HIV corrupts the Cdc42-IQGAP1 axis of actin regulation to promote cell-cell viral spread

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Abstract

F-Actin remodelling is important for the spread of HIV via cell-cell contacts, yet the mechanisms by which HIV corrupts the actin cytoskeleton are poorly understood. Through live cell imaging and focused ion beam scanning electron microscopy (FIB-SEM), we observed F-Actin structures that exhibit strong positive curvature to be enriched for HIV buds. Virion proteomics, gene silencing, and viral mutagenesis supported a Cdc42-IQGAP1-Arp2/3 pathway as the primary intersection of HIV budding, membrane curvature and F-Actin regulation. Whilst HIV egress activated the Cdc42-Arp2/3 filopodial pathway, this came at the expense of cell-free viral release. Importantly, release could be rescued by cell-cell contact, providing Cdc42 and IQGAP1 were present. From these observations we conclude that out-going HIV has corrupted a central F-Actin node that enables initial coupling of HIV buds to cortical F-Actin to place HIV at the leading cell edge. Whilst this initially prevents particle release, maturation of cell-cell contacts signals back to this F-Actin node to enable viral release & subsequent infection of the contacting cell.

Introduction

Actin is a major component of the cellular cytoskeleton and is present in both monomeric globular (G-actin) and polymeric filamentous (F-actin) forms in all eukaryotic cells. Specifically in human leukocytes, actin accounts for over 10% of the total protein content [1] and is a prerequisite for many pathways involved in communication of the immune response, such as chemotaxis of leukocytes through to the formation of supramolecular structures like the immunological synapse- a fundamental structure driving the primary immune response [2,3]. While cells encode a wide range of proteins that mediate F-actin remodelling, the critical ability to seed or 'nucleate' F-actin from the monomeric G-actin pool is limited to only a few protein families [4]. The two major classes of cellular actin nucleators are the Arp2/3 complex and formins [5]. The Arp2/3 complex is composed of 7 different subunits and allows formation of branched actin networks, through nucleation of a branch filament from an existing mother filament at an angle of 70 degrees [6,7]. In contrast, formins are large multidomain proteins that drive nucleation and/or elongation of unbranched linear actin filaments (reviewed in [4,8]. The activity of cellular Arp2/3 and formins is tightly regulated by a complex network of signalling pathways that primarily rely on the molecular switch properties of Rho-GTPases, such as Rac1 and Cdc42, for their activation [9,10].

HIV infection and spread proceeds primarily in CD4 positive leukocytes of our immune system [11]. Viral spread can be observed at two levels. Firstly, free virus release from infected cells, with virions travelling in a cell-free form until encountering a new target cell to infect. Secondly, HIV budding that occurs directly at sites of cell-cell contact. The supramolecular structure that enables the latter and highly efficient cell-cell viral transfer is referred to as the virological synapse (VS) [12,13]. In both cases, viral budding needs to proceed at the plasma membrane (PM) of infected cells and is initially driven by oligomerisation of the HIV structural protein Gag [14] and culminates with HIV particle abscission mediated by cellular proteins of the endosomal sorting complexes required for transport machinery (ESCRT) [15]. Several F-actin structures have been previously observed in association with HIV assembly and higher-level Gag oligormerisation. These include; the temporal formation of F-Actin asters/stars that appear just underneath the PM prior to particle release [16], and assembling HIV particles decorating the tips of finger-like filopodial structures [17-19]. It is however unclear how these events are mechanistically connected and how coupling to the F-actin cytoskeleton benefits HIV release/spread [20]. This can be considered at two-levels: firstly how does F-Actin regulation influence the assembly and release of cell free virus in infected cells? For instance, do cortical F-Actin structures facilitate assembly and release at the PM as observed for other viruses [21]? Secondly, how is F-actin regulated at cell-cell contacts involving HIV infected cells? Several studies have shown that functional actin dynamcis are required for cell-cell viral transfer [13,22], yet how HIV assembly and release are spatiotemporally coordinated during this process has not been clarified mechanistically.

With our primary aim to determine the role for F-Actin in cell-free HIV egress and cell-cell viral transfer, herein we peeled back the complexity of F-Actin regulation in leukocytes by successive depletion and/or knockout of key actin nucleators and other associated proteins that regulate their activity. In doing so, we biased the formation of different cortical F-Actin structures such as filopodia and lamellipodia. Herein we define these structures as outlined by Mattila and Lappalain, i.e. Filopodia are cylindrical finger like protrusions approximately 100–300nm in diameter and up to 1μm to 10μm or more in length, whereas lamellipodia are thin (100nm to 200nm thick) sheet/viel like cortical F-Actin protusions [23]. Whilst the regulation of lamellipodia is well understood and primarily depends on branched F-actin

nucleation by the Arp2/3 complex downstream of Rac1 and its effector Wave2 [24-29], various models have been proposed for filopodia formation and increasing evidence suggests this process may be cell-type specific [23,30]. Importantly, little is known about the mechanism of filopodia formation in cells of hematopoietic lineage, despite the fact that filopodia play important roles for immune cell function.

Using a combination of live cell imaging, focused ion beam scanning electron microscopy (FIB-SEM), virion mass spectrometry and viral infection assays, we observed the influence of HIV on cortical F-Actin at several levels. First, FIB-SEM revealed HIV budding to be relatively enriched in areas of high positive membrane curvature within Arp2/3-dependent cortical F-Actin structures, including filopodia and lamellipodia. Second, virion mass spectrometry identified a cortical F-Actin signalling node comprising of the Arp2/3 related GTPases Rac1 and Cdc42 and their binding partner, the scaffolding protein IQGAP1. Finally, while depletion of a number of dominant F-actin regulators was observed to affect free virus release, cell-cell viral transfer was only significantly impaired in cells depleted of Cdc42 or IQGAP1. Collectively these observations support a dominant role of the GTPase Cdc42 and IQGAP1 in the final stages of viral egress and cell-cell spread. In this setting we propose HIV manipulation of the Cdc42/IQGAP1 node to be important at two levels: firstly it enables HIV to be embedded and rettained in Arp2/3-dependent leading edge structures that are important during pre-synaptic events. Secondly, as the VS is engaged and matures, the same regulators likely coordinate F-Actin dynamics to enable conditions that facilitate en masse viral particle release.

Results

Moulding cortical F-Actin through formin and Arp2/3 depletion

A physical association of HIV with F-actin structures has been previously observed in all major HIV primary target cell types [18,31]. In infected CD4+ T-cells and dendritic cells this manifests in the form of HIV-Filopodia, which are F-actin rich finger-like structures with HIV assembly observed at their tips [18]. Since these structures are more prominent on dendritic cells and similarly enriched in U937 cells [18,32,33], this latter myeloid cell line provides an ideal model to dissect the link between F-Actin and HIV assembly in the specific context of hematopoietic cell lineages. Given the proposed role of Arp2/3 and formins for filopodia formation in other cell types, we initially focused on these key actin regulators for depletion. However, while Arp2/3 is ubiquitously expressed in eukaryotic cells, there are at least 15 different formins in vertebrates [34,35]. Since Diaph1, Diaph2 and FMNL1 are the most abundantly expressed in leukocytes [36], we tailored our initial shRNA screening to depletion of these formins. Disruption of filopodial networks was assessed by measuring filopodial abundance (average number of filopodia per cell) and length (average filopodial length measured from the PM to the tip). To this end we used several imaging techniques at increasing levels of resolution, including; i) live cell imaging, ii) fixed cell fluorescence imaging followed by 3D deconvolution, and iii) the power of correlative FIB-SEM to finely resolve F-Actin structures closer to the PM. In brief, FIB-SEM represents a method where iterative cycles of finely tuned ion abrasion milling are followed by high-resolution scanning electron microscopy of heavy-metal stained, resin-embedded cell samples [37,38]. The end result is the recording of a stack of 2D back-scatter electron images, which are then processed and converted to a 3D image volume, typically at ~ 10 nm pixel sampling (Fig.1 B-E). This method provides a powerful imaging tool for cell biology and virology, as it gives users the

ability to resolve nanoscale ultrastructural features in cellular samples that may appear in association with viral particles [39,40].

Initial experiments revealed filopodial lengths to be dependent solely on the formin Diaph2 and not the other leukocyte-enriched formins (Fig. S1). Silencing of Diaph2 by shRNA achieved >95% depletion at the protein level (Fig. S3), and in these cells cortical F-Actin coalesced into a network of abundant and short (1 to 3µm) filopodia (Fig. 1E&H; Movie S1). Since we could confirm this phenotype in CRISPR/Cas9-generated and clonally expanded Diaph2 homozygous knockout cells, our observations suggest that filopodial length but not seeding is dependent on Diaph2. Subsequent shRNA codepletion of other expressed formins in addition to Diaph2 did also not disrupt this short filopodial network (Fig. S1). Therefore, to test if the shorter but more abundant filopodia were Arp2/3-dependent, we disrupted both Diaph2 and the Arp2/3 complex by shRNA. Co-depletion led to short filopodia converging into an extensive lamellipodial network (Fig. 1A,E&H; Movie S2). To conclude, we could readily control cortical F-Actin within this leukocyte landscape, and generate three unique cell types with a continuum of cortical F-actin structures; i) long filopodia, ii) abundant short filopodia, and iii) an extensive network of large lamellipodia. Furthermore, our observations indicate that seeding of filopodia in myeloid cells requires Arp2/3-mediated actin nucleation, whereas filopodial elongation is dependent on the formin Diaph2 (Fig. 1. F-H).

