Effect of Calcium Modulating Cyclophilin Ligand (CAML) on HIV-1 particle release and Tetherin cell-surface expression

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Running Title: Effect of CAML on HIV-1 particle release

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Abstract

The HIV-1 accessory protein Vpu enhances virus particle release by counteracting a host factor that retains virions at the cell surface of infected cells. It was recently demonstrated that cellular protein BST2/ CD317/Tetherin restricts HIV-1 release in a Vpu-dependent manner. CAML was also proposed to be involved in this process. We investigated whether CAML is involved in Tetherin cell-surface expression. Here, we show that CAML over-expression in permissive Cos-7 cells or CAML depletion in restrictive HeLa cells has no effect on HIV-1 release nor on Tetherin surface expression, indicating that CAML is not required for Tetherin-mediated restriction of HIV-1 release.

Human immunodeficiency virus type-1 (HIV-1) viral protein U (Vpu) is an accessory protein expressed late during the HIV-1 life cycle (2, 15). Vpu enhances virus particle release in a cell-type dependent manner (6, 11, 14, 16). Some model cell lines (including 293T, Cos-7 and HT1080) are capable of supporting HIV-1 particle release independently of Vpu expression (permissive cell lines). In contrast, in other cells (such as HeLa cells, monocyte-derived macrophages, and primary CD4+ T lymphocytes) efficient HIV-1 release requires expression of Vpu (restrictive cells).

It was recently demonstrated that the interferon (IFN)-regulated cellular protein Tetherin (BST-2, HM1.24, and CD317) is able to restrict HIV-1 particle release by retaining virions at the cell-surface of infected cells (13, 19). Evidence supporting a role of Tetherin in HIV-1 particle release restriction is well documented. Tetherin is constitutively expressed in restrictive HeLa cells but not in permissive 293T and HT1080 cells (13, 19). Expression level of Tetherin can be induced by IFN-α treatment of 293T and HT1080 cells and enhanced in Jurkat and primary CD4+ T cells (13). Moreover, over-expression of Tetherin into 293T and HT1080 cells inhibits the release of Vpu-defective HIV-1 particle without affecting Gag expression or processing (13). Finally, HeLa cells are able to efficiently release HIV-1 particles in absence of Vpu if Tetherin is depleted (13, 19).

By the same time Tetherin was identified as a HIV-1 host restriction factor, another protein, Calcium Modulating Cyclophilin Ligand (CAML), was linked to Vpu-regulated HIV-1 particle release (20). CAML is a highly conserved and

ubiquitously expressed protein that is essential for cell viability (8, 17). Initial functional studies demonstrated that CAML over-expression in Jurkat T cells causes a rise in intracellular calcium, followed by NF-AT transcription factor activation (1, 7). Later work linked CAML with the intracellular trafficking of diverse receptors and signaling proteins (17, 18, 21, 22). Yet, several properties of CAML do not fulfill the criteria expected for the putative HIV-1 tethering protein. Mainly, CAML is essential for cellular viability, is ubiquitously expressed, and is not IFN-inducible (1, 8). In an attempt to reconcile these discrepancies, we sought to investigate whether CAML is involved in regulating Tetherin cell-surface expression, thus explaining its proposed role in restricting HIV-1 particle release.

Over-expression of human CAML in permissive Cos-7 cells. We started by testing the effect of human CAML (hCAML) on Tetherin cell-surface expression in Cos-7 cells. The restrictive effect of CAML on HIV-1 particle release was previously revealed by over-expressing hCAML in HIV-1- or Gag virus-like-particle (VLP)-producing African green monkey (Agm) Cos-7 cells in presence or absence of Vpu (20). The hCAML open reading frame (ORF) was amplified by PCR from 293T cell line DNA and cloned into pCMV-HA plasmid (Clontech) to generate pCMV-HA-hCAML. The sequence of HA tagged-hCAML and the integrity of the expression plasmid were confirmed by automated sequencing. A complete analysis of HIV-1 particle release and Tetherin cell-surface expression was performed in Cos-7 cells after transfection of HxBH10*vpu_wt* or HxBH10*vpu*- provirus and increasing concentrations of pCMV-HA-hCAML.

