

Citron Kinase, a RhoA Effector, Enhances HIV-1 Virion Production by Modulating Exocytosis

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RhoGTPases play important roles in the regulation of protein transport and membrane recycling. Little is known, however, about how RhoGTPases affect HIV-1 virion production, which is dependent on the endosomal sorting pathway. We report that ectopic expression of citron kinase (citron-K), a RhoA effector, preferentially enhances HIV-1 virion production. Depletion of endogenous citron-K inhibits HIV-1 virion production. Citron-N, which lacks the kinase domain, also enhances HIV-1 virion production. The leucine zipper, Rho-binding and zinc finger domains of citron-N are necessary for the enhancement activity. Citron-K also enhances murine leukemia virion production and the HIV-1 late domain is not required for the citron-K-mediated enhancement. Ectopic expression of citron-K leads to the formation of cytoplasmic structures containing citron-K and HIV-1 Gag proteins. HIV-1 and citron-K cooperatively enhance acidic endosome and lysosome compartments. Finally, citron-K promotes exocytosis of microvesicles or exosomes that co-purify with HIV-1 virions. We conclude that citron-K enhances HIV-1 virion production by stimulating the endosomal compartments and exocytosis.

Key words: citron kinase, exocytosis, exosome, HIV-1 egress, RhoA

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HIV-1 replication is modulated by a number of cellular signaling pathways regulated by both host and viral factors (1). HIV-1 assembly and release occur in a series of essential steps mediated by the viral Gag precursor pro-

tein, Pr55^{Gag} (2,3). HIV-1 Gag is organized into four distinct regions: matrix, capsid, nucleocapsid and late (L) domain. The L domain catalyzes the pinching off and detachment of virus particles from the cell surface and each other (2). The L domain is a highly conserved Pro-rich motif found in enveloped viruses. There are three classes of tetrapeptide motifs: PT/SAP (4,5), PPXY (6–8) and YPXL (9). In the case of HIV-1, the L domain is encoded by a small peptide motif, Pro-Thr/Ser-Ala-Pro (PTAP) in the C-terminal, p6 domain of Pr55^{Gag}. The L domains of retroviruses, despite differences in amino acid sequence and location within their respective viral structural proteins, are functionally interchangeable, suggesting commonality of function, perhaps as docking sites for host proteins (10–12). Mutations in the PTAP motif of p6 or deletion of the p6 domain produced a striking defect in the production of virus particles where viral particles remain tethered to each other and the plasma membrane (4,5), thereby identifying the p6 domain as playing a critical role in HIV-1 budding.

Viruses like HIV-1 do not encode their own machinery for viral budding and must usurp existing cellular pathways to facilitate viral release. The PTAP motif of HIV-1 Gag^{p6} was found to bind to the ubiquitin enzyme 2 variant domain of Tsg101 (13–16). Tsg101 is a component of the endosomal sorting complex required for transport (ESCRT-I), a 350 kDa cellular complex essential in the vacuolar protein-sorting (VPS) pathway. Point mutations in HIV-1 PTAP motif block virus release at late stages (4,5) and disrupt binding to Tsg101 (14). Small inhibitory RNA-mediated Tsg101 depletion potently blocks HIV-1 release resulting in the virus forming stalks of tethered virus at the plasma membrane (14). Moreover, overexpression of the dominant-negative form of VPS4, an ESCRT recycling factor, inhibits particle release of HIV-1 and other enveloped viruses, such as murine leukemia virus (MLV) (14). Moreover, a second region of HIV-1 Gag^{p6} has been defined to contribute to viral release and interacts with AIP1, a host protein (17,18). AIP1 interacts with Tsg101 and CHMP proteins of ESCRT-III complex, coupling HIV-1 Gag^{p6} to the early and late-acting endosomal sorting complexes and binds directly to HIV-1 Gag^{p6} Leu-Arg-Ser-Leu (LRSL) motif (17,18). Taken together, these data suggest that the VPS machinery and perhaps other host factors are involved in facilitating the budding of retroviruses.

The endosomal sorting pathway controls a variety of cellular processes and plays a role in the sorting of ubiquitinated cargo proteins into the lumen of the multivesicular bodies (MVB) (19,20). Ubiquitinated proteins are recognized on the limiting endosomal membrane and

sorted resulting in either MVB fusion with the lysosome to degrade contents or release of material into the extracellular environment via exosomal vesicles (21–23). ESCRT-I, composed of Tsg101, Vps28 and Vps37, recognize the ubiquitinated protein cargo and recruit two more class E protein cargos (ESCRT-II/III) that participate in protein sorting and vesicle formation (24–26). HIV-1 may bind Tsg101 and AIP1 to gain access to downstream machinery involved in catalyzing MVB vesicle budding, a mechanism topologically similar to viral budding from the plasma membrane.

RhoGTPases play a pivotal role in the dynamic regulation of actin cytoskeleton (27–29) and through this, control cell morphology (30,31), motility, adhesion and activation of transcription factors such as NF- κ B (32) and serum response factor (33). Although RhoGTPases have been implicated in various steps of T-cell activation (34–36), little is known about how RhoGTPases affect HIV-1 replication. We have previously shown that the cytoplasmic tail of the HIV-1 transmembrane glycoprotein interacts with the carboxy-terminus of p115RhoGEF (37), a guanine nucleotide exchange factor and activator of RhoA. Activation of RhoA by p115RhoGEF or G α 13 leads to inhibition of *HIV-1* gene expression in a RhoA-dependent manner (38). The RhoA effectors involved in modulating *HIV-1* gene expression are not defined.

To investigate how RhoA-signaling pathways modulate HIV-1 replication, we tested various RhoA effectors in 293T and human T cells. We determined that citron kinase (citron-K), a Ser/Thr kinase, enhances HIV-1 virion production with no significant effect on *HIV-1* gene expression. Knockdown of citron-K by short-hairpin RNA (shRNA) reduced HIV-1 virion production but not *HIV-1* gene expression. Citron-K also enhanced MLV production, as well as virion production from a HIV-1 Gag Δ p6 construct, suggesting that citron-K mediates virion production independently of the HIV-1 L domain. Citron-K induced intracellular compartments and colocalized with Gag in these compartments. HIV-1 and citron-K cooperatively enhanced acidic (late) endosome and lysosome compartments. Furthermore, citron-K enhanced exocytosis of microvesicles or exosomes that co-purify with HIV-1 virions.