The influence of shifting F-Actin structures on the location of HIV budding

In the context of HIV infected cells, we used our high-resolution imaging approaches to probe for a possible link between HIV assembly and specific F-Actin structures and/or pathways in leukocytes. Previously we have observed live cells with long filopodia to have significant numbers of HIV positive tips [18], and readily concluded that in untreated cells HIV assembly is enriched to this site. However, detection of HIV buds in cells with short filopodia (i.e. depleted of Diaph2) was constrained by the inability to resolve F-Actin structures proximal to the PM by fluoresecence microscopy alone. Thus, we applied FIB-SEM imaging to HIV-infected Diaph2^{-ve} cells (short filopodia) and observed HIV buds in routine association with the tip and sides of these structures (Fig. 2A,B,D&H). In cells with prominent lamellipodia (Diaph2^{-ve}Arp2/3^{-ve}), FIB-SEM imaging revealed abundance of HIV buds along the ridges of lamellipodia (Fig. 2F&K; Movie S2). Therefore one common feature of each F-Actin structure was the appearance of HIV preferentially in areas of positive membrane curvature. Given the large topological differences between filopodia and lamellipodia, we assessed viral-bud density by accounting for the surface area available for budding within each distinct F-Actin structure. This revealed HIV buds to be significantly enriched in areas of high positive membrane curvature (Table I; Fig. 2 G-L): Lamellipodial ridges and filopodia were the most active areas of viral assembly, with a distinct preference for the latter (filopodia tips outscored lamellipodial ridges by 5-fold, when surface area was considered). This observation supports two potential mechanisms. First, HIV assembly is facilitated by areas of positive curvature or alternatively, HIV assembly recruits/influences cellular protein(s) at the PM that can lead to positive curvature.

Filopodia dominated by the formin Diaph2 present positive curvature at the plasma membrane but exclude assembling HIV particles

To assess whether strong positive membrane curvature alone was sufficient to position HIV buds at filopodial tips, we induced long filopodia using a constitutively active (C/A) mutant of Diaph2. Diaphanous-related formins exist in an autoinhibited conformation mediated by the interaction between their N-proximal inhibitory domain (DID) and C-terminal autoregulatory domain (DAD) [36,41]. Disruption of this autoinhibitory state can be achieved

by formins binding to Rho-GTPases or, as in our case, by deletion of their C-terminal DAD domain [42,43]. Importantly, in both cases the central actin polymerization domain of the formin is rendered constitutively active.

If HIV assembly was directly promoted by positive membrane curvature, filopodia induced by Diaph2$^{C/A}$ would incorporate assemblying viral particles. However, while Diaph2$^{C/A}$ expression readily induced the formation of long straight filopodia with Diaph2 accumulating at the filopodial tips (Fig. 3A & Movie S3), in HIV infected cells we also observed complete exclusion of HIV particles from the tips of these structures (Fig. 3A-C & Movie S3). Therefore, the strong membrane curvature in filopodial tips alone is not sufficient to recruit HIV assembly to this region. Since long filopodia in WT cells are routinely HIV positive, whereas straight C/A Diaph2 driven filopodia are not, it is unlikely that formins represent the link of HIV to the F-actin cytoskeleton. We then turned our attention to the Arp2/3 complex, given that previous observations propose this as the dominant F-actin nucleator at the cell cortex, with formin activity being restricted to filament elongation post F-actin nucleation [44,45]. To confirm if Arp2/3 was the dominant filopodial nucleator in WT versus Diaph2$^{C/A}$ cells, we immunostained filopodia for Arp2/3, and examined the footprint of this nucleator along the filopodial body and tip. In both cell types, the filopodial bases (3µm from the membrane) were all Arp2/3 positive (Fig. 3D & E). In contrast, the filopodial tips of Diaph2$^{C/A}$ cells were negative for Arp2/3 antigen (Fig. 3D), whereas Arp2/3 was frequently observed along the entire shaft and at the tip of wildtype filopodia (Fig. 3E). To quantify the extent of Arp2/3 tip exclusion, we measured the distance from the tip of filopodia to the first detectable Arp2/3 signal and observed a significantly greater distance of Arp2/3 from the filopodial tip in Diaph2$^{C/A}$ cells relative to WT cells (6.2µm versus 1.4µm; p>0.0001; n = 50). In summary, by mapping the HIV budding sites at high resolution we could reach several conclusions . Firstly, HIV buds primarily enrich to cortical F-Actin structures with positive curvature. Secondly, positive curvature and/or Diaph2 activity alone are not responsible for the enrichment of HIV buds to these sites. Finally, Arp2/3-dependent cortical F-actin structures are primarily HIV positive.

HIV Gag can influence Arp2/3 dependent F-Actin pathways

Since HIV assembly at the PM is primarily driven by HIV-Gag, we turned to strategic Gag mutagenesis in an attempt to resolve the link of HIV assembly with cortical F-Actin structures. The HIV Gag mutant panel covered several well characterised mutants that could maintain HIV particle assembly and also binding to membrane Phosphatidylinositol (4,5)-bisphosphate (PIP2) (i.e. Able to maintain Gag assembly at the plasma membrane). Deletion of HIV Gag p6 and mutagenesis of the PTAP motif in p6, was used to block the recruitment of TSG101 and related ESCRT proteins involved in viral particle abscission (Fig. 4A-B). We also deleted the Nucleocapsid (NC) domain, as this has been previously proposed to mediate the interaction between HIV-Gag and F-actin [46,47]. However, since NC is required to facilitate higher order oligomerisation of Gag [48], we replaced NC with the Leucine Zipper (LZ) domain from the *Saccharomyces cerevisiae* GCN4 protein (Fig. 4C), as this rescues Gag oligomerisation and ensures particle assembly proceeds in the absence of NC [49]. Finally, given the enrichment of HIV buds on F-Actin structures with positive curvature, we further generated two HIV Gag Capsid mutants $P_{99}A$ and $EE_{75,76}AA$ (Fig. 4D & E), both of which inhibit Gag curvature at the PM but not high order Gag oligomerisation [50,51]. As Diaph2 cannot recruit HIV to F-Actin and depletion of Diaph2 actually enriched HIV-positive filopodia (Fig. S1), we utilised Diaph2^{-ve} cells and simply scored the number of filopodia per cell that were HIV positive for each viral Gag mutant. Using this approach, we observed no significant difference in viral filopodia when deleting p6, the PTAP motif in p6 or NC (Fig.

4F). However when using the $P_{99}A$ and $EE_{75,76}AA$ HIV capsid mutants (HIV curvature mutants), we observed Diaph2-depleted cells to not only lack any evident HIV buds at the PM but also their characteristic short filopodia. Instead, these cells resembled the lamellipodial phenotype observed in Diaph2^{-ve}Arp2/3^{-ve} co-depleted cells (Fig. 4G-I). This is consistent with HIV Gag curvature mutants acting as dominant negatives for the Arp2/3-dependent short filopodial pathway. To further test that HIV curvature mutants were specifically interrupting Arp2/3 F-actin pathways and not broadly influencing all pathways that may lead to filopodial formation (eg. Formin-induced filopodia), we infected Diaph2$^{C/A}$ cells with these mutants. In this setting we observed an ability of Diaph2$^{C/A}$ to partially rescue filopodia formation (Fig. 4F; Movie S4). Thus filopodial pools nucleated by Arp2/3 are most affected by HIV curvature mutants and this further supports the hypothesis that HIV assembly primarily influences elements of Arp2/3 F-Actin nucleation pathway.

The HIV proteome reveals a GTPase node associated with Arp2/3 F-Actin regulation

HIV has been previously observed to incorporate F-Actin, various actin nucleators and numerous upstream/downstream regulators within virions [52-56]. Thus, we turned to mass spectrometry analysis of purified virions to observe the footprint of cytoskeletal proteins that are present at HIV assembly sites. For this analysis we also leveraged the three distinct F-Actin cell types generated above (i.e. long-filopodia, extensive short filopodia and large lamellipodia) as across each cell type they shared the feature of HIV buds being enriched in positively curved F-Actin structures. Using this approach we identified several Arp2/3 complex subunits, alongside two major Arp2/3 regulators, the Rho-GTPases Rac1 and Cdc42, as well as their interaction partner IQGAP1. These regulators were observed across all viral proteomes, irrespective of producer cell type (Fig.5 A&B). In addition to this F-Actin signalling node, HIV virions also acquired members of the integrin and cadherin families (Fig. 5 A-B; see nodes 3 and 4, respectively). These proteins, which are involved in cell-cell adhesion, are connected to the cortical F-actin cytoskeleton both physically and via signalling pathways [57,58]. Of interest was a depletion of the cadherin node in (Fig. 5 A; node 4), as well as an increase in Arp2/3 and Cdc42 content (Fig. 5 A; node 1) in virions produced by Diaph2-deficient cells. The latter observation is not only consistent with HIV assembly preferentially proceeding alongside short Arp2/3-dependent filopodia (as observed by FIB-SEM), but also suggests that these structures are dependent on Cdc42, which is a well known filopodial regulator [10,23]. Furthermore, it further supports a role for the formin Diaph2 in cadherin recruitment/co-ordination. Of note, the observed decrease of Arp2/3 components in virions produced in Diaph2 and Arp2/3 co-depleted cells (Fig. 5B, node 1) is both expected and consistent with depletion of these proteins at the cellular level (Fig. S3).

HIV exploits the Cdc42-Arp2/3 filopodial pathway to position virus at the leading edge of cell-cell contacts.

Since Cdc42 is an important regulator of Arp2/3, a master regulator of filopodia, and it was incorporated at higher levels in virions from our Diaph2^{-ve} cells (more abundant short filopodia), we next targeted this protein for depletion. As a functional control, we targeted the homologous Rho-GTPase Rac1, best known for its role in lamellipodial regulation. We also investigated the scaffolding protein IQGAP1, which; i) is a binding partner and effector of both Cdc42 and Rac1 [59,60], and ii) plays an increasingly recognized role in actin cytoskeleton regulation [61,62], and iii) was consistently incorporated in virions in our experiments (Fig.5). While we succeeded in generating a viable Cdc42 homozygous knockout cell line using CRISPR/Cas9 (Figure S4). Attempts at knocking out Rac1 led to multinucleated cell populations with reduced viability, which is consistent with previous reports of Rac1 being an essential gene [63,64]. To circumvent this, we partially depleted

Rac1 by shRNA, and also generated a Wave2 -/- cell line (Figure S4), since Wave is the main downstream effector of Rac1 in F-actin regulation [65,66]. While obtaining homozygous IQGAP1 knockout clones via CRISPR-Cas9 proved challenging, we were able to establish a line stringently depleted of IQGAP1 using shRNA (>99% depletion at the protein level, Fig. S3).

