The level of Nck rather than N-WASP determines the rate of actin-based motility of Vaccinia

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Running title: Quantitative imaging of vaccinia virus stimulating actin polymerization

ABSTRACT

Vaccinia virus exiting from host cells activates Src/Abl kinases to phosphorylate A36, an integral membrane viral protein. Phosphorylated A36 binds the adaptors Nck and Grb2 which
18 recruit N-WASP to activate Arp2/3-driven actin polymerisation to promote viral spread. A36 also recruits intersectin, which enhances actin polymerization via AP-2/clathrin and Cdc42. How many viral and host molecules does vaccinia hijack to induce actin polymerization? To advance our quantitative understanding of this process, we now determined absolute numbers of the essential molecules in the vaccinia signalling network using fluorescent molecule counting approaches in live cells. There are 1156±120 A36 molecules on virus
24 particles inducing actin polymerization in HeLa cells. This number, however, is over 2000 in mouse embryonic fibroblasts (MEFs), suggesting that A36 levels on the virion are not fixed. In MEFs, viruses recruit 1032±200 Nck and 434±10 N-WASP molecules, suggesting a ratio of 4:2:1 for the A36:Nck:N-WASP signalling network. Loss of A36 binding to either secondary adaptors Grb2 or intersectin results in a 1.3- and 2.5-fold reduction in Nck respectively. Curiously, despite recruiting comparable numbers of the Arp2/3 activator, N-WASP (245±26
30 and 276±66), these mutant viruses move at different speeds that inversely correlate with the number of Nck molecules. Our analysis has uncovered two unexpected new aspects of Vaccinia virus egress, numbers of the viral protein A36 can vary in the virion membrane and the velocity of virus movement depends on the levels of the adaptor protein Nck.

INTRODUCTION

36 Vaccinia virus is the most studied member of the poxvirus family and best known for being used as the vaccine to protect against smallpox. It is a large double stranded DNA virus, which undergoes a complex replication cycle in perinuclear cytoplasmic viral factories in infected cells (Leite & Way 2015). Replication results in the formation of infectious intracellular mature virions (IMV) which undergo kinesin-1 driven microtubule transport to disperse throughout the cell before being released when the infected host lyses (Sanderson et al. 2000; Ward
42 2005; Xu et al. 2022). Prior to cell lysis, some IMV are also capable of becoming enveloped by a Golgi cisterna to form intracellular enveloped virions (IEV) (Leite & Way 2015; Schmelz et al. 1994; Tooze et al. 1993). This envelopment process, which is still not fully understood, is dependent on several viral proteins including the integral membrane protein B5 and the lipid-anchored F13 (Blasco & Moss 1991; Engelstad & Smith 1993; Smith et al. 2002; Wolffe et al. 1993). Once formed, IEV undergo microtubule-based transport from their perinuclear
48 site of assembly to the plasma membrane (Geada et al. 2001; Hollinshead et al. 2001; Rietdorf et al. 2001; Ward & Moss 2001a), where they fuse their outermost membrane to release extracellular enveloped virions (EEV) (Leite & Way 2015; Smith et al. 2002). EEV are thought to promote the long-range spread of infection, but some virions can remain attached to the outer surface of host cell after fusion. Known as cell-associated extracellular virions (CEV), these viruses can induce Arp2/3 complex dependent actin polymerisation beneath the virion
54 (Fig 1), (Cudmore et al. 1995, 1996; Frischknecht et al. 1999; Hollinshead et al. 2001). The power of actin polymerization subsequently drives the CEV away from the host cell to enhance its spread into non-infected neighbouring cells (Cudmore et al. 1995, 1996; Doceul et al. 2010; Ward & Moss 2001b).

The ability of IEV to move on microtubules as well as the stimulation of actin polymerization
60 by CEV is dependent on A36, an integral viral membrane protein present in the Golgi that envelopes IMV during IEV formation (Frischknecht et al. 1999; Rietdorf et al. 2001; Röttger et al. 1999; Sanderson et al. 1998; Ward & Moss 2001a; Wolffe et al. 1998). In contrast to B5 and other integral viral membrane proteins in the Golgi that wrap IMV, A36 does not end up in EEV and is only retained in the plasma membrane beneath CEV (Fig 1), (Smith et al. 2002; van Eijl et al. 2000). A36 facilitates microtubule transport of IEV by recruiting kinesin-1 to the

66 virus (Rietdorf et al. 2001). It achieves this by interacting directly with the tetratricopeptide repeats (TPR) of the kinesin light chain via a bipartite tryptophan acidic motif that is also found in cellular kinesin-1 binding proteins (Dodding et al. 2011; Pernigo et al. 2013; Ward & Moss 2004). After IEV fuse with the plasma membrane, CEV attached to the outside of the cell locally activate Src and Abl family kinases to induce phosphorylation of tyrosine 112 and 132 in the cytosolic tail of A36 (Fig 1), (Frischknecht et al. 1999; Newsome et al. 2004, 2006;
72 Reeves et al. 2005). Phosphorylation of tyrosine 112 and 132, results in the recruitment of the adaptors Nck and Grb2 via their SH2 domains respectively (Frischknecht et al. 1999; Scaplehorn et al. 2002). Nck subsequently recruits N-WASP via WIP to stimulate the actin nucleating activity of the Arp2/3 complex beneath CEV (Donnelly et al. 2013; Moreau et al. 2000; Zettl & Way 2002). The resulting actin polymerisation drives CEV motility, enhancing cell-to-cell spread of infection (Ward & Moss 2001a). Not surprisingly, the amount of
78 phosphorylation competent A36 influences the length of actin tails as well as the speed of virus movement (Humphries et al. 2012). Grb2, in contrast to Nck, is not essential for this process, but its recruitment stabilises the signalling complex, making actin assembly more robust (Scaplehorn et al. 2002; Weisswange et al. 2009). In addition, three NPF motifs near the C-terminus of A36 interact with the RhoGEF intersectin to recruit Cdc42 and clathrin, further enhancing actin polymerisation beneath CEV (Fig 1) (Humphries et al. 2012, 2014;
84 Snetkov et al. 2016).

We know the identity of the viral and key host components as well as their interactions and roles in CEV-stimulated actin assembly. The turnover rates of Nck, Grb2, WIP and N-WASP beneath CEV have also been determined (Weisswange et al. 2009). However, to obtain a full quantitative understanding of virus-induced actin polymerization we need to establish the
90 number of molecules participating in this signalling network. Such an investigation will also allow comparative analyses with other networks in cell physiology to uncover basic signalling principles. Taking advantage of fluorescent nanocages with defined numbers of GFP molecules (Akamatsu et al. 2020), we recently determined that IEV recruit 320 kinesin-1 motor complexes (Xu et al. 2022). Using a similar approach, we have now determined the number of A36 molecules beneath CEV together with B5 and F13 which are essential for IEV
96 assembly, as well as Nck and N-WASP that are required for CEV induced actin polymerization.

RESULTS

Quantification of the number of A36 molecules associated with CEV

To measure the number of A36 molecules on virus-inducing actin tails using quantitative fluorescent imaging, we first generated a recombinant virus expressing an A36-TagGFP2
102 fusion from its genomic locus together with RFP-A3 to mark viral cores (Fig S1A). The A36-TagGFP2 recombinant virus formed plaques that were slightly smaller in diameter than the Western Reserve (WR) the parental strain (1.63±0.12 mm vs 1.95±0.07 mm) (Fig S1B). Importantly, A36-TagGFP2 was incorporated in late-stage virus particles which localized to the tip of actin tails (Fig 2A). The intensity of A36-TagGFP2 was substantially higher than membrane-tethered nanocages we have previously used to generate a fluorescence
108 calibration curve in live cells (Fig 2B, (Xu et al. 2022)). The upper limit of our calibration curve is 180 molecules (Fig S2A, (Xu et al. 2022)). To examine whether the curve we generated would be valid for higher fluorescence intensities, we acquired images of 60mer and 120mer nanocages at 4x and 3x exposure times respectively. The average measurements obtained from these experiments, were consistent with the expected values for a 240mer and 360mer nanocage (Fig 2C). To quantitively determine the number of A36 molecules on virus inducing
114 actin tails, we imaged and analysed confocal Z-stacks of HeLa cells infected with the A36-TagGFP2 virus from 8 hours post-infection, using LifeAct-iRFP and RFP-A3 as actin and viral markers respectively (Fig 2D). Co-localisation of A36-TagGFP2 and RFP-A3 signals occurs in IEV and CEV but the inclusion of an actin marker ensures that only CEV are analysed. This analysis revealed that CEV on the tips of actin tails have 1156 ± 120 A36 molecules. As this value is much higher than our experimentally established calibration curve, we also measured
120 A36-TagGFP2 intensities on actin tails at 0.5x and 0.25x exposure times (Fig 2D). The average intensity values obtained from these shorter exposure times lie along the extrapolated calibration curve, suggesting that our determination of A36 numbers is reliable.

Viral proteins F13 and B5 are present in high numbers on CEV

In addition to A36, the outer membrane of Vaccinia has additional viral proteins, A33, A34,
126 A56, E2, F12, B5 and F13, the latter two of which are required for formation of intracellular enveloped virions (IEV) the precursor to CEV (Dodding et al. 2009; Domi et al. 2008; Smith et al. 2002). B5, like A36 is an integral membrane protein, while F13 associates with the viral membrane via lipidation. Given the essential role of B5 and F13 in the assembly of IEV, the

precursor to CEV, we investigated how the abundance of B5 and F13 compares to A36. We therefore generated recombinant viruses expressing B5-TagGFP2 or TagGFP2-F13 at their
132 endogenous loci in a parental virus expressing RFP-A3 to determine the numbers of B5 and F13 molecules associated with CEV nucleating actin tails (Fig S1A). The B5-TagGFP2 and TagGFP2-F13 recombinant viruses also formed plaques that were slightly smaller than the parental Western Reserve (WR) strain (Fig S1B). B5-TagGFP2 and TagGFP2-F13 positive virus particles were observed at the tips of actin tails in infected cells, with TagGFP2-F13 appearing substantially brighter than B5-TagGFP2 (Fig 3A). To quantify their absolute numbers, we
138 imaged and analysed Z sections of infected cells as described for A36-TagGFP2. Based on comparisons of the fluorescence intensities obtained with our calibration curve, we calculated that CEV particles in HeLa cells inducing actin tails have 3107±602 and 8729±688 B5 and F13 molecules respectively (Fig 3B,C). These values are considerably higher than that of A36, however, B5 and F13 unlike A36 are present in the plasma membrane beneath CEV as well as the virion membrane (Fig 1).

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What is the ratio of A36:Nck:N-WASP on virions nucleating actin?

Once phosphorylated, A36 recruits a signalling network involving the adaptor Nck and N-WASP to activate the Arp2/3 complex to induce an actin tail. Nck and N-WASP are essential for this process, but how many molecules of each do Vaccinia CEV particles recruit? To address this question, we generated cell lines stably expressing TagGFP2-Nck1 in mouse
150 embryonic fibroblasts (MEFs) lacking Nck1 and Nck2, as well as TagGFP2-N-WASP in N-WASP null MEFs (Fig S3). Using these cells ensures that all the Nck or N-WASP molecules are fluorescently labelled. However, these are a different host cell type from HeLa, which were used to determine the number of A36 molecules associated with CEV.

