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ITCH E3 Ubiquitin Ligase Interacts with Ebola Virus VP40 To Regulate Budding

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ABSTRACT

Ebola virus (EBOV) and Marburg virus (MARV) belong to the *Filoviridae* family and can cause outbreaks of severe hemorrhagic fever, with high mortality rates in humans. The EBOV VP40 (eVP40) and MARV VP40 (mVP40) matrix proteins play a central role in virion assembly and egress, such that independent expression of VP40 leads to the production and egress of virus-like particles (VLPs) that accurately mimic the budding of infectious virus. Late (L) budding domains of eVP40 recruit host proteins (e.g., Tsg101, Nedd4, and Alix) that are important for efficient virus egress and spread. For example, the PPxY-type L domain of eVP40 and mVP40 recruits the host Nedd4 E3 ubiquitin ligase via its WW domains to facilitate budding. Here we sought to identify additional WW domain host interactors and demonstrate that the PPxY L domain motif of eVP40 interacts specifically with the WW domain of the host E3 ubiquitin ligase ITCH. ITCH, like Nedd4, is a member of the HECT class of E3 ubiquitin ligases, and the resultant physical and functional interaction with eVP40 facilitates VLP and virus budding. Identification of this novel eVP40 interactor highlights the functional interplay between cellular E3 ligases, ubiquitination, and regulation of VP40-mediated egress.

IMPORTANCE

The unprecedented magnitude and scope of the recent 2014-2015 EBOV outbreak in West Africa and its emergence here in the United States and other countries underscore the critical need for a better understanding of the biology and pathogenesis of this emerging pathogen. We have identified a novel and functional EBOV VP40 interactor, ITCH, that regulates VP40-mediated egress. This virus-host interaction may represent a new target for our previously identified small-molecule inhibitors of virus egress.

Filoviruses continue to cause severe outbreaks of hemorrhagic fever in humans, and there are currently no approved vaccines or therapeutics to combat Ebola virus (EBOV) and Marburg virus (MARV) infections. A better understanding of the interplay between EBOV and host cells will provide new insights into EBOV pathogenesis and identify novel targets for antiviral intervention. EBOV VP40 (eVP40) is the major virion structural protein that plays a crucial role in the assembly and budding of both virus-like particles (VLPs) and infectious virions. Indeed, eVP40 recruits multiple host proteins to facilitate late stages of virion assembly and egress (1–9). For example, the well-described late (L) budding domain motifs (PTAP and PPxY) of eVP40 mediate the recruitment of ESCRT and ESCRT-associated proteins that facilitate complete and efficient virus-cell separation (2, 4, 10–16).

The PPxY core motif recruits WW domain-bearing proteins with diverse functions (17–25). In previous work, we and others demonstrated that the viral PPxY motif within eVP40, MARV VP40 (mVP40), and other viral matrix proteins interacts specifically with WW domains of host Nedd4, a HECT family E3 ubiquitin (Ub) ligase that is linked with the cellular ESCRT machinery (1, 3, 5, 8, 9, 26–43). In general, viral PPxY/Nedd4 WW domain interactions promote the ubiquitinylation of viral matrix proteins, which is beneficial for efficient virus production (3, 8, 9, 28–31, 35–38, 40–50).

Although the PPxY core motif is important for this interaction, there is a built-in degree of specificity of PPxY/WW domain binding such that specific PPxY-containing proteins will interact physically and functionally with only select WW domain partners (51). In addition, although it is clear that Nedd4 is important for budding, the importance of other WW domain-containing host proteins for virus budding remains to be determined.

To further identify the complement of WW domain proteins capable of binding to the eVP40 PPxY motif, we used an unbiased approach in which we assessed the ability of an EBOV PPxY-containing peptide to bind to a glutathione *S*-transferase (GST) array of 115 mammalian proteins known to contain one or more WW domain modules (52). Using this technique, we identified ITCH, a HECT family E3 ubiquitin ligase. We used coimmunoprecipitation (co-IP) to confirm the previously undescribed PPxY-dependent physical interaction between eVP40 and ITCH and, importantly, demonstrated a functional role for ITCH in eVP40 budding. Indeed, the expression of ITCH not only led to the ubiquitination of eVP40 but also was required for efficient PPxY-mediated egress of eVP40 VLPs and live recombinant vesic-

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ular stomatitis virus (VSV) expressing the EBOV VP40 PPxY L domain motif (VSV-M40) (14).

MATERIALS AND METHODS

Cell lines, plasmids, reagents, and viruses. HEK293T and BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml)-streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator. Human wild-type (WT) HAP1 cells (kindly provided by K. Chandran, Albert Einstein College of Medicine, New York, NY) and HAP1-ITCH$^{-/-}$ cells (Horizon Discovery) were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and penicillin (100 U/ml)-streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 incubator.

Plasmids expressing wild-type eVP40 (eVP40-WT) and eVP40-ΔPT/PY (PTAPPEY deletion mutant) were described previously (3, 32, 53). Plasmids expressing c-*myc*–ITCH-WT or the enzymatically inactive ITCH-C830A mutant were kindly provided by G. Melino (Leicester University, UK) and were described previously (54). ITCH-specific or random small interfering RNAs (siRNAs) were purchased from Dharmacon. the Jun N-terminal kinase 1 (JNK1) inhibitor SP600125 was obtained from Sigma-Aldrich. Mouse anti-c-Myc monoclonal antibody (MAb) clone 4A6 (catalog number 05-724) was purchased from EMD Millipore. Rabbit anti-ITCH (catalog number SAB4200036), mouse anti-GST (catalog number SAB4200237), mouse antihemagglutinin (anti-HA) (catalog number H9658), and mouse anti-β-actin (catalog number A1978) antisera were obtained from Sigma-Aldrich. Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5) was obtained from Abcam (catalog number ab8245). Anti-VSV-M MAb 23H12 was kindly provided by D. Lyles (Wake Forest School of Medicine, NC). Recombinant VSV-M40 was propagated in BHK-21 cells and was described previously (14).