Initial Rac1 depletion via shRNA, revealed a greater frequency of filopodia in infected cells and secondly the generation of significantly longer and thicker filopodia when cells were infected (Fig. 6B vs A). Similarly, WAVE2$^{k/o}$ cells infected with HIV had greater propensity to form filopodia (two-fold), and these were significantly longer and thicker compared to WT cells (Fig. 6C vs A and Fig. S2), but also uninfected WAVE2$^{k/o}$ cells (Fig. S2). Together these observations suggest that HIV infection stimulates a pathway of filopodial formation that is unchecked in Rac1^{-ve} and WAVE2$^{k/o}$ cells, where the lamellipodial F-actin arm is disabled. Given the known role of Cdc42 in filopodia formation and its competing nature with the Rac1 pathway, we turned our attention to this Rho-GTPase. Importantly, Cdc42$^{k/o}$ cells were devoid of filopodia and coalesced cortical F-Actin into prominent lamellipodia, with no evident influence on F-actin when cells were HIV infected (Fig. 6D). Since IQGAP1 has been previously reported to articulate Cdc42 signaling to the cytoskeleton [62], we also assessed the role of this regulator in the filopodial context. IQGAP1-deficient cells displayed a collapse in filopodial lengths (Fig. 6E), with maintenance of HIV at the leading edge of remaining filopodia, similar to that observed in Diaph2-depleted cells. We therefore conclude that IQGAP1 can influence filopodial networks but, like Diaph2, is not required for the seeding of filopodia. To summarize our combined observations from mass spectrometry, gene silencing and high-resolution imaging, reveal that HIV infection augments a pathway of filopodia formation, and this is most evident when the lamellipodial regulators are inactivated. In contrast, removing Cdc42 completely blocked filopodia formation in a manner similar to Arp2/3 and Diaph2 co-depletion, whereas depletion of IQGAP1 or Diaph2 led to shorter filopodia. Together, our data indicates that HIV-assembly hijacks a cellular pathway that is dependent on Cdc42-Arp2/3 F-actin nucleation for filopodial seeding and IQGAP1/Diaph2 for filopodial elongation, in order to position itself at the tips of filopodia.

HIV cell-cell transfer is dependent on an intact Cdc42-IQGAP1-Arp2/3 pathway

Given the continuum of phenotypes observed in our abovementioned observations, we tested their impact on the late stages of the viral life cycle in the context of viral spread. For free virus release, we enumerated HIV particles accumulating in the supernatant as a measure of budding. As HIV spread can also proceed through direct cell-cell contacts, we further tested the ability of HIV to spread cell to cell by coincubating infected donor cells with permissive target cells. Using this approaches, we could determine if the generic lack of a cortical F-Actin structure or a specific F-Actin pathway is essential for HIV budding and/or cell-cell transfer.

Disruption of either the Rac1-WAVE2 pathway (lamellipodia) or Cdc42-IQGAP1 pathway (filopodia) both impaired free HIV budding, as indicated by significantly lower viral particle counts in the supernatant from cells depleted of these regulators, compared to untreated cells (Fig. 7 B). However, impaired release of free HIV did not predict outcomes for cell-cell HIV transfer. For cells with disabled Rac1/WAVE2 (Rac1^{-ve}, Wave2$^{k/o}$ cells) cell-cell HIV transfer persisted (Fig. 7 C-D), despite the decreased free virus budding ability. In contrast, disruption of the Ccd42/IQGAP1 axis (Cdc42k/o and IQGAP1-ve cells) impacted both HIV budding and cell-cell transfer (Fig. 7 C-D). These observations suggest that while normal actin dynamics are important for free virus release, Cdc42 and IQGAP1 are specifically

required for cell-cell HIV transfer, whereas Rac1/Wave2 are not. To further test this hypothesis in the setting of primary CD4 T cell targets, we focussed cell-cell transfer assays with disruption of the Rac1-WAVE2 pathway versus disruption of Cdc42-IQGAP1. In this setting, we further tested the efficiency of cell-cell spread by limiting dilution of the infected donors into primary CD4 T cell co-cultures. Using this approach, we observed almost complete loss of cell-cell HIV transfer in $Cdc42^{k/o}$ and $IQGAP1^{-ve}$ clones, whereas cell-cell transfer persisted in $Rac1^{-ve}$, $Wave2^{k/o}$ clones, albeit lower than in WT cells. To address the paradox of persistent cell-cell HIV transfer, despite reduced viral budding in cells with augmented filopodia, we turned to live imaging of HIV infected $Wave2^{k/o}$ clones to observe F-Actin dynamics during viral transfer. As expected, HIV infected $Wave2^{k/o}$ clones displayed extensive and dynamic filopodial networks (Movies S5 & S6). Donor-target cell interactions were often guided by filopodial activity, suggesting a probing/tethering function for HIV-filopodia. Whilst filopodial activity persisted in early cell-cell conjugates, we routinely observed collapse of filopodial networks immediately preceding VS formation and HIV-GFP transfer to the opposing target cell (Fig. 7 E and Movie S6).

To conclude, in regard to F-Actin-dependent HIV spread we make two important observations. Firstly, whilst viral budding is sensitive to depletion of the Arp2/3 regulators Rac1/WAVE2 and Cdc42/IQGAP1, cell-cell spread is primarily dependent on Cdc42/IQGAP1. Secondly, although HIV manipulates and enhances Cdc42/IQGAP1-dependent filopodia, which help mediate early donor-target cell contacts, Cdc42 may be inactivated at later stages of cell-cell HIV transfer, as suggested by collapse of filopodial networks during VS progression.

Discussion

The elaborate corruption of cortical F-Actin by HIV has remained elusive, with evidence both for and against its role in budding and cell-cell viral spread [16,20,33,67]. Through systematic depletion of various F-Actin regulators, combined with viral mutagenesis and high-resolution imaging, we were able to illuminate the intersection of HIV egress with cortical F-Actin and conclude this is primarily associated with the Cdc42-IQGAP1-Arp2/3 pathway.

In exploiting the power of FIB-SEM imaging, we observed HIV buds to be enriched to areas of the PM where cortical F-actin structures induce strong positive curvature. However, curvature and/or formin activity alone were not sufficient to position HIV at the tips of filopodia. Thus, other actin regulators associated with membrane curvatrue must be involved. Our observations herein that HIV infection specifically enhances Cdc42-Arp2/3-dependent filopodia, supports a mechanism of action that corrupts this pathway of actin nucleation. While it is conceivable that the curvature provided by HIV during budding could itself drive filopodia formation (e.g by direct recruitment/activation of Cdc42/Arp2/3 [68,69], we deem more likely that the virus hijacks a pre-existing cellular pathway that is dependent on curvature and that involves these regulators. This is because uninfected myeloid cells can generate similar long filopodia (albeit uncapped with HIV), whereas most filopodia in infected cells are HIV-capped [18]. If HIV would provide an independent mechanism of filopodia formation, both structures would be expected to coexist, whereas in reality HIV-capped filopodia predominate. Furthermore, HIV curvature mutants had a dominant negative effect on all filopodia, indicating i) a functional overlap of the viral and cellular pathways of filopodia formation, and ii) a critical role of Gag in hijacking of these structures. While we initially suspected the latter to be dependent on Gag's ability to interact with F-actin (i.e. via

its NC domain [46,47]), we show here that cells infected with Δ-NC virus readily form HIV-filopodia. Thus, other properties of Gag must be at play in this process.

A likely alternative link between the cellular and viral pathways of filopodia formation could be provided by PIP2. This is because both Gag and numerous cortical actin regulators are targeted to PIP2-rich domains of the PM [70-74]. Furthermore, several studies have shown that both PIP2 and positive membrane curvature can lead to Cdc42 activation and subsequent Arp2/3-mediated actin nucleation through a mechanism of curvature cascade amplification [68,69]. Of note, the ability of PIP2 to orchestrate actin cytoskeleton changes is exploited by HIV during viral entry [75,76], and from our observations herein also supports its importance for key stages in later viral spread. Based on our experimental observations and the available literature, we propose a model in which Gag, Cdc42, IQGAP1 and formins congregate at the PM due to their interactions with PIP2 (Fig. 8 A). Gag oligomerization may locally increase PIP2 concentration [51] and induces positive membrane curvature (Fig. 8 B). The latter may be further enhanced by Gag-mediated recruitment of BAR-domain proteins like Angiomotin and Pascin2 [77,78]. Both curvature and PIP2 activate Cdc42 [68,69], which in turn leads to activation of the Arp2/3 complex and branched F-actin polymerisation immediately below the PM. Growing actin filaments further bend the membrane and push the nascent viral bud away from the cell's surface, seeding a short Arp2/3-dependent filopodium (Fig. 8 C). IQGAP1 binds to Cdc42 and stabilizes it in its active conformation [62], while also facilitating assembly of multiprotein complexes that spatially link Cdc42, Arp2/3 and formins [61,79,80]. Activation of Diaph2 (e.g. via Cdc42 [81]) results in rapid and linear formin-mediated elongation of free F-actin barbed ends. Our model therefore proposes that immune cell filopodia are dependent on Arp2/3 mediated-actin nucleation for initial seeding, and on formin activity for their elongation. This is compatible with both the 'convergent elongation' and 'clustering-outgrowth' models of filopodia formation. In the former, filopodial birth involves reorganization of pre-existing filaments from the branched cortical F-actin network and their subsequent linear elongation [82], whereas the latter requires Arp2/3 activity to seed a small protruding F-actin precursor structure that is then extended by Dia formins and/or ENA/VASP proteins [83]. Importantly, Lee et al showed that filopodia formation by the latter model was strongly dependent on the presence of PIP2 and Cdc42, and could be most efficiently disrupted by simultaneous inhibition of Arp2/3 and Dia formins [83]. Our observations are therefore strongly consistent with this model, and our use of living human cells of hematopoietic lineage (as opposed to a cell-free animal protein system) extends the relevance of this biological meachanism to the medical immunology research field. In addition, our observations with CA-Diaph2 show that, although not the default mechanism, it is also possible for filopodia in immune cells to form via the 'tip nucleation' model, which depends on formin-mediated F-actin nucleation directly below the PM [23,84]. These formin-driven filopodia contained less Arp2/3 molecules and displayed formin-rich tip complexes (Fig. 3 D), as proposed in [84].

