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► To cite this version:

Jamal Alfaisal, Alice Machado, Mathilde Galais, Véronique Robert-hebmann, Laetitia Arnauné-pelloquin, et al.. HIV-1 Vpr inhibits autophagy during the early steps of infection of CD4 T cells. *Biology of the Cell*, 2019, 111 (12), pp.308-318. 10.1111/boc.201900071 . hal-02997501

HAL Id: hal-02997501

<https://hal.science/hal-02997501>

Submitted on 16 Nov 2020

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HIV-1 Vpr inhibits autophagy during the early steps of infection of CD4 T cells

Running title: Incorporated HIV-1 Vpr inhibits autophagy

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Key words: autophagy, HIV-1, Vpr, FOXO3a, LC3

Abbreviations

- ANOVA: analysis of variance
- ATG: autophagy related protein
- BNIP3: BCL2/adenovirus E1B 19kDa protein-interacting protein 3
- CQ: chloroquine
- Env: HIV-1 envelope

- FOXO3a: forkhead transcription factor 3a
- HIV-1: human immunodeficiency virus type 1
- MAPLC3B: microtubule-associated protein light chain 3B, hereafter referred to as LC3
- PBS: phosphate buffer saline
- ROS: reactive oxygen species
- RT-PCR: reverse transcriptase polymerase chain reaction
- To: torin 1
- ULK1 (Unc-51 like autophagy activating kinase 1)
- UPS: ubiquitin proteasome system
- Vpr: HIV-1 viral protein R

Acknowledgments

We thank S. Benichou for providing plasmids expressing Vpr and M. Mougel for technical advises and very helpful scientific discussions. The authors have no financial interests to declare.

Funding

This work was supported by institutional funds from the Centre National de la Recherche Scientifique (CNRS) and the Montpellier University and grants from the Agence Nationale de Recherches sur le SIDA et les Hépatites Virales (ANRS) and Sidaction. J.A. was benefiting from the Dunia Beam Erasmus Mundus scholarship, funded with the support of the European Commission.

Abstract

Background information: Autophagy is induced during HIV-1 entry into CD4 T cells by the fusion of the membranes triggered by the gp41 envelope glycoprotein. This anti-HIV-1 mechanism is inhibited by the viral infectivity factor (Vif) neosynthesized after HIV-1 integration to allow viral replication. However, autophagy is very rapidly controlled after HIV-1 entry by a still unknown mechanism. As HIV-1 viral protein R (Vpr) is the only auxiliary protein found within the virion in substantial amount, we studied its capability to control the early steps of HIV-1 envelope-mediated autophagy.

Results: We demonstrated that ectopic Vpr inhibits autophagy in both the Jurkat CD4 T cell line and HEK.293T cells. Interestingly, Vpr coming from the virus also blocks autophagy in CD4 T cells, the main cell target of HIV-1. Furthermore, Vpr decreases the expression level of two essential autophagy proteins (ATG), LC3B and Beclin-1, and an important autophagy-related protein, BNIP3 as well as the level of their mRNA. We also demonstrated in HEK.293T cells that Vpr degrades the FOXO3a transcription factor through the ubiquitin proteasome system.

Conclusion: Vpr, the only well expressed HIV-1 protein incorporated into viruses, is able to negatively control autophagy induced during HIV-1 entry into CD4 T cells.

Significance: We provide insights of how HIV-1 controls autophagy very early after its entry into CD4 T cells and discovered a new function of Vpr. These results open the route to a better understanding of the roles of Vpr during HIV-1 infection through FOXO3a degradation and could be important to consider additional therapies that counteract the role of Vpr on autophagy.

1. Introduction

Autophagy is a lysosomal degradation mechanism involving autophagy related proteins (ATGs). Sequential ATG complexes, including the ULK1 complex, the Beclin-1/class III PI3K complex and the ATG5/ATG12 and ATG8/LC3 conjugation systems, orchestrate and mediate the formation of double membrane vesicles (autophagosomes) that encompass cytoplasmic material (Wesselborg and Stork, 2015). The autophagosomes then fuse with lysosomes for cargo degradation. BNIP3 (BCL2/adenovirus E1B 19kDa protein-interacting protein 3), a member of the BH3-only subfamily of Bcl-2 family proteins, is a potent inducer of autophagy in different cell types (Bellot et al., 2009; Hamacher-Brady et al., 2007; Kanzawa et al., 2005). BNIP3 can thus be defined as an ATG by regulating autophagy induction (Fullgrabe et al., 2016). BNIP3 triggers also mitophagy (Ney, 2015) and apoptosis (Landes et al., 2010), and its precise functions are not yet clearly defined (Burton and Gibson, 2009).

Autophagy is regulated by several transcription factors, including the forkhead transcription factor FOXO3a (Warr et al., 2013). Many autophagy genes are under the control of FOXO3, including MAPLC3B (microtubule-associated protein light chain 3B, hereafter referred to as LC3), ATG9A, ATG4B, ATG5, ATG10, Beclin 1, BNIP3 (BCL2/adenovirus E1B 19kDa protein-interacting protein 3) and ULK1 (Unc-51 like autophagy activating kinase 1), depending on the cell type studied (Audesse et al., 2019; Fullgrabe et al., 2016; Li et al., 2017; Mammucari et al., 2007; Sanchez et al., 2012)

Besides its role in maintaining homeostasis, autophagy plays a crucial role in the immune responses against pathogens. Autophagy is induced into CD4 T cells by HIV-1 envelope (Env), via the fusogenic function of gp41 (Denizot et al., 2008). In bystander CD4 T cells, which are not productively infected, autophagy is uncontrolled and leads to their death by apoptosis (Espert et al., 2006). This mechanism is thus responsible, at least in part, for HIV-1 pathogenesis. Autophagy is differentially controlled in HIV-1-infected cells (Espert et al., 2009). In macrophages, autophagy is required for HIV-1 replication,

and IRGM (immunity-associated GTPase family M) plays an important role through its binding to Nef (Gregoire et al., 2011). In THP-1 macrophages, transfected viral protein R (Vpr) can also trigger autophagy (Zhou et al., 2017). However, the autophagy flux can degrade HIV-1 and Nef acts as an anti-autophagic maturation factor through interaction with beclin-1 (Kyei et al., 2009) and through transcription factor EB sequestration (Campbell et al., 2015). In CD4 T cells, autophagy is an anti-HIV-1 process by selectively degrading the viral transactivator Tat, an essential protein for viral transcription (Sagnier et al., 2015). We and others have previously reported that autophagy is blocked in CD4 T cells that were productively infected by HIV-1 (Espert et al., 2009; Zhou and Spector, 2008), thus allowing HIV-1 replication to occur. We already know that neo-synthesized Vif blocks autophagy in CD4 T cells infected by HIV-1 for at least 4 days (Borel et al., 2015). However, the Env-induced autophagy level is very rapidly controlled in infected CD4 T cells, before its complete inhibition in the late steps of infection (Sagnier et al., 2015). This result suggests that another viral component, present in the incoming viral particles, acts very early after HIV-1 entry, before integration.