Results

Citron-K preferentially enhances HIV-1 viral replication

To investigate how RhoA-signaling pathways modulate viral replication, we tested various RhoA effectors for their effect on HIV-1 viral replication in 293T and human T cells. Ectopic expression of citron-K, a RhoA effector involved in cytokinesis (39) and membrane vesicle transport (40–42), was shown to preferentially enhance HIV-1 virion production without significantly affecting *HIV-1* gene expression in 293T (Figure 1A–D), Jurkat T (Figure 1E–G) and

HeLa cells (data not shown). Both infectious virions (Figure 1A, E) and total virions (virion-associated RT activity or p24; Figure 1B and data not shown) demonstrated a similar enhancement (7- to 15-fold), while expression of *HIV-1* reporter gene (Figure 1C, F) and cell-associated viral proteins (Figure 1D, G) showed minimal enhancement (<2.5-fold in 293T cells and no change in Jurkat T cells). The C-terminal truncation mutant of citron-K, citron Δ 1, did not enhance HIV-1 virion production. We conclude that citron-K preferentially enhances HIV-1 virion production with minimal effect on *HIV-1* gene expression and that activity depends on the C-terminal domains of citron-K.

To evaluate a role for endogenous citron-K in HIV-1 virion production, we depleted endogenous citron-K from 293T and Jurkat T cells using shRNA constructs that targeted various regions of citron-K (Figure S1). The 293T cells were transfected twice, first with control or citron-targeted shRNA constructs only and 24 h later, cells were co-transfected with citron-targeted shRNA constructs and pNL4GFP. Supernatant and cells were harvested 48 h after the second transfection and analyzed. We saw efficient knockdown of citron-K protein (65–90%; Figure 2A) and a 75–85% reduction in virion production by all three constructs as determined by p24 enzyme-linked immunosorbent assay (Figure 2B). *HIV-1* gene expression, as measured by cell-associated Gag, was not affected by depletion of citron-K (Figure 2A). Jurkat T cells were also co-transfected with pNL4GFP and the citron-specific shRNA or control constructs. Supernatants and cells were collected 48 h post-transfection. Virion production was inhibited by 60% in citron-K-depleted cells compared with the control (Figure 2C). The inhibition of viral particle release correlated with reduction in expression of endogenous citron-K (Figure 2D). Therefore, citron-K is required for efficient HIV-1 virion production.

The leucine zipper, Rho-binding and zinc finger domains, but not kinase activity, of citron-K are necessary for enhancing HIV-1 virion production

Deletion mutants were generated to map the domain(s) of citron-K involved in the enhancement of HIV-1 virion production. Citron-N, a naturally occurring splice variant of citron-K lacking the N-terminal kinase domain, showed similar enhancement activity as citron-K, indicating that the kinase domain was dispensable (Figure 3A). Next, we made a series of C-terminal truncation mutants in the *citron-N* gene. The PH, SH3 and PDZ domains were not required for the activity. The zinc finger motif of citron was necessary for the enhancement of virion production because deletion of the zinc finger domain from citron-N (CN Δ ZnF) abolished its ability to enhance virion production (Figure 3A). The zinc finger domain alone was unable to enhance virion production (Figure 3A). Further deletion of the N-terminal α -helix demonstrated that two copies of the leucine zipper motif and the Rho-binding domain (RBD), in addition to the zinc finger, were sufficient for citron-mediated viral

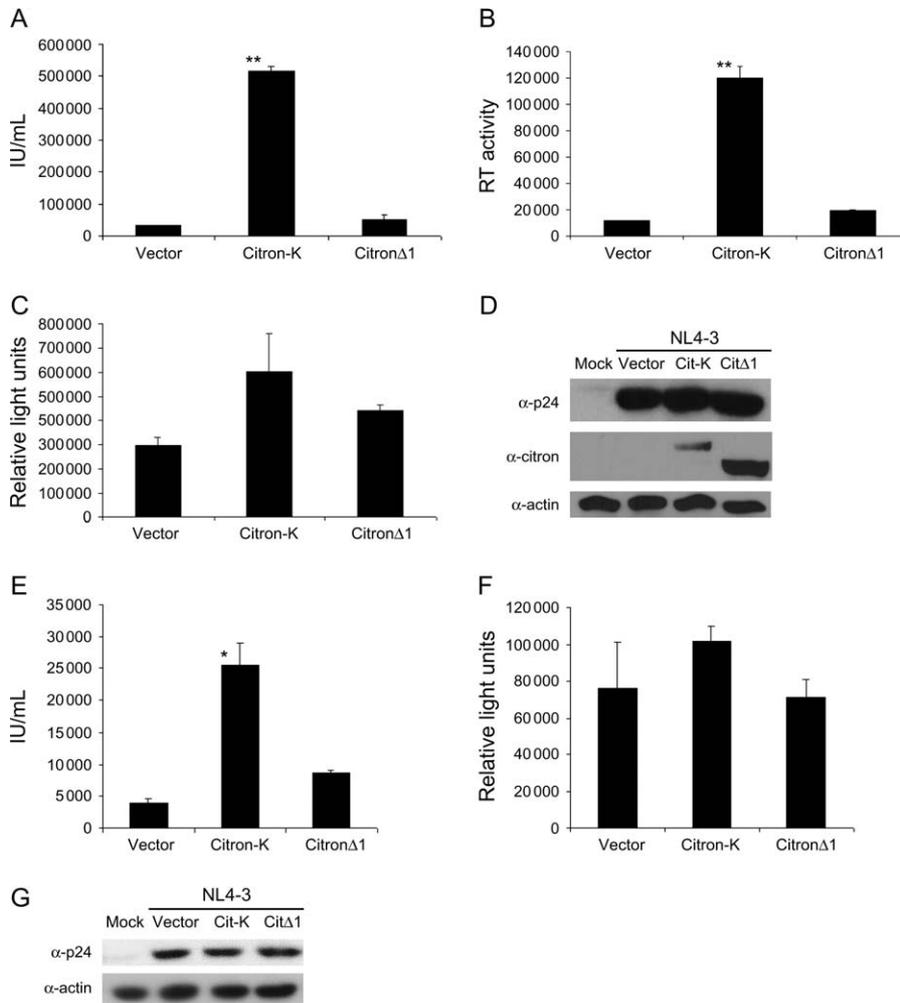


Figure 1: Citron-K preferentially enhances HIV-1 virion production. (A and B) 293T cells were co-transfected with proviral DNA NL4-3 and vector, citron-K or citronΔ1. Supernatant was harvested at 48 h post-transfection to determine infectious units/mL (A) or virion-associated RT activity (B). (C and D) 293T cells were co-transfected with pNL4.Luc and vector, citron-K or citronΔ1. Cells were harvested at 48 h post-transfection to determine luciferase activity (C). (D) Cell-associated viral Gag proteins, Myc-tagged citron-K and actin in 293T cells were analyzed using anti-p24, anti-Myc and anti-actin antibodies, respectively. (E and F) Jurkat T cells were co-transfected with pNL4-3 (E) or pNL4.Luc (F) and vector, citron-K or citronΔ1. Supernatants and cells were harvested at 48 h post-transfection to determine infectious units/mL (E) or luciferase activity (F). (G) Cell-associated viral Gag proteins (p55) and actin in Jurkat T cells were analyzed using anti-p24 and anti-actin antibodies. Error bars are standard deviation of duplicate samples and at least three independent experiments were performed. * $P < 0.05$, ** $P < 0.005$.

enhancement (Figure 3). All citron truncation mutant proteins were efficiently expressed (Figure S2A, B).