HxBH10*vpu_wt* and HxBH10*vpu-* are two infectious HIV-1 molecular clones that are isogenic except for the expression of Vpu (16). As a positive control, transfected cells were treated with 10,000U/ml of human IFN-α, since IFN-α was previously shown to induce a restriction of HIV-1 release in Cos-7 cells (12). Cells and virus-containing supernatants were collected 48hr post-transfection, as previously described (5). Protein lysates were analyzed by western blotting to detect steady-state levels of HA-tagged CAML and viral products (processed Gag forms and Vpu) in the case of cell lysates, or p24 in the case of virus lysates. HIV-1 release efficiency was evaluated by determining the ratio of virion-associated p24 signal over the total intracellular Gag signal measured by scanning densitometry of western blots. These results were confirmed by measuring the levels of released infectious virus using HeLa-TZM indicator cells (AIDS Research and Reference Program, NIH). A second fraction of the transfected Cos-7 cells was stained for cell-surface Tetherin and analyzed by flow cytometry as described elsewhere (10).

We were unable to detect an effect of hCAML on Vpu-defective HIV-1 particle release either by determining the ratio between virion- and cell-associated Gag signals by western blot or by infectious particle release (Fig. 1A, compare lanes 7 and 2; quantified in Fig. 1B). Indeed, the release efficiency of the Vpu+ and Vpu- defective viruses was comparable in presence or absence of hCAML (Fig.1A, compare lanes 7 and 8 with lanes 2 and 3; quantified in Fig.1B). As previously reported (12), IFN-α–treated Cos-7 cells restricted HIV-1 release independently of Vpu expression (Fig. 1A, compare lanes 2 and 3 with lanes 4 and 5; quantified

in Fig.1B). Similar results were obtained when increasing amounts of pCMV-HA-hCAML were transfected (Fig. 1C). Interestingly, HA-hCAML over-expression resulted in the detection of lower levels of Gag products and Vpu proteins in a dose-dependent manner (Fig. 1C).

Consistent with their permissive phenotype, Cos-7 cells did not express Tetherin at their cell-surface (Fig. 2A, solid line). Importantly, no significant change in the expression of the restriction factor was detected in Cos-7 cells transfected with pCMV-HA-hCAML alone or together with Vpu+ or Vpu-defective proviruses (Fig. 2A, 2B, and 2C, compare dashed and solid line). In contrast, IFN-α treatment of Cos-7 cells significantly up-regulated agm Tetherin cell-surface expression (Fig. 2A, dotted line); however, this up-regulation was not significantly affected by the presence of Vpu (compare Fig. 2B and 2C, dotted line).

Depletion of CAML in restrictive HeLa cells. It was previously shown that siRNA-mediated depletion of endogenous CAML relieved the Vpu-responsive block to Gag or Vpu-defective HIV-1 particle release in HeLa cells (20). Using RNAi technology, we tested whether CAML down-regulation affected basal cell-surface Tetherin expression levels and had an impact on HIV-1 virus particle release. HeLa cells were transfected with siRNA specific to CAML or non-targeting siRNA following previously described methods (4). As a control, endogenous Tetherin was depleted by siRNA. Twenty four hours post siRNA transfection, cells were transfected with either HxBH10*vpu_wt* or HxBH10*vpu*- proviruses. Cells and virus-containing supernatants were collected and analyzed

as described previously. An additional fraction of transfected cells was collected for RNA extraction. Depletion of CAML was evaluated by RT-PCR analysis of endogenous CAML mRNA. As shown in figure 3A, endogenous CAML mRNA levels were reduced significantly in presence of the CAML siRNAs while control β-actin mRNA levels remained constant. Despite several attempts, we were unable to achieve complete siRNA-directed depletion of CAML mRNA. In this regard, it was recently reported that CAML-depleted cells are not viable (8, 17).