Virion composition and structure is not expected to vary depending on the host cell type, but
156 this has never formally been investigated. To determine whether A36 numbers are comparable between MEF and HeLa cells, we quantified A36-TagGFP2 molecules on virions inducing actin tails in Nck+/+ and N-WASP+/+ parental MEFs. Surprisingly, we find that the number of A36 molecules in both MEF cell lines is considerably higher than in HeLa cells (Fig 4A). We confirmed that nanocage intensities were comparable between these cell types (Fig S2B), suggesting that A36 incorporation into CEV is indeed higher in MEFs. We wondered

162 whether this difference relates to the cell type and/or host species. To test whether this is the case, we determined A36 numbers on virus inducing actin polymerization in human lung epithelial A549 cells. We found that CEV forming actin tails in A549 cells have comparable numbers of A36 molecules as we measured in HeLa (A549 1165±126 vs HeLa 1156 ± 120). We next sought to determine if these host-dependent differences are also true for B5, another integral membrane Vaccinia protein. Measuring the intensity of B5-TagGFP2 on CEV in HeLa,
168 A549 and the two MEF cell lines reveals that, in contrast to A36, the numbers of B5 molecules are comparable between the four different cell lines (Fig 4B).

As the Vaccinia life-cycle appears to proceed faster in HeLa cells than MEFs, our lab typically analyses actin tails at 8 and 16 hours post-infection in these cell types respectively (Weisswange et al. 2009). To investigate whether A36 levels on CEV depends on time post-
174 infection, we compared A36-TagGFP2 intensities in HeLa and MEF cells at 8 and 16 hours post infection. We find that A36 numbers on CEV particles do not vary with time in HeLa cells. In contrast, in MEFs, the level of A36 associated with CEV increase as the infection progresses, but even at 8 hours post infection, more A36 molecules are present than in HeLa (Fig 4D). The reason for this difference is not immediately clear although it is striking that the bulk levels of A36, but not B5 are considerably higher in N-WASP+/+ parental MEFs than HeLa cells (Fig 4E).
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Having determined the number of A36 molecules on CEV in MEF cells, we measured the number of Nck and N-WASP molecules associated with the virus. TagGFP2 tagged Nck and N-WASP were both recruited to CEV inducing actin tails (Fig 5A). Quantification of the fluorescence intensity of the TagGFP2 signals in Z stacks indicates that each CEV recruits on average 1032 ± 200 and 434 ± 10 Nck and N-WASP molecules respectively (Fig 5C, D). Taken
186 together with our data on A36 numbers on actin tail forming particles in MEFs in Fig 4C, this suggests that A36, Nck and N-WASP are present in a ~4:2:1 ratio.

The level of Nck and not N-WASP determines the velocity of CEV

While Nck and N-WASP are essential for CEV induced actin polymerisation, the virus also recruits Grb2 and intersectin which act as secondary factors to enhance actin tail formation
192 (Fig 1). Our previous FRAP analysis demonstrates that Grb2 helps stabilize Nck and N-WASP beneath CEV during virus induced actin polymerization (Weisswange et al. 2009). To

determine whether these factors also impact on the absolute number of Nck and N-WASP molecules, we infected cells with either the A36-Y132F or A36△NPF mutant viruses recruited to CEV. Our measurements show that mutation of Tyr132 to phenylalanine (loss of Grb2) reduced the level of Nck and N-WASP by ~24% and 44% respectively N-WASP (Fig 6A,B).
198 However, in the absence of the A36 NPF motifs, both Nck and N-WASP numbers were significantly reduced (~61 and 36%) (Fig 6A, B). Interestingly, for both the A36-Y132F and A36 △NPF mutant viruses, the number of N-WASP molecules were reduced to similar levels (245 ± 26 and 276 ± 66 respectively).

Nck and N-WASP association with CEV generates Arp2/3-mediated actin polymerisation that
204 drives the motility of the virus. As N-WASP directly activates the Arp2/3 complex, we would predict that N-WASP numbers alone ultimately determine the speed of the virion. To examine if this is indeed the case, we measured the speed of wild-type, A36-Y132F and A36 △NPF viruses in HeLa cells stably expressing LifeAct-iRFP. Surprisingly, despite having similar levels of N-WASP, the A36-Y132F and A36△NPF viruses move at different speeds (Fig 6C). The A36-Y132F virus is slightly faster than the wildtype WR strain (0.2 ± 0.01 compared to
210 0.17 ± 0.01 μm/sec), but the speed of A36△NPF virus is significantly increased (0.24 ± 0.02 μm/sec). It is striking that these changes in virus speed are inversely proportional to the number of Nck molecules recruited by the virus.

DISCUSSION

Over the years, Vaccinia has provided a powerful model to understand the function and
216 regulation of Nck:N-WASP signalling and its role in stimulating Arp2/3 driven actin polymerization. Our current analysis provides new quantitative information concerning the signalling network driving this process. We found that Nck is twice as abundant as N-WASP but half as much as A36, the viral protein ultimately initiating actin tail formation. Overall, there appears to be a 4:2:1 ratio for the A36:Nck:N-WASP signalling network in MEFs. These values are consistent with the 2:1 ratio for Nck:N-WASP determined from modelling and live
222 cell imaging of artificially clustered Nck SH3 domain on the plasma membrane (Ditlev et al. 2012). Our analysis goes beyond ratios as it now provides absolute numbers for Nck and N-

WASP as well as A36, the activator of the signalling network. This constitutes the first such characterisation of a host-viral signalling network.

Speed of virus depends on the level of Nck and not N-WASP

228 Previous studies have taken advantage of the known structure of viruses to generate fluorescence calibration standards by tagging defined numbers of viral capsid proteins with (Dundr et al. 2002; Jose et al. 2015; Murray 2017). More recently, engineered nanocages with fixed numbers of GFP molecules have been used to provide quantitative insights into N-WASP and Arp2/3-driven endocytosis in yeast and human cells (Akamatsu et al. 2020; Sun et al. 2019). Using a similar imaging approach, we find that CEV inducing actin polymerization in
234 MEFs recruit just over 400 molecules of N-WASP. This value is considerably higher than the maximum N-WASP numbers measured for endocytic patches in yeast (budding yeast 102 and fission yeast 138) (Sun et al. 2019). Interestingly, however, in human cells, ~200 copies of the Arp2/3 complex are recruited during clathrin-mediated endocytic events (Akamatsu et al. 2020). Previous biochemical and structural analysis demonstrate that N-WASP dimers are more efficient at activating Arp2/3 and that the complex contains two N-WASP binding sites
240 (Higgs & Pollard 2000; Padrick et al. 2011; Zimmet et al. 2020). This would suggest that an endocytic site in human cells will also recruit ~400 N-WASP molecules, which is similar to the values we have observed associated with CEV. This is surprising, as the area under a CEV is considerably larger than an invaginating endocytic pit which are typically 60-100 nm in diameter in human cells (Vigers et al. 1986; Yang et al. 2022). Based on our recent measurements of Vaccinia virion dimensions in cryo-electron tomograms of infected cells, the
246 area under a CEV, which corresponds to the outer membrane of IEV, is 405,037 nm^2 (Hernandez-Gonzalez et al. 2022). This value is ~ 13x higher than the surface of an endocytic pit (based on a radius of 50 nm). This suggests that the density of the signalling network beneath Vaccinia CEV is significantly less than that of an endocytic patch.

Loss of Grb2 or intersectin recruitment leads to even lower levels of N-WASP (245±26 and
252 276±66 respectively) at CEV. However, the system does not collapse, and virus motility even increases. Moreover, while the recruited levels of N-WASP in the two mutants are similar, in the absence of intersectin (A36△NPF) the virus moves significantly faster than when Grb2 is absent (A36-Y132F). The striking difference between these two mutant viruses is the level of

Nck recruited to the CEV, with the numbers being 61% lower for the A36△NPF mutant compared to the wild-type virus. Nck and N-WASP are both essential for Vaccinia-induced
258 actin polymerization, but how their relative levels impact on the rate of actin-based motility is not established. Previous analysis reveals the density of artificially clustered VCA, the N-WASP domain responsible for activating Arp2/3, does not affect downstream actin-based motility (Borinskaya et al. 2016). Interestingly, the same group also found that increasing the density of artificially clustered Nck SH3 domains on the plasma membrane increases the motility of actin structures (Ditlev et al. 2012). In their model system, Nck is permanently
264 attached to the plasma membrane and not free to exchange as under CEV. This may underlie the difference in the trends of our observations. However, together with these previous studies, our data indicate that Nck numbers and density are a critical determinant of actin-based motility in cells.

Why do Nck numbers impact Vaccinia motility? N-WASP/WASP activates Arp2/3 mediated
270 actin polymerization more efficiently when acting as a dimer (Higgs & Pollard 2000; Padrick et al. 2008; Padrick et al. 2011; Zimmet et al. 2020). Previous simulations based on results from *in vitro* experiments suggests that when a protein that would dimerize N-WASP is high, the probability of two N-WASP molecules engaging the same dimerizer is low (Padrick et al. 2008). Namely, each dimerizer would have a single N-WASP, which would be less efficient at stimulating the actin nucleating activity of the Arp2/3 complex. When the dimerizer
276 concentration is optimal, it is more likely that two N-WASP molecules will interact with the same dimerizer making actin nucleation is more efficient. Our previous *in vitro* pulldown experiments demonstrated that two of the three SH3 domains in Nck were capable of binding to N-WASP suggesting that this adaptor can function as a dimerizer (Donnelly et al. 2013). Thus, it is possible that the increase in the velocity of the A36△NPF virus is due to the concentration of Nck being closer to the optimal level required to fully dimerize N-WASP,
282 making actin nucleation more efficient. It remains to be established why the lack of intersectin recruitment by A36 affects the levels of Nck so profoundly. The loss of the A36 NPF motifs results in reduced clustering of A36 (Humphries et al. 2012), which is expected to reduce the local density of Nck under CEV. However, it should not reduce the total number

of Nck molecules. We therefore favour a hypothesis that the absence of intersectin results in the loss of an unknown Nck stabilizing protein in addition to Cdc42 and AP2/clathrin.

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Implications of A36 density under CEV

A36 is present in the outer IEV membrane which becomes incorporated into the plasma membrane below CEV during Vaccinia egress from the host cell. A36 contains a single helical transmembrane domain, which is estimated to be approximately 1.2 nm wide. Assuming that no membrane is lost, and proteins do not diffuse significantly when IEVs fuse with the plasma
294 membrane, our data indicate that A36 is present at a density of 0.2-0.5 molecules per 100 nm^2 based on the outer surface of 405,037 nm^2 for IEV. Interestingly, it has been determined that 5800 molecules of the actin nucleating protein ActA of Listeria are present on the surface of the bacterium (Footer et al. 2008). The bacterial surface area is substantially larger than the membrane beneath CEV, however, the density of ActA molecules (0.09-0.3 molecules/100nm^2) is in a similar range to that of A36 (Footer et al. 2008).

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Unexpectedly, we found that the number of A36 molecules associated with CEV varies depending on the cell type. It can also be influenced by the length of infection. This is not the case with B5, which appears to remain relatively constant. The reason for the variation in A36 numbers is not clear, but it might be related to its level of expression in the infected cell (Fig 4E). Moreover, the variation in A36 numbers suggests that viral protein composition
306 in membranes around IEV and subsequent forms of the virus, may not be fixed for all viral proteins. Consistent with this, previous analysis reveals that ~ one third of extra-cellular enveloped virions (EEV) lack A56, another integral IEV membrane protein (Krauss et al. 2002; Smith et al. 2002).