Citron-K enhances release of MLV and of HIV-1 Gag lacking the L domain

The L domain is important for retroviral release (4,5). HIV-1 viral release is dependent on the unique HIV-1 L domain to interact with the endosomal sorting protein Tsg101 and subsequently the endosomal sorting pathway (14). Release of MLV, which contains a different L domain tetrapeptide sequence (PPPY) than HIV-1 (PTAP), is unaffected by depletion of Tsg101. To determine if citron-K enhancement of virion production is HIV-1 specific, we evaluated whether citron-K was able to enhance MLV production. Citron-K enhanced MLV virion production to comparable levels as HIV-1 (Figure 4A), suggesting that citron-K has a general enhancement activity on retroviral production.

To further determine the role of HIV-1 L domain in citron-K-mediated virion production, we transfected 293T cells with Gag or GagΔp6 constructs (43). The GagΔp6 construct, which lacks the entire L domain, showed significantly

reduced virion production compared to full-length Gag (Figure 4B). Citron-K enhanced virus-like particle (VLP) production similarly from HIV-1 Gag proteins with or without the L domain (5–7 fold; Figure 4B, C). These results demonstrate that citron-K enhances HIV-1 virion production independent of the viral L domain.

Citron-K induces formation of intracellular compartments and co-localizes with HIV-1 Gag

To define a possible mechanism by which citron-K enhances HIV-1 virion production, we evaluated the subcellular localization of citron-K and HIV-1 Gag in 293T cells by confocal microscopy. Citron-K localized to unique compartments that accumulated in the cytoplasm [data not shown, previously described in (44)]. When GagGFP was expressed alone or GagGFP and Gag were co-expressed in 293T cells (Figure 5A, C), Gag exhibited cytoplasmic and plasma membrane localization, the primary site for HIV-1 release. However, when GagGFP or GagGFP/Gag were co-expressed with citron-K, Gag localized to the same dense protein compartments that expressed citron-K (Figure 5B, D). Citron-K mediated relocalization

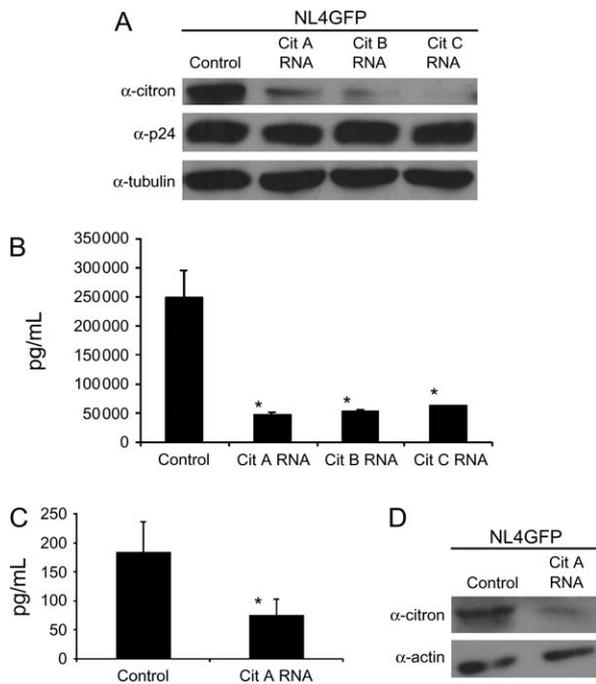


Figure 2: Depletion of endogenous citron-K inhibits HIV-1 virion production. (A and B) Knockdown of endogenous citron-K using citron-targeted shRNA constructs in 293T cells inhibited HIV-1 virion production. 293T cells were transfected with citron-targeted shRNA or control plasmid constructs, 24 h later, cells were co-transfected with pNL4GFP and citron-targeted shRNA or control plasmid constructs. Supernatant and cells were harvested 48 h after the second transfection and analyzed for cell-associated gene expression of citron-K, p24 and actin (A) and relative HIV-1 virion production in the supernatant (p24 pg/mL) (B). (C and D) Knockdown of endogenous citron-K in T cells inhibited HIV-1 virion production. Jurkat T cells were transfected with pNL4GFP and citron-targeted shRNA or control plasmid constructs. Supernatant and cells were harvested at 48 h post-transfection. The effect of citron-K knockdown on HIV-1 virion production in the supernatant was measured by p24 enzyme-linked immunosorbent assay (C). * $P < 0.05$. Cells were lysed and analyze with anti-citron and anti-actin antibodies (D).

of Gag to these citron-K-induced intracellular structures may mediate enhancement of virion production.

Citron-K enhances HIV-1 virion production by modulating late endosomal compartments and exocytosis

Next, we determined the effect of citron-K on the late endosomal and lysosomal compartments during HIV-1 replication because all retroviruses are dependent on the late endosomal sorting pathway for virion release. Cells were co-transfected with citron-K and/or HIV-1 proviral DNA (pNL4-3) and labeled with LysoTracker, a dye that marks acidic endosomal/lysosomal compartments (i.e. low internal pH). Citron-K or HIV-1 alone showed no significant effect on these compartments (Figure 6A–C). Citron-K and HIV-1, however, cooperatively enhanced the acidic late endosomal

and lysosomal compartments (Figure 6D). The results from confocal microscopy were supported by fluorescence-activated cell sorter (FACS) analysis of the LysoTracker signal (Figure 6E). These findings suggest that citron-K interacts with HIV-1 to enhance activity of the late endosomal sorting pathway to promote HIV-1 virion production.

In the endosomal sorting pathway, the MVB either fuses with the lysosome to degrade its contents or is directed to the plasma membrane where its contents are released via an exocytic pathway. Interestingly, HIV-1 virions have been shown to resemble exosomes (or exocytosed microvesicles) in both size and components (45–48). We hypothesized that citron-K modulates the exocytic pathway to promote HIV-1 virion production as well as MVB exocytosis. To test this, we purified microvesicles or exosomes secreted from cells transfected with HIV-1 proviral DNA in the presence and absence of citron-K. Our results indicate that citron-K enhanced the secretion of microvesicles with exosomal markers (i.e. hsc70, CD82 and LAMP-1), as well as HIV-1 virions, in HeLa (Figure 7A) and 293T cells (Figure 7C). The intracellular exosomal markers in the presence of citron-K and/or HIV-1 were not significantly altered (Figure 7B, D). We conclude that citron-K enhances HIV-1 virion production by promoting exocytosis of microvesicles/exosomes, including HIV-1 virions.