Depletion of CAML did not rescue the release of HIV-1 particle in HeLa cells in absence of Vpu as indicated by the very low levels of mature p24 only detectable in the supernatant of cells producing HxBH10*vpu*- virus particles upon prolonged exposure (Fig. 3A, compare lanes 3 and 6). In contrast, the presence of Vpu overcame the block to HIV-1 particle release in presence of CAML siRNAs or non-targeting siRNAs (Fig. 3A, lanes 2 and 5). Quantification of virus particle release efficiency evaluated by western blot quantification or released infectious virus (Fig. 3B), both confirmed that depletion of CAML did not rescue HIV-1 particle release in HeLa cells in absence of Vpu. Furthermore, as shown in figure 3C, depletion of CAML did not affect Tetherin levels at the cell-surface. In contrast, as previously reported (13, 19), depletion of Tetherin did indeed rescue HIV-1 particle release in absence of Vpu, as indicated by the similar amounts of virion-associated p24 detected in the supernatant of HeLa cells producing HxBH10*vpu_wt* or HxBH10*vpu*- (Fig. 4A compare lanes 5 and 2 as well as lanes 5 and 6; quantified in Fig. 4B).

Discrepancies were noted between non-targeting and CAML-specific siRNAs. Levels of viral products were significantly increased when CAML expression was reduced (Fig. 3A, compare lanes 2-3 to lanes 5-6). It is unlikely that this effect was due to non-targeting siRNAs, since this effect was not observed when these same non-targeting siRNAs were tested against Tetherin-specific siRNAs (Fig. 4A, compare lanes 2-3 to lanes 5-6). To control for the amounts of total protein and gel loading, β-actin was probed in the same membrane used to detect Gag products in cells. No significant difference was noted in the β-actin control to account for the difference in viral products expression (Fig. 3A).

Concluding remarks. Over-expression of hCAML in permissive Cos-7 cells did not modulate Tetherin cell-surface expression and was not sufficient to generate a HIV-1 restrictive phenotype in these cells. Furthermore, significant depletion of CAML in restrictive HeLa cells did not affect Tetherin cell-surface expression and did not overcome the requirement of Vpu for HIV-1 particle release. In contrast, depletion of Tetherin in HeLa cells restored the release of Vpu-defective HIV-1 particle to levels comparable to those observed in presence of wild type Vpu. Overall, we conclude that Tetherin restricts HIV-1 particle release and does not require CAML. Furthermore, these results do not support an important function of CAML in HIV-1 particle release.

Interestingly, CAML over-expression resulted in lower levels of Gag products and Vpu proteins, while in CAML depleted cells, levels of viral proteins were found to be significantly higher. It was reported that CAML over-expression can affect the

NF-AT transcription factor pathway (1, 7) a pathway that was also reported to be important in modulating HIV-1 LTR promoter activation (3, 9). It is therefore possible that the effect observed in the previous report linking CAML to HIV-1 particle release restriction (20) is not connected to Tetherin cell-surface expression modulation, but rather with an earlier event that affect HIV-1 production, such as LTR promoter activity modulation.

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Figure legends

Figure 1: Effect of CAML over-expression on HIV-1 particle release in permissive Cos-7 cells. (A-B) Cos-7 cells were seeded on 6-well plates and transfected using Lipofectamine 2000 (Invitrogen), following manufacturer recommendations. Triplicate wells received fixed amounts of HxBH10*vpu_wt* or HxBH10*vpu*- proviral DNA (2 µg) and pCMV-HA-hCAML (1 µg) and completed to the same amount of final total DNA with pCMV-HA empty vector. **A-** Western blot for Cos-7 cells transfected with either the HxBH10*vpu*- (lanes 2, 4 and 7) or the HxBH10*vpu_wt* (lanes 3, 5 and 8) proviral DNA plasmids or mock transfected (labeled as m; lanes 1 and 6). Samples from lanes 4 and 5 were treated with 10,000U/ml of IFN-α, while samples from lanes 6 to 8 were co-transfected with the human HA-tagged CAML-expressing plasmid. Cells and supernatant-containing viral particles were harvested 48 h post-transfection and lysates analyzed by western blotting to detect steady-state levels of target proteins. Cell lysates were analyzed to detect HA-tagged CAML (using anti-HA antibodies) as well as Gag products, Vpu and cellular actin using specific antibodies. Virus lysates were analyzed for the presence of p24 using specific antibodies. **B-** Quantification of virus particle release efficiency. Upper panel, densitometric quantification of HIV-1 particle release efficiency in presence of hCAML or upon IFN-α-treatment was evaluated by determining the ratio between virion-associated Gag signal (mature p24) and all cell-associated Gag signals (p24/25, and precursors p55 and p41) by western blot. Bands corresponding to all Gag products in cells and virus particles were scanned by laser densitometry and