Previous biochemical pulldowns and immunoprecipitations approaches have demonstrated
312 that there are multiple interactions between IEV membrane proteins including A33:B5 (Chan & Ward 2012a,b; Perdiguero & Blasco 2006), A34:B5 (Earley et al. 2008; Monticelli et al. 2019; Röttger et al. 1999), A34:A36 (Röttger et al. 1999), A33:A36 (Röttger et al. 1999; Ward et al. 2003; Wolffe et al. 2001), A36:F12 (Johnston & Ward 2009) and F12:E2 (Dodding et al. 2009; Van Vliet et al. 2009). These multiple associations suggest that the viral proteins likely form an immobile lattice within the membrane. Our measurement of the number of A36, B5 and

318 F13 molecules in CEV is an important step in fully determining the precise organization of proteins in the viral membrane. B5 and F13 are present in the membrane surrounding CEV as well as together with A36 in the plasma membrane beneath CEV. Assuming B5 and F13 are equally partitioned between the two membranes, then the levels of B5 and F13 are 1.3 and 3.8 fold higher than A36 beneath the CEV. It is striking that the levels of A36 and B5 are similar given there in no evidence that these proteins associate with each other (Van Vliet et
324 al. 2009). Given their interactions, it will be interesting to measure the levels of A34 and A33. If the levels of A33 and A34, which are also integral membrane proteins are comparable to A36 and/or B5 it would suggest that the viral protein density in membranes around IEV is surprisingly low and that they do not form rigid protein lattices. In addition, the absence of an immobile viral protein lattice may explain why clathrin is able to cluster A36 after IEV fuse with the plasma membrane (Humphries et al. 2012).

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In summary, our quantitative approach in determining precise numbers of molecules at CEV has unexpectedly revealed that A36 levels in the viral membrane are variable. It has also demonstrated that the velocity of virus movement depends on the adaptor protein Nck rather than N-WASP which activates Arp2/3 complex dependent actin polymerization.

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354 **FIGURE LEGENDS**

Figure 1: CEV and the signalling network that induces actin polymerisation

A schematic showing localisation of the vaccinia virus proteins A36, B5 and F13 in cell associated enveloped virions (CEV) on the plasma membrane. A36 and B5 are integral membrane proteins, while F13 associates with the membrane via lipidation. Inset shows the signalling network recruited by the cytoplasmic tail of A36 inducing actin polymerisation.
360 Tyr112 and Tyr132 in A36, when phosphorylated by Src and Abl family kinases, recruit the adaptor proteins Nck and Grb2. Nck and Grb2 interact with N-WASP, which results in the activation of the Arp2/3 complex and stimulation of actin polymerisation. NPF motifs in the C-terminus of A36 recruit the RhoGEF intersectin which recruits AP2/clathrin and also activates Cdc42 so it can bind N-WASP. While Nck is essential for actin tail formation, Grb2 and Cdc42 are not. Molecules labelled in green have been quantitatively analysed in this
366 study.

Figure 2: Determining the number of A36 molecules on CEV inducing actin polymerisation

A. Representative single-plane spinning disc confocal image of a live HeLa cell stably expressing LifeAct-iRFP and infected with a recombinant virus expressing A36-TagGFP2 and RFP-A3 at their endogenous locus. The image is acquired at 9 hours post-infection. Scale bars
372 = 10 µm. **B.** Representative single-plane confocal images comparing fluorescence intensity of A36-TagGFP2 at virus particles with TagGFP2-labeled 60mer nanocages in live HeLa cells. Scale bar = 3 µm. **C.** Representative inverted average-intensity Z-projections of TagGFP2-tagged nanocages transiently expressed in live HeLa cells treated with 500 nM AP21967. Images were acquired at 100ms exposure. 60mers were imaged at 4x exposure to generate "240mer" intensities and 120mers were imaged at 3x exposure to generate "360mer"
378 intensities. Scale bar = 2 µm. The graph shows quantification of background-subtracted raw integrated fluorescence intensities of nanocages. Linear line of regression is fitted to data from 24-, 60-, 120- and 180mers. A total of 47-110 membrane-tethered nanocages were measured per condition over three independent experiments. A detailed version of the

experimental data used to generate the calibration curve (green shaded area) is shown in Fig S2A. **D.** Representative inverted average-intensity Z-projections of A36-TagGFP2 at virus
384 particles generating actin tails in live HeLa cells. Images were acquired at indicated exposure times. Coloured arrowheads indicate individual virus particles. Single-plane images in the lower panel show positions of each particle 5 seconds after the Z-stack acquisition. Actin is labelled with LifeAct-iRFP, and all virus particles are labelled with RFP-A3. Scale bar = 2 µm. The graph shows quantification of background-subtracted raw integrated A36-TagGFP2 intensities at actin-polymerising virus particles, overlaid on the calibration curve generated in
390 panel C. A total of 50-57 virions were measured per condition over three independent experiments. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". The table shows the calculated number of A36 molecules on CEV inducing actin tails in HeLa cells (s.e.m. = standard error of mean).

Figure 3: Determining the number of B5 and F13 molecules on CEV inducing actin tails

396 **A.** Representative single-plane confocal images of live HeLa cells infected with a recombinant virus expressing B5-TagGFP2 or TagGFP2-F13 at their endogenous loci. Actin is labelled with LifeAct-iRFP, and all virus particles are labelled with RFP-A3. Images are acquired at 9 hours post-infection. Scale bar = 10 µm. **B.** Representative inverted average-intensity Z-projections of A36-TagGFP2, B5-TagGFP2 and TagGFP2-F13 at virus particles generating actin tails in live HeLa cells. Coloured arrowheads indicate individual virus particles. Single-plane images in the
402 right panel show positions of each particle 5 seconds after the Z-stack acquisition. Actin is labelled with LifeAct-iRFP, and all virus particles are labelled with RFP-A3. Scale bar = 2 µm. **C.** The top graph shows quantification of background-subtracted raw integrated TagGFP2 intensities at actin-polymerising virions. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 39-54 particles were measured per recombinant virus over three independent experiments. The lower graph
408 shows average measurements for each molecule superimposed on the calibration curve generated in Fig 2C. The table shows the calculated number of A36, B5 and F13 molecules at actin-polymerising virus particles in HeLa cells (s.e.m. = standard error of mean).

Figure 4: Comparing numbers of A36 and B5 at CEV across host cell types

A and **B.** Representative inverted average-intensity Z-projections of A36-TagGFP2 (**A**) and B5-
414 TagGFP2 (**B**) at virus particles generating actin tails in the indicated cell lines. Coloured arrowheads indicate individual virus particles. Single-plane images in the lower panels show positions of these particles 5 seconds after the Z-stack acquisition. Actin is labelled with LifeAct-iRFP (stably expressed in HeLa and transiently expressed in other cell types). All virus particles are labelled with RFP-A3. Scale bar = 2 µm. The graphs show quantification of background-subtracted raw integrated TagGFP2 intensities at actin-polymerising virus
420 particles. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 29-62 (A36) or 26-41 (B5) particles were measured per condition over three independent experiments. Tukey's multiple comparison's test was used to determine statistical significance; ns, $p > 0.05$; * $p \leq 0.05$. **C.** Table showing the calculated number of A36 and B5 molecules at virions inducing actin tails in the different host cell lines. **D.** Graph showing quantification of background-subtracted raw integrated A36-TagGFP2
426 intensities at actin-polymerising virus particles at 8- and 16-hours post-infection (hpi) in HeLa and N-WASP MEF host cells. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 48-62 particles were measured per condition over three independent experiments. Tukey's multiple comparison's test was used to determine statistical significance; ns, $p > 0.05$; ** $p \leq 0.01$. **E.** Immunoblot analyses of total cell lysates from WR-infected HeLa and N-WASP MEF cells at 8- and 16-hours post-infection
432 (hpi) respectively. N1 and vinculin represent viral and host protein loading controls.

Figure 5: Determining the number of Nck and N-WASP molecules on CEV

A. Representative immunofluorescence images showing the recruitment of N-WASP to actin tails in Vaccinia-infected MEF cells expressing TagGFP2-N-WASP in a N-WASP-null background or parental wildtype cells at 16 hours post-infection. N-WASP was detected with an antibody
438 and actin is stained with phalloidin. Scale bar = 1 µm. **B.** Representative immunofluorescence images showing the recruitment of Nck to actin tails in Vaccinia-infected MEF cells expressing TagGFP2-Nck in a Nck-null background or parental wildtype cells at 16 hours post-infection. Nck was detected with an antibody and actin is stained with phalloidin. Scale bar = 1 µm. **C.** Representative inverted average-intensity Z-projections of TagGFP2-Nck and TagGFP2-N-WASP in live Vaccinia WR-infected MEF cells stably expressing the corresponding tagged
444 protein in Nck1/Nck2 -/- and N-WASP-/- backgrounds respectively. Images are taken at 16

hours post-infection. Scale bar = 10 µm. **D.** The graph shows quantification of background-subtracted raw integrated TagGFP2 intensities at actin-polymerising virus particles. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 71-85 virions were measured per condition over three independent experiments. The table shows the calculated number of Nck and N-WASP molecules at actin-
450 polymerising virus particles in MEF cells.

Figure 6: Nck and N-WASP are reduced on CEV in absence of Grb2 and intersectin

A. Representative inverted average-intensity Z-projections of TagGFP2-Nck and TagGFP2-N-WASP at virus particles generating actin tails in live MEF cells infected with Vaccinia Western Reserve virus (WR) or the indicated A36 mutants. Pink arrow heads indicate particles
456 generating actin tails as discerned by a transiently expressed LifeAct-iRFP and RFP A3 which labels viral cores. The LifeAct-iRFP and RFP-A3 channels are not shown for clarity. Scale bar = 10 µm. **B.** Graphs showing quantification of background-subtracted raw integrated intensities of TagGFP2-Nck and TagGFP2-N-WASP at actin-polymerising A36 mutant virus particles. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 34-49 (Nck) or 41-85 (N-WASP) particles were measured per condition
462 over three independent experiments. Dunnett's multiple comparison's test was used to determine statistical significance; ns, $p > 0.05$; * $p \leq 0.05$. The table shows the calculated number of Nck and N-WASP molecules at actin-polymerising virus particles in the indicated A36 mutants. **C.** Temporal colour-coded representation of time-lapse movies tracking the motility of the indicated RFP-A3-labelled virus over 50 seconds in HeLa cells at 8 hours post infection. Images were recorded every second and the position of virus particles at frame 1
468 (open triangles) and frame 50 (white triangles) are indicated. Scale bar = 3 µm. The graph shows quantification of virus speed. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 31-51 tracks were analysed per condition over three independent experiments. Dunnett's multiple comparison's test was used to determine statistical significance; ns, $p > 0.05$; ** $p \leq 0.01$.

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SUPPORTING INFORMATION

Supplement 1: Validation TagGFP2-labelled recombinant viruses

A. Immunoblot analyses of total cell lysates from HeLa cells infected with Vaccinia WR or the indicated TagGFP2 fusion viruses at 9 hours post-infection. **B.** Representative images of plaques produced by the indicated viruses in confluent BS-C-1 cells at 72 hours post-infection.
486 Scale bar = 2 mm**.** The graph shows quantification of plaque diameter of the recombinant viruses compared with WR. The graph on the right shows that the RFP-A3 tag that labels the virus core does not confer a change in plaque diameter. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". A total of 36 plaques per virus were measured over three independent experiments. Dunnett's multiple comparison's test (left graph) or Welch's t test (right graph) was used to determine statistical
492 significance; ns, $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$.

Supplement 2: Validation of fluorescent nanocages

A. The graph shows quantification of background-subtracted raw integrated fluorescence intensities of nanocages transiently expressed in live HeLa cells treated with 500 nM AP21967. Linear line of regression is fitted to the data. A total of 47-110 membrane-tethered
498 nanocages were measured per condition over three independent experiments. **B.** Quantification of background-subtracted raw integrated intensity of 60mer TagGFP2-nanocages transiently expressed in HeLa cells or N-WASP+/+ parental MEFs treated with 500 nM AP21967. A total of 66-84 membrane-tethered spots were measured over three independent experiments. All error bars represent S.D and the distribution of data from each experiment is shown using a "SuperPlot". Welch's t test (panel B) was used to determine
504 statistical significance; ns, $p > 0.05$.

Supplement 3: Validation of stable cell lines expressing TagGFP2-Nck and TagGFP2-N-WASP

A. Immunoblot analyses of total cell lysates from Nck1/Nck2 null MEFs with and without stable expression of TagGFP2-Nck together with parental wild-type cells. **B.** Immunoblot

analyses of total cell lysates from N-WASP null MEFs with and without stable expression of
510 TagGFP2-N-WASP together with parental wild-type cells. Immunoblot analyses was performed with the indicated antibodies. ** indicates the band corresponding to the TagGFP2-labelled protein.