Discussion

We investigated the role of citron-K, a RhoA effector, in modulating HIV-1 replication. Ectopic expression of citron-K and the kinase-deficient splice variant citron-N preferentially enhanced HIV-1 virion production but not *HIV-1* gene expression. Knockdown of endogenous citron-K by shRNA inhibited HIV-1 virion production, demonstrating an important role for endogenous citron-K in viral budding. Citron-K also enhanced MLV production and the HIV-1 L domain was not required for the enhancement. Citron-K forms cytoplasmic structures (44) and, when both HIV-1 Gag and citron-K are co-expressed, Gag is colocalized to these citron-K-induced cytoplasmic structures. Further analysis of the endosomal sorting pathway indicated that citron-K, together with HIV-1, enhanced the compartments of acidic late endosomes and lysosomes. We demonstrated that production of microvesicles or exosomes and HIV-1 virions were both enhanced by citron-K. These results suggest that citron-K is involved in modulating exocytosis and HIV-1 virion production.

RhoGTPases play a central role in the dynamic regulation of the actin cytoskeleton and through this, control cell morphology, motility and adhesion (49,50). RhoGTPases act locally to control individual trafficking events, but also act globally to control the spatial organization of membrane traffic in response to cues from the extracellular environment. In addition to interacting with the endocytic pathway to modulate clathrin-independent internalization from the

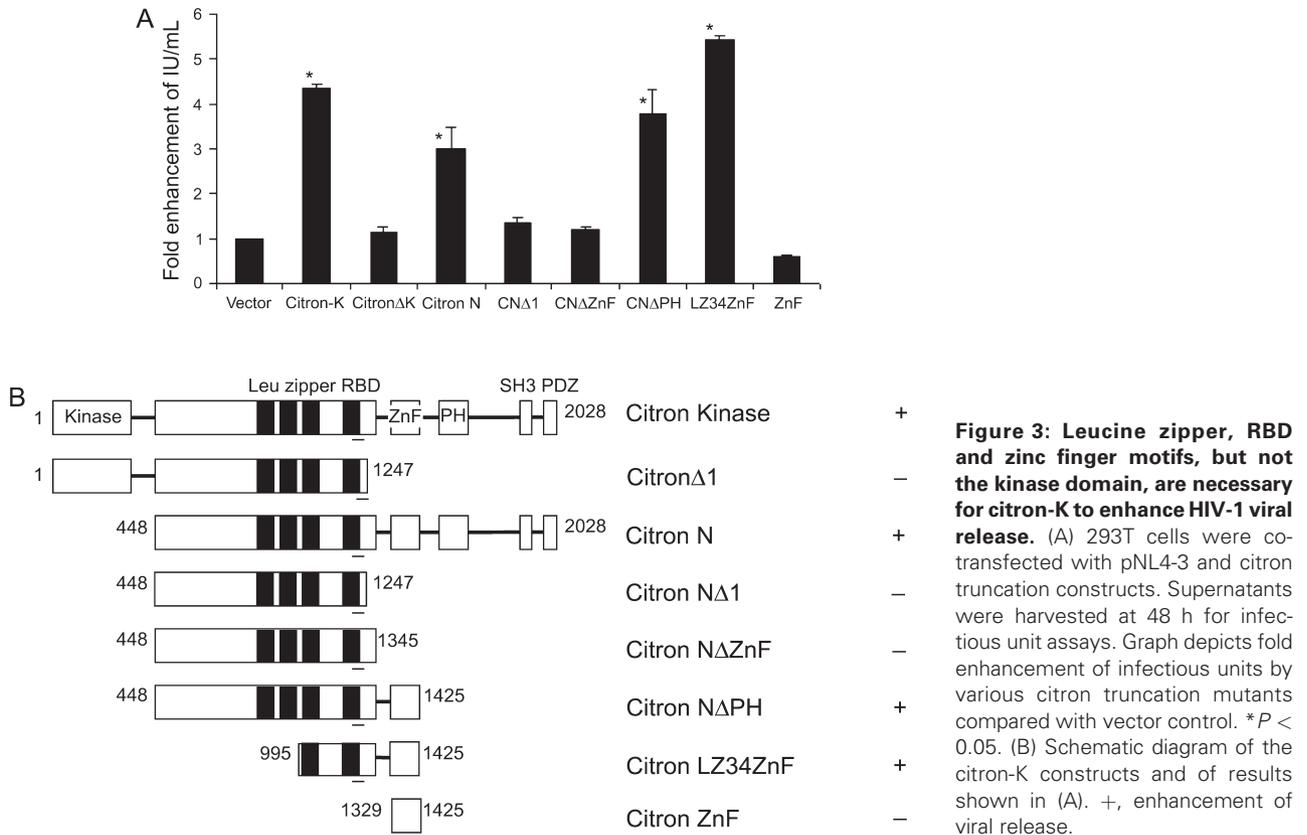


Figure 3: Leucine zipper, RBD and zinc finger motifs, but not the kinase domain, are necessary for citron-K to enhance HIV-1 viral release. (A) 293T cells were co-transfected with pNL4-3 and citron truncation constructs. Supernatants were harvested at 48 h for infectious unit assays. Graph depicts fold enhancement of infectious units by various citron truncation mutants compared with vector control. * $P < 0.05$. (B) Schematic diagram of the citron-K constructs and of results shown in (A). +, enhancement of viral release.