quantified using ImageQuant 5.0. Lower panel, levels of released infectious virus using HeLa-TZM indicator cells. HeLa-TZM indicator cells (NIH, AIDS Research and Reference Program) were inoculated with an aliquot of virus-containing supernatant. After 48h, cells were lysed and luciferase activity was determined using the Promega Luciferase Assay System. For both panels, the release efficiency of HxBH10*vpu_wt* was arbitrarily set at 100%. Error bars indicate the standard deviations of the means of the results from two independent experiments. **C-** Effect of increasing amounts of HA-hCAML on HIV-1 particle release. Cos-7 cells were seeded on 6-well plates and transfected as in A. The cells received fixed amounts of HxBH10*vpu_wt* or HxBH10*vpu*- proviral DNA (2 µg) and increasing amounts of pCMV-HA-hCAML (from 2 to 8µg) and completed to the same amount of final total DNA with pCMV-HA empty vector. Cells and virus-containing supernatants were harvested 48 h post-transfection and lysates analyzed by western blotting to detect Gag products and Vpu using specific antibodies as indicated. Western blot for Cos-7 cells transfected with either the HxBH10*vpu*- (lanes 2, 4, 6, 8 and 10) or the HxBH10*vpu_wt* (lanes 1, 3, 5, 7 and 9) proviral DNA plasmids. Samples from lanes 3 to 10 were co-transfected with the human HA-tagged CAML-expressing plasmid.

Figure 2: Effect of CAML over-expression on Tetherin cell-surface expression in permissive Cos-7 cells. (A-C) Cos-7 cells were mock-transfected (A) or transfected with HxBH10*vpu_wt* (B) or HxBH10*vpu*- (C) proviral constructs. Forty-eight hours post-transfection tetherin cell-surface expression was evaluated after surface staining using anti-Tetherin antibodies