MATERIALS AND METHODS:

Expression constructs and targeting vectors

516 The expression vector pE/L-LifeAct-iRFP670 and the plasmid for the 180-mer nanocage was previously made in the Way lab (Galloni et al. 2021; Xu et al. 2022). Plasmids for 12-, 24-, 60- and 120-mer TagGFP2 nanocage expression were obtained from the Drubin lab (Akamatsu et al. 2020). All other expression constructs generated for this study were made using Gibson Assembly (New England Biolabs) according to manufacturer's instructions. All primers used in cloning are listed in Table 1. The lentiviral expression constructs pLVX-TagGFP2-Nck and
522 pLVX-TagGFP2-N-WASP were generated by cloning a TagGFP2 fragment into ApaI/NotI sites of pLVX-GFP-Nck (Basant & Way 2022) and pLVX-GFP-N-WASP (Donnelly et al. 2013) respectively. The A36-TagGFP2 construct was made by assembling the fusion fragment and cloning into NotI/HindIII sites of a A36 targeting vector (Basant & Way 2022). The B5-TagGFP2 construct was made by cloning a TagGFP2 fragment into NotI/XbaI sites of a B5-YFP targeting vector (Dodding et al. 2009). To generate the TagGFP2-F13 targeting vector, the F13 gene and
528 423bp of upstream and downstream sequences were amplified from the WR strain of Vaccinia virus genomic DNA. This was assembled with a TagGFP2 fragment and cloned into NotI/HindIII sites of pBS SKII. SnapGene software (from Insightful Science; available at snapgene.com) was used to plan and visualise cloning strategies, and to analyse sequencing results.

534 **Table 1. Primers**

No.	***Sequence***	***Construct(s)***
AB123	TCGACGGTACCGCGGGCCCATGGGAGGCGAAGAGCTGTTTGCTG	pLVX-TagGFP2-Nck pLVX-TagGFP2-N-WASP
AB124	TGCCATTCCTCCGCGGCCGCCTGTCCCGGAACCTGCCCT	pLVX-TagGFP2-Nck
AB125	GCCGCCGCCGCGGCCGCCTGTCCCGGAACCTGCCCT	pLVX-TagGFP2-N-WASP

AB084	GTCGTATCATTGGTGGGGTCGGGGTCGGATCCACCGGTCGCCACCG GAGGCGAAGAGCTGTTT	pBS SKII A36-TagGFP2
AB085	GTCGTATCATTGGTGGGGTCGGGGTCGGATCCACCGGTCGCCACCG GAGGCGAAGAGCTGTTT	pBS SKII A36-TagGFP2
AB086	GCAGGTTCCGGGACATAATTAGTTTCCTTTTTATAAAATTGAAG	pBS SKII A36-TagGFP2
AB052	GGTCGACGGTATCGATAAGCTTTATCTATAGAGATAACAC	pBS SKII A36-TagGFP2
MHG055	ATTGGAGCTCCACCGCGGTGGCGGCCGCCGCCATCGTCGGTGTGTT GTC	pBS SKII TagGFP2-F13
AB135	GCAAACAGCTCTTCGCCTCCCATTTAGTTAACATAAAAACTTA	pBS SKII TagGFP2-F13
AB136	TAAGTTTTTATGTTAACTAAATGGGAGGCGAAGAGCTGTTTGC	pBS SKII TagGFP2-F13
AB137	GTACCGATGCAAATGGCCACATGCGGCCGCCTGTCCCGGAACCTGC CCT	pBS SKII TagGFP2-F13
AB131	AGGGCAGGTTCCGGGACATAAATATAAATCCGTTAAAATAA	pBS SKII B5-TagGFP2
AB132	TCCCCGCGGGTGGGCGCTCTAGAACTAGTGGGATCCTATACCATT	pBS SKII B5-TagGFP2
AB133	ATTGCTACCGGGCGGCGCGGCCGCCGGAGGCGAAGAGCTGTTTGC	pBS SKII B5-TagGFP2
AB134	TTATTTTAACGGATTTATATTTATGTCCCGGAACCTGCCCT	pBS SKII B5-TagGFP2

Cell lines

HeLa cells were maintained in minimal essential medium (MEM) and MEFs and A549 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). All cells were supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO_2. The HeLa
540 cell line stably expressing LifeAct-iRFP670 (Snetkov et al. 2016) was previously generated in the Way lab. Nck -/- MEFs (Bladt et al. 2003) and N-WASP-/-MEFs (Snapper et al. 2001) were provided by the late Tony Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada) and Scott Snapper (Harvard Medical School, Boston, MA) respectively. A549 cells were obtained from ATCC. For this study, lentiviral expression vectors were used to stably express TagGFP2-Nck in Nck-/- MEFs and TagGFP2-N-WASP in N-WASP-/- MEFs. All cell lines were
546 generated using the lentivirus Trono group second generation packaging system (Addgene) and selected using puromycin resistance (2 µg/ml) as previously described (Abella et al. 2016). Expression of the relevant fusion proteins was confirmed by live imaging and immunoblot analysis (Fig S3). The following primary antibodies were used: anti-Nck (BD transduction; 1:1000), anti-vinculin (Sigma #V4505; 1:2000), anti-N-WASP (Cell Signalling #4848S; 1:1000),

anti-TagGFP2 antibody (Evrogen #AB121; 1:3000). HRP-conjugated secondary antibodies
552 were purchased from The Jackson Laboratory.

Viral plaque assays

Plaque assays were performed in confluent BS-C-1 cell monolayers. Cells were infected with the relevant Vaccinia virus at a multiplicity of infection (MOI) = 0.1 in serum-free MEM for one hour. The inoculum was replaced with a semi-solid overlay consisting of a 1:1 mix of MEM
558 and 2% carboxymethyl cellulose. Cells were fixed with 3% formaldehyde at 72 hours post-infection and subsequently visualized with crystal violet cell stain as previously described (Humphries et al. 2012). To determine plaque size, the diameter of well-separated plaques was measured using the Fiji line tool (Schindelin et al. 2012).

Construction of recombinant Vaccinia viruses

564 In this study, recombinant Vaccinia viruses expressing A36-TagGFP2, B5-TagGFP2 or TagGFP2-F13 at their endogenous loci were generated in a parental WR viral strain expressing RFP-A3. Recombinants were isolated by selecting viral plaques positive for TagGFP2 fluorescence as described previously (Basant & Way 2022). To introduce relevant constructs into their genomic loci, HeLa cells infected with WR (RFP-A3) at MOI = 0.05 were transfected with the appropriate pBS SKII targeting vectors using Lipofectamine2000 (Invitrogen) as described by
570 the manufacturer. When all cells displayed cytopathic effect at 48-72 hours post-infection, they were lysed, and serial dilutions of the lysates were used to infect confluent BS-C-1 cell monolayers in a plaque assay (see above). RFP and TagGFP2 positive plaques were picked, and plaque lysates were used to infect fresh BS-C-1 cell monolayers over at least three rounds of plaque purification. Successful recombination at the correct locus, loss of the parent variant and virus purity were verified by PCR, sequencing and immunoblot. The following
576 primary antibodies were used: anti-vinculin (Sigma #V4505; 1:2000), anti-TagGFP2 (Evrogen #AB121; 1:3000), anti-N1 ((Postigo & Way 2012); 1:3000), anti-A36 ((Röttger et al. 1999); 1:3000), anti-B5 ((Schmelz et al. 1994); 1:5000), anti-F13L-N ((Rietdorf et al. 2001); 1:5000). HRP-conjugated secondary antibodies were purchased from The Jackson Laboratory. For all experiments, recombinant viruses were purified through a sucrose cushion before use and storage.

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Vaccinia virus infection for imaging

For live and fixed cell imaging, cells plated on fibronectin-coated MatTek dishes (MatTek corporation) or coverslips were infected with the relevant Vaccinia virus recombinant in serum-free MEM at MOI = 1. After one hour at 37°C, the serum-free MEM was removed and replaced with complete MEM. Cells were incubated at 37°C until further processing.

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Transient transfection and drug treatment

All transfections prior to imaging were carried out using FUGENE (Promega) as described by the manufacturer. To visualize filamentous actin in live cells, Vaccinia-infected A549 cells and MEFs were transiently transfected with pE/L-LifeAct-iRFP670 one hour after infection. Plasmids expressing nanocage constructs were transfected into HeLa cells or MEFs at least 16
594 hours prior to imaging along with a plasmid expressing cytosolic mCherry to easily identify transfected cells. To induce nanocage tethering to the plasma membrane, 500nM AP21967 (Takara) was added to the media at least 30 minutes prior to imaging.

Immunofluorescence

At 8 hours (HeLa) or 16 hours (MEFs) post-infection, cells were fixed with 4%
600 paraformaldehyde in PBS for 10 min, blocked in cytoskeletal buffer (1 mM MES, 15 mM NaCl, 0.5 mM EGTA, 0.5 mM $MgCl_2$, and 0.5 mM glucose, pH 6.1) containing 2% (vol/vol) fetal calf serum and 1% (wt/vol) BSA for 30 min, and then permeabilized with 0.1% Triton-X/PBS for 5 min. To visualize cell-associated enveloped virions (CEV), cells were stained with, with a monoclonal antibody against B5 (19C2, rat, 1:1000; (Schmelz et al. 1994)) followed by an Alexa Fluor 647 anti-rat secondary antibody (Invitrogen; 1:1000 in blocking buffer) prior to
606 permeabilization of the cells with detergent. Other primary antibodies used were anti-Nck (Millipore #06-288; 1:100) and anti-N-WASP (Cell Signalling #4848S; 1:100) followed by Alexa Fluor 568 conjugated secondary antibodies (Invitrogen; 1:1000 in blocking buffer). Actin tails were labeled with Alexa Fluor 488 phalloidin (Invitrogen; 1:500). Coverslips were mounted on glass slides using Mowiol (Sigma). Coverslips were imaged on a Zeiss Axioplan2 microscope equipped with a 63x/1.4 NA Plan-Achromat objective and a Photometrics Cool Snap HQ
612 cooled charge-coupled device camera. The microscope was controlled with MetaMorph 7.8.13.0 software.

Live-cell imaging

Live-cell imaging experiments were performed starting at 8 hours or 16 hours post-infection in complete MEM (10% FBS) in a temperature-controlled chamber at 37°C. For all molecule
618 counting experiments, cells were imaged on a Zeiss Axio Observer spinning-disk microscope equipped with a Plan Achromat 100×/1.46 NA oil lens, an Evolve 512 camera, and a Yokagawa CSUX spinning disk. The microscope was controlled by the SlideBook software (3i Intelligent Imaging Innovations). To generate a calibration curve using TagGFP2 nanocages, image stacks of ten Z-slices 0.1 µm apart were acquired using an exposure of 100ms (unless stated otherwise) for 10 seconds at 1 sec intervals. When quantifying TagGFP2 molecules at virus
624 particles, a single Z stack was acquired followed by a single plane image of RFP (virus) and iRFP (LifeAct) channels to help identify virus particles generating actin tails. To determine virus speed, images were acquired using a 63x/1.4 Ph3 M27 oil lens in RFP (virus) and iRFP (LifeAct) channels for 50 seconds at 1Hz.

Image analysis and quantitation

630 For molecule counting analysis, average intensity Z projections of GFP channel images were generated in Fiji. The ComDet v.0.5.5 plugin for ImageJ (https://github.com/UU-cellbiology/ComDet) was used to identify fluorescent spots and measure their background-subtracted integrated intensity. Only tethered nanocages, that remained in frame over 10s of imaging, were used for analysis. Intensities obtained from 24-, 60-, 120-, 180-mers imaged at 100ms exposure were plotted as a function of predicted TagGFP2 number per nanocage. A
636 line of linear fit through the origin was applied by linear least-squares fitting to generate the calibration curve used in this study. To calculate TagGFP2 numbers at Vaccinia CEV, fluorescent spots identified by the ComDet v.0.5.5 plugin were overlaid with RFP (virus)/iRFP (actin) images to only select particles associated with actin tails.