Manipulation of the Cdc42-Arp2/3 actin regulatory axis is likely not a unique feature of HIV. Nascent viral buds have also been observed at the tips of filopodia-like structures for other types of viruses [85-90], and numerous intracellular pathogens are known to exploit Rho-GTPases and the unique ability of the Arp2/3 complex to promote formation of specialized cortical F-actin membrane protrusions that facilitate cell-cell infection spread [91-95]. Similarly, IQGAP1 is a prominent target of microbial manipulation and this is closely related to its ability to modulate the actin cytoskeleton (reviewed in [96]). Several viruses bind IQGAP1 either directly (via interactions with the viral matrix protein) or indirectly (via common binding partners) and this has important consequences for viral assembly, budding

and/or pathogenesis [21,87,97-100]. For HIV, a similar interaction could be mediated by the known binding of Gag and IQGAP1 to the ESCRT proteins TSG101/ALIX [101,102], which participate in the final stages of viral particle abscission. This in turn would imply a role for IQGAP1 in HIV-budding, which is consistent with our observations in IQGAP1-deficient cells. Hijacking of IQGAP1 therefore likely represents a common mechanism of viral egress and could be an interesting pharmacological target for wide-spectrum antiviral activity.

In terms of HIV spread, free viral particle release was susceptible to inhibition of both lamellipodial and filopodial regulators. In contrast, cell-cell spread of HIV was mainly dependent on Cdc42 and IQGAP1 but showed tolerance to depletion of Rac1/Wave2, despite a similar impact of all regulators on free virus budding. Together, these observations suggest that long filopodia specifically contribute to cell-cell HIV spread, whereas lamellipodia are less important for this process. This is consistent with findings from other enveloped viruses where filopodia have been associated with the ability to mediate cell-cell viral transfer [103-107]. Indeed, we have previously observed HIV-Filopodia to mediate hundreds of contacts per hour between relevant primary HIV target cells, with filopodial activity often preceding VS formation [18]. The latter is in agreement with previous observations that filopodia and/or dendrites may commonly serve as precursors for biological synapses [108-112]. It has also been proposed that, as synapses mature, filopodia must be “supressed to allow a smooth and broad cell-cell interface” [112]. This is consistent with our observations herein that early HIV donor-target cell contacts are characterized by abundant filopodial activity, whereas filopodia are often lost during late stages of VS formation, when polarization and transfer of Gag is most evident. These remarkable cell-shape changes reveal that VS progression involves extensive cytoskeletal remodelling and suggest a clear switch of actin-manipulation strategy as the synpase matures. Loss of filopodia likely requires inactivation of Cdc42, and this could be important to allow synchronised budding and large-scale viral release at the VS, in a similar manner to how Cdc42 inactivation promotes mechanistically analogous (i.e. ESCRT-dependent) abscission events during late stage cytokinesis. In the latter context, Cdc42, IQGAP1 and TSG101 are all recruited to the midbody of dividing cells. Cdc42 activity is initially important for maturation of the contractile actin ring, but inactivation of this Rho-GTPase is then required to allow F-actin clearance from the midbody and the final step of daughter cell abscission [113-115]. The interaction between IQGAP1 and Cdc42 likely plays a pivotal role in regulating cytokinesis [116,117], whereas the interaction between IQGAP1 and ESCRT proteins such as TSG101 may be required to complete abscission [101,118]. Thus, IQGAP1 may serve as a scaffolding center that enables coordination and cross-signaling of F-actin remodelling and abscission events during both cytokinesis and the VS. A hypothetical model for the role of IQGAP1 at the VS is provided in Figure 9. However, as with other synapses, maturation of the VS is a temporally and mechanistically complex process, and further studies will be required to fully elucidate the mechanisms involved.

Overall, we propose that HIV has evolved to highjack a specific node of F-actin regulation that positions outgoing virus at the leading edge of cortical F-actin structures. Since filopodia play an important role in scanning of the microenvironment and mediating immune cell interactions, their corruption is beneficial to the virus because it biases the first line of cell-cell contacts towards HIV spread, e.g. by providing enhanced adhesion and specificity to CD4+ target cells. However, as these contacts form and the VS matures, the relationship of HIV with F-Actin must change to enable switching from target-cell “catch mode” to viral “release mode”. We conclude that manipulation of the Cdc42-IQGAP1-Arp2/3 actin regulatory node is essential for corruption of cellular filopodia to facilitate cell-cell HIV

spread, and propose a key role for IQGAP1 in integrating signals of F-actin regulation and viral abscission to strategically enhance HIV egress at sites of productive cell-cell contact.

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Figure legends

Figure 1. Depletion of F-actin regulators reveals long filopodial networks to be driven by Arp2/3 and Diaph2. A. F-Actin staining by phalloidin Alexa-647 reveals extensive and long filopodia in uninfected scramble shRNA control cells (Scr-CTL), short abundant filododia upon deletion of the formin Diaph2, and extensive lamellipodia when both Diaph2 and Arp2/3 are co-depleted. B. Schematic representation of FIB-SEM imaging data. C-H. FIB-SEM 2D images and 3D reconstructions of C-D non-depleted control cells (Scr-CTL), E-F Diaph2-depleted cells and G-H Diaph2 and Arp2/3 co-depleted cells. All scale bars are 5µm. F-H. Schematic representation of phenotypes induced by loss of F-Actin regulators. F. Wildtype scenario, G. Diaph2 deficiency, H. Diaph2 & Arp2/3 deficiency. Note in H. we represent reduced levels of Arp2/3, given its high cellular abundance and residual levels of Arp2/3 in our knockdown cells (Fig. S3).

Figure 2. HIV budding enriched to positively curved cortical F-Actin.
A-D Representative FIB-SEM images of HIV virions associated with B. Filopodia and C-D plasma membrane of a HIV infected cell depleted of Diaph2. E.-F. 3D rendering FIB-SEM images of HIV infected E. Diaph2^{-ve} and F. Diaph2^{-ve}Arp2/3^{-ve} cell clones. HIV buds are shaded in red to highlight their location. G&J. enumeration of HIV-buds in association with positively curved cortical F-Actin structures. G. Filopodia and J. lamellipodia are pseudocolored using a colour spectrum from blue (positive curvature), green (neutral curvature) to red (negative curvature). H-I Enumeration of total HIV buds in association with the filopodia in Diaph2^{-ve} cells. H. Absolute viral bud counts and I. HIV bud density per µm^2. K-L. Enumeration of total HIV buds in association with the lamellipodia in Diaph2^{-ve} Arp2/3^{-ve} cells. K. Absolute viral bud counts and L. HIV bud density per µm^2. All scale bars are 0.5µm with the exception of B-D, which is 0.1µm. Bar graphs in H-I represent mean and standard deviations of virion counts from n = 15 filopodial structures. Whereas K-L bar graphs represent mean and standard deviations of virion counts from n = 15 lamellipodial structures.

Figure 3. Constitutively active Diaph2 driven Filopodia are not associated with HIV buds.

A & B. Live still images from supplement Movie S3; A. $^{C/A}$Diaph2-mCherry (red) positive cells, infected with B. HIViGFP (green). Note Diaph2 at the tips of filopodia are negative for HIV. Scale bars are at 5µm. C. Quantification of HIV postive filopodia per cell in HIV infected live cell cultures. HIV filopodia counts pools from three independent HIV infections ***p<0.0001. D&E. Immunofluorescent Arp2/3 staining of D. $^{C/A}$Diaph2-mCherry (red) positive cells and E. Untreated cells. Asterisks highlight the terminal ends of filopodia that are either Diaph2 positive & Arp2/3 negative (for $^{C/A}$Diaph2-mCherry) or Arp2/3 positive (for untreated cells).

Figure 4. HIV Gag curvature mutants can impact Arp2/3 dependent cortical F-Actin

A-E HIV Gag mutants used. A. Wild type HIV Gag. B. HIV Gag late domain mutant with p6 deleted. C. NC deletion mutant with the Leucine Zipper (LZ) derived from *Saccharomyces cerevisiae* GCN4 to rescue Gag oligomerisation. D&E. HIV Gag curvature mutants D. P99A and E. EE75,76AA. F. Enumeration of filopodia per cell in Diaph2^{-ve} cell clones. ***=p<0.0001. G-H Representative FIB-SEM 3D rendered images of G. WT versus curvature mutants H. P99A and I. EE75,76AA.

Figure 5. Cortical F-Actin regulators enriched at the final stages of HIV egress are revealed through HIV proteomics.

A-B Viral proteome analysis of proteins associated with cortical F-Actin regualtion. Proteins with increased abundance relative to untreated cells are shown in green, whereas those with relative decreased abundance are shown in red. A. Virions produced in Diaph2^{-ve} cells. 1. Highlights the Arp2/3 complex node where the amounts of ACTR2 and Cdc42 are increased in virion proteomes. 2. Indicates a GTPase node in association with IQGAP1. 3. Highlights a node of integrins and related proteins. 4. Highlights a node involved in cadherin adhesion that is downregulated upon Diaph2 depletion. B. Virions produced in Diaph2^{-ve}Arp2/3^{-ve} cells. Note that in 1. Arp2/3 components are predictably depleted compared to untreated cells, whilst in 2. the GTPase node and IQGAP1 remain unchanged.

Figure 6. HIV infection and its influence on cortical F-Actin.

