Flavivirus internalization is regulated by a size-dependent endocytic pathway

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Flaviviruses enter host cells through the process of clathrin-mediated endocytosis, and the spectrum of host factors required for this process is incompletely understood. Here we found that lymphocyte antigen 6 locus E (LY6E) promotes the internalization of multiple flaviviruses, including West Nile virus, Zika virus, and dengue virus. Perhaps surprisingly, LY6E is dispensable for the internalization of the endogenous cargo transferrin, which is also dependent on clathrin-mediated endocytosis for uptake. Since viruses are substantially larger than transferrin, we reasoned that LY6E may be required for uptake of larger cargoes and tested this using transferrin-coated beads of similar size as flaviviruses. LY6E was indeed required for the internalization of transferrin-coated beads, suggesting that LY6E is selectively required for large cargo.

Cell biological studies found that LY6E forms tubules upon viral infection and bead internalization, and we found that tubule formation was dependent on RNASEK, which is also required for flavivirus internalization, but not transferrin uptake. Indeed, we found that RNASEK is also required for the internalization of transferrin-coated beads, suggesting it functions upstream of LY6E. These LY6E tubules resembled microtubules, and we found that microtubule assembly was required for their formation and flavivirus uptake. Since microtubule end-binding proteins link microtubules to downstream activities, we screened the three end-binding proteins and found that EB3 promotes virus uptake and LY6E tubularization. Taken together, these results highlight a specialized pathway required for the uptake of large clathrin-dependent endocytosis cargoes, including flaviviruses.

Endocytosis | flavivirus | entry | virus | LY6E

Clathrin-mediated endocytosis is a major endocytic route by which cells sample their environment and take up nutrients. Thus, many viruses, including flaviviruses, hijack this endocytic pathway to gain access to internal compartments of host cells (1–3). Flaviviruses are a large group of reemerging viruses transmitted to humans by mosquito species of global significance (4). West Nile virus (WNV) is widespread in the Americas, neurotropic, and can cause encephalitis (5, 6). Zika virus (ZIKV) is newly emerging in the Americas and can also be neurotropic (7–9). Dengue virus (DENV) is reemerging globally, infecting 350 million people annually (10). Presently, there are no specific antiviral treatments or vaccines approved to treat these viruses.

Clathrin-mediated endocytosis is a tightly orchestrated pathway whereby cargoes bound to surface receptors are internalized using a number of well-characterized cellular factors (reviewed in ref. 11). Indeed, for those viruses that depend on clathrin-mediated endocytosis for entry, the classically known canonical components are required to facilitate this process (12, 13). However, most of our understanding of this cellular pathway comes from studies on endogenous cargoes, such as transferrin, which are an order of magnitude smaller than viral particles. Studies on vesicular stomatitis virus found that actin, which is dispensable for transferrin uptake, is required for internalization of this virus (14). Therefore, it is likely that there are additional requirements for the internalization of flavivirus particles. Indeed, RNASEK, a recently identified ~100-aa transmembrane protein, was found to be required for the internalization of flaviviruses, but was dispensable for transferrin, suggesting that RNASEK may represent a protein required for particular cargo (15, 16). However, it is unknown how RNASEK facilitates the internalization of these viruses and whether there are other factors that selectively promote viral endocytosis.

Here we report that lymphocyte antigen locus 6 E (LY6E) is required for flavivirus infection at the level of entry. LY6E is a small (101 aa) GPI-anchored protein of the Ly6/uPAR superfamily of proteins that can be IFN-inducible (17, 18). Ectopic expression of LY6E can promote replication of the flavivirus yellow fever virus (19, 20). Additionally, a genome-wide siRNA screen revealed that knocking down LY6E reduces susceptibility to West Nile virus infection (21). However, the mechanism by which LY6E promotes flavivirus infection was unknown. We found that LY6E facilitates infection of flaviviruses but not parainfluenza virus 5 (PIV5), which fuses at the plasma membrane. Further, we find that LY6E is required for the internalization of flavivirus virions, but is dispensable for the internalization of canonical clathrin cargo, transferrin. To directly test the role of size in this process, we coated beads of similar size of flaviviruses with transferrin and found that LY6E is specifically required for the internalization of this large cargo. Since we had previously found that RNASEK promoted internalization of virions, but not transferrin, we also tested the requirement for this factor in transferrin-bead uptake. We found that RNASEK is also necessary for the uptake of these large cargos. Morphological studies revealed that virus infection induces tubularization of LY6E, which is dependent on RNASEK and microtubules. Moreover, disruption of microtubules, or the plus-end-binding, microtubule-associated protein EB3 abrogates LY6E tubule formation and attenuates viral entry. Taken together, these data demonstrate that large cargoes, including viruses, are dependent on additional cellular factors for their internalization and which may be amenable to therapeutic interventions.
uptake and that elucidation of this process may aid in the future development of new classes of broad-spectrum antiviral therapies.