To analyse virus motility, two-colour time-lapse movies of HeLa cells stably expressing LifeAct-
642 iRFP670 infected with the relevant recombinant virus labelled with RFP-A3 were used. The velocity of virus particles in the RFP channel was measured using a Fiji plugin developed by David Barry (the Francis Crick Institute) as previously described (Basant & Way 2022).

Whole-cell lysate immunoblot

HeLa cells and MEFs infected with WR were lysed at 8- and 16-hours post-infection
648 respectively in PBS containing 1% SDS, a cocktail of protease and phosphatase inhibitors (cOmplete (Roche), PHOSstop (Roche)) and Benzonase (Millipore). Proteins from these lysates were resolved on an SDS-PAGE and the levels of total B5 and A36 were determined by immunoblot analysis using anti-A36 ((Röttger et al. 1999); 1:3000) and anti-B5 ((Schmelz et al. 1994); 1:5000). As loading controls, anti-vinculin (Sigma #V4505; 1:2000) and anti-N1 ((Postigo & Way 2012); 1:3000) were used. HRP-conjugated secondary antibodies were
654 purchased from The Jackson Laboratory.

Statistical analysis and figure preparation

All data are presented as means ± S.D. For all experiments, means of at least three independent experiments (i.e. biological replicates) were used to determine statistical significance by a Welch's t-test (comparing only two conditions), Tukey's multiple
660 comparisons test (comparing multiple conditions with each other) or a Dunnett's multiple comparisons test (comparing multiple conditions with a control). All data are represented as SuperPlots to allow assessment of the data distribution in individual experiments (Lord et al. 2020). SuperPlots were generated using the SuperPlotsOfData webapp (Goedhart 2021) and GraphPad Prism 9. All data were analyzed using GraphPad Prism 9 or the SuperPlotsOfData webapp. Schematics were created with BioRender.com. Final figures were assembled using
666 Keynote software.

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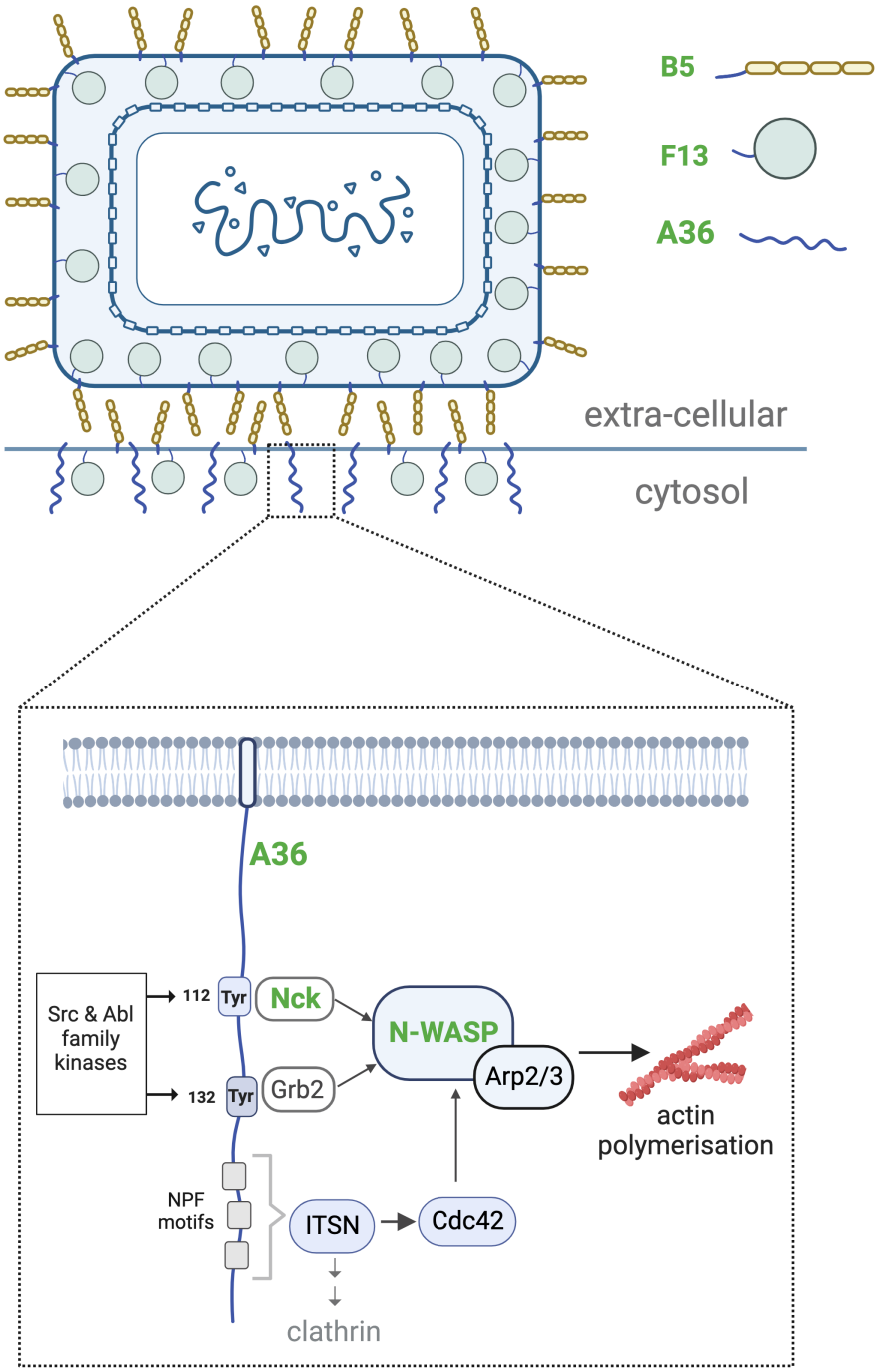
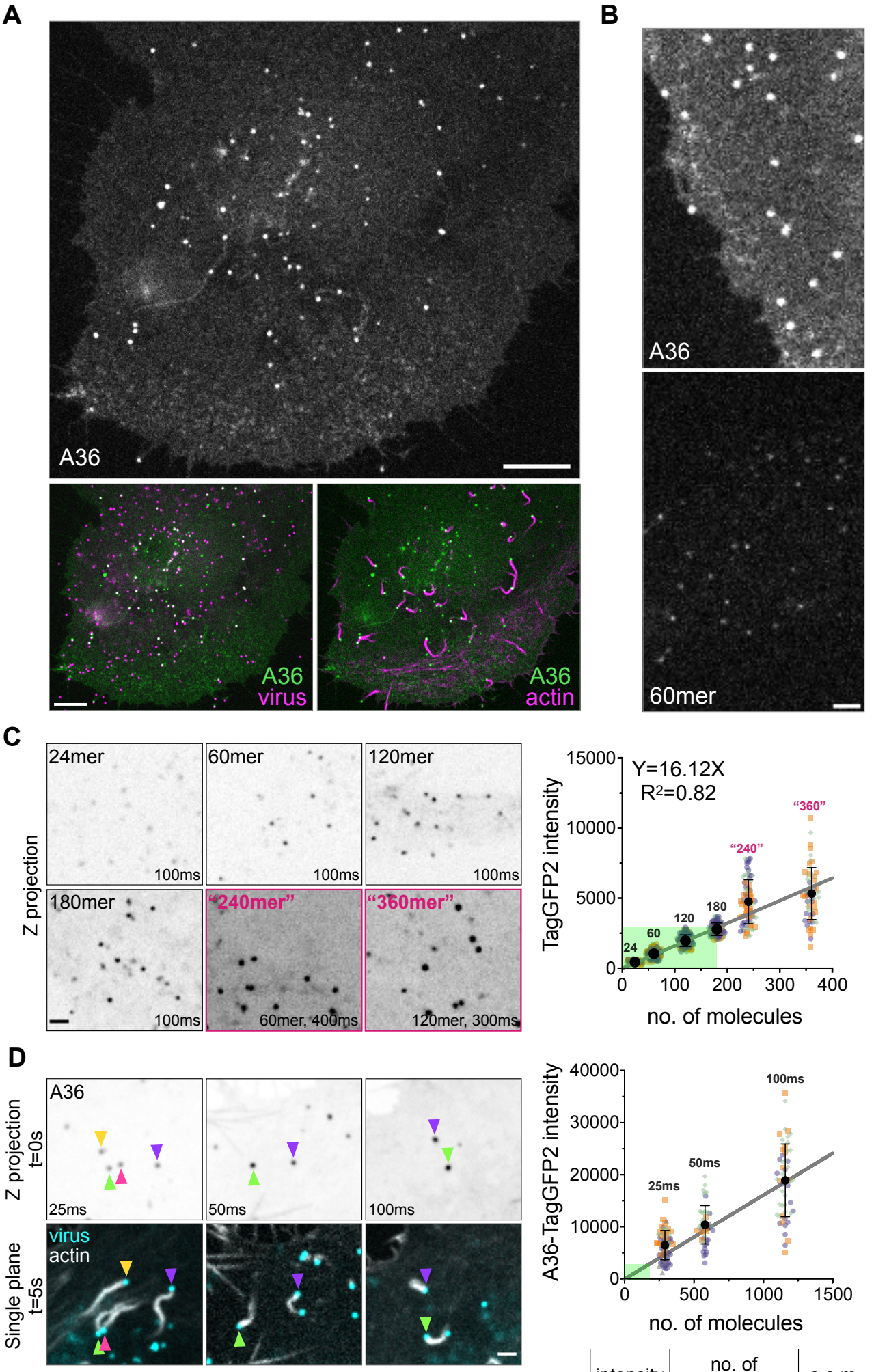

B5
F13
A36
extra-cellular
cytosol
A36
Src & Abl family kinases
112
Tyr
Nck
132
Tyr
Grb2
N-WASP
Arp2/3
actin polymerisation
NPF motifs
ITSN
Cdc42
clathrin