A. From viral proteomics, we identified a common node of actin regulators sassociated with Arp2/3-dependent filopodia and lamellipodia. Through shRNA depletion or CRISPR-Cas9 knockout, we generated clonal cell populations depleted of various actin regulators. B-C Lamellipodial regulators. B. Rac1^{-ve} and C. WAVE2$^{k/o}$. D. Cdc42$^{k/o}$ (filopodial regulator). E. IQGAP1^{-ve}. A. Represents the untreated control. All cells were infected with HIV iGFP (green) and then counterstained with phalloidin Alexa-647 (white). All scale bars are at 5µm. Inset magnifications reveal HIV at the leading edge of filopodial structures. Note in Rac1^{-ve} and WAVE2$^{k/o}$ images the extensive filopodial networks only present in HIV infected (green) cells (see Fig. S2 for formal quantification).

Figure 7. HIV spread is dependent on Cdc42 and IQGAP1

A. A functional protein interaction network is presented, summarising the F-Actin regulators associated with HIV assembly. This is based on the sum of observations from Fig. 1 through to 6. Of note IQGAP1 is central to this interaction node. TSG101 is also presented, as it provides the link between not only HIV Gag and this network, but also HIV abscission. From this network, clonal cells derived from shRNA depletions or CRISPR Cas9 knockout (single cell sorted, expanded, and verified) are presented in B.-C. B. All cells are infected with pHIVNL43iGFP and normalised to 5% infection on day 3. After normalisation, cell

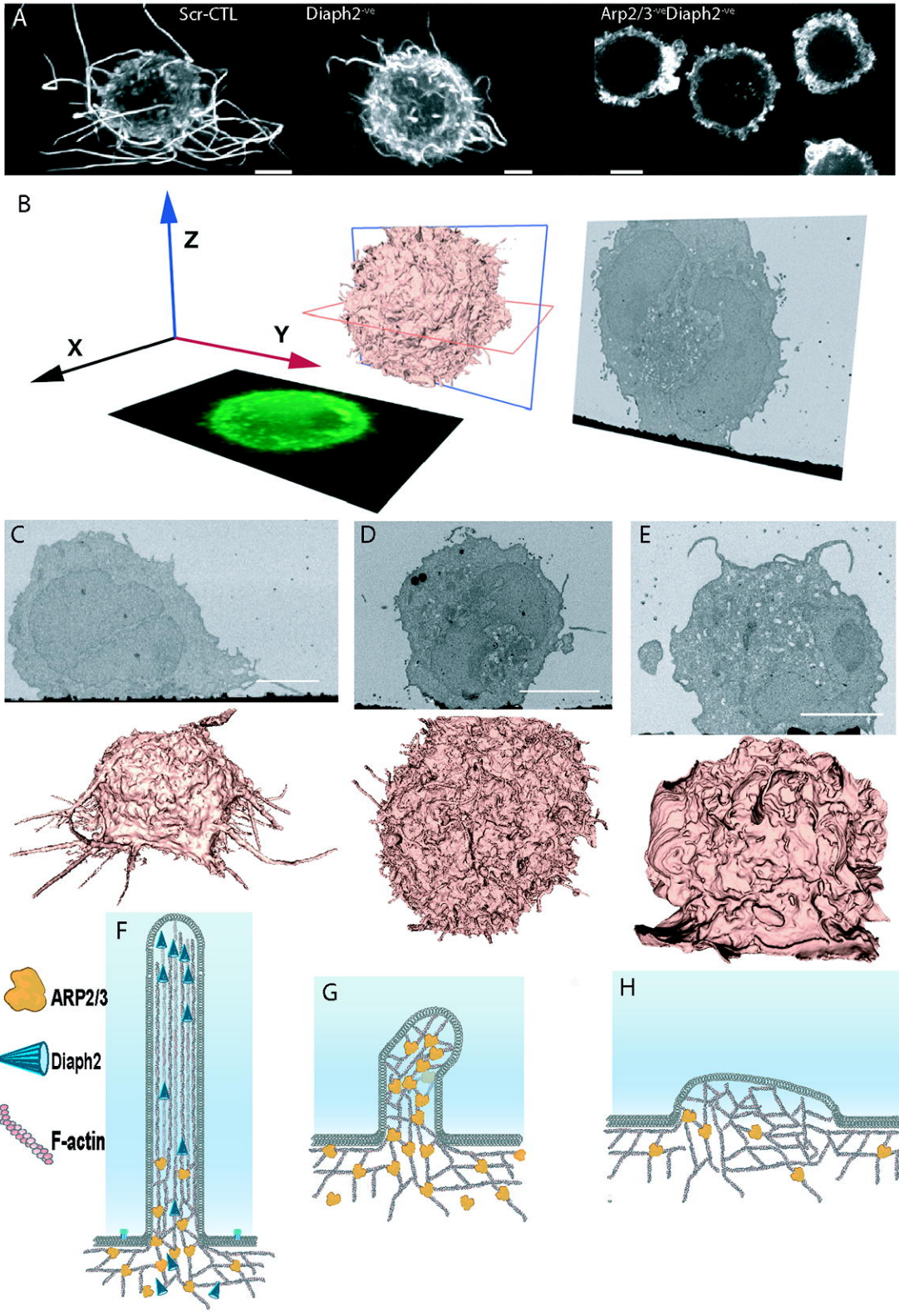
supernatants are collected over a 24 hour period and GFP positive HIV particles are spinoculated onto 96-well glass plates coated with poly-L-lysine. Absolute viral particle counts are determined by high-resolution fluoresecence microscopy per 4 fields of view. Herein the data is presented as a relative count [(virion count/virion count in WT control)*100]. C. HIV infected cells as normalised in B. are then co-cultured at a ratio of 1:5 with HIV-permissive TZMBl targets. D. Infected cells are co-cultured with primary CD4 target T-cells at limiting dilutions. Dilution steps correspond to 5%, 1% and 0.2% infected cells in the donor population. Exposure to virus from infected cells is limited to 24 hours, after which an entry inhibitor BMS806 is added to prevent further viral spread. B-D. Data indicates the mean and standard deviation from 3 independent experiments. In D, primary recipient CD4 T cells were sourced from independent blood donors. * $p<0.01$, ** $p<0.001$, *** $p<0.0001$.

Figure 8. Summary of F-actin remodelling during HIV-assembly associated with filopodial formation

A. Both HIV Gag and F-actin nucleation machinery accumulate at PIP2-rich regions of the PM. B. The presence of PIP2 and curvature driven by Gag assembly activates Cdc42 driving filopodial formation. C. IQGAP1 is recruited by active Cdc42, as well as ESCRT proteins (not illustrated) which are recruited by Gag. D. In the absence of the formin Diaph2, small curved filopodia dominate. E. In the presence of Diaph2, the scaffolding activity of IQGAP1, drives the collaboration between Arp2/3 and formins to enable the formation of long filopodia (averaging 10µm in length). In all settings, HIV buds accumulate alongside Arp2/3 dependent F-Actin structures that exhibit positive curvature at the plasma membrane. As a consequence, most filopodia in infected cells have their terminal ends replaced with HIV buds.

Figure 9. Proposed model of spatio-temporal regulation of F-Actin during HIV egress.

A. With probing and tethering activity corrupted by HIV buds, the F-actin structures at this phase enable pre-synpatic events (cell catching and adhesion). Note, only a subset of HIV buds are engaged in this process, with many undergoing the final stages of egress to contribute to cell-free virus. B. Following cell-cell engagement, the viral synapse matures. This leads to two important outcomes. Firstly, similar to that observed at the immunological synapse [119] Cdc42-F-Actin activity is altered and transitions from filopodial biogenesis to cell-cell adhesion required for HIV release (as observed when cells collapse filopodia just prior to HIV fusion). During this change this signals the final stages of abscission to generate the concentration of mature particles at the synaptic cleft. Of note, we hypothesise IQGAP1 being directly involved through providing a scaffolding center needed temporally coordinate and feedback to the Cdc42-F-Actin pathway and the TSG101-ESCRT abscision node.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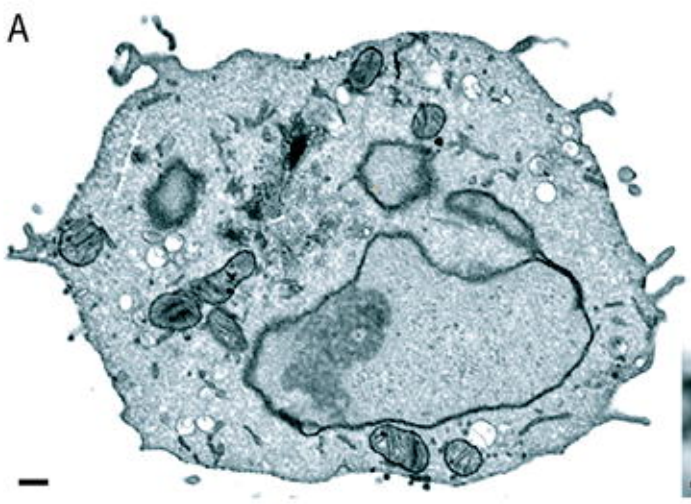