Among the HIV-1 auxiliary proteins, Vpr is the only one packaged into virions in large quantity (Cohen et al., 1990). It is a multifunctional viral protein essential for viral replication in macrophages (Heinzinger et al., 1994), but it also facilitates viral replication in CD4 T cells (Hohne et al., 2016; Rucker et al., 2004; Zhou et al., 2012). Vpr is known to play multiple roles at different stages of the HIV-1 viral life cycle such as arresting the cell cycle at the G2/M phase, increasing the activity of the HIV-1 long terminal repeat and regulating apoptosis (Guenzel et al., 2014) by hijacking E3 ubiquitin ligases. Indeed, Vpr binds to DCAF1 (DDB1 (DNA damage-binding protein 1) and CUL4-associated factor 1), also known as viral protein R binding protein (VprBD), which is part of two E3 ubiquitin ligases, DCAF1-DDB1-CUL4-ROC1 and DCAF1-DDB1-DYRK2-EDD. Thus, Vpr targets multiple cellular proteins to proteasomal degradation and in consequence causes global remodeling of the cellular proteome (Greenwood et al., 2019).

We demonstrate here that ectopic Vpr inhibits the basal and induced autophagy flux in both the Jurkat CD4 T cell line and HEK.293T cells. It is worth noting that HEK.293 cells expressing the receptors of HIV-1 also undergo Env-mediated autophagy (Espert et al., 2006). In the context of HIV-1 infection of CD4 T cells, Vpr coming from the viruses decreases autophagy triggered during viral entry. Interestingly, Vpr decreases the expression of three ATGs, LC3, Beclin-1 and BNIP3, at their transcriptional level. Furthermore, Vpr induces the degradation of the transcription factor FOXO3a.

2. Results

Vpr inhibits the autophagy process. Basal autophagy and chemically-induced autophagy (Torin 1, To) were first analyzed by immunofluorescence in the Jurkat CD4 T cell line expressing, or not, HA-Vpr, by counting the number of LC3 dots per cell. The autophagy flux was also studied using chloroquine (CQ), which blocks the maturation step of the process. As expected, autophagy is induced by To when Vpr is not expressed (Fig. 1a, white bars). However, the number of LC3 puncta remains very low in cells expressing HA-Vpr (red cells) whatever the conditions used, i.e. cells treated by To, CQ, or To + CQ (Fig. 1a, black bars). This result was reproduced with the ectopically expressed DsRed-Vpr in Jurkat cells (Fig. 1b), indicating that the tag has no influence on the inhibitory effect of Vpr on autophagy. It is important to note that cell nucleofection with a plasmid expressing the tag alone does not affect the autophagy flux (data not shown). Ectopic expression of Vpr in HEK.293 cells also inhibits autophagy induced by To (Suppl. Fig. 1)

To confirm these results, we analyzed the effect of Vpr on autophagy by western blot by following the level of lipidated LC3 (LC3-II) in Jurkat cells expressing, or not, HA-Vpr. As shown in figure 1c, LC3-II expression increases in presence of To + CQ without HA-Vpr, whereas the level of LC3-II stays very low in the presence of HA-Vpr. Altogether, these results confirm that Vpr inhibits both basal and induced autophagy. Interestingly, we observed that Vpr also decreases the expression of LC3-I, the non-

lipidated form of LC3 (Fig. 1c). In accordance, red cells (Vpr positive cells) have a low LC3-I labeling (diffuse green fluorescence) in the cell cytoplasm (Fig. 1a and Fig. 1b).

Vpr decreases the expression of several ATGs and acts at the transcriptional level. As we showed that Vpr decreases the LC3-I expression level, we then analyzed the expression of two others ATGs, i.e. Beclin-1 and BNIP3. As presented in Figure 2, Vpr triggers a significant decrease in the expression of the three ATGs analyzed. It is worth noting that LC3 and HA-Vpr cannot be revealed on the same blot due to their very similar molecular weight. This result suggests that Vpr may either trigger degradation of the three proteins, or, more probably, regulate the expression of these ATGs upstream by acting at their transcriptional level.

To analyze whether Vpr could modulate the transcription of the LC3, Beclin-1 and BNIP3 genes, we studied the expression of their mRNA by RT-PCR in Jurkat cells expressing, or not, HA-Vpr. As shown in Figure 3, the mRNA level of these three genes is strongly decreased in presence of HA-Vpr. Expression of HA-Vpr was controlled by Western blot (data not shown). These results demonstrate that the Vpr-mediated decrease in the expression of the ATGs tested is transcriptionally regulated.

Vpr, coming from the virion, inhibits HIV-1-induced autophagy. As Vpr is incorporated into the viral particles, we next analyzed the capability of Vpr coming from virions to block Env-induced autophagy at the early steps of infection, before the integration of the provirus into the genome of the host cells (Mohammadi et al., 2013). To this aim, we infected Jurkat cells with HIV-1 (NL4-3 or NL4-3ΔVpr strains) and analyzed autophagy after 8h of infection, in presence or absence of CQ to follow the autophagy flux. The quantity of viruses were adjusted to have the same percentage of infected Jurkat cells (40%) by flow cytometry at 24h of infection. Autophagy was analyzed in HIV-1-infected Jurkat cells by counting the number of endogenous LC3 dots in cells expressing the viral capsid (p24 positive cells). As shown in Figure 4, the number of autophagosomes is significantly higher in HIV-1ΔVpr-infected cells compared to wt HIV-1-infected cells, in presence or absence of CQ. These results demonstrate that

Vpr, coming from the virion, negatively controls autophagy at the early step of infection, before *de novo* expression of the viral proteins.

The FOXO3a transcription factor is a target of Vpr. Several transcription factors are known to trigger autophagy by regulating the expression of autophagy-related genes (Lapierre et al., 2015). Among them, FOXO3a hold our attention because its activation increases the abundance of mRNA and protein levels of LC3 and BNIP3 in several cell types, including cardiomyocytes, mouse primary renal proximal tubular cells or skeletal muscle cells (Kume et al., 2010; Mammucari et al., 2007; Sengupta et al., 2009). FOXO3a is also known to induce expression of Beclin-1 in muscle cells (Mammucari et al., 2007; Webb and Brunet, 2014) and kidney tubular cells (Li et al., 2017). As Vpr is known to target several cellular proteins to degradation by the ubiquitin-proteasome system (UPS), we analyzed whether Vpr could also trigger FOXO3a degradation. We thus analyzed the expression level of Flag-FOXO3a in presence or absence of HA-Vpr and MG132, a UPS inhibitor. As shown in Figure 5a, the expression level of FOXO3a is strongly decreased in presence of HA-Vpr without MG132, and this expression is almost completely restored in presence of the UPS inhibitor. This result indicates that Vpr triggers the UPS-mediated FOXO3a degradation.