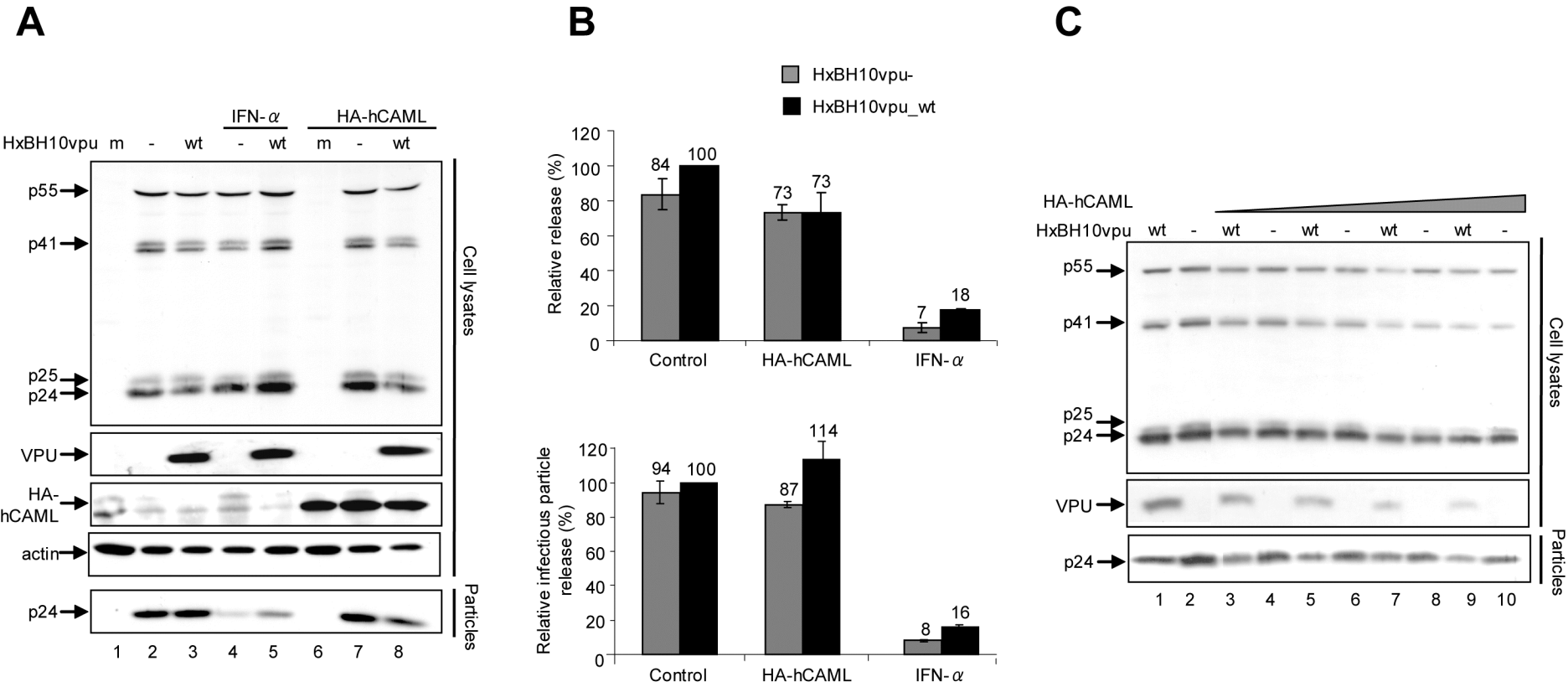
followed by flow cytometry. Tetherin antiserum was produced in rabbits using a GST-Tetherin fusion protein produced in bacteria composed of Tetherin amino acids 40 to 181, thus generating a polyclonal antibody against the extracellular portion of Tetherin. The grey filled histogram represents mock-transfected cells stained with the pre-immune rabbit serum (unstained control), while the other histograms represent cells stained with anti-Tetherin polyclonal rabbit serum. The Histogram with a solid line represents mock-transfected cells; the histogram with a dot line corresponds to mock-transfected cells treated with 10,000U/ml of IFN-α, while data from the dashed line histogram represents cells expressing hCAML. Mean fluorescence intensity (MFI) values after unstained control subtraction are indicated for each sample. Stained cells were analyzed on a FACSCalibur (BD Biosciences Immunocytometry Systems), and data analysis was performed by using CellQuest Pro (BD Biosciences) and Flow Jo software v. 7.25 (Tree Star).

Figure 3: Effect of CAML depletion on HIV-1 particle release and Tetherin cell-surface expression in non-permissive HeLa cells. A- HeLa cells were transfected with non-targeting siRNA (siGENOME control siRNA, Dharmacon D-001210-02-20; lanes 1 to 3) or specific siRNA against CAML (siGENOME SMART pool, Dharmacon M-011601-01; lanes 4 to 6). Subsequently, cells were mock-transfected (labeled as m; lanes 1 and 4) or transfected with either the HxBH10*vpu_wt* (lanes 2 and 5), or HxBH10*vpu*- (lanes 3 and 6) proviral plasmids. Cells and supernatant-containing viral particles were harvested 24 h post-transfection and lysates analyzed by western blotting. Cell lysates were analyzed to detect Gag products, Vpu and β-actin using specific antibodies. Virus