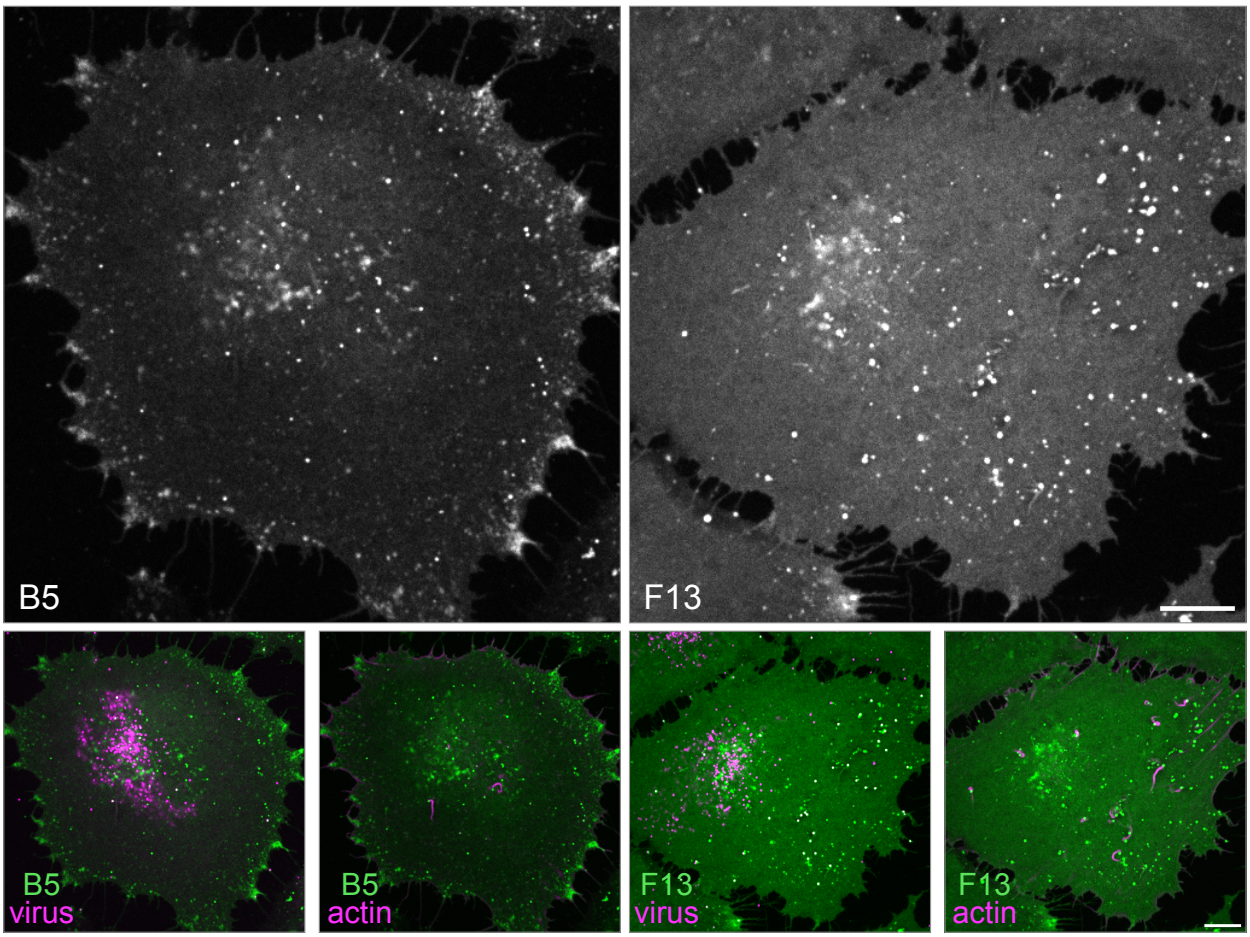
Figure 1



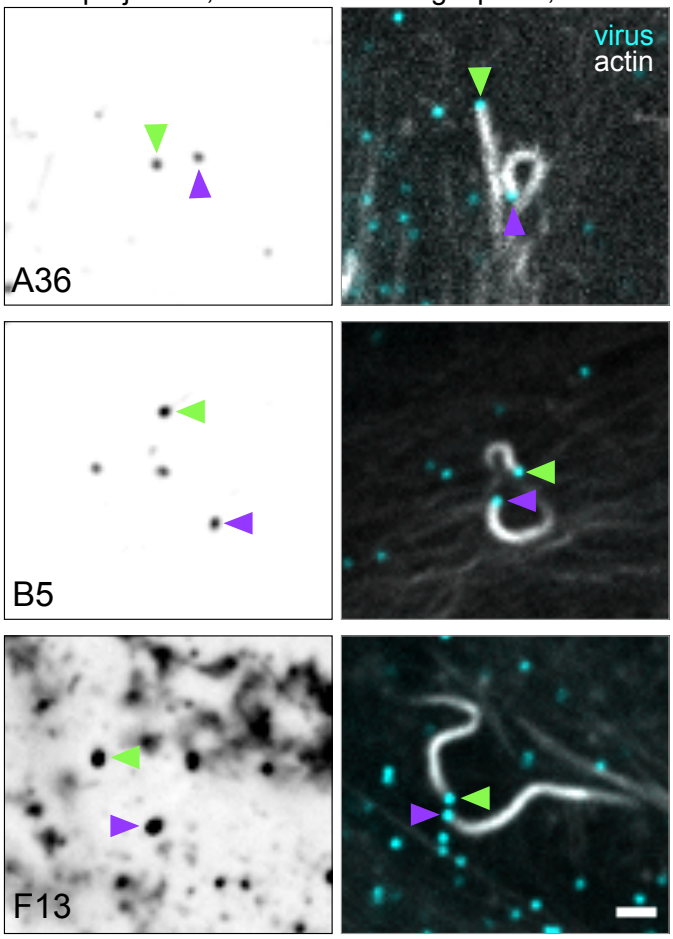
	intensity	no. of molecules	s.e.m.
A36	18632	1156	±120

Figure 2:

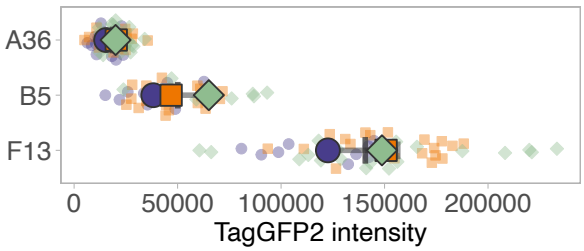
A

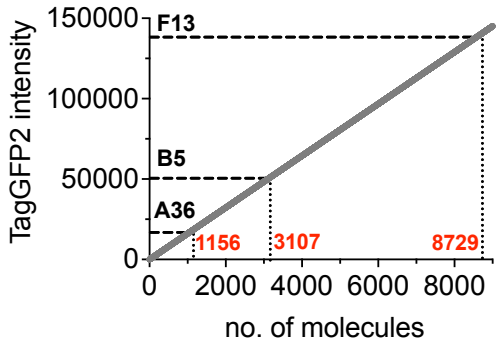


B



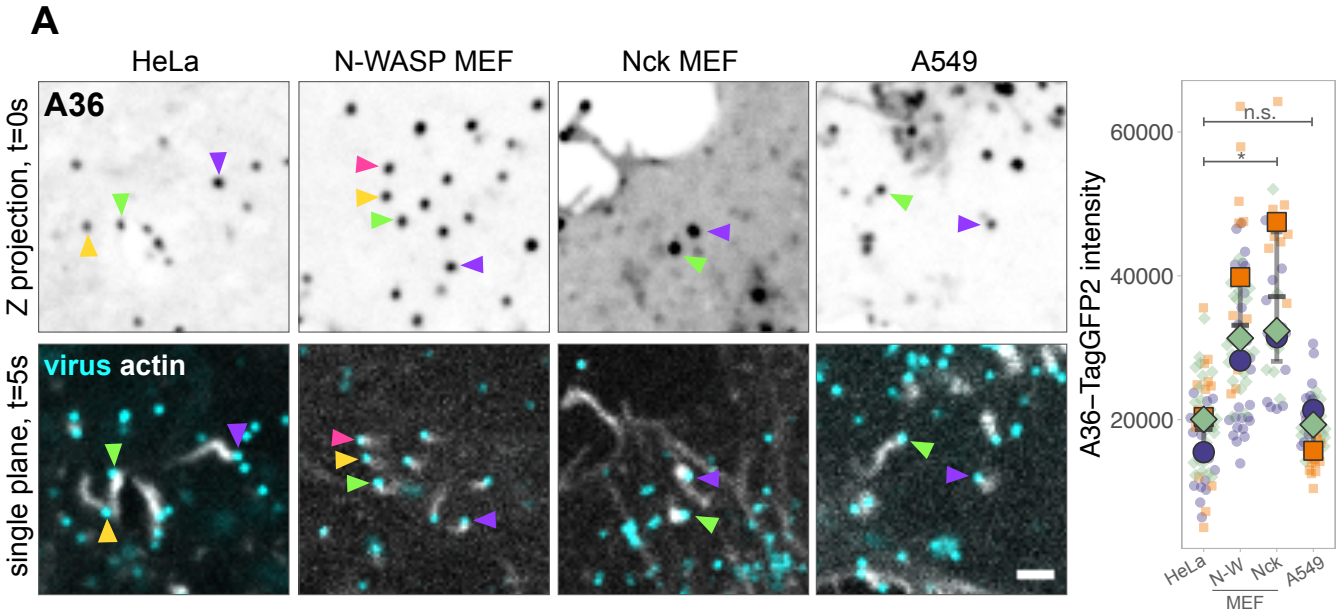
C

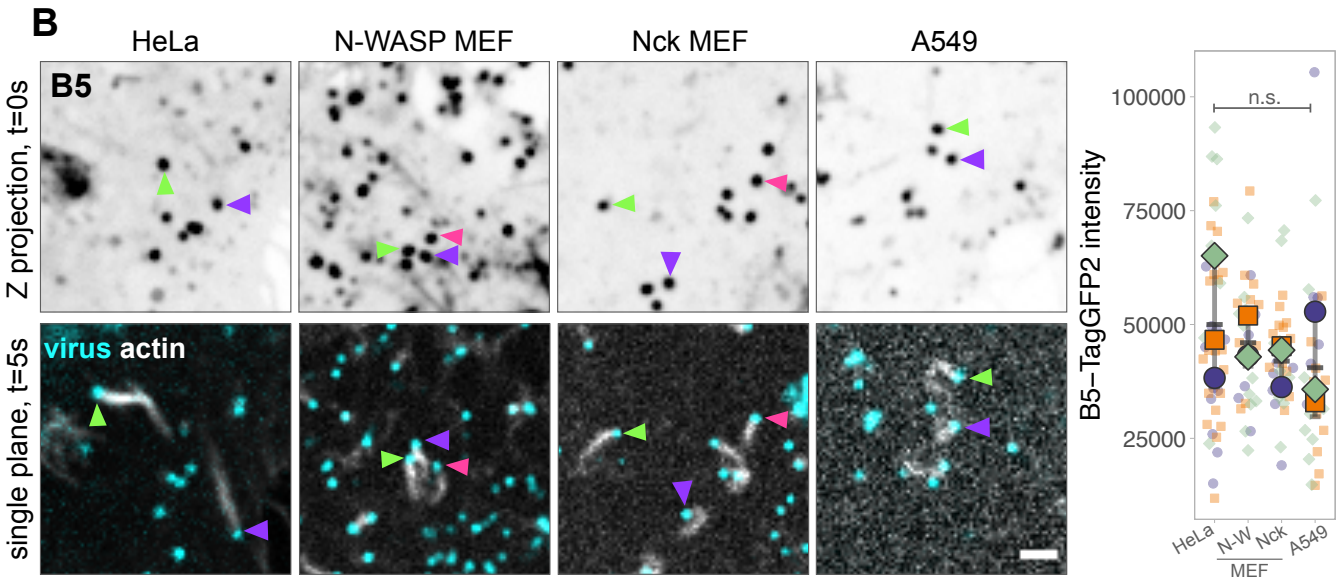




	intensity	no. of molecules	s.e.m.
A36	18632	1156	±120
B5	50079	3107	±602
F13	140707	8729	±688

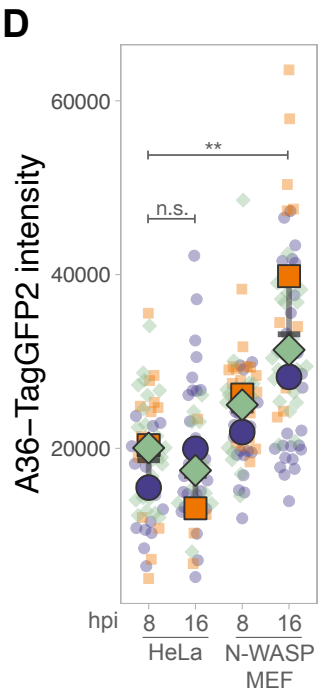
Figure 3:

