abscission until it was degraded, released or until the time-lapse ended. For BST2-GFP time-lapse fluorescent microscopy, images were acquired with an inverted Eclipse TiE Nikon microscope equipped with a CSU-X1 spinning disk confocal scanning unit (Yokogawa) and with an EMCCD Camera (Evolve 512 Delta, Photometrics). MBRs present at t0 were followed until they were degraded, released or until the time-lapse ended.

For EDTA treatment (Figure 4B), the medium was replaced with either DMEM, DMEM + 40 μM RADfV or DMEM + 40 μM RGDfV, and time-lapse sequences were recorded every 15 min for 6 h, using an inverted Eclipse TiE Nikon microscope equipped with a CSU-X1 spinning disk confocal scanning unit (Yokogawa) and with an EMCCD Camera (Evolve 512 Delta, Photometrics). MBRs present at t0 were followed until they were degraded, released or until the time-lapse ended.
followed until they were degraded, released or until the time-lapse ended. Midbodies in intercellular bridges were excluded from the analysis using a tubulin staining, Sir-tubulin (Tebu-bio, #SC002).

**SIM microscopy**
SIM was performed on a Zeiss LSM 780 Elyra PS1 microscope (Carl Zeiss, Germany) using C Plan-Apochromat 63 x /1.4 oil objective with a 1.518 refractive index oil (Carl Zeiss). The fluorescence signal was detected on an EMCCD Andor Ixon 887 1K. Raw images were composed of fifteen images per plane per channel (five phases, three angles), and acquired with a Z-distance of 0.091 μm. SIM images were corrected for chromatic aberration using 100-nm TetraSpeck microspheres (ThermoFisher Scientific) embedded in the same mounting media as the sample. The SIMcheck plugin in ImageJ was used to analyze the quality of the acquisition and the processing in order to optimize parameters for resolution, signal-to-noise ratio, and reconstruction pattern.

**Western blots**
Western blots were carried out as follows: cells were lysed in NP-40 extract buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40) containing protease inhibitors. 20 μg of lysate was migrated in 4%–15% gradient SDS–PAGE gels (BioRad Laboratories), transferred onto PVDF membranes (Millipore) and incubated with indicated primary antibodies in PBS, 1% low-fat milk and 0.1% Tween20. The membranes were incubated with HRP-coupled secondary antibodies (1:10,000, Jackson ImmunoResearch) and revealed by chemiluminescence (GE Healthcare). For western blots against deglycosylated BST2, the cell extracts were deglycosylated with PNGase F (New England Biolabs, #P0704S) for 3 h at room temperature before processing with the SDS-PAGE.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
All values are displayed as mean ± SD (standard deviation) for at least three independent experiments (as indicated in the figure legends). Significance was calculated using paired, two-sided t tests or ANOVA tests, as indicated. In all statistical tests p > 0.05 was considered as non-significant. p values are indicated in each individual graph.