lysates were analyzed for the presence of p24 using specific antibodies. Depletion of CAML mRNA was confirmed by RT-PCR, using CAML mRNA specific primers (Forward primer: 5' GGTGATTCAGTCAGTACAGG 3', Reverse primer: 5' CTGACTCCAAGAGCAAGAAG 3'); as a control actin mRNA levels were analyzed by RT-PCR using actin specific primers (Forward primer: 5'ACTCCTGCTTGCTGATCCAC 3', Reverse primer: 5' TGGCTACAGCTTCACCACC 3'). **B-** Quantification of virus particle release efficiency. Upper panel: densitometric quantification of HIV-1 particle release efficiency after endogenous hCAML depletion. Virus release efficiency was evaluated as in Fig. 1B. The virion-associated p24 signals were evaluated using longer blot exposure to reveal the HxBH10*vpu*- associated bands. Lower panel: the levels of released infectious virus were evaluated using HeLa-TZM indicator cells as in Fig.1B. For both panels, the release efficiency of HxBH10*vpu_wt* was arbitrarily set at 100%. Error bars indicate the standard deviations of the means of the results from two independent experiments. **C-** Tetherin cell-surface expression was measured by flow cytometry as described in Fig. 2. Comparison of Tetherin cell-surface expression between HeLa cells transfected with non-targeting siRNA (solid line histogram) and HeLa cells transfected with specific siRNA against CAML (dashed line histogram). As a negative control, unstained HeLa cells transfected with non-targeting siRNA were included (grey-filled histogram). MFI values are indicated for each sample.

Figure 4: Effect of Tetherin depletion on HIV-1 particle release in non-permissive HeLa cells. A- HeLa cells were transfected with non-targeting