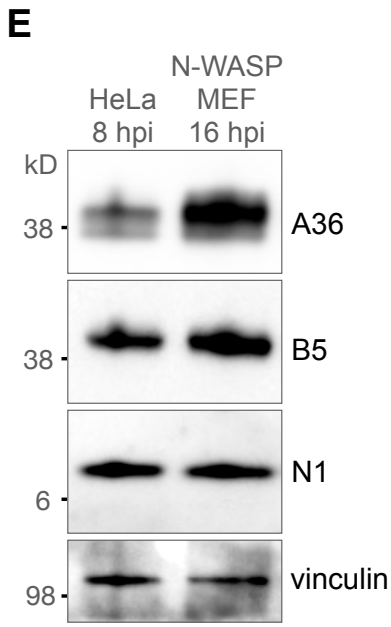




C

	cell type	intensity	no. of molecules	s.e.m.
A36	HeLa	18632	1156	±120
A36	N-WASP MEF	33128	2055	±263
A36	Nck MEF	37111	2302	±395
A36	A549	18782	1165	±126
B5	HeLa	50079	3107	±602
B5	N-WASP MEF	45999	2854	±227
B5	Nck MEF	41957	2603	±217
B5	A549	40504	2513	±472





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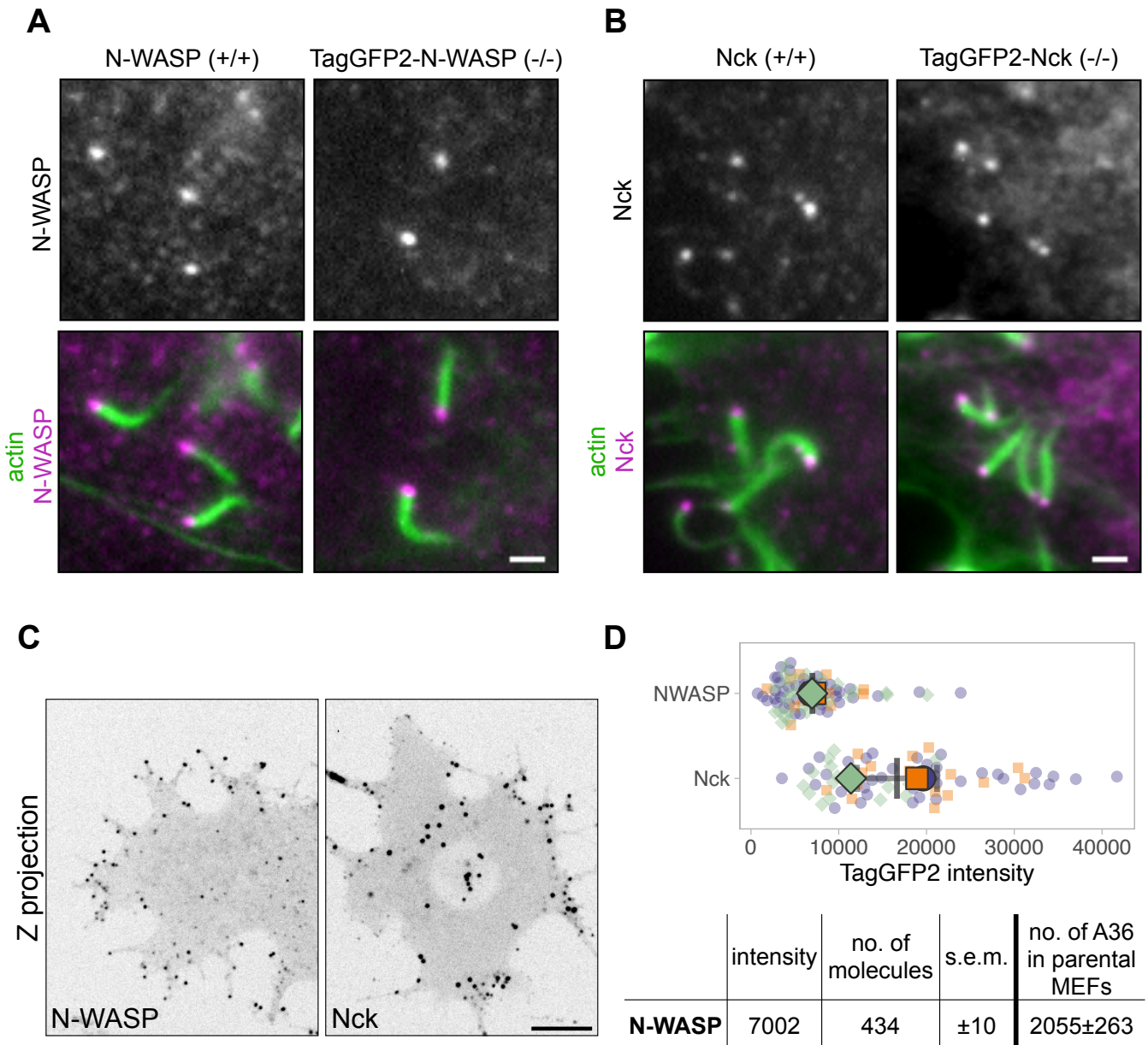
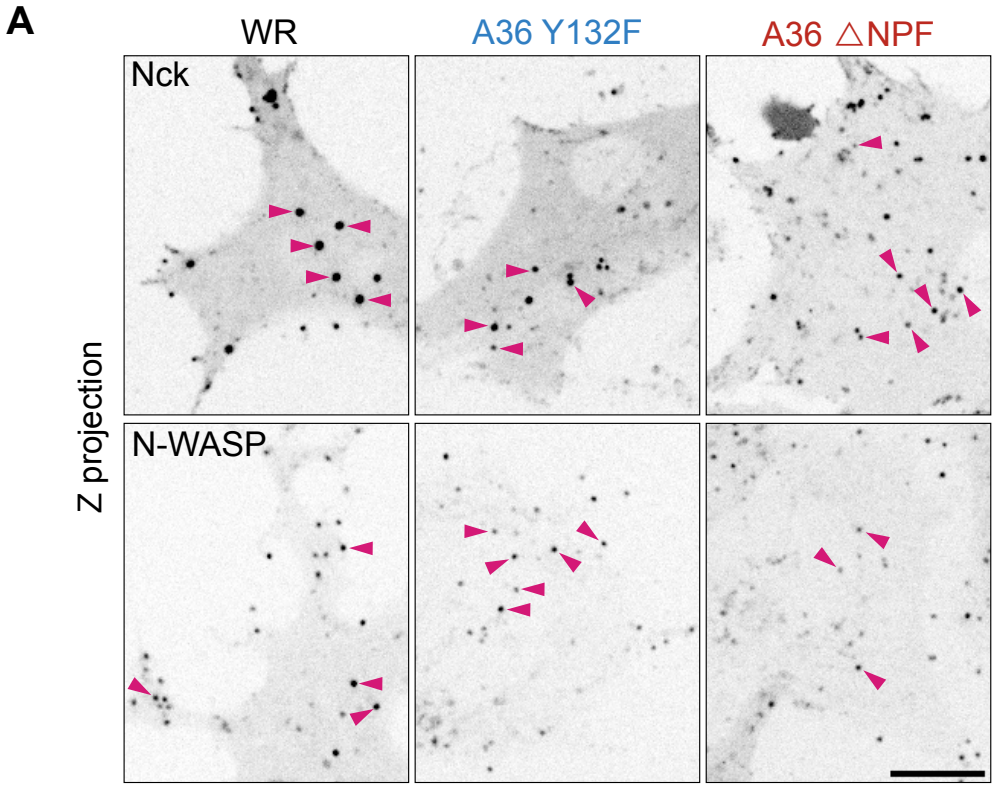
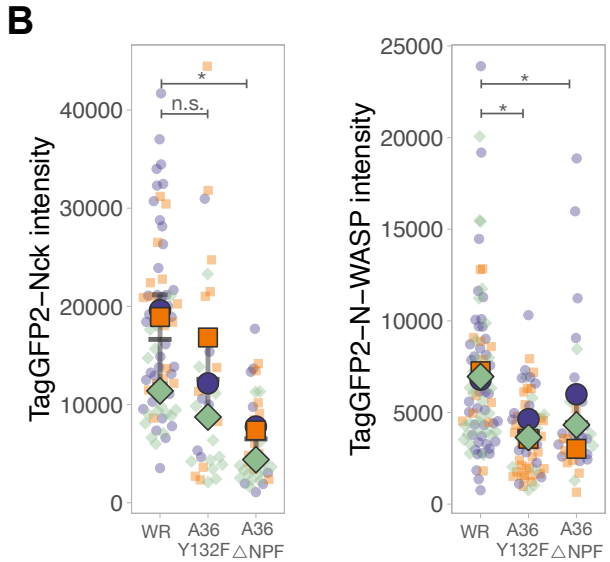


	intensity	no. of molecules	s.e.m.	no. of A36 in parental MEFs
N-WASP	7002	434	±10	2055±263
Nck	16637	1032	±200	2302±395

Figure 5:





virus		intensity	no. of molecules	s.e.m.
WR	Nck	16637	1032	±200
A36 Y132F	Nck	12573	780	±178
A36 △NPF	Nck	6504	403	±55
WR	N-WASP	7002	434	±10
A36 Y132F	N-WASP	3948	245	±26
A36 △NPF	N-WASP	4447	276	±66