Protein array experiments. To generate the "proline-rich" reading array, the WW and SH3 domains were codon optimized for bacterial expression and cloned into a pGex vector. All WW and SH3 domains were expressed as GST fusions in *Escherichia coli* and purified on glutathione-Sepharose beads. The recombinant domains were arrayed onto nitrocellulose-coated glass slides (OncyteAvid slides; Grace Bio-Labs, Bend, OR), using an Aushon 2470 arrayer with solid pins, as described previously (52). Fluorescence labeling of the biotinylated peptide probe and slide binding were also described previously (52). Two peptides were tested on the array: eVP40-WT (MRRVILPTAPPEYMEAI[Lys-biotin]) and eVP40 mutant (MRRVILPTAAAEAMEAI[Lys-biotin]) peptides. The fluorescent signal was detected by using a GeneTac LSIV scanner (Genomic Solutions).

Expression and purification of GST fusion proteins. GST-WW domain fusion proteins were purified from *E. coli* BL21(DE3) cells grown in LB broth with appropriate antibiotics at 37°C. GST-WW domain fusion proteins were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM) for 3 h at 30°C. Bacterial cultures were centrifuged at 5,000 rpm for 10 min at 4°C, and lysates were extracted by using B-PER bacterial protein extract reagent according to the protocol supplied by the manufacturer (Pierce). GST-WW domain fusion proteins were purified with glutathione-Sepharose 4B and eluted with elution buffer (100 mM Tris-Cl [pH 8.0], 120 mM NaCl, 30 mM reduced glutathione). Purified proteins were analyzed on SDS-PAGE gels and stained with Coomassie blue.

GST peptide pulldown. Streptavidin-agarose beads (25 μl) (Millipore) were prewashed once with 1× mild buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40 [NP-40], 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$), and 15 μg of biotinylated peptide was incubated with prewashed streptavidin beads in 500 μl of 1× mild buffer for 1 h at 4°C with rocking. The beads were then washed three times with mild buffer. Two to three micrograms of the indicated GST-WW domain fusion protein was incubated with the bound peptides in 500 μl of 1× mild buffer for 1 h at 4°C with rocking, and the beads were then washed three times with 1× mild buffer. The beads were suspended with 30 μl of 2× loading buffer with boiling. Ten microliters of supernatants was analyzed by SDS-PAGE and Western blotting with mouse anti-GST antiserum followed by horseradish peroxidase (HRP)-conjugated anti-mouse IgG.

siRNA analysis. HEK293T cells in Opti-MEM in collagen-coated 6-well plates were transfected twice with either control siRNAs or ITCH-specific siRNAs at a final concentration of 200 nM by using Lipofectamine (Invitrogen) at 2-day intervals. A total of 0.5 μg of eVP40 plasmid DNA was transfected with the second round of siRNAs. Cell extracts and VLPs were harvested at 24 h posttransfection, and the indicated proteins were detected in cell and VLP samples by Western blotting using specific antisera.

IP/Western analysis. Human HEK293T cells were transfected with the indicated plasmids by using Lipofectamine reagent (Invitrogen) according to the supplier's protocol. Cells were harvested and lysed in nondenaturing buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 1.0% NP-40, 2.0 mM EDTA, 2.0 mM EGTA, and 10% glycerol) at 18 to 20 h posttransfection. Cell lysates were clarified for 10 min at 3,000 rpm. Supernatants were incubated with anti-eVP40 or normal IgG (Cell Signaling) for 5 h at 4°C. Protein A-agarose beads (Invitrogen) were added to the samples and incubated with agitation overnight at 4°C. The beads were washed five times in nondenaturing lysis buffer, suspended in loading buffer with boiling, and then fractionated by SDS-PAGE. The indicated proteins were detected in precipitates by Western blotting using specific antisera.

VLP budding assays. Filovirus VLP budding assays using HEK293T cells and eVP40 only were described previously (3, 6, 29, 32, 55).

Virus infection and titration. ITCH-WT or HAP1 ITCH knockout (KO) cells were infected with recombinant VSV-M40 at a multiplicity of infection (MOI) of 0.1 for 1 h. The inoculum was removed, cells were washed three times with phosphate-buffered saline (PBS), and cells were then incubated in serum-free Opti-MEM for an additional 7 h. At 8 h postinfection, virions were harvested from the medium, and titers were determined by standard plaque assays on BHK-21 cells. Briefly, BHK-21 cells in 6-well plates were washed once with PBS, inoculated with 200 μl of 10-fold serial dilutions of virus in serum-free DMEM in triplicate, and incubated for 1 h. The inoculum was removed, and cells were washed three times with PBS and then incubated with 2 ml of Eagle's MEM containing 5% fetal bovine serum (FBS) and 1% methylcellulose at 37°C for 36 to 48 h until plaques were observed. Cells were washed twice with PBS, fixed with methanol, and stained with a crystal violet solution. Infected cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). VSV M protein was detected by SDS-PAGE and Western blotting using anti-VSV-M monoclonal antibody 23H12.