siRNA (siGENOME control siRNA, Dharmacon D-001210-02-20, lanes 1 to 3) or specific siRNA against Tetherin (ON-TARGET plus SMART pool, Dharmacon L-011817-00; lanes 4 to 6). Subsequently, cells were mock-transfected (labeled as m; lanes 1 and 4) or transfected with either the HxBH10*vpu*- (lanes 2 and 5) or the HxBH10*vpu_wt* (lanes 3 and 6) proviral plasmids as indicated. Cells and supernatant-containing viral particles were harvested 24 h post-transfection and lysates analyzed by western blotting. Cell lysates were analyzed to detect Gag products and endogenous cellular Tetherin using specific antibodies (*asterisk denotes an unspecific band used as an internal loading control). Virus lysates were analyzed for the presence of p24 using specific antibodies. **B-** Densitometric quantification of HIV-1 release efficiency after endogenous Tetherin depletion. HIV-1 particle release efficiency was evaluated as in Fig. 1B. The release efficiency of HxBH10*vpu_wt* was arbitrarily set at 100%. Error bars indicate the standard deviations of the means of the results from two independent experiments.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7 18
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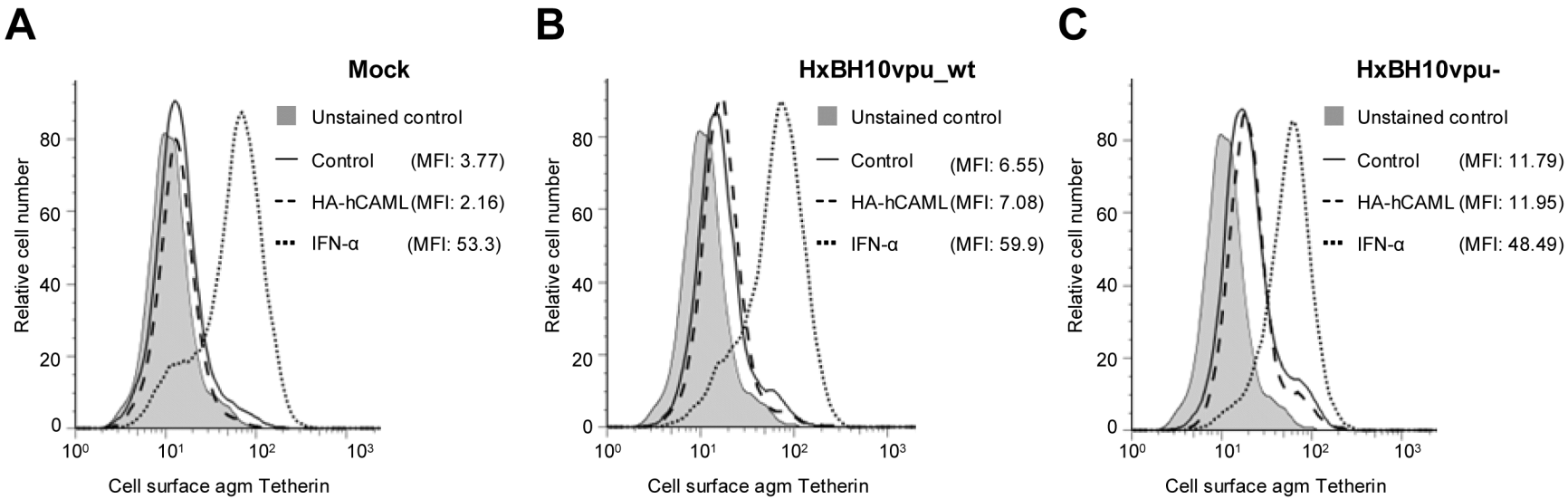
Figure 2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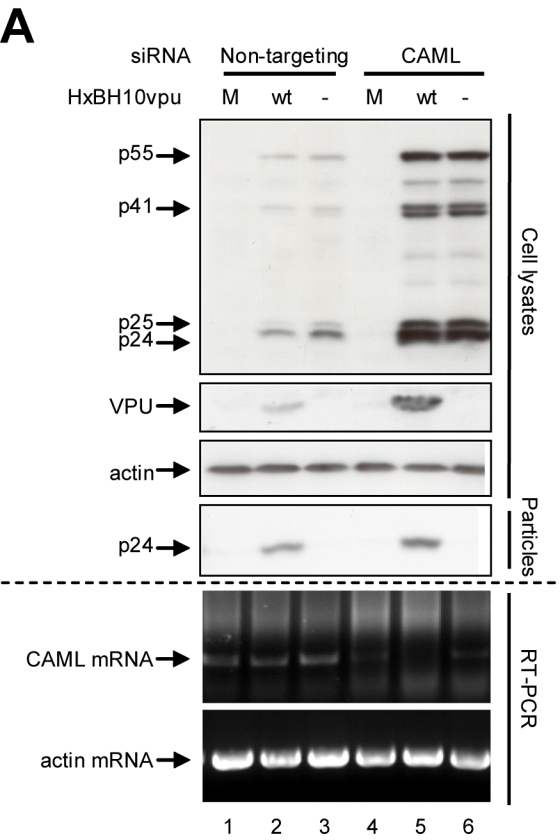
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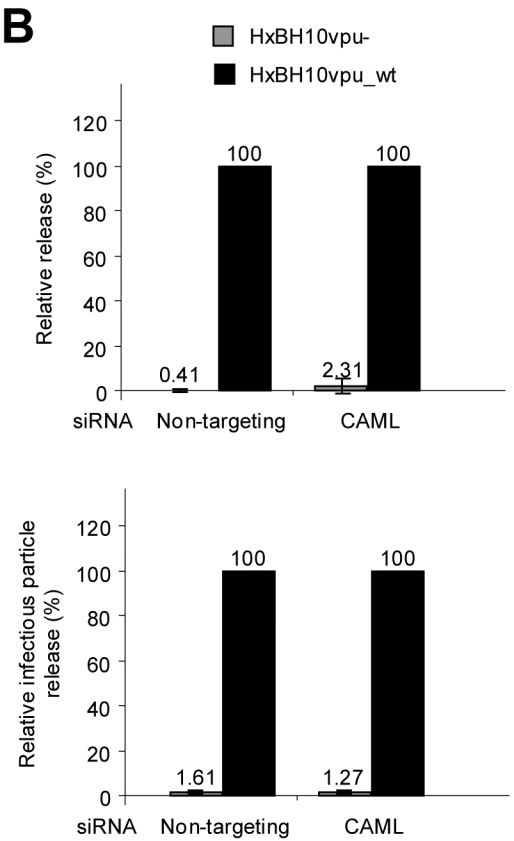
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Non-targeting
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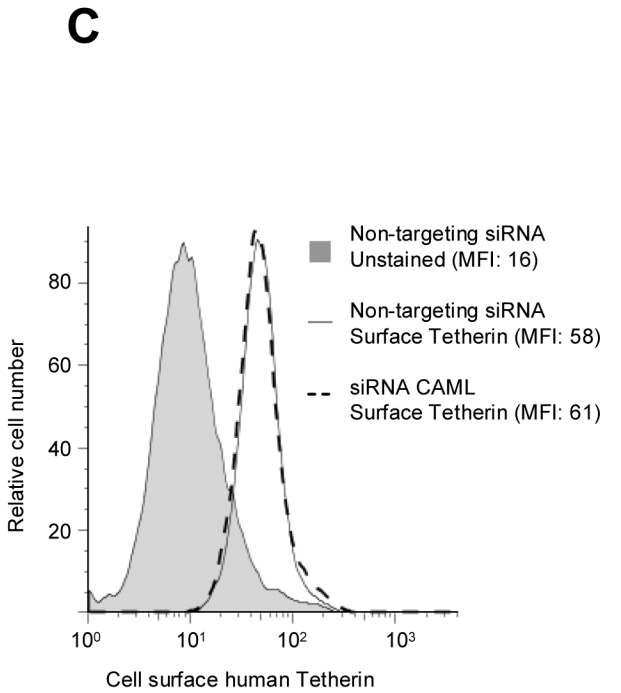
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10¹
10²
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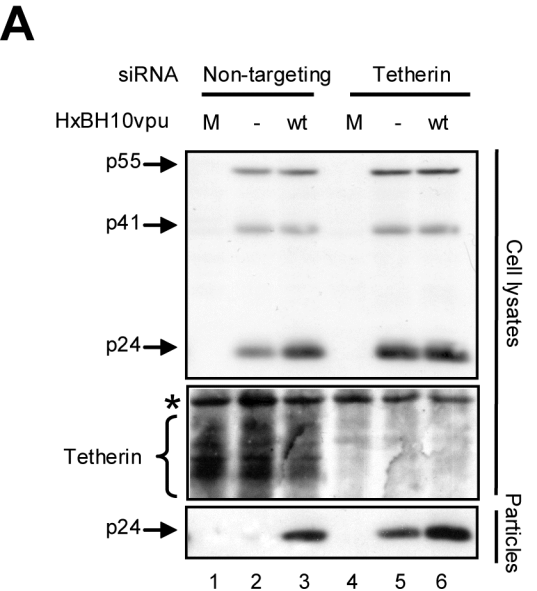
Cell surface human Tetherin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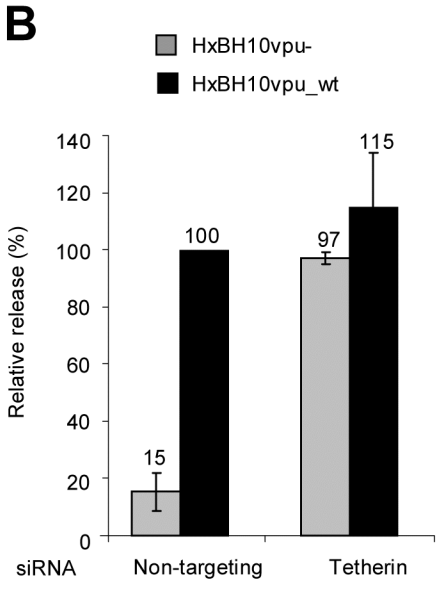
M
-
wt
M
-
wt
p55
p41
p24
Cell lysates
*
Tetherin
p24
Particles
1
2
3
4
5
6


B
HxBH10vpu-
HxBH10vpu_wt
Relative release (%)
140
120
100
80
60
40
20
0
100
97
115
15
siRNA
Non-targeting
Tetherin