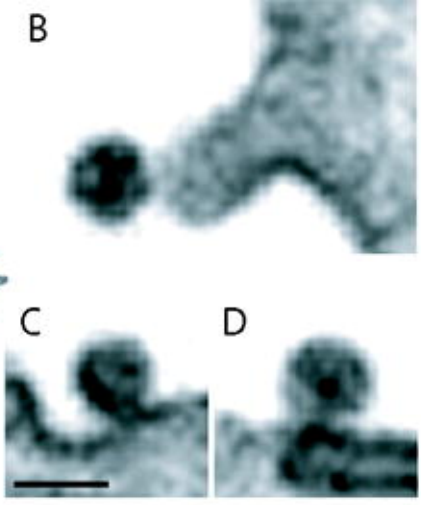

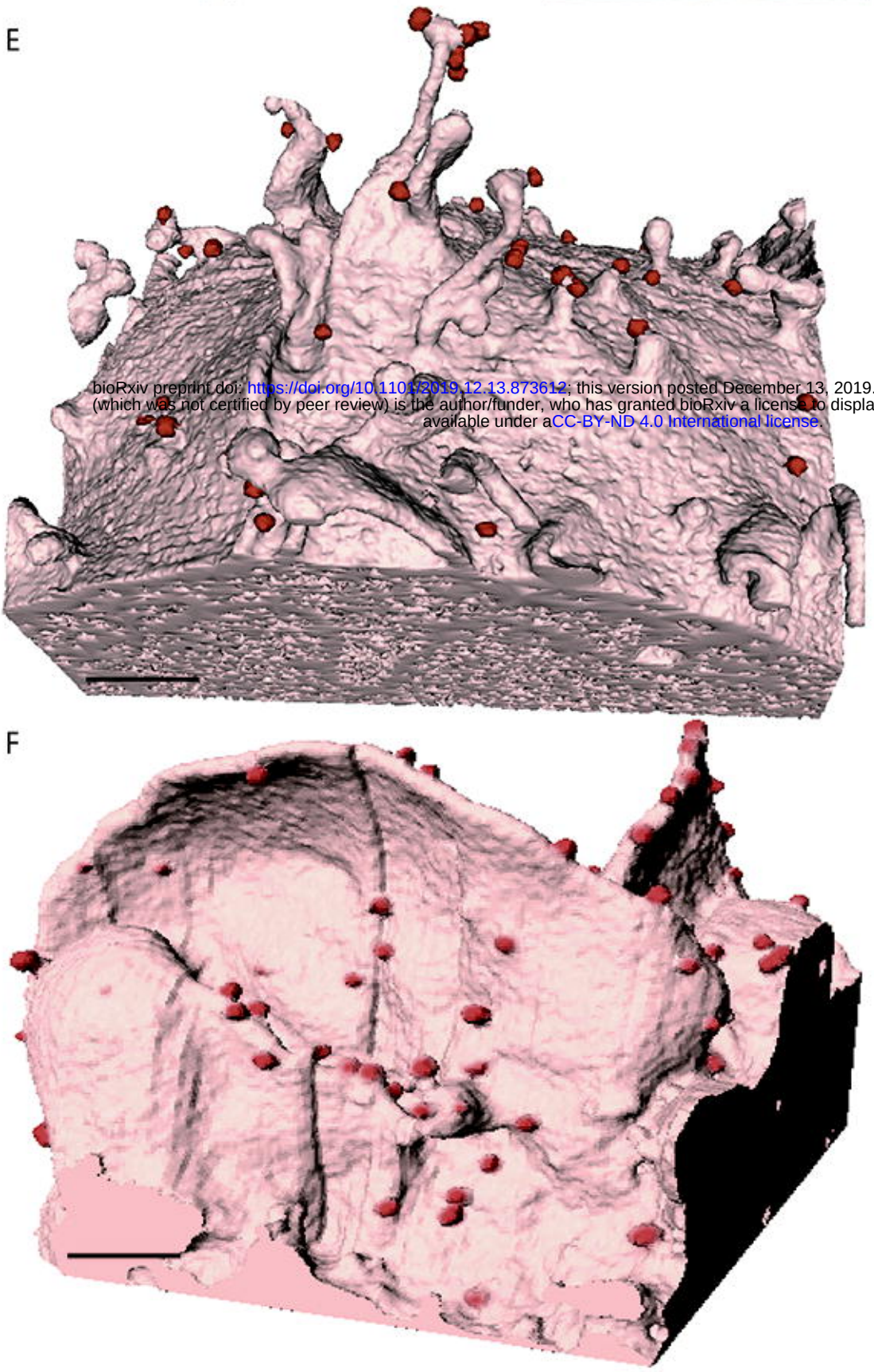

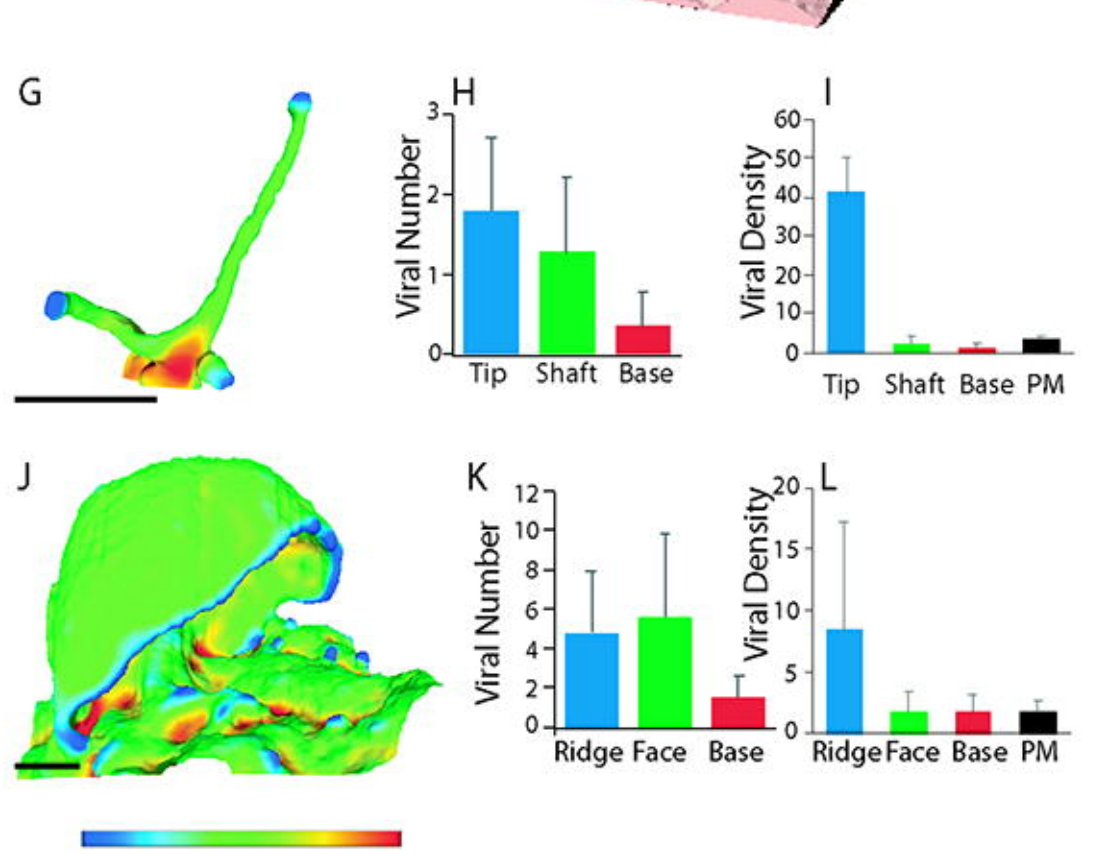

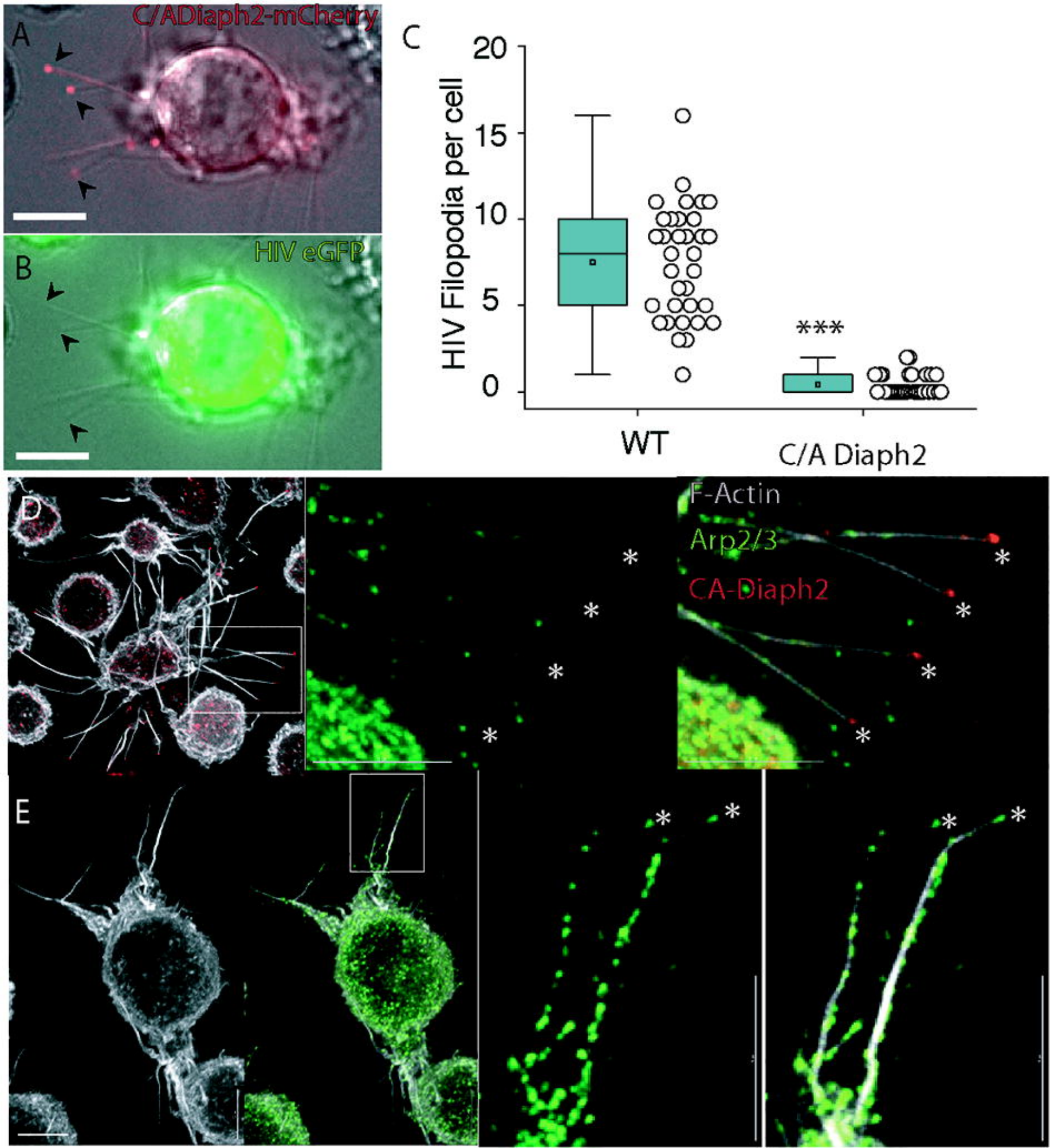
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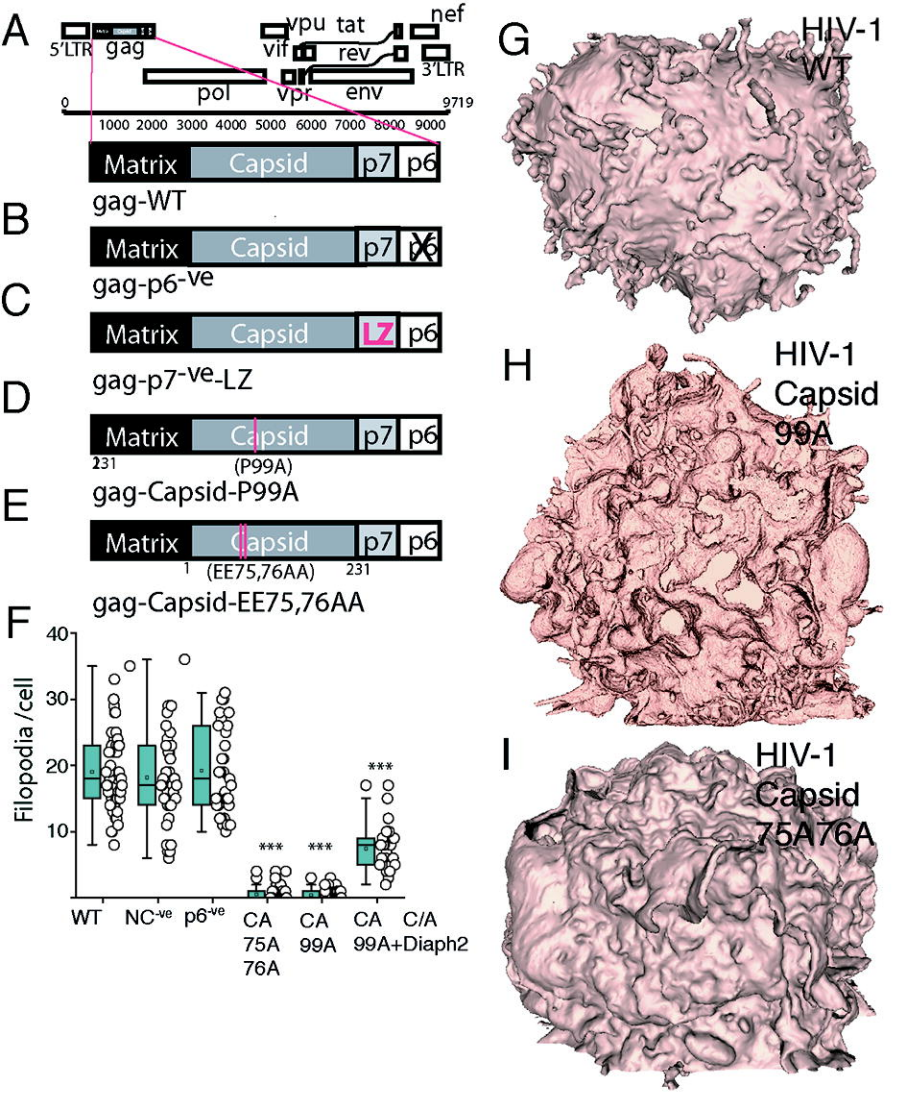
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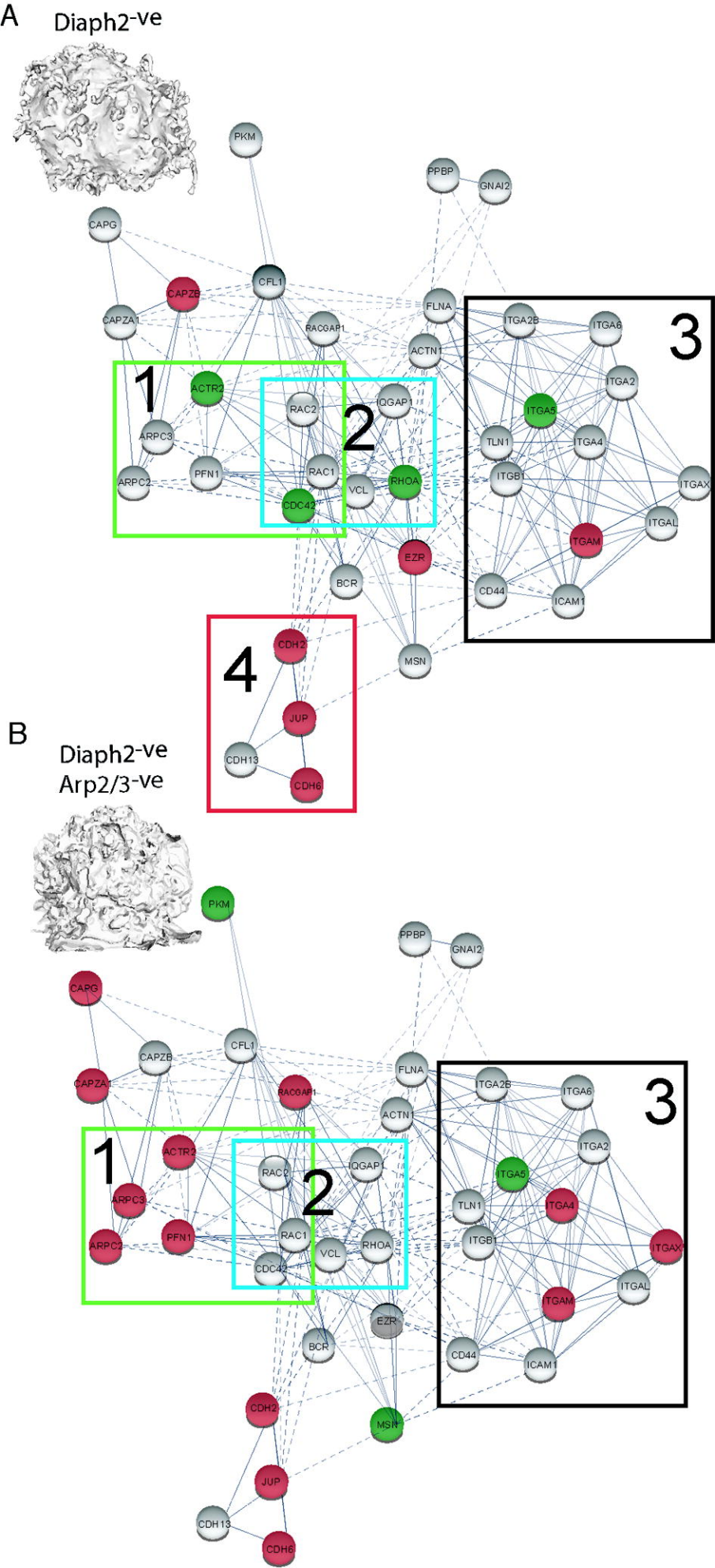
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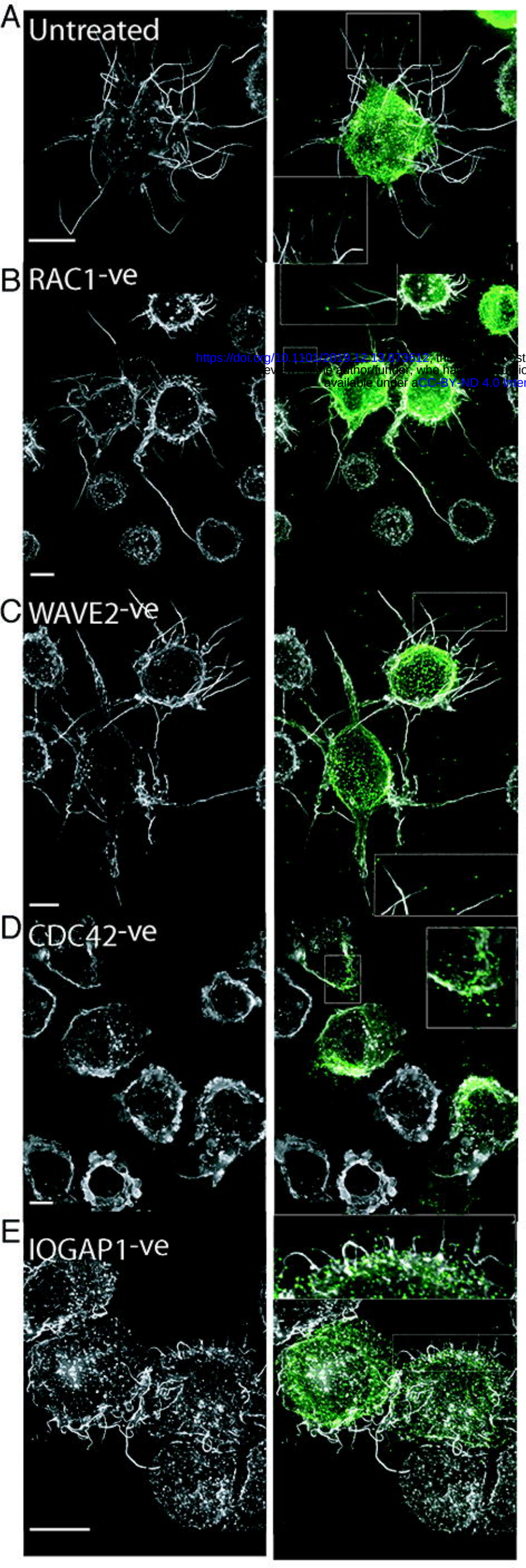

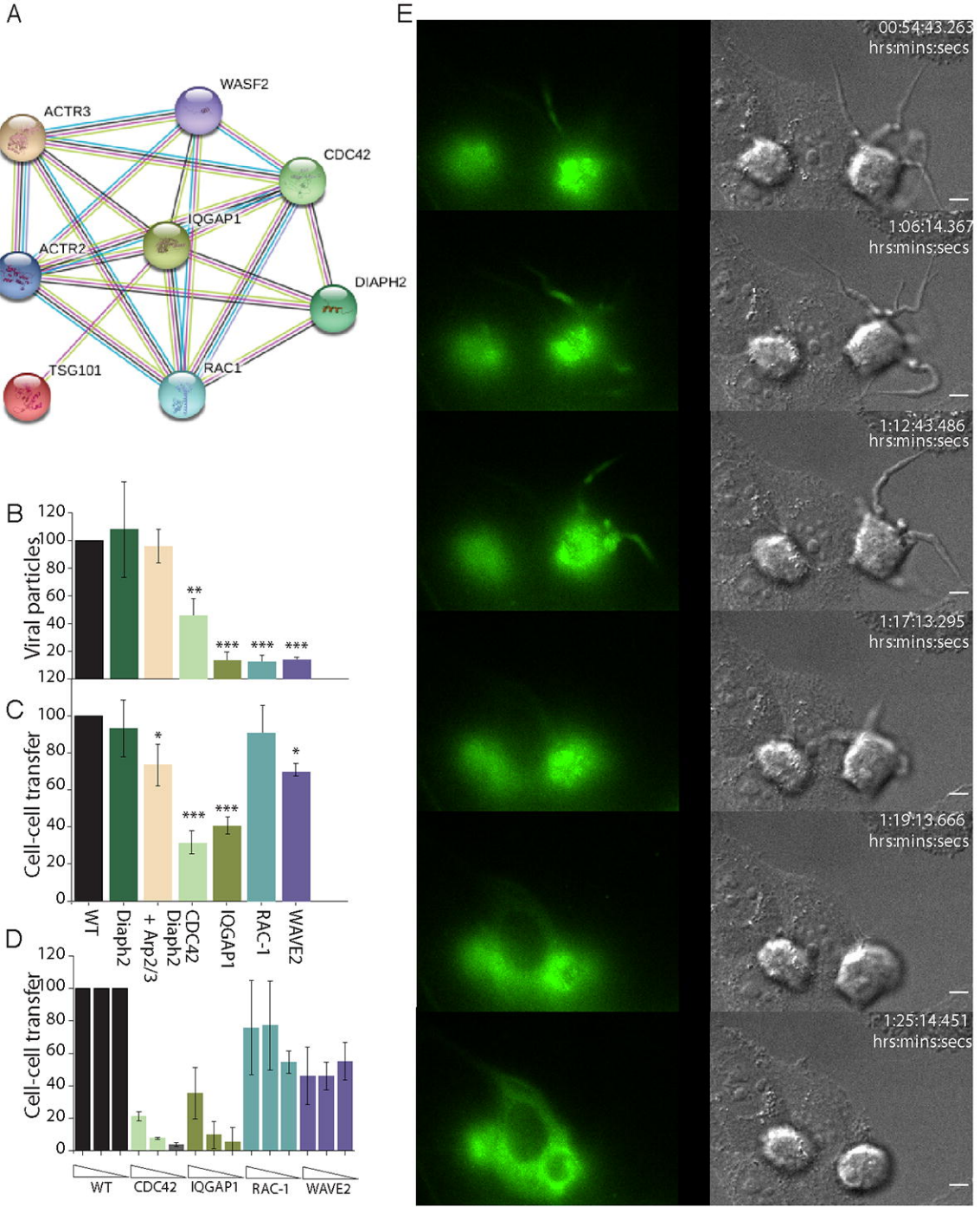
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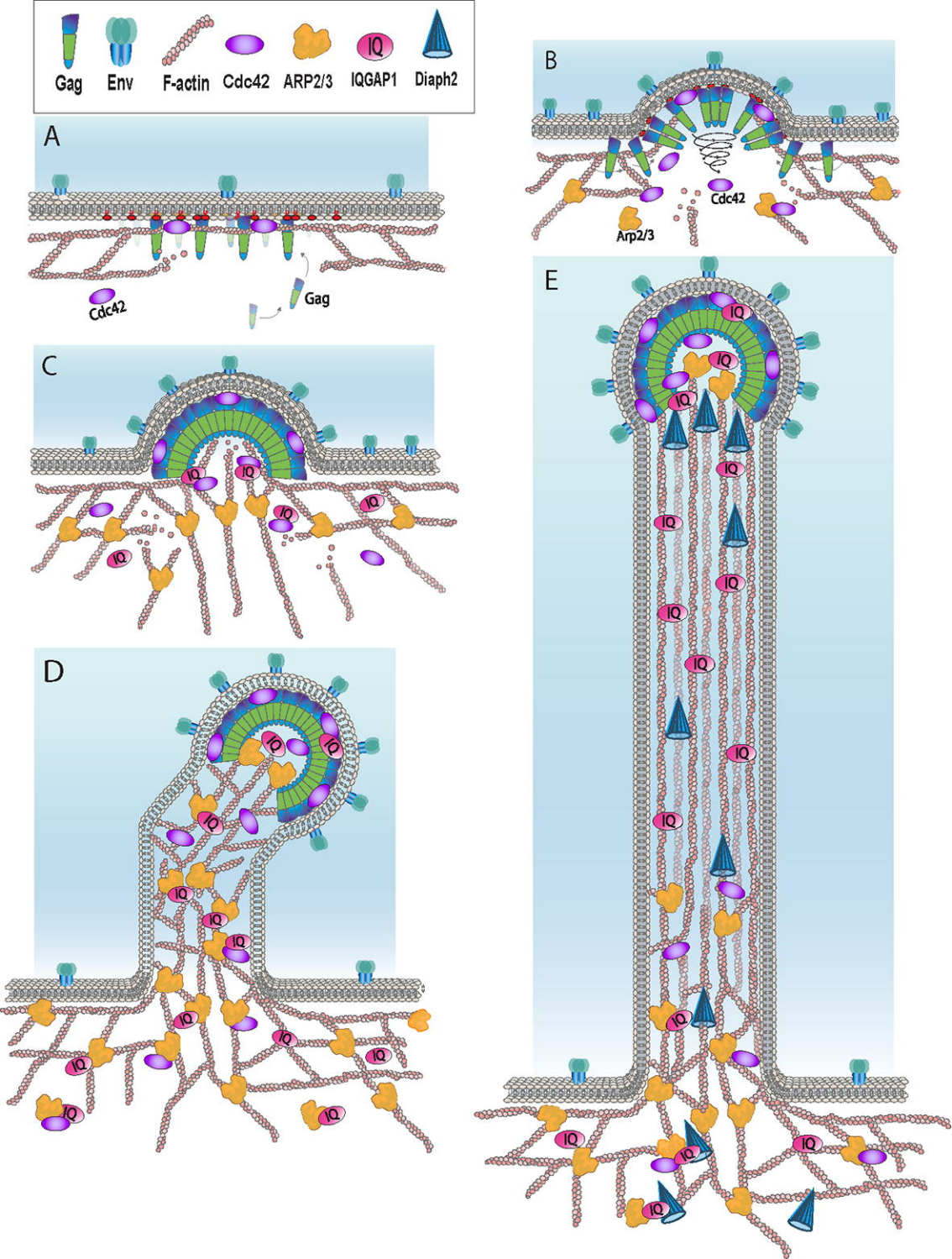
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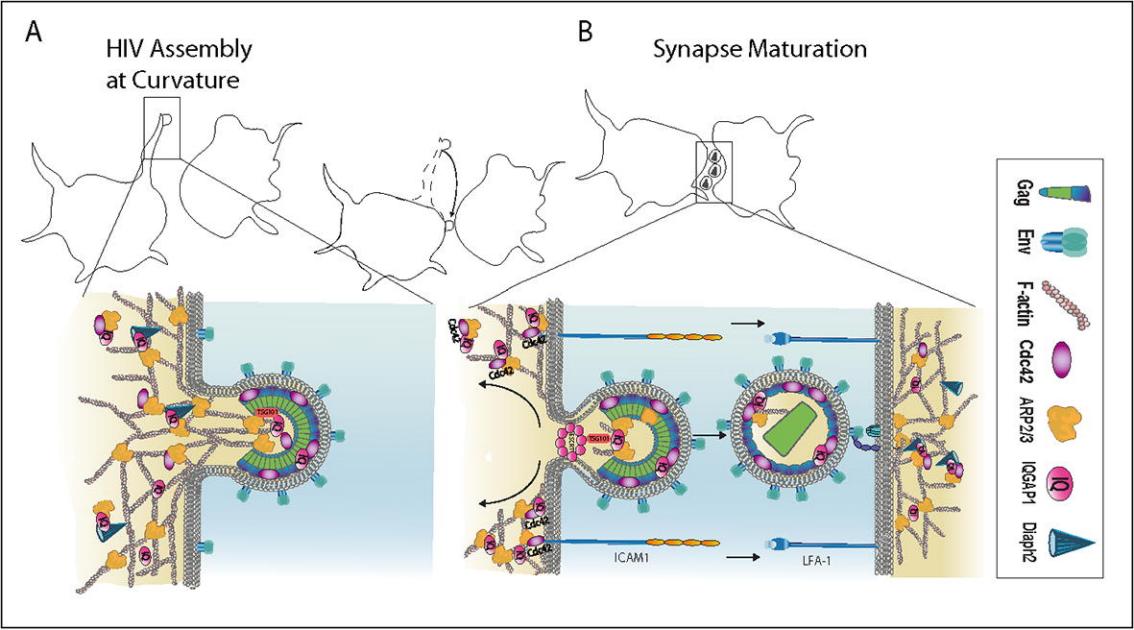
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