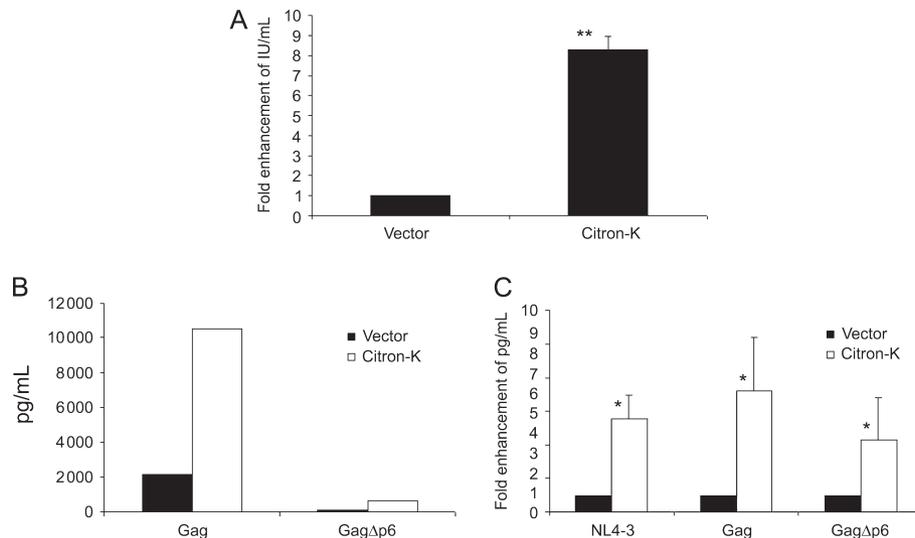


Figure 4: Citron-K enhanced release of MLV and HIV-1 GagΔp6. (A) MLV virus-like particle production was similarly enhanced by citron-K. 293T cells were co-transfected with MLV Gag-Pol, VSV-G-env and HSCG (an MLV-based retroviral vector expressing GFP) with citron-K or control vector. Supernatant was collected at 48 h post-transfection and titered for GFP expression on 3T3 cells to determine infectious units/mL. (B and C) Citron-K enhancement of virion production is L domain independent. 293T cells were co-transfected with Gag or GagΔp6 and vector or citron-K. Supernatant and cells were harvested 48 h post-transfection for analysis. (B) Citron-K enhances virion production of both HIV-1 Gag and GagΔp6. Virion production of GagΔp6 alone is significantly reduced compared with Gag but is enhanced by citron-K. (C) Citron-K similarly enhances virion production of HIV-1 Gag, GagΔp6 and NL4-3. Shown is relative enhancement of virion production of Gag, GagΔp6 and NL4-3 from two independent experiments. * $P < 0.05$, ** $P < 0.005$.

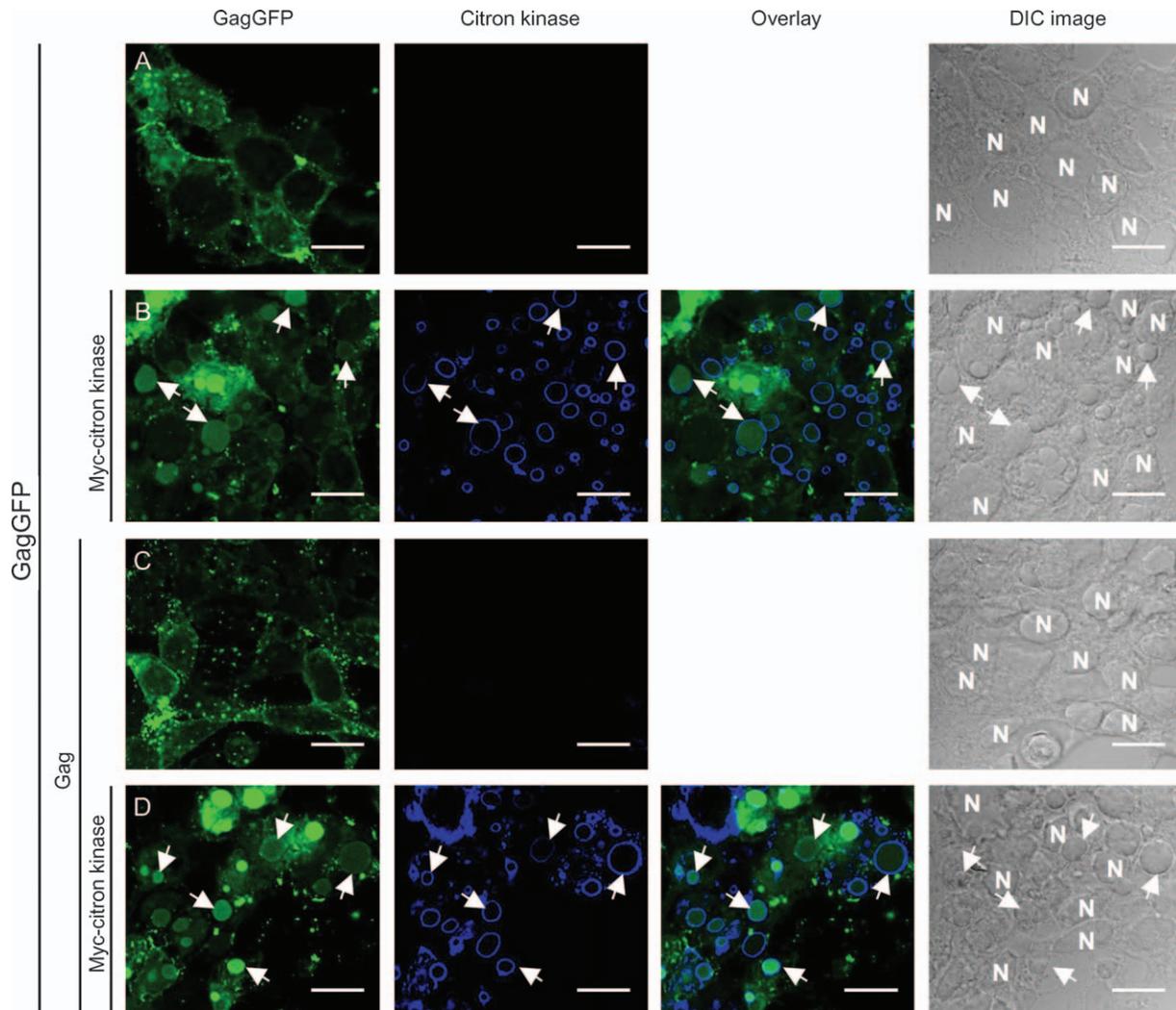


Figure 5: Citron-K and Gag colocalize to citron-K-induced cytoplasmic compartments. (A and C) 293T cells were transfected with GagGFP or GagGFP/Gag in a 10:1 ratio and protein localization was determined by confocal microscopy. GagGFP localizes primarily to the plasma membrane in the absence of exogenous citron-K. (B and D) 293T cells were co-transfected with GagGFP or GagGFP/Gag combination and citron-K. The localization of GagGFP is altered in the presence of citron-K. Instead of localizing to the plasma membrane, GagGFP localized intracellularly with citron-K into 'citron-induced cytoplasmic compartments' (indicated by arrows). N; nucleus. Bars represent 17.5 μm .

cell surface (51,52), RhoGTPases are involved in mediating trafficking/sorting decisions at a number of distinct endocytic subcompartments. For example, RhoD is localized to both the plasma membrane and early endocytic vesicles, resulting in activation of an isoform of Diaphanous and tyrosine kinase *c-Src* (53). RhoB, which is highly homologous to RhoA, is localized to the plasma membrane and the bounding membrane of MVBs (54,55) and is activated by internalized epidermal growth factor receptors, as they enter the late endosomal compartment (56). These findings suggest that RhoGTPases interface the actin cytoskeleton with endocytic trafficking events. Activated RhoA mediates a number of effectors that modulate distinct cellular processes. For example, an undefined RhoA effector inhibits *HIV-1* gene expression (38), making it difficult to evaluate

the effect of activating citron-K specifically via RhoA on *HIV-1* virion budding or production.