Results

**LY6E Promotes Flavivirus Infection.** Previous studies suggested that LY6E promotes infection of multiple flaviviruses (20, 21). LY6E is ubiquitously expressed and in some studies has been shown to be IFN-inducible (17, 18). However, in human osteosarcoma cells (U2OS) we found that LY6E is basally expressed and largely insensitive to IFN stimulation (Fig. S1). We next tested the role of LY6E in WNV infection. First, we transfected either nontargeting control or LY6E-directed siRNAs and found that we could deplete LY6E mRNA (Fig. 1A). We then challenged these cells with WNV–Kunjin, as it is closely related to circulating strains of WNV in the United States (98% identical) and can be used under BSL2 conditions (22). We found that there is a significant decrease in WNV-KUN RNA at 8 hours post-infection (hpi) as measured by RT-qPCR (Fig. 1B) and protein at 24 hpi as measured by immunoblot (Fig. 1C). We next took advantage of cells that express a WNV replicon with a GFP reporter in place of the structural proteins (23). We found that while targeting GFP with siRNAs significantly reduces the reporter signal, LY6E knockdown has no apparent impact on viral replication in this system (Fig. S1 B and C). This result suggested that LY6E may be required upstream, at the level of entry.

Since many flaviviruses use a similar pathway for entry, we next tested whether other flaviviruses were dependent on LY6E and found that dengue virus [Serotype 2, New Guinea C (DENV2-NGC)] and ZIKV (MR766) are also dependent on LY6E for efficient infection as measured by RT-qPCR (Fig. 1D). In contrast, the paramyxovirus PIV5, which enters at the plasma membrane, does not require LY6E, and there is a trend toward increased permissivity (Fig. 1D). These data suggest that LY6E promotes flavivirus entry.

To directly assay entry we implemented a RT-qPCR-based assay we previously developed that allows us to quantitatively assess viral binding and uptake (15). Briefly, we prebind virus to cells at 4 °C, wash off unbound virus, and monitor the levels of bound virus by RT-qPCR. Using this assay, we found that WNV-KUN binding to cells is not affected by RNAi against LY6E or clathrin light chain (CLTC), which is known to be required for internalization (Fig. 1E). Next, we monitored virus internalization in cells prebound with virus by subsequently incubating the cells at 37 °C for 4 h. We remove the surface-bound virus with trypsin and quantify the internalized virus. Under these conditions we found that depletion of either LY6E or the control CLTC leads to significant reduction in virus internalization (Fig. 1F).

**LY6E and RNASEK Facilitate Internalization of Large Endocytic Cargo.** Since LY6E promotes WNV internalization, which is dependent on clathrin-mediated endocytosis, we reasoned that LY6E may also play a role in canonical clathrin-mediated endocytosis. Therefore, we tested whether LY6E is required for uptake of transferrin—a well-characterized endogenous cargo for clathrin-mediated endocytosis. While depletion of CLTC attenuated transferrin uptake, as expected, loss of LY6E had no effect (Fig. 2 A and B).

One clear difference between transferrin and viral cargoes is the significantly larger size of virions. Flaviviruses are ~50 nm, while transferrin is ~6 nm. We thus developed an assay to directly test whether cargo size alters LY6E dependence, taking advantage of the fact that transferrin uptake is not dependent on LY6E. For these studies we conjugated transferrin to streptavidin-labeled 40-nm microspheres that are green fluorescent (schematic in Fig. 2C). We developed a confocal microscopy-based assay whereby extracellular beads could be detected using red fluorescent streptavidin, while internalized particles were not bound by the red fluorophore since we did not permeabilize the cells. Given the fact that the beads are constitutively green, they are observed both inside and outside of the cells. This assay results in extracellular yellow beads and green intracellular beads. Using this assay, we assessed whether LY6E plays a role in internalizing the same cargo (transferrin) that differs in size. We added transferrin-bound beads to cells and allowed uptake for 45 min where we observed ~50% internalization in control cells (Fig. 2 D and E). As a positive control we depleted CLTC and found that there was almost a complete block in internalization (Fig. 2 D and E). Likewise, LY6E was required for the internalization of transferrin-bound beads (Fig. 2 D and E). Since globular transferrin uptake was independent of LY6E, our data suggest that LY6E is selectively required for clathrin-mediated endocytosis of this larger form of cargo.

RNASEK is another small protein that is required for WNV internalization but dispensable for transferrin uptake (refs. 15 and 16 and Fig. 2 A and B). We next tested whether RNASEK was required for the internalization of this transferrin-bound bead cargo and found that, indeed, depletion of RNASEK also blocked transferrin-bound bead uptake (Fig. 2 B and C), demonstrating a requirement for both LY6E and RNASEK in the internalization of large cargo. We also tested ~20 nm beads coupled to transferrin and found that uptake of these beads was not dependent on LY6E or RNASEK (Fig. S2). These data suggest that cargo of the size of virions is dependent on this pathway.

**LY6E Adopts a Microtubule-Like Organization upon Flavivirus Infection.** We next examined the subcellular localization of LY6E during infection. We found that LY6E is diffuse in uninfected cells and becomes tubular within 20 min of infection with
either WNV-KUN or ZIKV infection (Fig. 3A and Fig. S3A).

Furthermore, tubularization of LY6E is also induced by transferrin-coated beads (Fig. S4A). Moreover, in cells ectopically expressing RNASEK-Flag (RK-F), LY6E is more tubular and no longer relocates upon infection (Fig. 3B and Fig. S3B), suggesting a potential link between these proteins. Because the tubular pattern of LY6E resembles microtubules, we also tested whether this tubularization is dependent on microtubule integrity. Pretreatment of U2OS cells with the microtubule inhibitor nocodazole drastically reduces WNV-induced LY6E tubularization and prevents virus-induced relocalization (Fig. 3C and Fig. S3C). These data suggest that microtubules may play a role in WNV entry.

Flaviviral Uptake and Large-Cargo Internalization Are Dependent upon Microtubules. It has been previously established that flaviviruses remodel the cytoskeleton at late stages postinfection and that treatment with microtubule inhibitors can impact multiple steps in the viral lifecycle (24–27). We specifically set out to determine whether flavivirus uptake is dependent on microtubules. We first monitored viral uptake using a microscopy-based assay we previously developed (15). After binding the virus to cells for 1 h at 4 °C (0 hpi) and washing off unbound virus, samples are either fixed to monitor virus binding or subsequently shifted to 37 °C for 4 h (4 hpi) to allow for uptake before fixation. We performed the immunostaining in the absence of permeabilization to specifically monitor extracellular virions using an antibody to

![Fig. 2. LY6E and RNASEK facilitate size-dependent endocytosis. (A and B) Biotin-conjugated transferrin (25 μg/mL) was added to siRNA-treated U2OS cells for the indicated times. (B) Quantification of transferrin (TF) uptake; n = 3, mean ± SEM, **P < 0.01. (C) Schematic of bead uptake assay. (D and E) Transferrin-conjugated GFP beads were added to siRNA-transfected U2OS cells for 45 min before fixation. Non-permeabilized cells were then treated with streptavidin-594. (D) Images are representative of three independent experiments. Arrows show internalized beads (green only). (E) Quantification of transferrin-conjugated bead uptake (Mander’s colocalization analysis); n = 3, mean ± SEM, *P < 0.05. n.s., not significant. (Magnification: 63×.)](https://www.pnas.org/cgi/doi/10.1073/pnas.1720032115)
the glycoprotein (4G2). In vehicle-treated cells (DMSO), we observe WNV-KUN virions bound at 0 hpi and internalization at 4 hpi since no surface-bound virus is detected at this time point (Fig. 4A). As a control, we treated cells with the flavivirus entry inhibitor nanchangmycin, which robustly blocks internalization (28). As expected, WNV remains on the surface of nanchangmycin-treated cells at 4 hpi. Similarly, we found that the microtubule inhibitor nocodazole blocks uptake of WNV (Fig. 4A). We found that nocodazole and nanchangmycin also inhibit uptake of ZIKV (Fig. S5). To confirm that nocodazole impacts virus uptake, we also monitored internalization of WNV-KUN using our RT-qPCR assay and found that treatment with either nanchangmycin or nocodazole attenuates WNV internalization (Fig. 4B). This effect was specific for flavivirus entry, as PIV5, which fuses at the plasma membrane, was insensitive to either nanchangmycin or nocodazole treatments (Fig. 4B).

Next we set out to determine if microtubules play a role in canonical clathrin-mediated endocytosis. As has been previously shown, we found that microtubule polymerization is not required for transferrin uptake but rather for downstream trafficking as the intracellular pattern of staining is distinct in nocodazole treated cells, while the dynamin inhibitor dynasore completely abrogated uptake (Fig. S6 and ref. 29). This led us to test if microtubules are required for large-cargo uptake. Using transferrin-bound beads as the cargo, we found that both nocodazole and nanchangmycin block this internalization equivalently to dynasore, an inhibitor of dynamin (Fig. 4 C and D). This requirement is specific, as treatment with latrunculin A, which leads to actin depolymerization, does not block viral uptake as measured by RT-qPCR (Fig. S7A) or the internalization of transferrin-coated beads (Fig. S7B).

**EB3, a Microtubule-Associated Protein, Is Essential for Flavivirus Infection.** Because microtubules are necessary for virus-induced LY6E reorganization and the internalization of viral cargo, we reasoned that microtubule end-binding proteins, particularly plus-end-tracking proteins (+TIPs), which influence the rate of microtubule growth, may link virus internalization to the microtubule network. The core components of +TIP complexes are the end-binding (EB) family, EB1, EB2, and EB3 (30, 31). Recent studies found that EB1 plays roles in the early stages of viral replication of several viruses, whereas roles for EB2 or EB3 have not been described (4, 32). We used RNAi to deplete these EBs and validated mRNA knockdown (Fig. S8A). We screened these EB-depleted cells for their impact on WNV infection and found that depletion of EB3, but not EB1 or EB2, significantly attenuates WNV-KUN infection (Fig. 5A). Furthermore, we found that EB3, but not EB1 or EB2, promotes DENV and ZIKV infection, while all three EBs were dispensable for PIV5 infection (Fig. 5B and Fig. S8 B and C). Interestingly, depletion of EB2 exacerbated infection of WNV-KUN and trended toward

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**Fig. 4.** Flavivirus entry and uptake are dependent on microtubule polymerization. (A) U2OS cells were pretreated with the indicated drug 45 min before infection with WNV-KUN (MOI 15) and fixed at 0 hpi or 4 hpi before processing for microscopy in the absence of permeabilization and stained for the viral glycoprotein (anti-E, 4G2). Representative image of three experiments shown. (B) U2OS cells were pretreated with the indicated drug for 45 min, and WNV-KUN or PIV5 internalization at 4 hpi was measured by RT-qPCR; n = 3, mean ± SEM, *P < 0.05. (C and D) U2OS cells were pretreated with the indicated drug for 45 min and subsequently treated with transferrin-conjugated beads for 45 min. Nonpermeabilized cells were then probed with streptavidin-594. Arrows show internalized beads (green only). (C) Representative images shown; n = 3. (D) Quantification of transferrin-conjugated bead uptake (Mander’s colocalization analysis); n = 3, mean ± SEM, *P < 0.05. n.s., not significant. (Magnification: 63×.)
increased infection for the other flaviviruses (Fig. 5A and Fig. S8C).

We next explored whether EB3 promotes entry using our RT-qPCR assay. We found that EB3 is dispensable for WNV binding to cells but required for uptake (Fig. 5C and D). We then set out to link LY6E to EB3. We find that depletion of EB3 reduces the LY6E tubulization upon WNV infection (Fig. 5E–H). Further, we found that silencing of RNASEK also attenuates LY6E tubulization (Fig. 5E–H). We explored the possibility that EB2 may impact LY6E tubulization and found that depletion of EB2 led to increased LY6E tubules at baseline (Fig. S9). This may suggest a negative regulatory role of EB2, which may explain the increased infection.

Our data suggest a link between LY6E, RNASEK, and EB3. Furthermore, the small-molecule entry inhibitor nanchangmycin phenocopies the depletion of these genes. Therefore, we explored whether there was an interaction between these entry factors and the inhibitor. To test this, we determined if depletion of these entry factors impacted the antiviral activity of nanchangmycin against WNV-KUN. The IC50 of nanchangmycin in control cells was 158 nM, and that was significantly reduced upon depletion of LY6E, RNASEK, or EB3 (Fig. 5I, P < 0.05, t test on pIC50s). Altogether, these data functionally link the microtubule-associated protein EB3 to LY6E- and RNASEK-dependent viral uptake, providing evidence for microtubules in viral endocytosis and the uptake of large cargoes.

**Discussion**

Many viruses gain entry into cells using clathrin-mediated endocytosis. This pathway is important for several aspects of cell biology, including nutrient uptake, and thus, clathrin-mediated endocytosis has been extensively studied. Indeed, many of the factors required for cargo internalization have been identified. However, it is clear that numerous unknowns remain, including cellular factors that may be required for specific cargoes and, in particular, large cargo. As viruses are typically much larger than most endogenous cargoes, it remains unclear whether there are particular proteins that facilitate this process. Here we found that LY6E promotes flavivirus entry at the level of internalization. Further study found that this requirement for internalization was not general for all clathrin-mediated endocytosis cargoes as LY6E was dispensable for the uptake of the endogenous cargo transferrin. This led us to test if this difference was due to the size of the cargo. We developed an assay where we compared internalization of transferrin to that of transferrin-coated beads, which are similar in size to virions, and found that LY6E was specifically required for the larger cargo.

To our knowledge, the only other cellular gene known to promote internalization of flaviviruses yet dispensable for transferrin uptake is RNASEK, another small-membrane–associated protein (15). Using the transferrin bead uptake assay, we found that RNASEK also facilitated internalization of this large cargo, suggesting that RNASEK may work with LY6E to promote viral entry. Cell biological studies revealed that LY6E relocalizes during flavivirus uptake to form tubular structures. Furthermore, this relocalization is dependent on RNASEK. Loss of RNASEK attenuates virus-induced tubulization of LY6E, suggesting that RNASEK is upstream of LY6E.

This tubulization of LY6E resembled microtubules, and we found that microtubule assembly is required for this virus-induced change in LY6E morphology. This led us to explore the role of microtubules in flavivirus uptake. Microtubules have been shown to play important roles in downstream aspects of flavivirus infection, including trafficking of endosomes and viral release (24–27, 33). Therefore, we tested whether flavivirus uptake was dependent on microtubules and found that indeed flavivirus...
internalization is blocked by microtubule inhibitors, while transferrin internalization is not. Furthermore, we found that nodocazole blocked transferrin-coated bead uptake. These data suggest that this large-cargo internalization requires changes in the microtubule compartment.

Microtubule plus-ends polymerize rapidly and are the sites where microtubule elongation occurs. Microtubule stabilization is controlled by plus-end-tracking proteins whose accumulation is facilitated by microtubule end-binding proteins (EBs), which are the core components of these complexes (30, 31). We screened the three mammalian EBs and found a role for EB3 in flavivirus infection. EB3 is required for WNV uptake as well as the tubularization of LY6E, suggesting a role for EB3 in large-cargo uptake. While EB1 and EB3 are thought to play similar roles and can heterodimerize, some processes are selectively dependent on EB3 (34, 35). We found that depletion of EB1 did not impact infection suggesting a specific requirement for EB3. Interestingly, EB2, which is not known to directly interact with EB1 or EB3, may play a negative role in this process. Silencing of EB2 leads to increased WNV infection concomitant with altered LY6E localization. It is unclear how EB2 could negatively regulate this process; however, previous studies have shown that loss of EB2 leads to more stable microtubule ends (36). Future studies will explore potential interactions between the EBs and virus-induced structures.

Our findings suggest an additional level of regulation on the internalization of larger cargo. LY6E, RNASEK, and EB3 are defining a pathway required for the endocytosis of viruses as well as our model cargo, transferrin-coated beads, but dispensable for canonical transferrin cargo. Additionally, our discovery that depletion of these factors increases the potency of the entry inhibitor nanchangmycin suggests that nanchangmycin targets this size-dependent pathway. The full spectrum of cargoes that are dependent on these proteins is unclear. Future studies will define the rules of engagement. Nevertheless, given the potentially narrow list of cargoes dependent on these genes, the development of inhibitors active against this pathway, such as nanchangmycin, may represent a class of antiviral compounds, which may be effective against flaviviruses or other viruses that use clathrin-mediated endocytosis for entry.

Materials and Methods
Please see SI Appendix, SI Materials and Methods for detailed description of cell culture, viral infections, siRNA transfections, immunoblotting, RT-qPCR, microscopy, internalization assays, transferrin uptake, and IC50 analysis. siRNA and primer sequences are found in SI Appendix, Tables S1 and S2.

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