FOXO3a is mainly regulated through its subcellular localization. Indeed, its transcriptionally active form is located in the nucleus, while its inactive form is sequestered in the cytoplasm. To analyze whether Vpr could influence the FOXO3a localization into cells, we analyzed its expression level after cell fractionation in presence or absence of HA-Vpr. The Figure 5b shows that Flag-FOXO3a and HA-Vpr are present in both fractions, but the latter is more expressed into the nucleus. In presence of HA-Vpr, expression of Flag-FOXO3a is dramatically decreased in the cytoplasm but is also strongly reduced in the nucleus, indicating that the overall pool of FOXO3a is subjected to Vpr-mediated degradation (Fig. 5b), and that Vpr does not trigger FOXO3a shuttling. Vpr-mediated FOXO3a degradation is UPS-dependent since addition of MG132 restores the expression level of FOXO3a in both fractions (Fig. 5c).

3. Discussion

During HIV-1 entry into CD4 T cells, autophagy is induced through the fusogenic function of gp41 (Denizot et al., 2008). Our previous results have shown that Env-mediated autophagy is an anti-viral mechanism by degrading specifically Tat (Sagnier et al., 2015). Thus, autophagy must be negatively regulated in infected CD4 T cells, the main target cells of HIV-1, for an efficient viral replication. After provirus integration, neosynthesized viral infectivity factor (Vif) blocks autophagy (Borel et al., 2015), but a first negative control of autophagy occurs earlier after HIV-1 entry, before synthesis of new viral proteins (Sagnier et al., 2015). Vpr was a good candidate for this action since it is the only auxiliary viral protein well incorporated into virions. Many functions of this viral protein are linked to its capability to bind to E3 ligases in order to degrade their substrates. In addition, several E3 ligases, and especially CUL-RING E3 ligases, can regulate the autophagy machinery and the upstream regulators of autophagy (Cui et al., 2016). We demonstrate here that the expression level of the proteins BNIP3, LC3 and Beclin-1 dramatically decreases in presence of Vpr. We thus investigated their level of transcription in the presence of Vpr. Interestingly, these three ATG genes were negatively regulated by Vpr at the transcriptional level, as well as the ULK1 gene (data not shown), suggesting that Vpr acts upstream, on a transcriptional factor. Several positive transcriptional regulators of ATGs are known, including transcription factor EB (TFEB), FOXO3a and E2F1 (Fullgrabe et al., 2016). All these factors induce the expression of LC3 (Polager et al., 2008; Sanchez et al., 2012; Settembre et al., 2011) whereas BNIP3 is overexpressed by both E2F1 and FOXO3a (Mammucari et al., 2007; Shaw and Kirshenbaum, 2008; Yurkova et al., 2008) and Beclin-1 only by FOXO3a (Sanchez et al., 2012). Very recent data also show that FOXO3 controls many autophagy genes in adult neural stem cells (Audesse et al., 2019). In addition, we already know that Env triggers the production of ROS (Molina et al., 2007) and numerous data demonstrate that FOXO3a is activated in response to oxidative stress (Storz, 2011). For all these reasons, we focused our work on FOXO3a. However, since Vpr drives DCAF1-dependent degradation

of many cellular proteins (Greenwood et al., 2019), we cannot exclude a role of other transcriptional factors in Vpr-mediated inhibition of autophagy.

FOXO3a is regulated by various post-translational modifications including phosphorylation that dictates its subcellular localization. One study have indirectly linked Vpr to FOXO3a, through its interaction with 14-3-3 (Kino et al., 2005b). 14-3-3 are a highly conserved family of molecules that inhibit FOXO3a by inducing its accumulation in the cytoplasm (Brunet et al., 1999; Brunet et al., 2002; Dobson et al., 2011; Lehtinen et al., 2006; Tzivion et al., 2011). In a context of insulin resistance/lipodystrophy syndrome, Vpr binding to 14-3-3 inhibits insulin/PI3K/Akt-1 signaling pathway, triggering FOXO3a translocation to the nucleus (Kino et al., 2005a). We demonstrated here another function of Vpr, where Vpr triggers the degradation of FOXO3a by the proteasome both in the cytoplasm and the nucleus of cells. Further studies are needed to know the E3 ligase involved in this process. However, as FOXO3a is involved in many essential cellular processes, including the control of the cell cycle, the protection from oxidative stress, apoptosis and longevity, these results are of major interest to understand the actions of Vpr at different steps of the HIV-1 life cycle. It would also be interesting to analyze the role of Vpr on FOXO3a in macrophages where ectopic Vpr was shown to activate autophagy (Zhou et al., 2017). This also underlines the complexity of HIV-1 infection depending on the cell type where autophagy is repressed in CD4 T cells but induced and regulated in macrophages (Espert et al., 2009).

In conclusion, these data demonstrate that during HIV-1 infection of CD4 T cells, incoming Vpr acts to inhibit the autophagy process activated during viral entry to “prepare” the cells for an efficient viral replication. These results also open the route to a better understanding of the roles of Vpr during HIV-1 infection through FOXO3a degradation.

4. Materials and methods

Cell lines, viruses and infections. The Jurkat CD4 T cell line and the adherent HEK.293T cell line were cultured in RPMI and DMEM, respectively, supplemented with 1% penicillin/streptomycin and 10%

fetal calf serum. HIV-1 infection of Jurkat cells was performed with normalized amounts of supernatant of HEK.293T cells that have been transfected with X4 viruses (NL4-3 or NL4-3ΔVpr strains) for two days. Follow-up of Jurkat cell infection was done by analysing the percentage of infected cells after 24h of infection by flow cytometry using the HIV KC57 FITC kit according to the manufacturer's instructions (Beckman Coulter). Briefly, cells were fixed and permeabilized, and the anti-p24 antibody was added to the cells. After staining for 30min, cells were washed with phosphate-buffered saline (PBS), and fluorescence intensity was measured on a Coulter Epics XL Flow Cytometer (Beckman Coulter).

Reagents, plasmids and antibodies. Torin 1 (To) was purchased from Tocris, chloroquine (CQ) and MG132 from Sigma-Aldrich. The pNL4-3 molecular clone and pNL4-3 deleted of Vpr were obtained from the NIH AIDS Reagent Program. Plasmids that express HA-Vpr and DsRed-Vpr were a gift from S. Benichou (Institut Cochin, Paris), and plasmid expressing Flag-FOXO3a was purchased from Addgene. Anti-LC3, anti-GAPDH, anti-Flag, anti-BNIP3 and anti-HA antibodies were obtained from Sigma-Aldrich; anti-Beclin-1 antibody was purchased from Santa-Cruz Biotechnologies. The monoclonal anti-p24 antibody was from the NIH AIDS Reagent program.

Western blotting. Cells were washed twice in PBS and the cell pellet was resuspended in Laemmli buffer. Cell lysates were electrophoresed in 12% ProSieve™ 50 gel (Lonza) and blotted to PVDF membranes. After a blocking step for 1 hour at room temperature in PBS containing 0.5% casein, blots were incubated with the primary antibody in the blocking buffer. After 3 washes with PBS and 0.05% Tween, the blots were incubated with peroxidase-coupled antiserum diluted in the blocking buffer. After further washes, the immune complexes were revealed by ECL (Biorad) and autoradiographed. Quantification of protein expression was performed by densitometry analysis using the ImageJ software and statistical analysis was performed using ANOVA.

Analysis of autophagy. The autophagy flux was analyzed by western blot by following the expression level of LC3-II and by immunofluorescence after labeling of endogenous LC3-II using in both cases the rabbit anti-LC3 antibody L7543 (1µg/ml, Sigma Aldrich). To analyze the flux, Torin 1 (To, 2µM, Sigma Aldrich) was added for 3h, in presence or absence of the autophagic flux blocker chloroquine (CQ, 50µM, Sigma Aldrich). Basal autophagy was also analyzed in parallel.

Immunofluorescence studies. Cells were fixed in 1% paraformaldehyde for 10min and then in 100% methanol for 5min at room temperature. After three washes in PBS, the first antibody was incubated for 1h at room temperature. After further washes, the fluorochrome-conjugated secondary antibody (Life Technologies) is then incubated for 30min, the nuclei were labeled with Dapi, and the cells were examined by epifluorescence using a Leica microscope. To analyze autophagy in HIV-1-infected CD4 T cells, cells were incubated with the rabbit anti-LC3 antibody L7543 (1µg/ml, Sigma Aldrich) and the mouse monoclonal anti-p24 antibody for 1h. After further washes, the cells were incubated with Alexa-488 and -568-conjugated secondary antibodies (Life Technologies) for 30min. The number of LC3 dots (green) per infected CD4 T cell (red) was counted. More than 100 CD4 T cells were analyzed for each culture condition by 2 independent investigators. Statistical analysis was performed using ANOVA.

Transfection. Jurkat cells were nucleofected using the Amaxa technology and HEK.293T cell transfection was performed using the Turbofect transfection reagent (Thermo Scientific) according to the manufacturer's instructions.

Reverse transcription PCR (RT-PCR). RNA was purified from 10^6 cells using the Nucleospin RNA plus (Macherey-Nagel), and RT-PCR was performed using the QIAGEN OneStep RT-PCR kit according to the manufacturer's instructions. The oligonucleotides used for LC3B, Beclin-1, BNIP3 and GAPDH mRNA are: LC3B FOR: GATGTCCGACTTATTCGAGAGC; LC3B BACK: TTGAGCTGTAAGCGCCTTCTA; Beclin-1 FOR: ACTGTGTTGCTGCTCCATGC; Beclin-1 BACK: CCCAAGCAAGACCCCACTTA; BNIP3 FOR:

GAAGTCCCTGAGGCGGGAAGAA; BNIP3 BACK: AGTGTTGCCGCTGTCAGTAC. GAPDH FOR: CCCATCACCATCTTCCAG; GAPDH BACK: CCTGCTTCACCACCTTCT. A control RT-PCR was done without RNA.

Cellular fractionation. Cells were lysed in hypotonic buffer (10mM Tris-HCl, pH 7.65, 1.5mM MgCl₂, 1mM DTT, 20mM *N*-ethylmaleimide, and protease inhibitors) and centrifuged at 500 × g for 15min. The supernatant constituted the cytoplasmic fraction and the pellet resuspended in Laemmli buffer constituted the nuclear fraction.

5. Author contribution

J.A. and A.M. performed experiments and analyzed data; M.G. and V.R-H. carried out experiments; L.A-P. and L.E. contributed to the interpretation of the results. M.B-P. supervised the project and wrote the manuscript with input from all authors. All authors discussed the results and contributed to the final manuscript.

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Figure Legends

Figure 1. Ectopic Vpr inhibits the autophagy flux in Jurkat cells. (a) Jurkat cells were transfected with a plasmid expressing HA-Vpr for 1 day. Torin 1 (To, 2 μ M), chloroquine (CQ, 50 μ M) or To + CQ was then added in the wells for 3h. Non-treated (NT) cells were analyzed in parallel. Cells were fixed, and LC3 and HA-Vpr were detected by immunofluorescence. Scale bars=10 μ m. The number of autophagosomes (LC3 dots) was counted by 2 investigators in more than 100 cells. Statistical significance was analyzed by one-way analysis of variance (ANOVA, ** P <0.01; *** P <0.001). (b) Jurkat cells were transfected with a plasmid expressing DsRed-Vpr for 1 day. To (2 μ M), CQ (50 μ M) or To + CQ was then added in the wells for 3h. Cells were fixed, and LC3 and DsRed-Vpr were detected by immunofluorescence. Scale bars=10 μ m. The number of autophagosomes (LC3 dots) was counted by 2 investigators in more than 100 cells. Results are from at least three independent experiments. (c) Jurkat cells were transfected with a plasmid expressing HA-Vpr or HA alone for 1 day. To (2 μ M), CQ (50 μ M) or To plus CQ was then added in the wells for 3h. Cells were harvested, and lysates were immunoblotted with anti-LC3 Ab and anti-GAPDH Ab as a loading control. LC3 levels (LC3-II and LC3-I) with or without Vpr were quantified by densitometry and normalized with GAPDH expression levels. Results are from at least three independent experiments.

Figure 2. Vpr decreases the expression level of LC3, Beclin-1 and BNIP3. Jurkat cells were transfected with a plasmid expressing HA or HA-Vpr for 1 day. Cells were harvested, and lysates were immunoblotted with the Abs of interest and the anti-GAPDH Ab as a loading control. Results are from at least three independent experiments.

Figure 3. Vpr acts at the transcriptional level. Jurkat cells were transfected with a plasmid expressing HA or HA-Vpr for 1 day. Total RNA was purified and expression of LC3B, BNIP3 and Beclin-1 mRNA, and GAPDH mRNA as a control, was analyzed by RT-PCR. A negative control was done without RNA. The figure is representative of three independent experiments.

Figure 4. Vpr coming from the viruses inhibits autophagy. Jurkat cells were infected by wt HIV-1 or HIV-1ΔVpr for 8h. Cells were treated, or not, with CQ for 3h before fixation, and LC3 and p24 were detected by immunofluorescence. Scale bars=10μm. The number of autophagosomes (LC3 dots) was counted by 2 investigators in more than 100 infected cells (p24 positive cells). Results are from at least three independent experiments. Statistical significance was analyzed by ANOVA (* $P < 0.05$).

Figure 5. Vpr triggers UPS-dependent degradation of FOXO3a. (a) HEK293T cells were transfected with plasmids expressing HA or HA-Vpr and Flag-FOXO3a and treated or not with MG132 for 5h at 20μM. Expression of Flag-FOXO3a was analyzed by western blot. Statistical significance was analyzed by ANOVA (*** $P < 0.001$; NS: non-significant difference). (b) HEK293T cells were transfected for one day with plasmids expressing HA or HA-Vpr and Flag-FOXO3a, and expression of Flag-FOXO3a was analyzed in both the cytoplasm and the nucleus. (c) In the same experimental conditions, subcellular expression of Flag-FOXO3a was analyzed in presence or absence of MG132. A ratio between expression of Flag-FOXO3a in absence and presence of Vpr as well as the x-fold difference was indicated. Results are from at least three independent experiments.

Supplemental Figure 1. Ectopic Vpr inhibits the autophagy flux in HEK.239T cells. Cells were transfected with a plasmid expressing HA-Vpr for 1 day. Torin 1 (To, 2μM), chloroquine (CQ, 50μM) or To + CQ was then added in the wells for 3h. Non-treated (NT) cells were analyzed in parallel. Cells were fixed, and LC3 and HA-Vpr were detected by immunofluorescence. Scale bars=10μm.

Figure 1

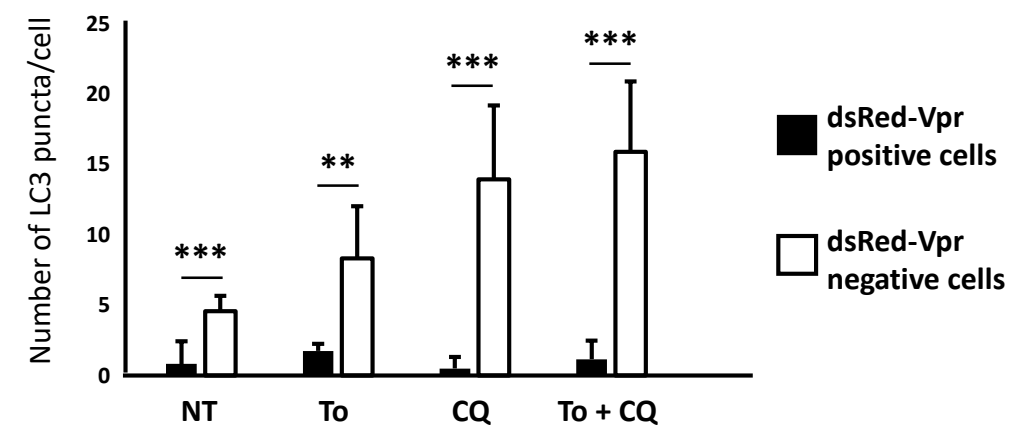
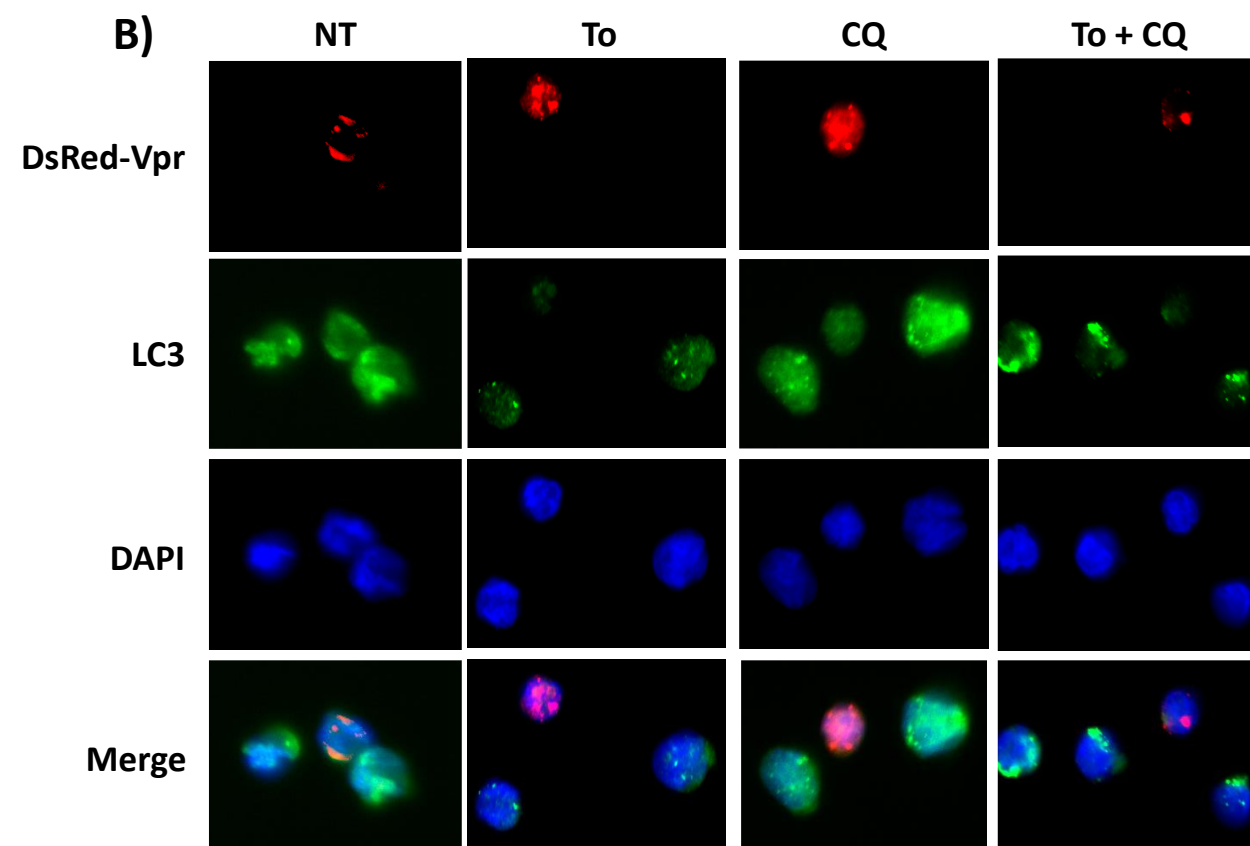
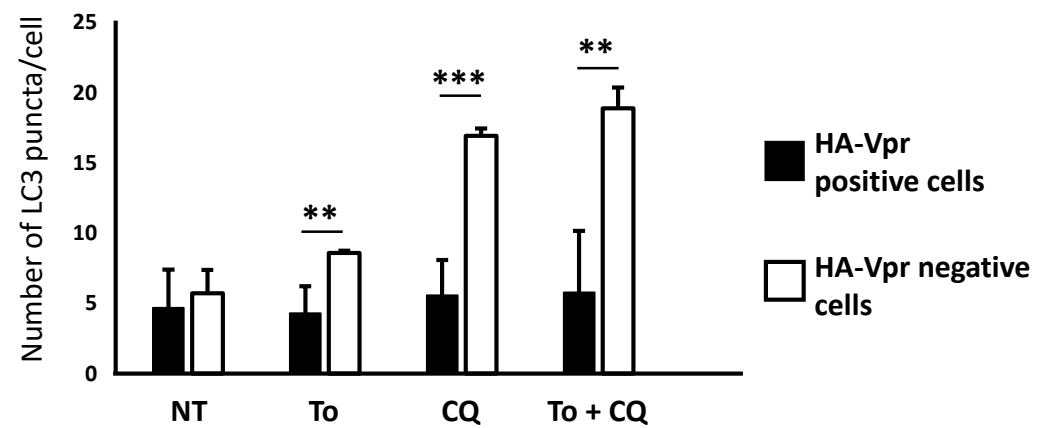
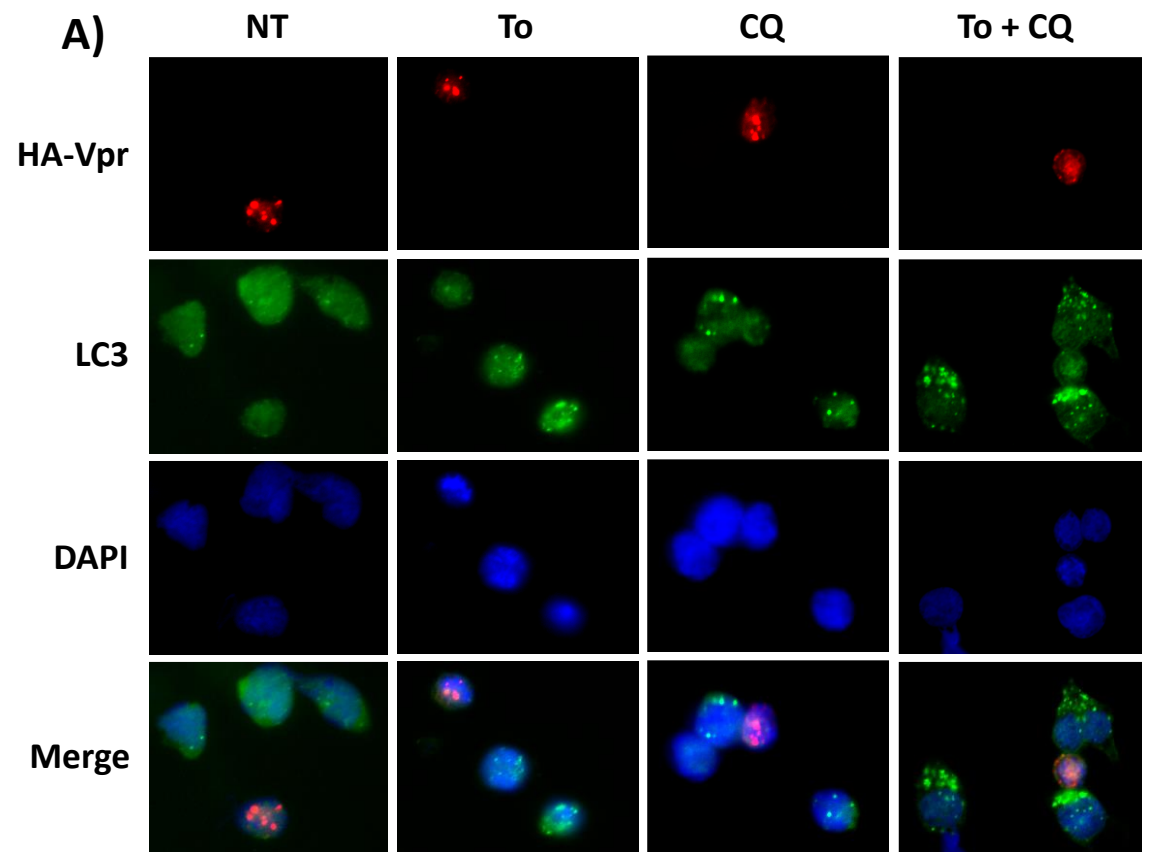


Figure 1

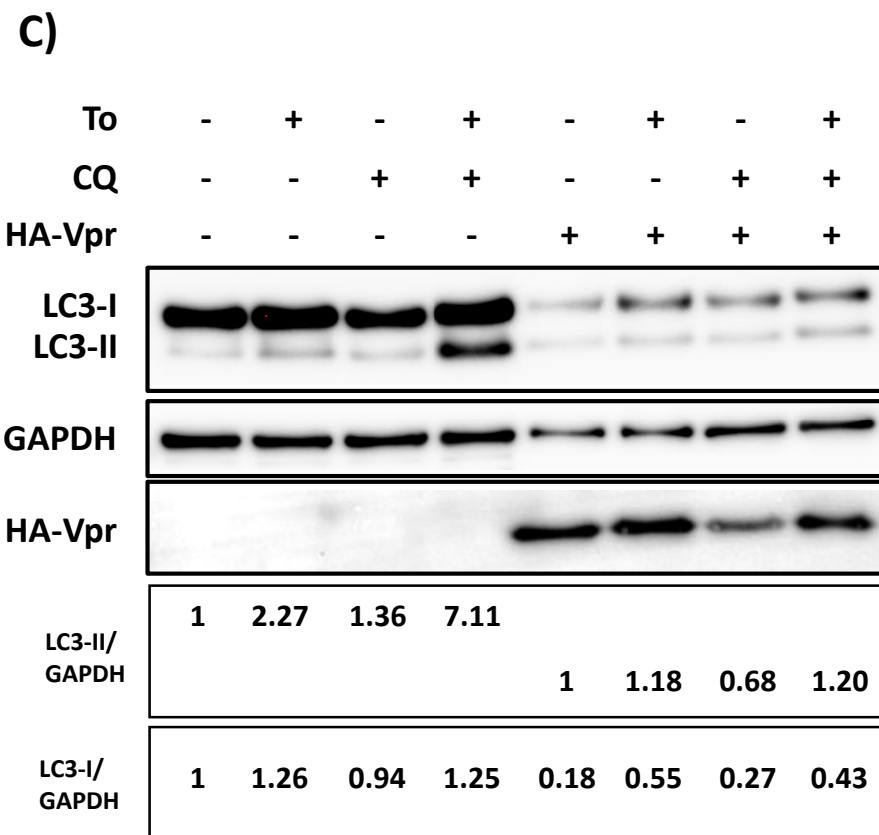


Figure 2

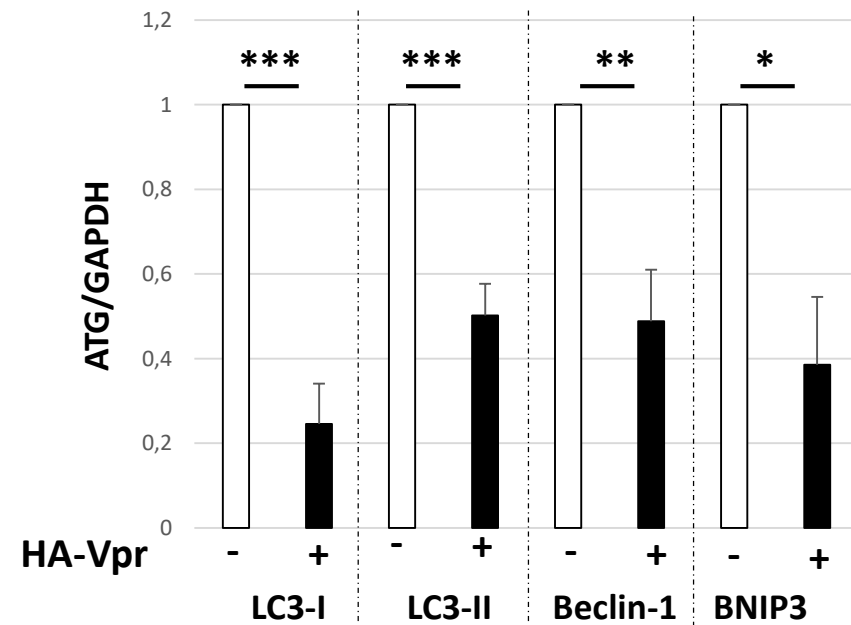
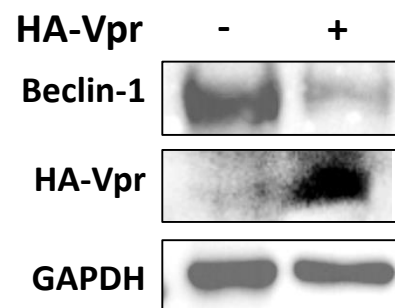
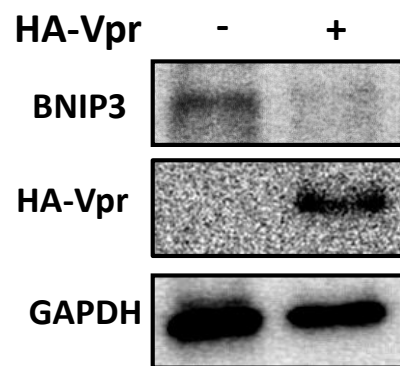
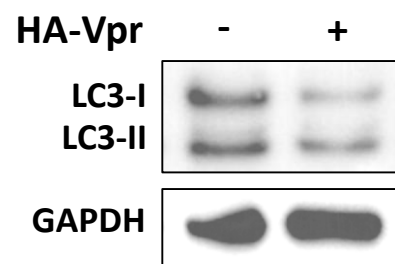


Figure 3

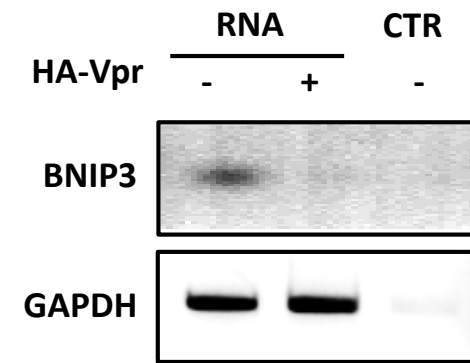
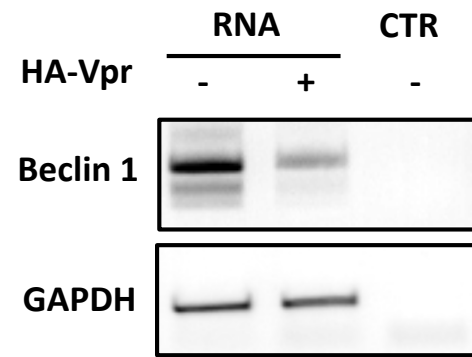
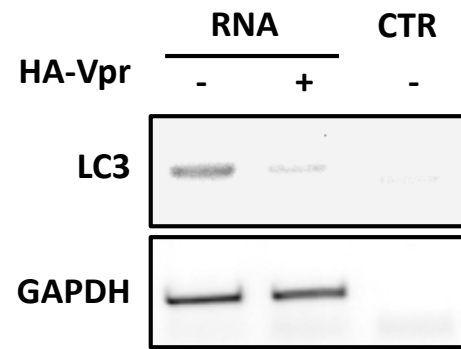


Figure 4

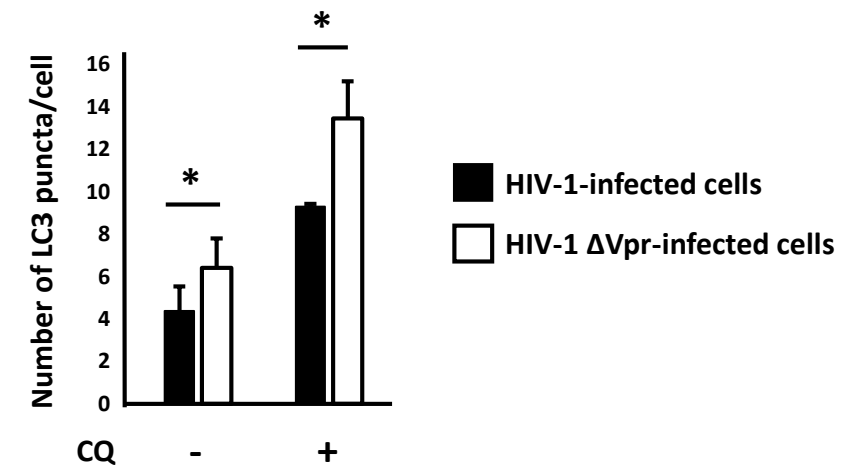
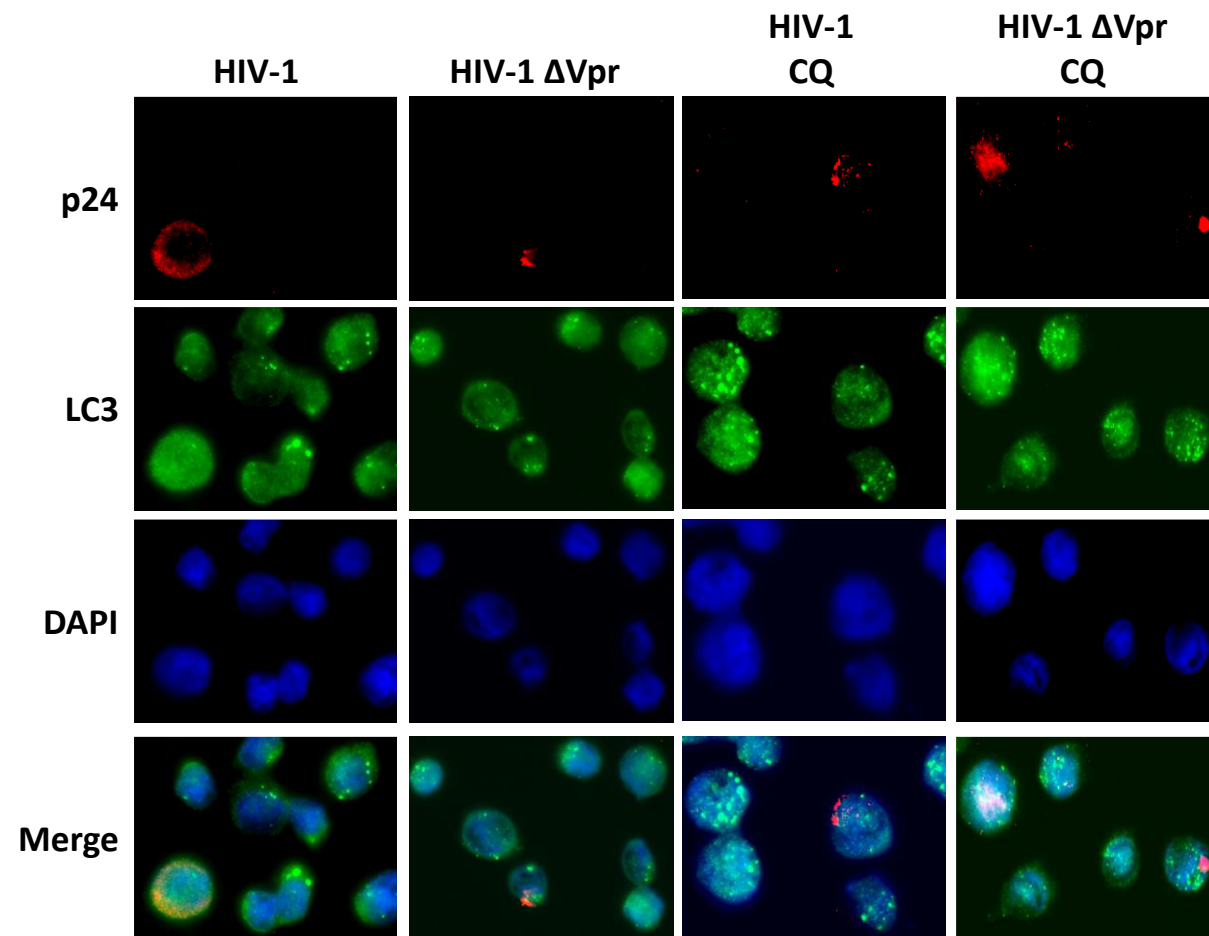
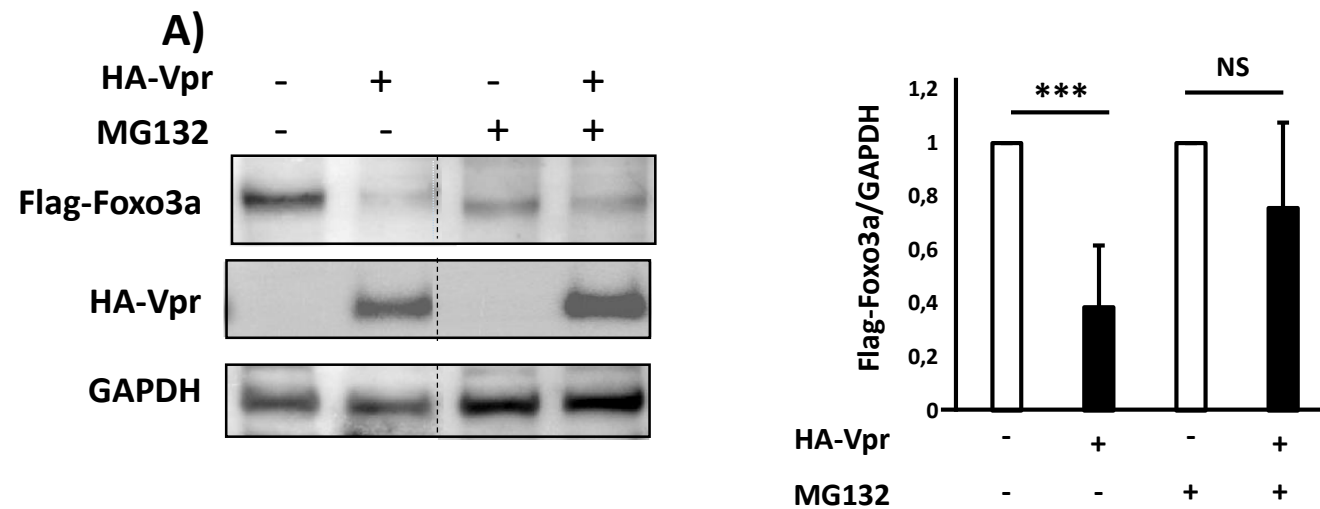
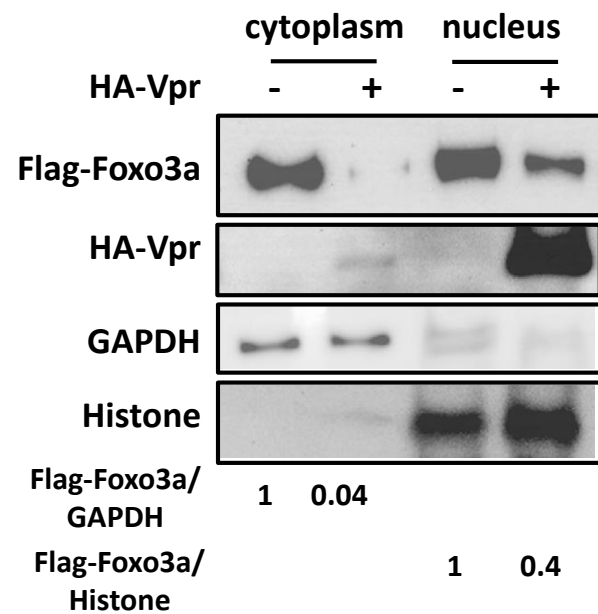


Figure 5



B)



C)