There are two endogenous forms of citron, the kinase form (citron-K) and the non-kinase form (citron-N). Citron-K is ubiquitously expressed in most cell types with cell cycle-dependent cellular localization (57). RhoA co-localizes with citron-K in the cell cortex during anaphase and both are enriched in the cleavage furrow and midbody during telophase (39). Citron-K modulates the contractile motion required for separation of the two daughter cells during cytokinesis through phosphorylation of the regulatory light chain of myosin II at both Ser-19 and Thr-18 (58). It is feasible that citron-K enhances virion budding from host cells in a similar process as cytokinesis. However, our

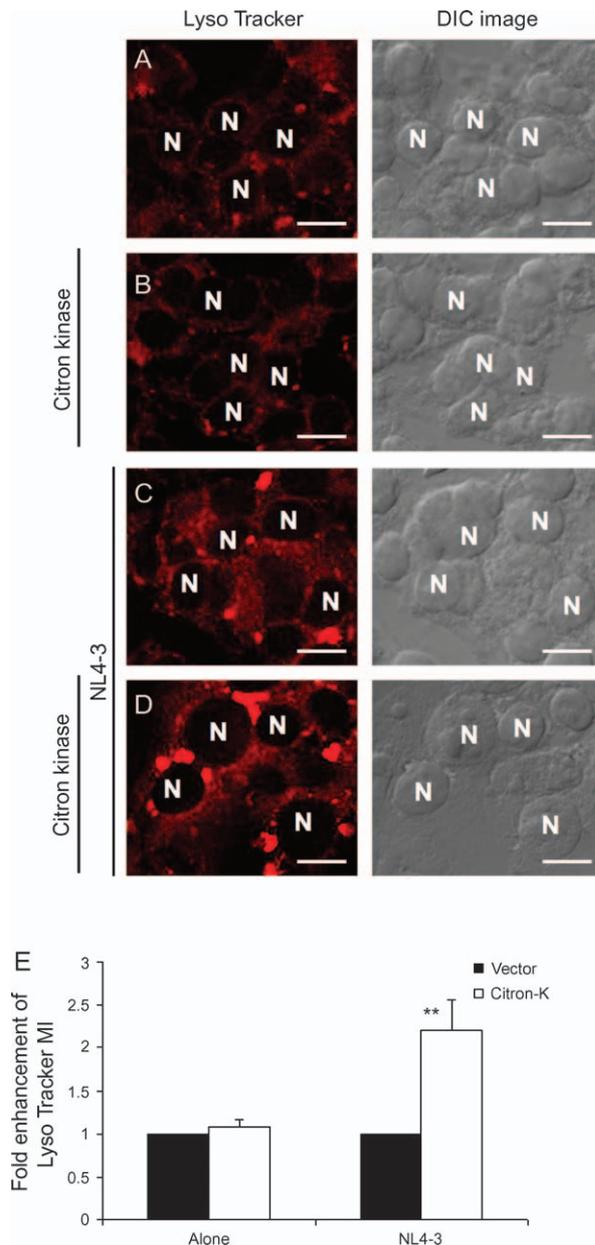


Figure 6: Citron-K and HIV-1 cooperatively enhance acidic (late) endosomal and lysosomal compartments. 293T cells were transfected with pNL4-3 and vector or citron-K. Cells were incubated with 60 μ M LysoTracker for 90 min before fixation at \sim 48 h post-transfection. (A–D) Images of LysoTracker signals were collected via confocal microscopy. N, nucleus. Bars represent 7 μ m. (E) Relative LysoTracker signals of 293T cells were measured by FACS analysis (average of four independent experiments). ** $P < 0.005$.

observation that the kinase domain is not required to enhance HIV-1 virion production indicates that a distinct mechanism is involved.

Citron-N is a splice variant of citron-K lacking the kinase domain (59). Actin polymerization and cytoskeleton are

regulated by RhoGTPases and are essential for the organization and dynamics of membrane organelles such as endosomes and the Golgi complex (40–42). Citron-N is enriched and associated with the Golgi apparatus of hippocampal neurons in culture (60). Suppression of citron-N or expression of a mutant lacking Rho-binding activity leads to dispersion of the Golgi apparatus (60), suggesting that citron-N functions as a scaffolding molecule on Golgi membranes, organizing Rho-mediated actin polymerization locally by assembling the actin polymerizing complexes together (ROCK II and profilin-Ila) (60). Citron-N also interacts with the postsynaptic scaffold protein, PSD-95/SAP-90, a member of the membrane-associated guanylate kinase protein family (MAGUK) (61,62). This interaction is important for neural plasticity and its localization at postsynaptic sites in all hippocampal neurons (61,62). Therefore, citron-N may play a role in organization of the endosomal sorting pathway and regulation of the actin cytoskeleton to facilitate exocytosis and viral release. However, neither monomeric nor polymerized actin appears to be altered by citron-K and/or HIV-1 in transfected 293T or HeLa cells (R. Loomis and L. Su, UNC-CH, Chapel Hill, NC, unpublished data).

We determined that the region of citron-K necessary for enhancement of virion production contained the leucine zipper, the RBD and the zinc finger domains. It has been speculated that the leucine zipper domain may be a scaffold for multimeric structures functioning through conformational changes and interactions with additional partners (59). The zinc finger (C_6H_2) in citron-K is believed to bind lipid second messengers or to recruit additional proteins (59). Taken together, citron-K may enhance virion production by binding or recruiting other cellular (perhaps endosomal sorting proteins) or viral components to facilitate viral release.

Recent evidence has shown that Tsg101, a component of ESCRT-I, binds specifically to the L domain of HIV-1 Gag to promote viral release (13–15). Small inhibitory RNA-mediated depletion of Tsg101 potentially blocked HIV-1 release but not MLV release (14). We showed that citron-K enhanced virion production of MLV. Citron-K also enhanced VLP production of HIV-1 Gag Δ p6, which lacks the L domain. Therefore, this citron activity probably affects a step of HIV-1 viral release that is distinct from the Tsg101-dependent step. Dominant-negative mutants of Vps4 inhibited viral release of both HIV-1 and MLV, indicating the essential role of the late endosomal pathway in mediating retroviral release (14). Co-expression of dominant-negative Vps4 mutants also blocked HIV-1 virion production in the absence or presence of citron-K (R. Loomis and L. Su, UNC-CH, Chapel Hill, NC, unpublished data). Therefore, citron-K enhanced virion production is still dependent on the late endosomal sorting pathway.

HIV-1 can bud from both the plasma membrane and internal endosomal membranes (14,18,43,63–67). Citron-K may either recruit late endosomal factors to the plasma

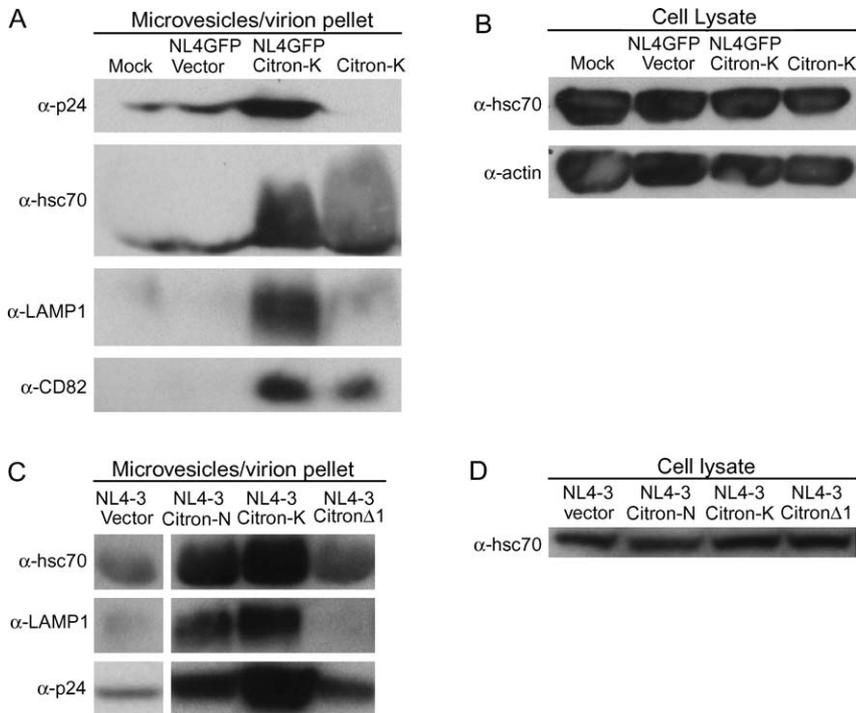


Figure 7: Citron-K enhances exocytosis. (A and B) HeLa cells or (C and D) 293T cells were co-transfected with pNL4-3 and vector or citron-K. Supernatant and cells were collected 48 h post-transfection for analysis. The microvesicles and virion pellets in the supernatant were purified and analyzed for late endosomal/exosomal markers and HIV-1 Gag p24 (A and C). Exosomal markers analyzed include hsc70, LAMP-1 and CD82. Cell-associated proteins were analyzed for late endosomal/exosomal markers (B and D).

membrane or promote utilization of the endosomal pathway for virion production. Cells transfected with both citron-K and HIV-1 lead to increased late endosome and lysosome compartments compared to cells transfected with HIV-1 or citron-K alone. Based on the localization of citron-K and colocalization of Gag, it is likely that citron-K promotes a step late in the endosomal sorting pathway to enhance virion production. HIV-1 virions share similar characteristics with exocytosed membrane microvesicles or exosomes (66,68). They are similar in size and contain exosome-specific markers, as well as common cellular membrane proteins. Although exosome production occurs more prominently in macrophages, B cells and dendritic cells, recent evidence indicates that the exosomal pathway operates in most blood cell types, including T cells (69). Our data suggest that citron-K is involved in modulating the exocytosis process to enhance HIV-1 virion production. In addition, HIV-1 may interact functionally with citron-K to modulate endosomal sorting and HIV-1 virion release.

Remarkably, citron-K^{-/-} mice are viable, although they grow at slower rates, are severely ataxic and die before adulthood as a consequence of fatal seizures (70). Since knockdown of endogenous citron-K expression in 293T and T cells inhibited HIV-1 virion production, citron-K may provide a new host target for the development of anti-HIV-1 therapeutics. In addition to affecting HIV-1 viral production, the altered release of exosomes/microvesicles may also have significant immunological consequences, as exosomes have been shown to function as antigen-presenting cells to interact with T cells (66,68,69,71).

Materials and Methods

Reagents, plasmids and cell lines

The pNL4-3 plasmid encodes the entire HIV-1 genome DNA in pUC18 (24). The pNL4.Luc.R-E plasmid was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (72). The pNL4GFP plasmid encodes Gag, Pol, tat and rev and was kindly provided by Dr Dan Littman, NYU, NY, NY. pCAG (vector), pCAG/citron-K, pCAG/citronΔ1 plasmids were previously reported (57). All citron truncation mutants were generated by polymerase chain reaction of the citron-N template with an N-terminal Myc-tag added and inserted into a retroviral vector, pHSCG (73). The control (pSUPER.Retro.Puro; OligoEngine, Seattle, WA, USA) and citron short-hairpin constructs (cit A, cit B and cit C) were a kind gift from Dr Alan Hall, University College London, London, UK. Gag and GagΔp6 constructs were a kind gift from Dr Paul Bieniasz, Aaron Diamond AIDS Research Center, NY, NY (43) and GagGFP was a kind gift from Dr Marilyn Resh, Sloan-Kettering Institute, NY, NY.

The 293T, HeLa and HeLa-MAGI cells [NIH AIDS Research and Reference Reagent Program; (73)] were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. Jurkat T cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin/streptomycin and L-glutamine. The NIH-3T3 cells were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin and L-glutamine.

HIV-1 production and replication in transfected human cells

Transient production of HIV-1 was performed by transfecting NL4-3 (0.1 μg) with pCAG, pCAG/citron-K, pCAG/citronΔ1 or citron truncation plasmids (0.2 μg) in 293T cells in 48-well plates at 2.5×10^4 cells/well using Effectene (Qiagen, Santa Clarita, CA, USA) or NL4-3 (0.3 μg) with pCAG, pCAG/citron-K, pCAG/citronΔ1 or citron truncation plasmids (0.6 μg) in Jurkat T cells in 48-well plates at 4×10^5 cells/well using GenePorter (Gene Therapy Systems, San Diego, CA, USA). At 48 h post-transfection, HIV-1 virions in cell supernatant were measured by p24 or RT assays (74) and infectious units were determined by titering the supernatant on HeLa-CD4-LTR-lacZ cells (MAGI) as described previously (73).

To analyze *HIV-1* gene expression in transfected cells, pNL4.Luc (0.1 μ g) was co-transfected into 2.5×10^4 293T cells/well together with pCAG, pCAG/citron-K, pCAG/citron Δ 1 or citron truncation plasmids (0.2 μ g) or pNL4.Luc (0.3 μ g) was co-transfected into 4×10^5 Jurkat T cells/well with pCAG, pCAG/citron-K, pCAG/citron Δ 1 or citron truncation plasmids (0.6 μ g). Cell extracts were analyzed at 48 h after transfection for luciferase activity (EG&G berthold, Oak Ridge, TN and FluoStar Optima; BMG LabTech, Durham, NC). In some experiments, we used pGag, pGag Δ p6 or pNL4GFP, in place of NL4-3 or pNL4.Luc.

Western blotting

Transfected 293T or Jurkat T cells were lysed and resolved (50–100 μ g of total protein) on a 10% SDS–PAGE. Gels were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ) and blocked with 5% non-fat dry milk. The membrane was probed with mouse anti-p24 (NIH AIDS Research and Reference Reagent Program), mouse anti-Myc (AbCam, Cambridge, MA, USA), rabbit anti-citron [a kind gift from Dr Lola Reid, UNC-CH, as described in (75)], mouse anti-actin (Sigma-Aldrich, St Louis, MO), mouse anti-tubulin, mouse anti-hsc70 (BD Biosciences, San Jose, CA), mouse anti-LAMP1 (Santa Cruz, Santa Cruz, CA) or mouse anti-CD82 (BD Biosciences, San Jose, CA) specific antibodies and visualized using an enhanced chemiluminescence Kit (Amersham Biosciences, Piscataway, NJ).

shRNA knockdown of citron-K

The citron-K shRNA and control constructs are in the pSUPER.Retro.Puro vector from OligoEngine. The citron-K shRNA constructs target different regions of citron-K. Cit A RNA targets the C-terminus of citron-K beginning at base pair 6085, Cit B and Cit C RNA target the N-terminus of citron-K beginning at base pair 359 and 244, respectively, (Figure S1). For experiments in 293T cells, 293T cells were plated in a 24-well plate at 5×10^4 cells/well and transfected with 0.4 μ g control or citron-targeted shRNA constructs using Effectene. Twenty-four hours later, another transfection was performed with 0.1 μ g pNL4GFP and 0.2 μ g control or citron-targeted shRNA constructs. Two days after the second transfection, supernatants and cells were harvested for further analysis. For experiments in Jurkat T cells, 0.3 μ g of pNL4GFP and 0.6 μ g of control or citron-targeted shRNA constructs were transfected via GenePorter into 4×10^5 cells/well. Supernatants and cells were harvested at 48 h post-transfection for further analysis.

MLV production in transfected human cells

The 293T cells were plated in a 6-well plate at 4×10^5 cells/well and transfected with 2 μ g HSCG (a retroviral vector with MSCV LTR, 34), 1.5 μ g vesicular stomatitis virus-glycoprotein (VSV-G), 1.5 μ g murine leukemia virus (MLV) Gag-Pol and 2 μ g vector or citron-K DNA using the calcium phosphate transfection method as described previously (76,77). Retrovirus was harvested 48 h after transfection. Virus was titered by seeding NIH-3T3 cells at 5×10^4 cells/well in a 24-well plate, adding serial dilution of virus and counting GFP-positive cells 48 h after infection on the Guava EasyCyte (Guava Technologies, Hayward, CA).

Confocal microscopy

The 293T cells were plated on coverslips at 1×10^5 cells/well in a 24-well plate and transfected as described previously (Effectene, Qiagen). For the GagGFP confocal experiments, we used 0.2 μ g of GagGFP, 0.02 μ g vector, 0.4 μ g vector or citron-K and for the samples with wild-type Gag – 0.2 μ g of GagGFP, 0.02 μ g of Gag and 0.4 μ g vector or citron-K were used in the transfections. At 48 h post-transfection, cells were fixed with 4% paraformaldehyde for 15 min at 4°C, washed once with cold 1 \times PBS, permeabilized with ice-cold methanol for 30 seconds, washed once with cold 1 \times PBS, washed three times with Quench Buffer (1% milk, 150 mM NaOAc pH 7, 1 \times PBS), washed three times with wash buffer (1% milk, 1 \times PBS), incubated with primary antibody for 1 h at room temperature, washed three times with wash buffer, incubated with secondary antibody for 1 h at room temperature, washed five times with wash buffer and mounted on slide with polyvinyl alcohol. The primary antibody used was chicken anti-Myc (Molecular Probes,

Carlsbad, CA) at 1:1000 dilution. The secondary antibody used was anti-chicken-Cy5 (Molecular Probes, Carlsbad, CA) at 1:200 dilution.

For LysoTracker experiment, cells were incubated with 60 μ M LysoTracker (Molecular Probes, Carlsbad, CA) for 90 min then fixed with 4% paraformaldehyde 48 h post-transfection. Images were collected using Olympus confocal microscope and software.

FACS

Each sample ($\sim 5 \times 10^5$ cells) that had been incubated with 60 μ M LysoTracker (Molecular Probes, Carlsbad, CA) for 90 min were washed in PBS with 2% FBS and stained with 7AAD (1 μ L/mL) for 15 min on ice, then rinsed and resuspended in PBS with 2% FBS. Cells were analyzed using FACScan (Becton Dickinson, San Jose, CA).

Purification of exosomal/microvesicles/HIV-1 virions

Transfections were performed as described previously (Effectene, Qiagen), except in 100-mm dishes using 1×10^6 cells/plate of 293T cells or 5×10^5 cells/plate of HeLa cells. The FBS used to culture the cells was depleted of exosomes or microvesicles by spinning at 100 000 $\times g$ overnight at 4°C. Supernatant (10 mL) were collected at 48 h post-transfection. The exosome purification protocol is similar to those described previously (66,71). At the time of collection, supernatants were spun at 800 $\times g$ for 10 min to remove cells and large cell debris. Supernatants were then added to Beckman Ultra Clear Centrifuges (11 \times 89 mm) with 2 mL 25% sucrose. Tubes were spun in the Beckman L7 Ultracentrifuge at 100 000 $\times g$ for 2 h at 4°C. Supernatant was discarded and exosomal pellet was resuspended in 1% Triton-X-100 and analyzed by SDS–PAGE.

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Supplementary Materials

Figure S1: Citron-K-targeted shRNA constructs. All citron-K shRNA constructs were made in the pSUPER.Retro.Puro vector. The start site base pairs are from the beginning of the coding sequence. Italics, target sequence, Underline, hairpin.

Figure S2: Protein expression of citron-K mutants. (A and B) Expression of citron constructs was verified by analyzing cell-associated proteins with anti-Myc, anti-p24 and anti-actin antibodies. All citron constructs are Myc-tagged at the N-terminus.

Supplemental materials are available as part of the online article at <http://www.blackwell-synergy.com>

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