RESULTS

Binding of eVP40 PPxY to host WW domain arrays. To identify WW domain-bearing host proteins capable of interacting with the proline-rich PPxY motif of eVP40, we prepared biotinylated peptides harboring either the WT PPxY motif (MRRVILPTA**PPEY**MEAI) or a mutated PPxY motif (MRRVILPTA**AAEA**MEAI) (motifs are indicated in boldface type). The biotinylated peptides were fluorescently labeled and used to screen a specially prepared proline-rich reading array composed of almost all known WW domain-containing (115 domains) and a large number of SH3 domain-containing (40 domains) proteins. The WT eVP40 PPxY motif-containing peptide bound robustly to select WW domains, including, but not limited to, Nedd4 (WW domain 3 [WW3], as expected), Nedd4L (WW3), and ITCH (WW1) (Fig. 1A). As expected, no interactions were observed between the WT eVP40 peptide and any of the SH3 domains (data not shown). In addition, no interaction was observed between the PPxY mutant peptide and any of the WW or SH3 domains (data not

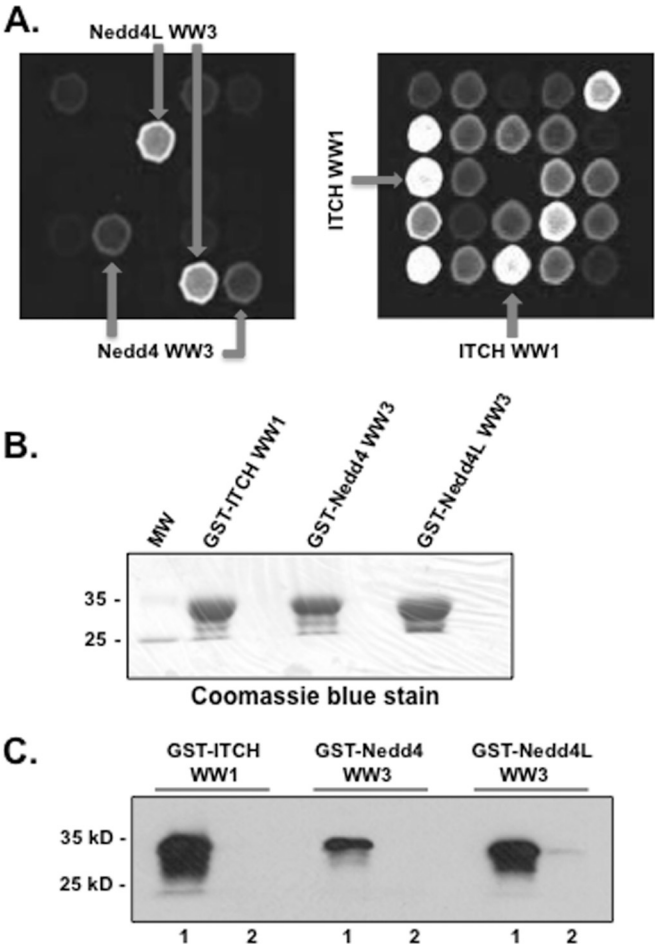

FIG 1 GST-WW domain pulldown of the eVP40 PPxY peptide. (A) Identification of eVP40 PPxY motif readers using fluorescently labeled biotinylated peptides to screen a proline-rich reading array. The GST-WW domain fusion proteins are arrayed in duplicate, at different angles. Robust interactions with multiple WW domains were observed, including those of Nedd4, Nedd4L, and ITCH (arrows). (B) The indicated GST-WW domain fusion proteins were purified from *E. coli* and analyzed in Coomassie-stained SDS-PAGE gels. MW, molecular weight (in thousands). (C) Biotinylated eVP40-WT (MRRVILPTA PPEYMEAI[Lys-biotin]) (lanes 1) or eVP40 mutant (MRRVILPTAAAEAME AI[Lys-biotin]) (lanes 2) peptides were used to pull down the indicated GST-WW domain fusion proteins. GST-WW domain fusion proteins were detected by Western blotting with mouse anti-GST antibody.

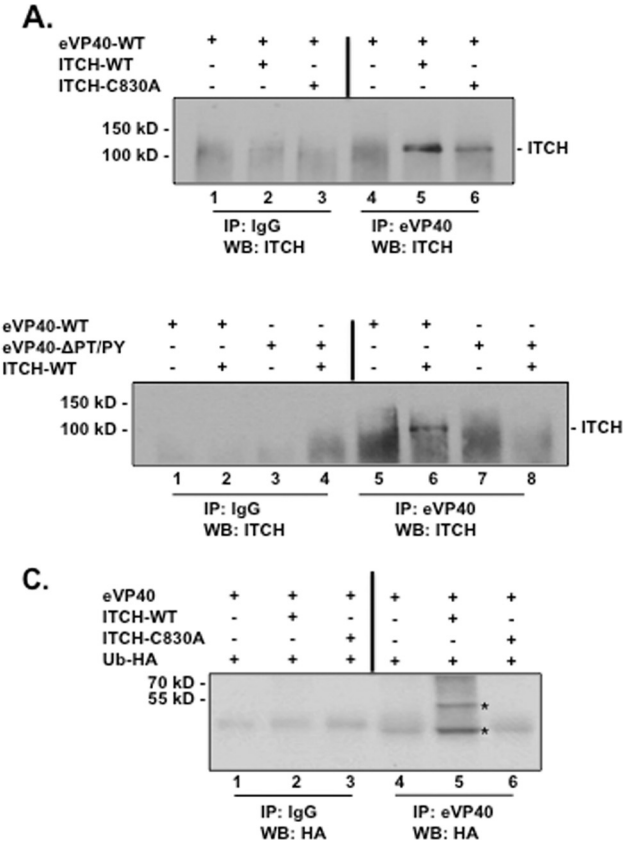

FIG 2 ITCH interacts with eVP40 in a PPxY-dependent manner. (A and B) Extracts from HEK293T cells transfected with the indicated plasmids were first immunoprecipitated (IP) with either rabbit preimmune serum (IgG) or polyclonal anti-eVP40 antiserum, and c-*myc*-tagged ITCH was then detected in precipitated samples by Western blotting (WB) using anti-c-*myc* antiserum. (C) Extracts from HEK293T cells transfected with the indicated plasmids were first immunoprecipitated with either rabbit preimmune serum (IgG) (lanes 1 to 3) or polyclonal anti-eVP40 antiserum (lanes 4 to 6), and proteins modified by HA-tagged ubiquitin (Ub) were then detected in precipitated samples by Western blotting using anti-HA antiserum.

shown). We confirmed these three specific interactions using purified GST-WW domain fusion proteins (Fig. 1B) and a GST pulldown assay to demonstrate that the WT eVP40 peptide (Fig. 1C, lanes 1), but not the PPxY mutant peptide (Fig. 1C, lanes 2), interacted with WW1 from ITCH, WW3 from Nedd4, and WW3 from Nedd4L. These findings underscore the high degree of specificity possessed by the eVP40 PPxY motif, suggesting that the newly identified eVP40 interactor ITCH is likely to play an important biological role in the EBOV life cycle, as the PPxY motif targets and binds to only select host WW domains.

Co-IP of eVP40 and ITCH. We next sought to confirm the above-described eVP40-ITCH interaction in mammalian cells. Briefly, HEK293T cells were transfected with eVP40-WT, ITCH-WT, and/or an enzymatically inactive ITCH-C830A mutant (Fig. 2A). Cell extracts were immunoprecipitated with either rabbit preimmune IgG (Fig. 2A, lanes 1 to 3) or polyclonal anti-eVP40 antiserum (Fig. 2A, lanes 4 to 6), and c-*myc*-tagged ITCH was detected in precipitated samples by Western blotting using anti-c-*myc* antiserum. Both ITCH-WT and ITCH-C830A were detected in eVP40 precipitates (Fig. 2A, lanes 5 and 6) but not in preimmune IgG precipitates of identical samples (Fig. 2A, lanes 2 and 3). These results indicated that eVP40-WT interacts with both WT and enzymatically inactive forms of ITCH in transiently transfected HEK293T cells, albeit the interaction between eVP40 and ITCH-C830A was reduced slightly compared to that between eVP40 and ITCH-WT (Fig. 2A).

We next sought to determine whether this interaction was dependent on the eVP40 PPxY motif. To this end, HEK293T cells were transfected with eVP40-WT, eVP40-ΔPT/PY, and/or ITCH-WT (Fig. 2B). Cell extracts were immunoprecipitated with either rabbit preimmune IgG (Fig. 2B, lanes 1 to 4) or polyclonal anti-eVP40 antiserum (Fig. 2B, lanes 5 to 8), and c-*myc*-tagged ITCH was detected in precipitated samples by Western blotting using anti-c-*myc* antiserum. ITCH was detected in eVP40 precipitates from cells expressing eVP40-WT and ITCH-WT (Fig. 2B, lane 6) but

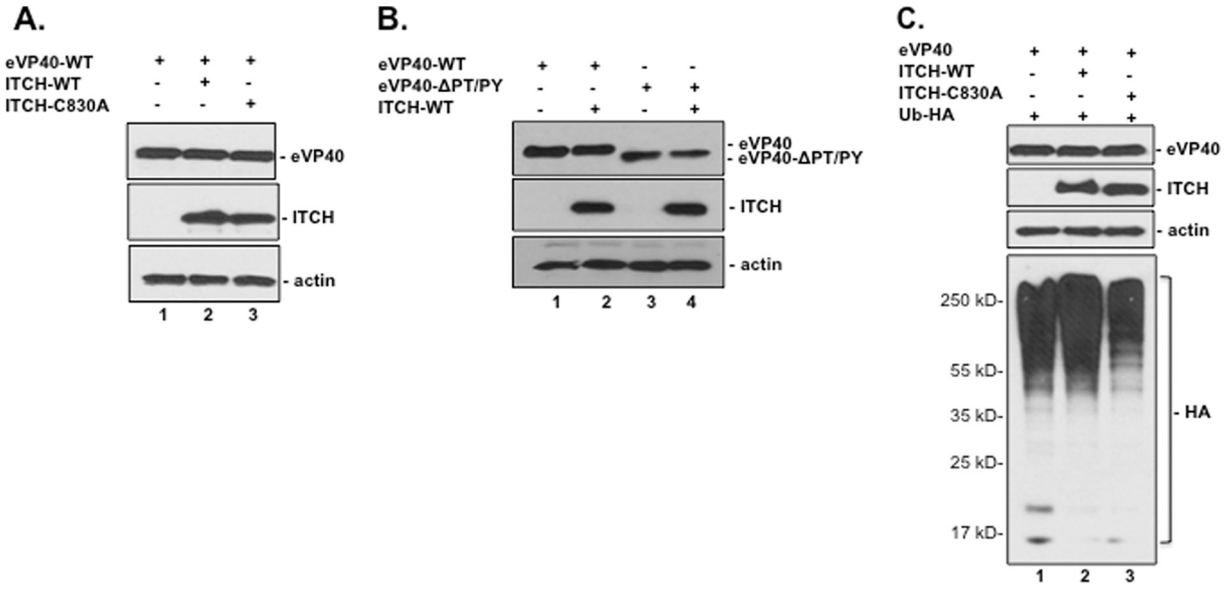

FIG 3 Western analysis of expression controls for the indicted proteins from cell extracts shown in Fig. 2. Extracts from HEK293T cells were transfected with the indicated plasmids.

not in those from cells expressing eVP40-ΔPT/PY and ITCH-WT (Fig. 2B, lane 8). As expected, ITCH was not detected in preimmune IgG precipitates of identical samples (Fig. 2B, lanes 2 and 4). These results indicate that the eVP40-ITCH interaction is PPxY dependent, which correlates well with GST pulldown results (Fig. 1).

Since ITCH interacts with eVP40, we asked whether eVP40 was ubiquitinated by ITCH. To test this, HEK293T cells were transfected with combinations of plasmids encoding eVP40, ITCH-WT, ITCH-C830A (inactive mutant), and/or Ub-HA (Fig. 2C). Cell extracts were immunoprecipitated with either rabbit preimmune IgG (Fig. 2C, lanes 1 to 3) or polyclonal anti-eVP40 antiserum (Fig. 2C, lanes 4 to 6), and HA-tagged ubiquitin was detected in precipitated samples by Western analysis. Ubiquitinated species of eVP40 that corresponded in size to mono- and diubiquitinated forms were detected in cells expressing eVP40, ITCH-WT, and Ub-HA (Fig. 2C, lane 5) but not in cells expressing either eVP40 and Ub-HA (lane 4) or eVP40, ITCH-C830A, and Ub-HA (lane 6). Together, these results demonstrate a physical and functional interaction between ITCH and eVP40 leading to the ubiquitination of eVP40. Western analysis of cell extracts corresponding to those shown Fig. 2A to C revealed appropriate expression levels of eVP40, eVP40-ΔPT/PY, ITCH-WT, ITCH-C830A, and actin (Fig. 3A to C).

Enzymatically active ITCH ligase enhances eVP40 VLP budding. Next, we sought to confirm that overexpression of enzymatically active ITCH enhances the egress of eVP40 VLPs. To this end, HEK293T cells were transfected with constant amounts of eVP40 and increasing amounts of either ITCH-WT or ITCH-C830A plasmid DNA (Fig. 4A). Cell extracts and VLPs were harvested, and the indicated proteins were detected by Western analysis (Fig. 4A). Interestingly, we observed a consistent 2- to 3-fold increase (Fig. 4B) in eVP40 VLP budding in the presence of exogenous ITCH-WT (Fig. 4A, compare lane 2 with lanes 3 and 4) but not in the presence of exogenous ITCH-C830A (compare lane 2 with lanes 5 and 6) compared to the eVP40-alone control. Indeed, overexpression of ITCH-C830A appeared to have a dominant negative effect on the budding of eVP40 VLPs, as levels of eVP40 in VLP samples expressing ITCH-C830A were reduced compared to those in samples expressing eVP40 alone (Fig. 4A). We also detected ITCH-WT, but not ITCH-C830A, in budding VLPs (Fig. 4A), which supports a functional role for ITCH in budding, as other host proteins important for budding (e.g., Nedd4 and Tsg101) are also packaged into VLPs (14, 32).

We used a pharmacological approach to demonstrate that the activation of ITCH ligase was critical for the enhanced egress of eVP40 VLPs. Activation of ITCH ligase depends on phosphorylation by Jun amino-terminal kinase (JNK) (56). As SP600125 is a well-described, selective inhibitor of JNK activity (57, 58), we reasoned that SP600125 may inhibit the egress of eVP40 VLPs by blocking JNK-mediated activation of ITCH. To test this, HEK293T cells were transfected with eVP40 in the absence or presence of increasing amounts of SP600125, and eVP40 in cell extracts and VLPs was detected by Western analysis (Fig. 4C). Indeed, we found that budding of eVP40 VLPs was reduced by ~10- and 20-fold in the presence of 5 and 10 μM SP600125, respectively (Fig. 4C, compare lane 2 with lanes 3 and 4). SP600125 had little (<2-fold) to no effect on the cellular expression of eVP40 and endogenous ITCH (Fig. 4C). Together, these data not only confirm that enzymatically active ITCH is required for the efficient egress of eVP40 VLPs but also imply that a pharmacological approach targeting this host pathway may represent a viable and novel strategy to target and inhibit virus egress.

ITCH is required for efficient eVP40 VLP egress. To further confirm that the interaction between eVP40 and ITCH was functionally relevant for eVP40 VLP budding, we used an siRNA knockdown/rescue approach. Briefly, HEK293T cells were mock transfected or transfected with eVP40-WT plus random or ITCH-specific siRNAs (Fig. 5A). Expression of eVP40-WT and efficient knockdown of endogenous ITCH (>90%) were confirmed by Western analysis (Fig. 5A). We found that the knockdown of en-

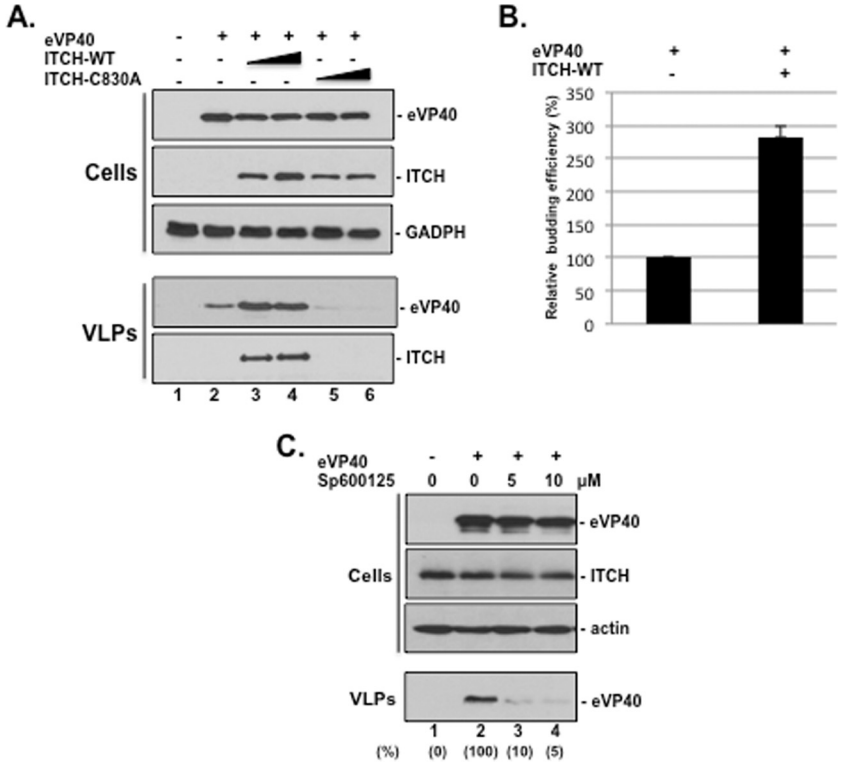

FIG 4 Enzymatically active ITCH enhances eVP40 VLP budding. (A) Western analysis of cell extracts and VLPs from HEK293T cells transfected with the indicated plasmids. (B) Budding efficiency of eVP40 VLPs in the presence of exogenous ITCH-WT relative to that with eVP40 alone. Error bars represent the standard deviations of the means from three independent experiments. C) Western analysis of cell extracts and VLPs from HEK293T cells mock transfected or transfected with eVP40 in the absence (0) or presence of SP600125 at a concentration of 5 or 10 μM. The budding efficiencies of eVP40 in VLPs relative to the control sample (lane 2) (100%) are shown in parentheses.

dogenous ITCH resulted in a 75 to 80% decrease in the egress of eVP40 VLPs compared to random siRNA controls (Fig. 5A).

To validate these siRNA results, we attempted to rescue eVP40 VLP budding in ITCH siRNA knockdown cells by transiently expressing exogenous ITCH-WT or ITCH-C830A (Fig. 5B). Western analysis was used to detect and confirm the expression of eVP40-WT and endogenous and exogenous ITCH in cell extracts (Fig. 5B). As described above, cells receiving ITCH-specific siRNAs showed an ~75% decrease in eVP40 VLP egress compared to random siRNA controls (Fig. 5B, compare lanes 2 and 3). Interestingly, budding of eVP40 VLPs was enhanced by ~3-fold in cells expressing exogenous ITCH-WT compared to control samples (Fig. 5B, compare lanes 2 and 4), whereas no enhancement of eVP40 VLP egress was observed in cells expressing exogenous ITCH-C830A (Fig. 5B, compare lanes 2 and 5). These results confirm that knockdown of endogenous ITCH in HEK293T cells resulted in a 4- to 5-fold decrease in eVP40 VLP egress in multiple experiments (Fig. 5C) and, importantly, that the enzymatic activity of ITCH ligase was required for the efficient egress of eVP40 VLPs.

Reduced budding of VLPs and virus from ITCH knockout cells. We next took advantage of the HAP1-ITCH$^{-/-}$ (KO) cell line as a genetic approach to determine whether the expression of endogenous ITCH is critical for the efficient egress of both eVP40 VLPs and live recombinant VSV expressing the eVP40 PPxY L domain motif and flanking residues. Briefly, parental HAP1 cells expressing endogenous ITCH (ITCH-WT) and HAP1 ITCH-KO cells were transfected with eVP40, and eVP40 was detected in cell extracts and VLPs at 24 h posttransfection by Western analysis (Fig. 6A). We found that budding of eVP40 VLPs was reduced in multiple independent experiments by up to 20-fold in ITCH KO cells compared to that in ITCH-WT cells (Fig. 6A and B). Equal expression of eVP40 and the absence of ITCH in the KO cell line were confirmed by Western analysis (Fig. 6A).

To confirm that the reduced levels of eVP40 VLP egress were due to the lack of ITCH expression in KO cells, ITCH KO cells were transfected with eVP40 alone or in combination with ITCH-WT, and eVP40 was detected in cell extracts and VLPs by Western analysis (Fig. 6C). Indeed, we observed an ~8-fold increase in eVP40 VLP budding from KO cells receiving exogenous ITCH-WT compared to that from KO cells expressing eVP40 alone (Fig. 6C and D). Appropriate levels of eVP40 and ITCH-WT in cell extracts were confirmed by Western analysis (Fig. 6C).

We next asked whether the decrease in eVP40 VLP budding from ITCH KO cells would also be observed for live-virus budding. To test this, we utilized our previously described recombinant VSV (VSV-M40) that was engineered to express the PPxY L domain motif and flanking residues from eVP40 in place of the PPxY L domain and flanking residues of VSV-M (14). Parental and ITCH KO cells were infected with VSV-M40 at an MOI of 0.1, and infected-cell extracts and supernatants were harvested at the peak budding time of 8 h postinfection (Fig. 7). Infectious virions released into the medium were quantified by standard plaque assays on BHK-21 cells, and these data revealed a >1-log decrease in

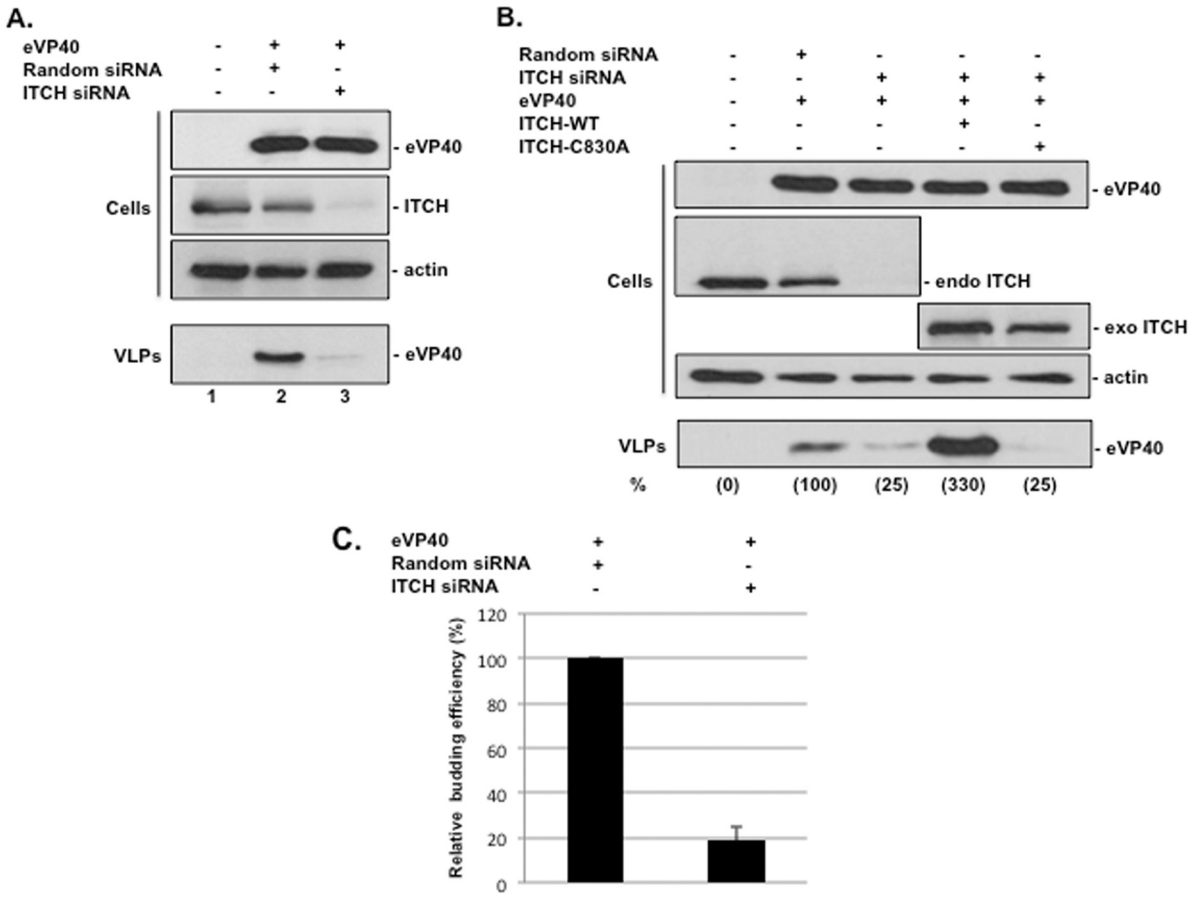

FIG 5 siRNA knockdown/rescue of ITCH regulates eVP40 VLP budding. (A) Western analysis of eVP40, endogenous ITCH, and actin in extracts and VLPs from HEK293T cells mock treated or treated with the indicated siRNAs. (B) HEK293T cells were transfected as indicated, and Western analysis was used to detect eVP40 and endogenous (endo) and exogenous (exo) ITCH in cell extracts and eVP40 in VLPs. The budding efficiencies of eVP40 in VLPs relative to the control sample (lane 2) (100%) are shown in parentheses. (C) Average budding efficiency of eVP40 VLPs in cells treated with ITCH-specific siRNA relative to that in cells treated with the random siRNA control. Error bars represent the standard deviations of the means from three independent experiments.

the average titer of VSV-M40 in ITCH KO cells compared to that in parental ITCH-WT cells (Fig. 7). Western analysis of infected-cell extracts confirmed the lack of ITCH expression in KO cells and that the expression levels of the VSV M protein in WT and KO cells were equivalent and unaffected by the lack of ITCH expression (Fig. 7). It should be noted that a similar degree of budding inhibition was observed for WT VSV, whose M protein contains a PPxY L domain motif (data not shown). These data suggest that, like Nedd4, ITCH plays a role in facilitating the budding of multiple negative-strand RNA viruses that depend on a PPxY-type L domain for efficient egress.

DISCUSSION

EBOV VP40 is the major viral structural protein that orchestrates virion assembly and egress. EBOV VP40 accomplishes this, in part, by hijacking or recruiting host proteins/pathways to facilitate efficient virus separation from the site of budding at the plasma membrane. Here we have identified ITCH as a novel eVP40 interactor that regulates the budding process.

A role for cellular ubiquitination and particularly the E3 ubiquitin ligase Nedd4 in promoting the efficient budding of many enveloped RNA viruses, including EBOV, has been well documented (3, 8, 9, 26–30, 32–43, 59, 60). ITCH is one of nine members of the Nedd4 family of HECT-type E3 ligases with canonical C2, WW, and HECT domains as their main functional components (61). Of all the mammalian WW domains tested here, we identified WW domain 1 (WW1) of ITCH as one of the strongest, specific interactors with the PPxY motif of eVP40 (Fig. 1). Indeed, the high degree of specificity of the eVP40 PPxY motif for binding to only select mammalian WW domains in our WW and SH3 domain array strongly suggests that the interacting host proteins likely play biologically meaningful roles in the life cycle of EBOV. As both Nedd4 and ITCH appear to be recruited to the eVP40 PPxY motif, our results raised the intriguing possibility that these related HECT-type E3 ligases may be recruited by eVP40 to provide analogous or perhaps cell type-specific functions to promote the late stages of virus assembly and egress. Indeed, other Nedd4-like E3 ligases have been implicated in the assembly and egress of several RNA viruses (28, 39, 41, 62, 63). It is possible that WW domain selectivity depends in part on the sequence of the WW domain itself and/or modifications of the PPxY ligand, such as tyrosine phosphorylation (64).

We confirmed both the physical and functional nature of the ITCH WW domain-eVP40 PPxY interaction using both VLP and live-virus budding assays (Fig. 5 to 7). Although a role for the ITCH E3 ubiquitin ligase as a novel host contributor to eVP40-

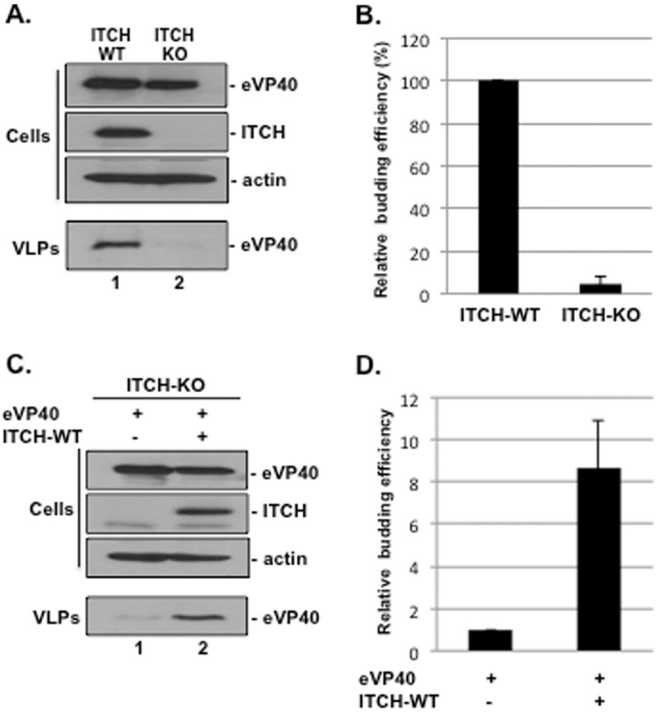

FIG 6 eVP40 VLP budding from ITCH WT and KO cells. (A) HAP1 ITCH-WT (parental) or HAP1 ITCH KO cells were transfected with an eVP40 expression plasmid, and the indicated proteins were detected in cell extracts and VLPs by Western analysis. (B) Budding of eVP40 VLPs in ITCH KO cells relative to eVP40 VLPs from control ITCH-WT cells. Error bars represent the standard deviations of the means from four independent experiments. (C) HAP1 ITCH KO cells were transfected as indicated, and the indicated proteins were detected in cell extracts and VLPs by Western analysis. (D) Budding of eVP40 VLPs from ITCH KO cells transfected with ITCH-WT relative to budding of eVP40 VLPs from control ITCH KO cells (set at 1). Error bars represent the standard deviations of the means from three independent experiments.

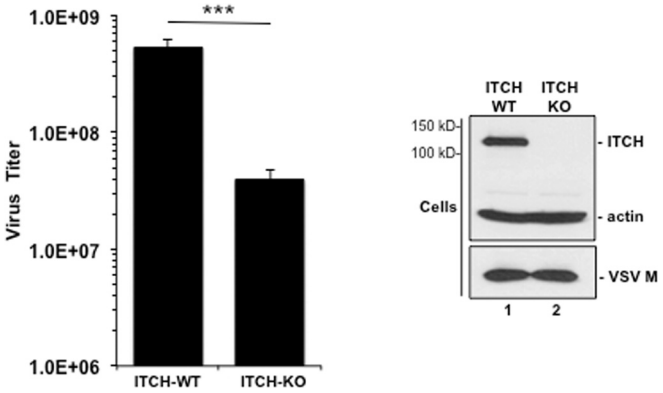

FIG 7 Decreased budding of live recombinant VSV-M40 in ITCH KO cells. HAP1 ITCH-WT (parental) or HAP1 ITCH KO cells were infected with recombinant VSV-M40, and virus titers were quantified by plaque assays at 8 h postinfection. Virus titers from ITCH KO cells were reduced >10-fold compared to those from ITCH-WT cells. ITCH, actin, and VSV M proteins were detected in the indicated infected-cell extracts by Western analysis. *** indicates a *P* value of <0.001, as determined by a Student *t* test.

mediated budding has not been reported previously, ITCH was identified previously as a functional contributor to the budding process of some retroviruses (63, 65–67). ITCH has been more widely studied for its role in immune regulation and inflammatory signaling, such as its ability to regulate lymphocyte activation, differentiation, and immune tolerance (for a review, see reference 68). Whether there is any link between the immune-regulatory activities of ITCH and its role in promoting virus egress remains to be determined, as does a role for ITCH in facilitating egress and modulating the pathogenesis of infectious EBOV. Nonetheless, we demonstrated that (i) the eVP40-ITCH interaction is PPxY dependent, (ii) enzymatically active ITCH ubiquitinated eVP40, (iii) siRNA or genetic knockdown of endogenous ITCH in HAP1 cells resulted in a decrease in eVP40 VLP and live-VSV-M40 egress, and (iv) the addition of enzymatically active ITCH rescued this budding defect.

While evidence for the "probudding" role of ubiquitination and E3 ligases such as Nedd4 and ITCH continues to accumulate, a counteracting and antagonistic "antibudding" role for host interferon-stimulated gene 15 (ISG15) and ISGylation (covalent modification of a target protein with ISG15) is emerging. ISG15 is an interferon (IFN)-induced ubiquitin-like protein that plays a key role as a central regulator of the innate immune response to a plethora of viral pathogens (69, 70; for reviews, see references 71 and 72). Indeed, we and others found that free/unconjugated ISG15 interferes with Nedd4 ligase activity, thereby preventing its functional interaction with eVP40 and indirectly preventing the budding of eVP40 VLPs (53, 72, 73). It will be of interest to determine whether this mechanistically novel function for ISG15 (53, 73) and the functional interplay between ubiquitination and ISGylation pathways extend to ITCH ligase as well.

In sum, our results provide new insights into the range of host proteins that regulate EBOV VP40-mediated egress. While we predict that ITCH should also interact with the PPxY motif of MARV VP40, this remains to be determined. A better understanding of this ongoing battle and interplay between the virus and early host innate immune defenses will be critical for our overall understanding of the biology and pathogenesis of the virus as well as for the future development of effective antiviral therapies. Indeed, we have identified small-molecule inhibitors of virus-host interactions, including those that target PPxY-WW domain interactions, with promising potential as broad-spectrum antiviral therapeutics (29, 55, 74).

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