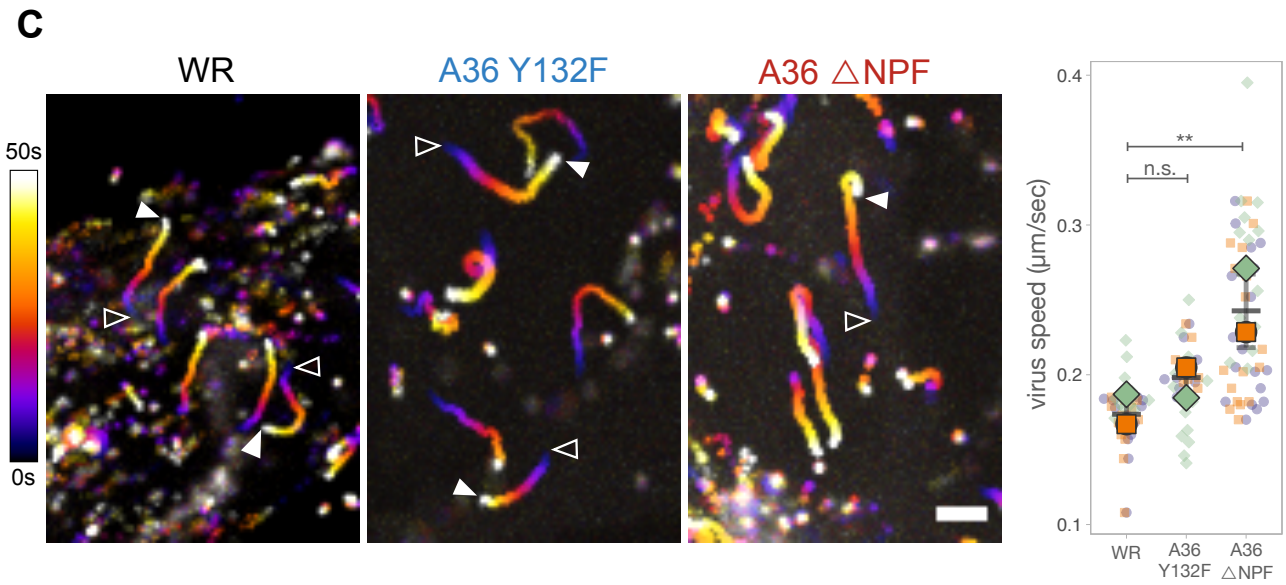
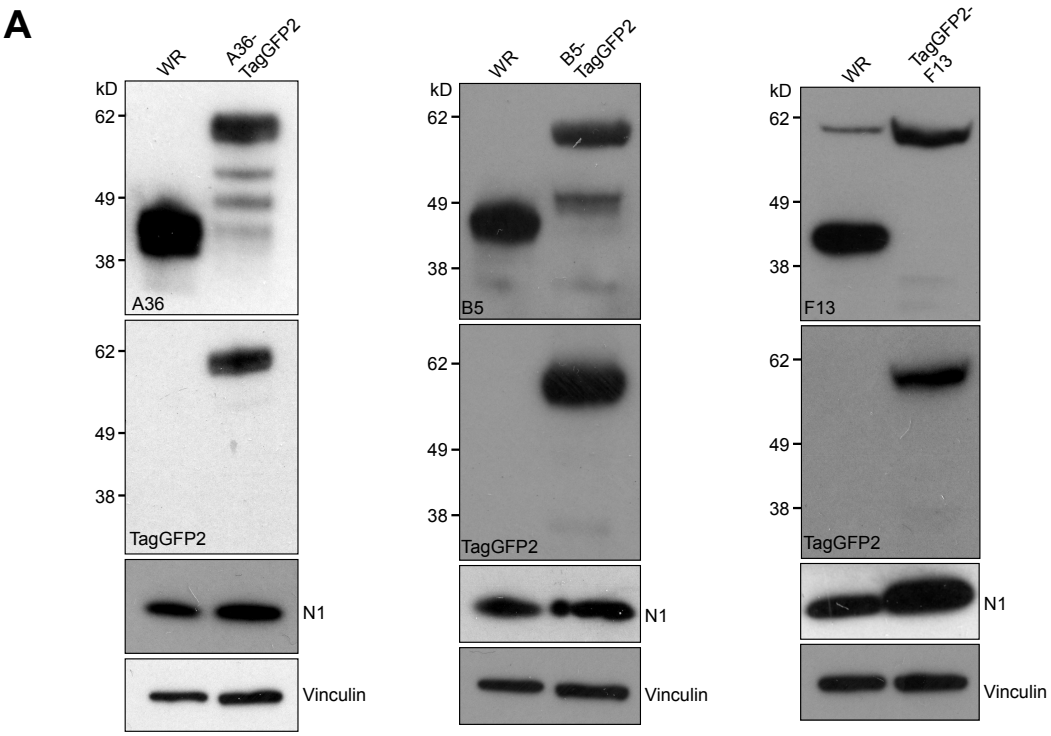
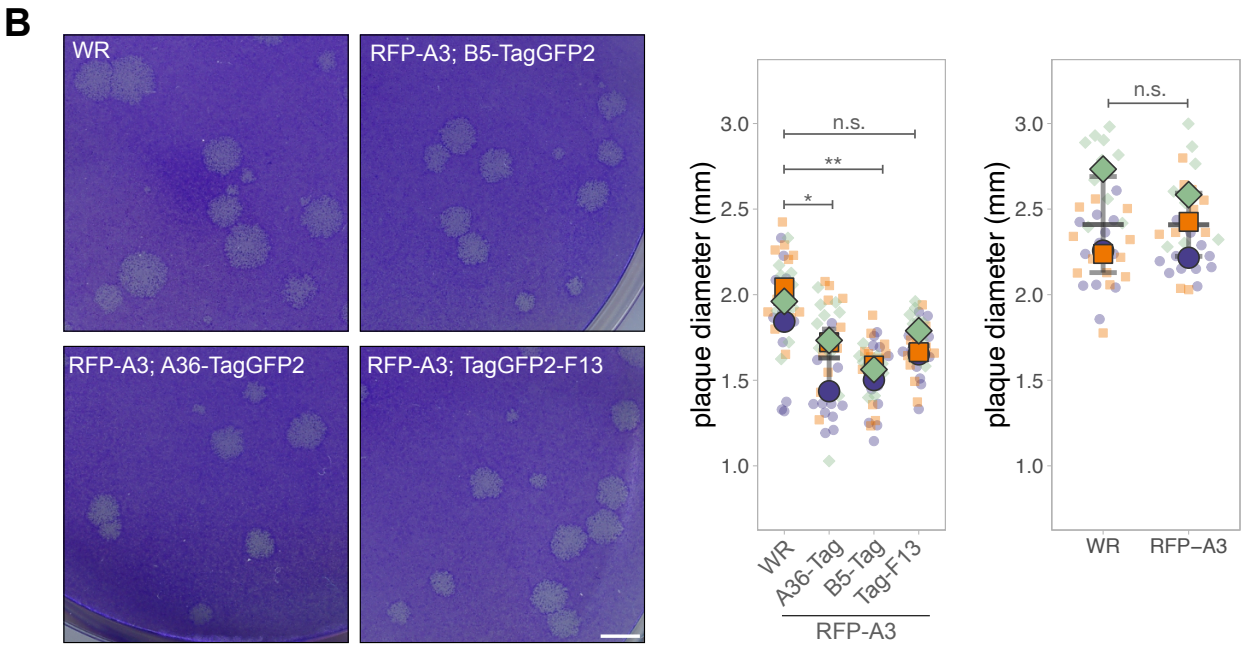
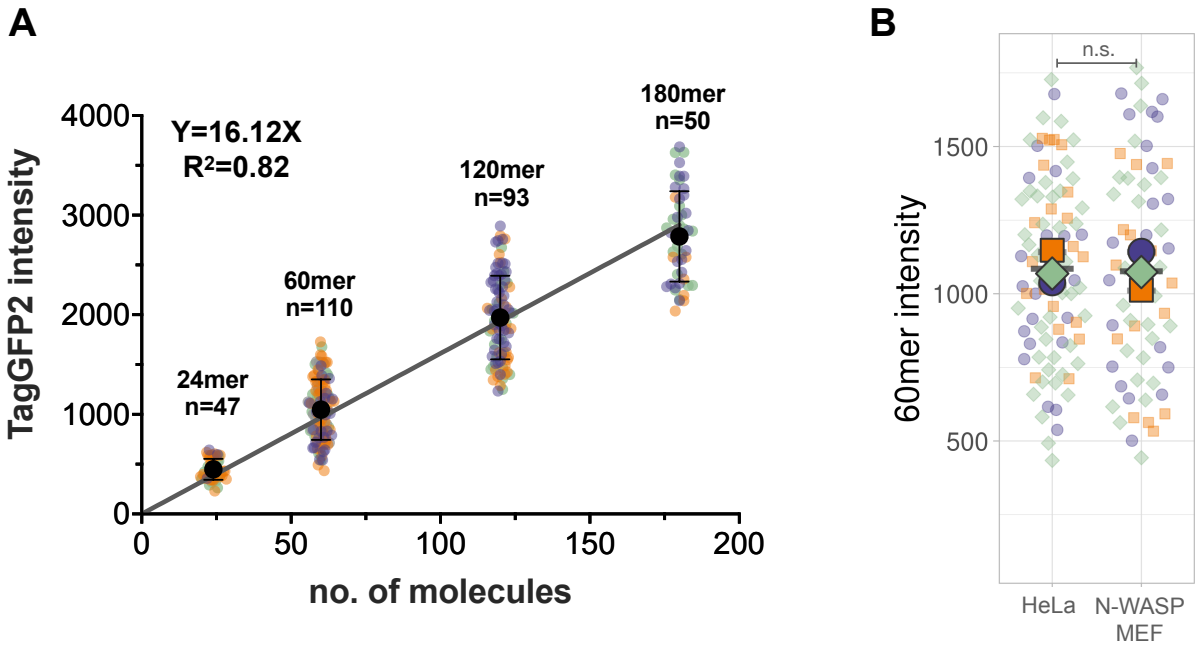


Fig 6:

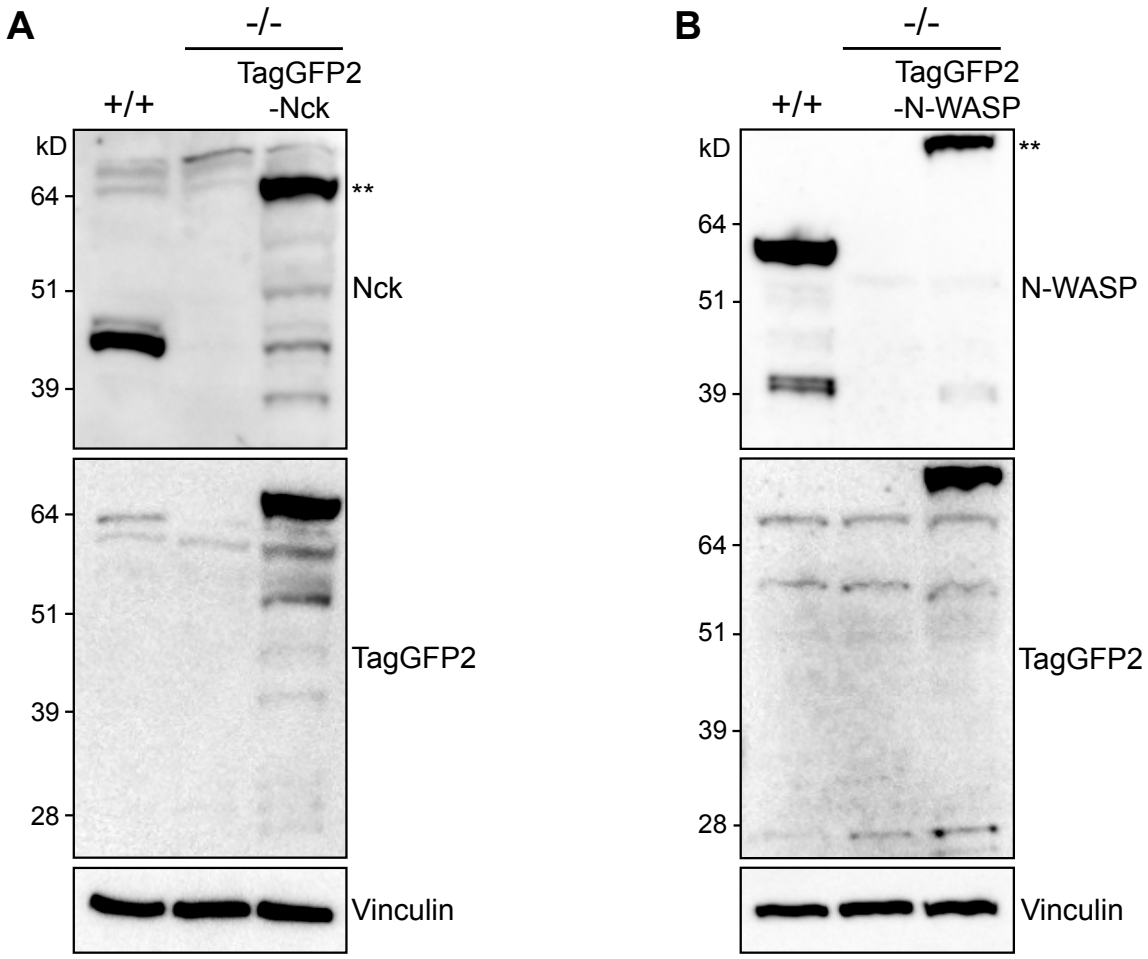

A
WR
A36-TagGFP2
B5-TagGFP2
TagGFP2-F13
kD
62
49
38
A36
B5
F13
TagGFP2
N1
Vinculin


B
WR
RFP-A3; B5-TagGFP2
RFP-A3; A36-TagGFP2
RFP-A3; TagGFP2-F13
plaque diameter (mm)
3.0
2.5
2.0
1.5
1.0
n.s.
**
*
WR
A36-Tag
B5-Tag
Tag-F13
RFP-A3
RFP–A3

Suppl1:


A
Y=16.12X
R²=0.82
TagGFP2 intensity
no. of molecules
24mer
n=47
60mer
n=110
120mer
n=93
180mer
n=50
0
1000
2000
3000
4000
0
50
100
150
200
B
n.s.
60mer intensity
1500
1000
500
HeLa
N-WASP
MEF

Suppl 2:


A
-/-
+/+
TagGFP2
-Nck
kD
64
51
39
**
Nck
64
51
39
28
TagGFP2
Vinculin
B
-/-
+/+
TagGFP2
-N-WASP
kD
64
51
39
**
N-WASP
64
51
39
28
TagGFP2
Vinculin

Suppl 3: