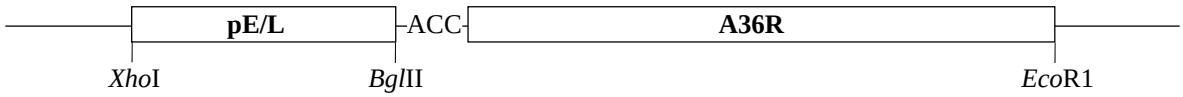


Figure 21: A schematic representation of the 'parental' contruct used for the transfection assay. pE/L denotes the synthetic viral early late promotor. Important restriction sites are shown in *italics*. See chapter 4.12 for cloning details.

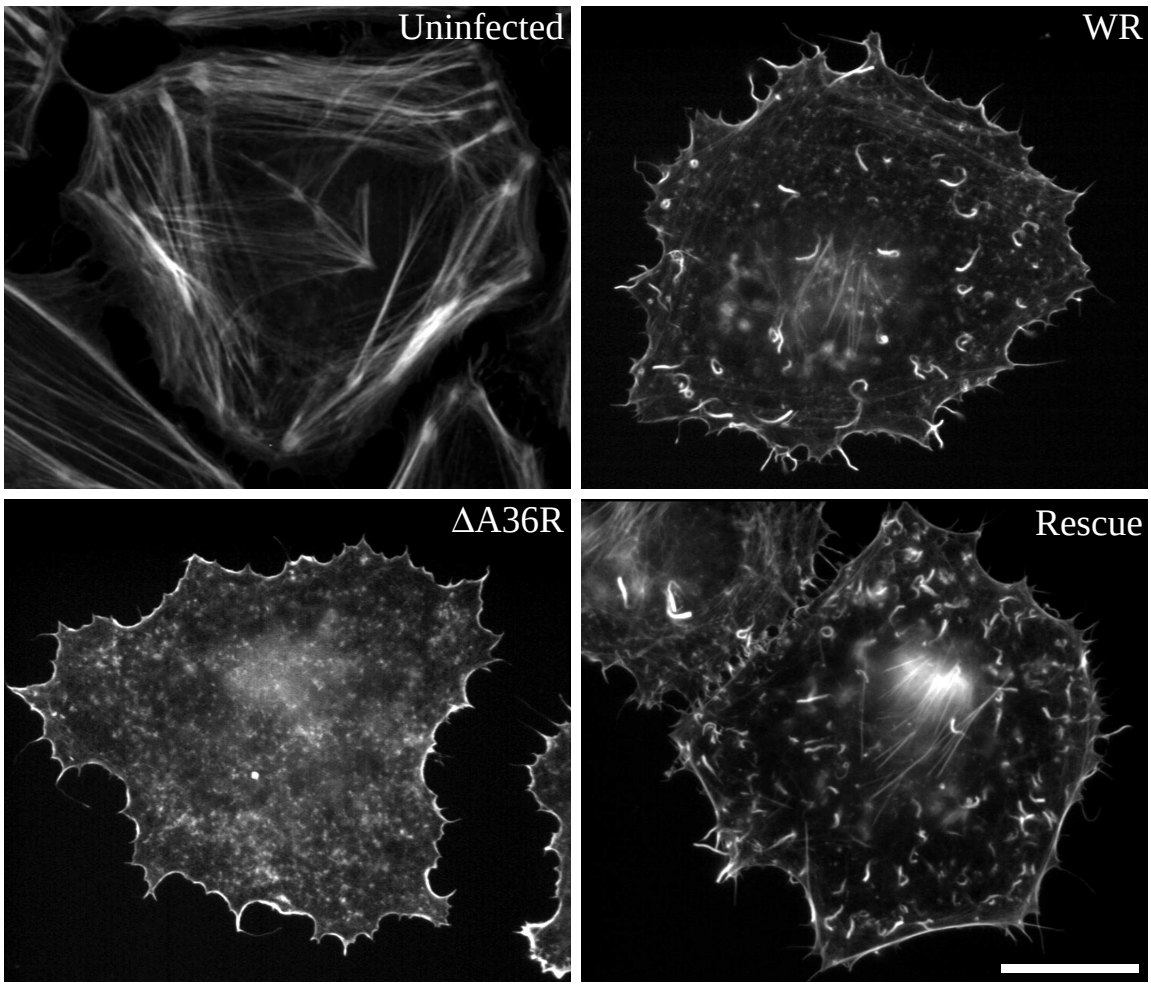
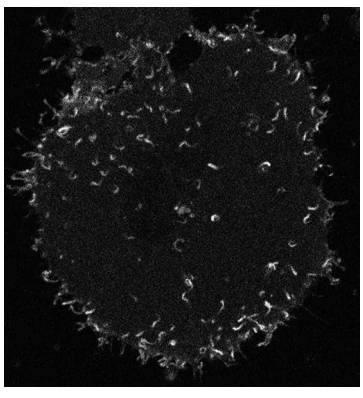


Figure 22: Transfection of A36R into cells infected with ΔA36R rescues actin tail formation. HeLa cells were infected with either WR or ΔA36R or with ΔA36R for 4 hours and then transfected with A36R under the control of the pE/L promotor (Rescue). Cells were fixed 8hpi and the actin cytoskeleton was stained with fluorecent phalloidin. Note the loss of actin stress fibers in all infected cells irrespective of actin tail formation. Scale bar: 20 μm.



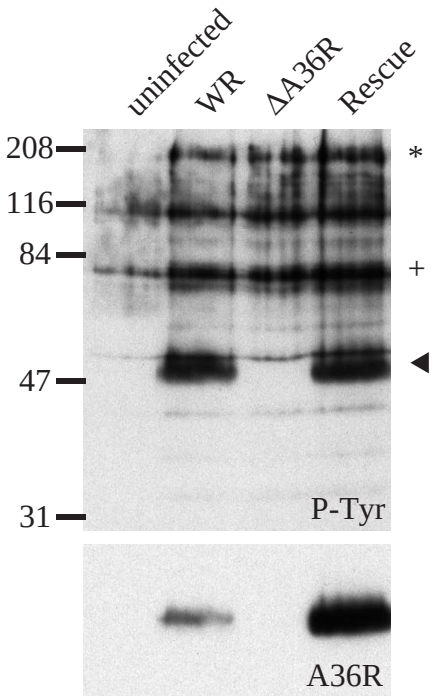


Figure 23: Ectopic expression of A36R in ΔA36R infected cells leads to overexpression of A36R and rescues phosphorylation of pTyr50 (arrowhead). The upper blot is probed with anti-phosphotyrosine antibody. The lower panel shows the same blot reprobed with anti-A36R antibody.

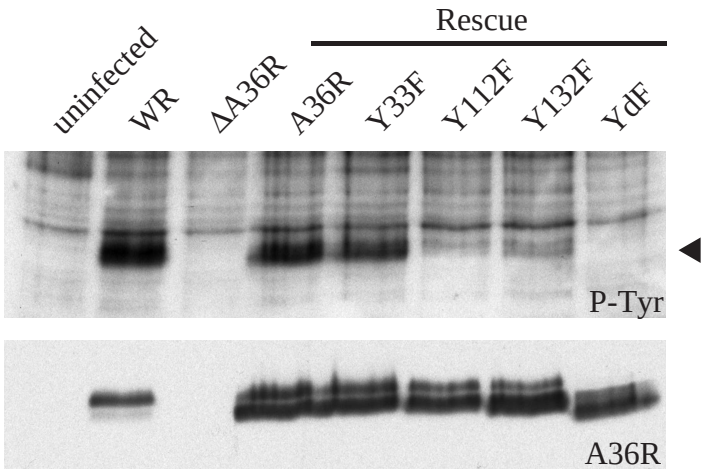


Figure 24: Western analysis of ectopically expressed A36R point mutants reveals that ΔA36R infected cells expressing Y112F or Y132F have a considerably weaker pTyr50 signal than cells infected with WR, or with ΔA36R and expressing wild-type A36R or Y33F. Y158F, Y166F and Y200F mutants are phosphorylated to a similar extent as A36R or Y33F (data not shown). No pTyr50 signal is detectable in ΔA36R infected cells expressing the YdF double mutant (Y112F and Y132F combined). The lower panel shows that YdF migrates slightly faster than the other A36R proteins consistent with an absence of tyrosine phosphorylation.

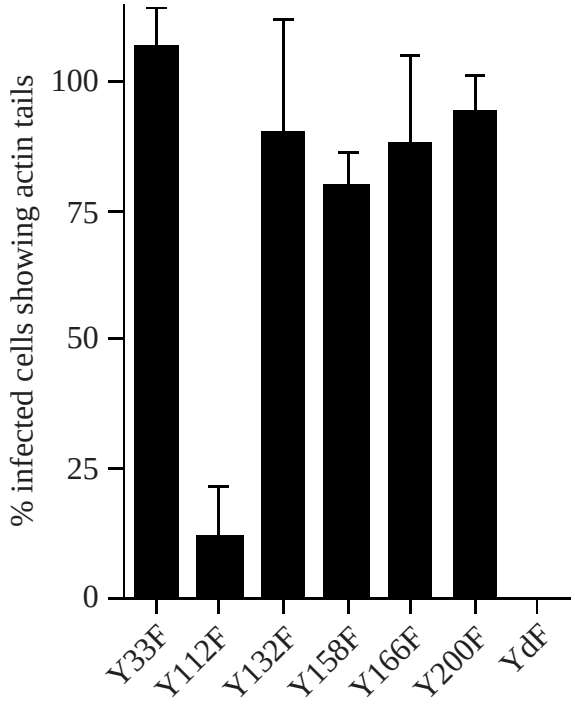
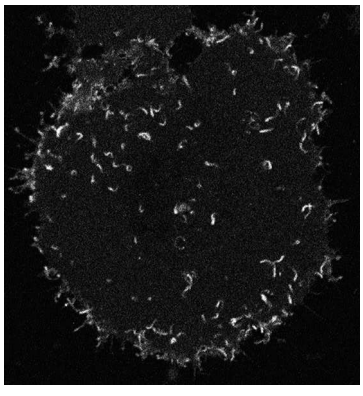
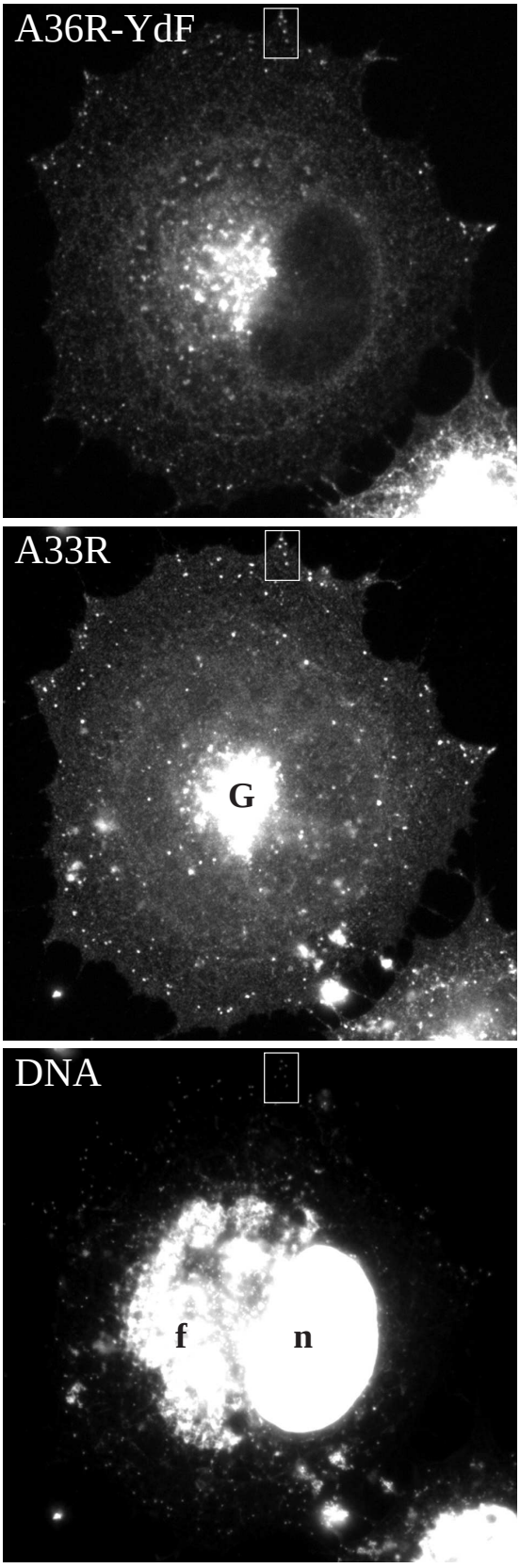


Figure 25: Quantification of actin tail rescue by tyrosine to phenylalanine mutants of A36R reveals that only Y112 is required for efficient actin tail formation. Only the A36R-YdF double mutant cannot form actin tails showing that phosphorylation of Y132 must play an additional role in actin tail formation. For each construct over 200 infected cells were counted in 4 independent experiments. Error bars represent standard deviation from the mean.





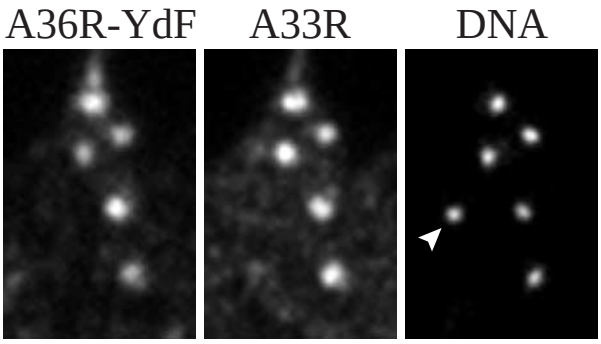
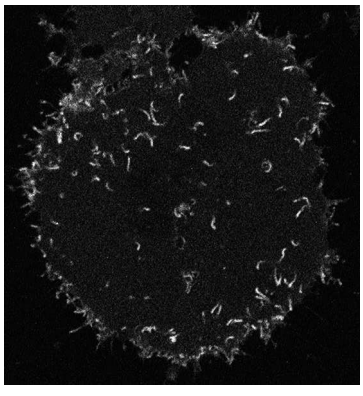


Figure 26: Transfected A36R proteins localize to IEVs. To check whether the tyrosine to phenylalanine mutation had any affect on the localization of the respective proteins, transfected cells were fixed in methanol and labelled with antibodies raised against the IEV specific proteins A33R and A36R. Labelling with DAPI, to reveal viral (and host cell) DNA, was used to distinguish between endogenous membrane structures, to which A33R and A36R localize, and fully formed IEVs. Here a cell is shown that was infected with ΔA36R and subsequently transfected with pE/L-A36R-YdF. The A36R protein with the double point mutations Y112F and Y132F localizes to the Golgi complex (perinuclearstaining) as well as to viral particles. Enlargements of the boxed areas show co-localization between A36R and A33R to several IEVs. The DNA dot which does not localize to A36R or A33R (arrowhead) represents most likely an IMV. Images are overexposed to allow visualization of individual viral particles. Note the perinuclear DNA staining marking the viral factories. n marks cell nucleus, f marks virus factories, G marks the Golgi apparatus.



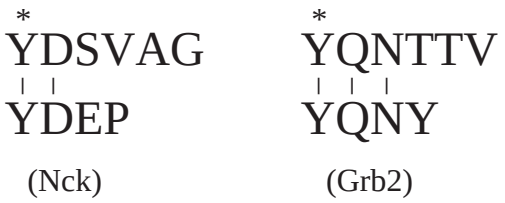


Figure 27: Alignement of the amino-acid sequence just C-terminal of A36R Y112 (top left) with the optimal peptide sequence required for binding to the SH2 domain of Nck and of the sequence C-terminal of Y132 with the optimal peptide sequence for Grb2 (right). Tyrosines 112 and 132 are marked by an asterix.

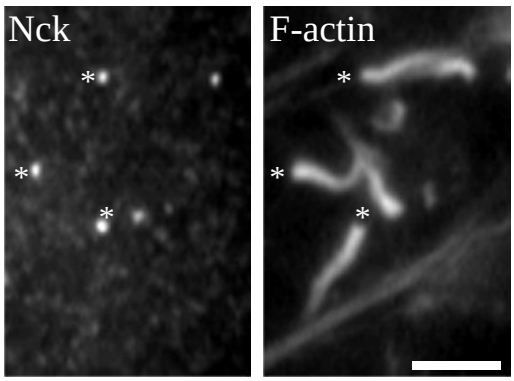


Figure 28: Localization of Nck to the tip of viral actin tails. Actin tails are stained with phalloidin and Nck is visualized by labelling with a polyclonal antibody. Asterixes indicate the localization of viral particles. Scale bar: 5 μm.

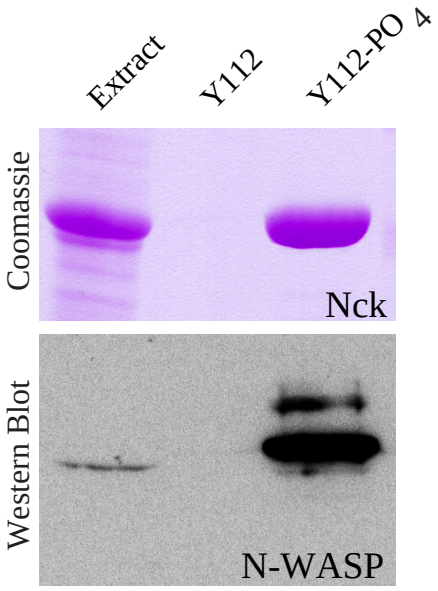


Figure 29: Nck interacts directly with phosphorylated Y112 of A36R and the complex of these proteins recruits N-WASP from cell extracts. Upper panel: the peptide residue 105-116 of A36R containing phosphorylated Y112 interacts directly with Nck as it retains the protein from the soluble *E. coli* extract. Lower panel: the peptide-Nck complex pellets N-WASP from an extract prepared from cells infected with vaccinia virus.

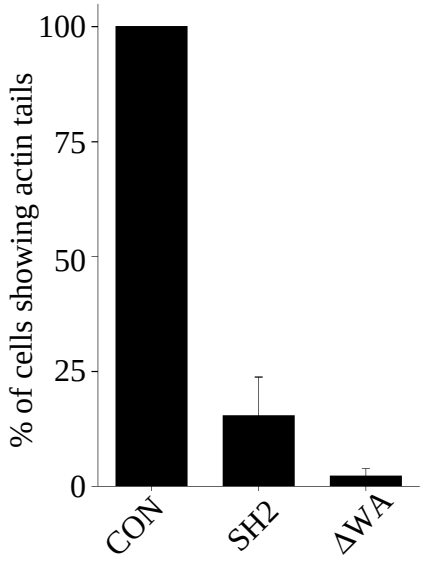


Figure 30: Overexpression of the Nck-SH2 domain (SH2) or an N-WASP construct lacking the WA domain (ΔWA) inhibits actin tail formation in WR infected cells. The control (CON) represents cells on the same coverslip that were infected with WR but not transfected. Over 200 cells were counted in 4 independent experiments. Error bars represent standard deviation from the mean.

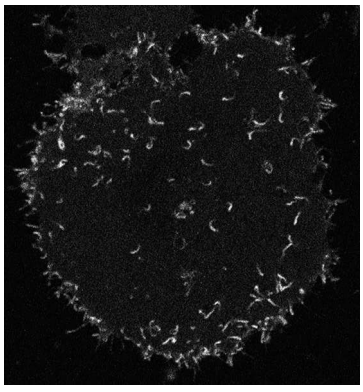


Figure 31: Alignment of the region around A36R Y112 (*) and the optimal substrate sequence for phosphorylation of a peptide by c-Src. Solid bars indicate identical residues while dotted lines indicate similar amino acids.

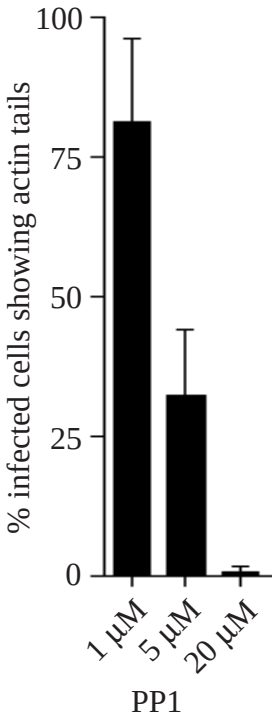


Figure 32: The Src-family kinase inhibitor PP1 inhibits actin tail formation in a dose dependent fashion. HeLa cells were infected with vaccinia virus strain WR in the presence of the indicated concentration of PP1. Cells were fixed 8 hpi and processed for immunofluorescence. Over 200 infected cells containing IEVs (Figure 33) were counted in 4 independent experiments. Error bars represent standard deviation from the mean.

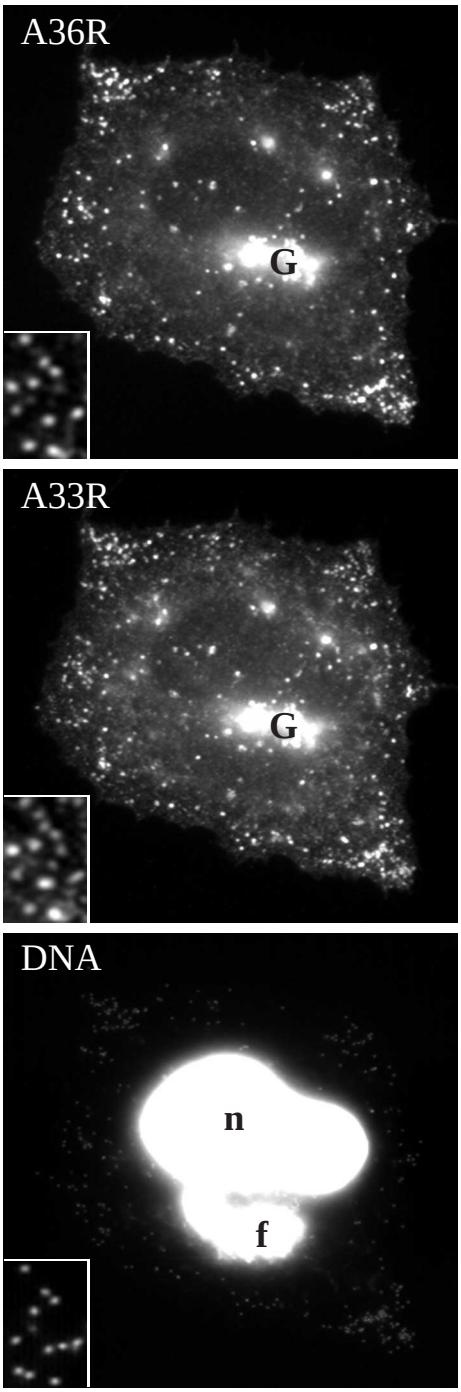
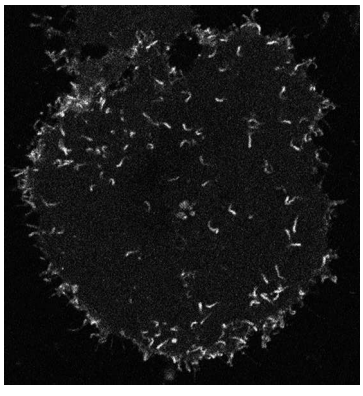


Figure 33: PP1 does not inhibit viral morphogenesis. Unlike other kinase inhibitors (e.g. herbimycine, data not shown) PP1 does not prevent assembly of IEVs. For details on the staining refer to legend of figure 27. PP1 was used at 20 µM.



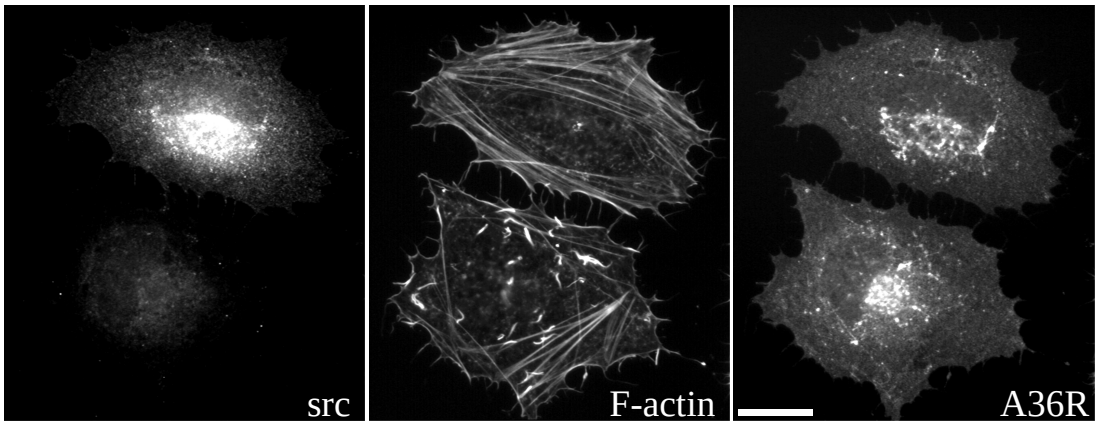


Figure 34: Over-expression of "dead open" c-Src inhibits vaccinia actin tail formation. Two infected cells, one expressing "dead open" c-Src (top), are shown. Bar: 2O μm.

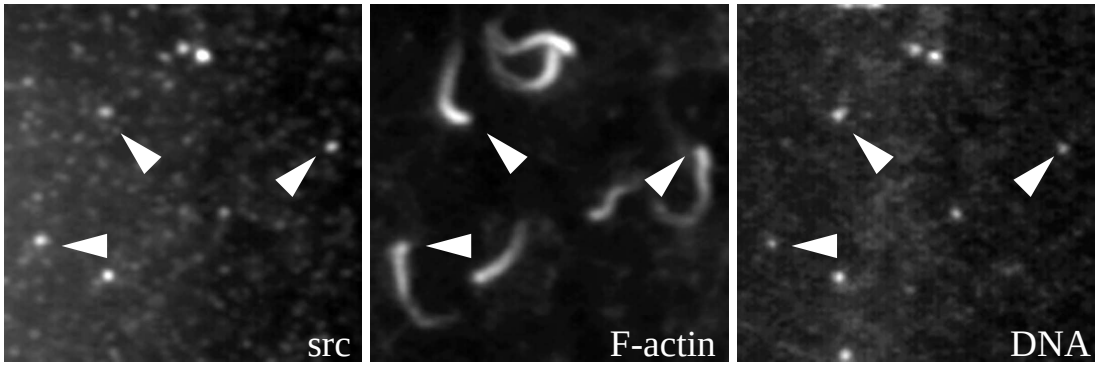


Figure 35: Closer examination of infected cells expressing low levels of "dead open" c-Src reveals that the protein is recruited to viral particles that have induced actin tails (arrowheads).

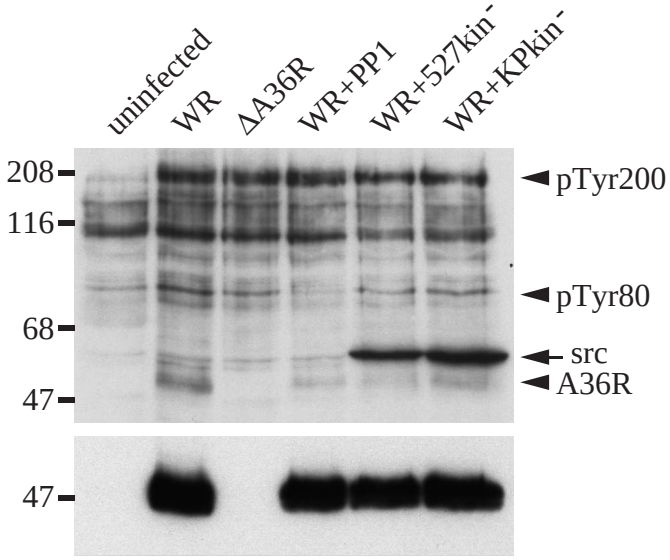
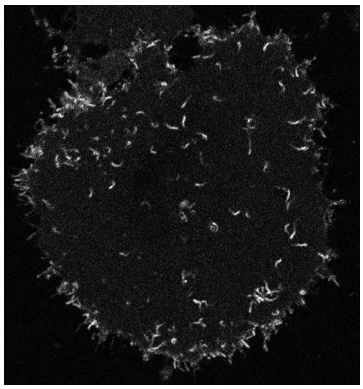


Figure 36: PP1 and "dead open" c-Src reduce phosphorylation of A36R. 20 μM PP1 also reduce phosphorylation of pTyr80. Neither PP1 nor "dead open" c-Src reduce phosphorylation of pTyr200. Upper panel: blot probed with anti-phospho-tyrosine antibody. Lower panel: same blot reprobed with A36R antibody.



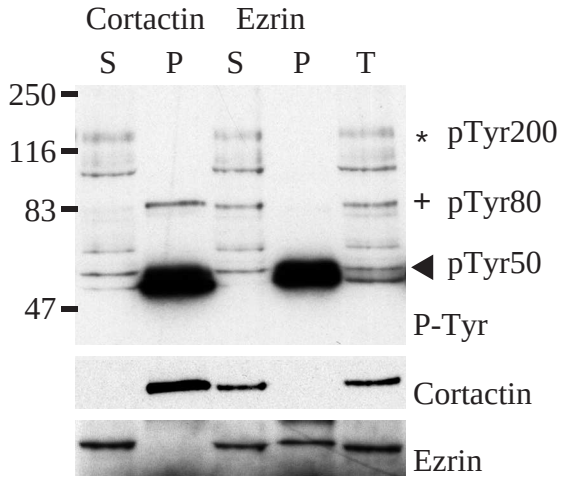


Figure 37: Cortactin is phosphorylated during vaccinia infection. Antibodies against cortactin but not against ezrin deplete pTyr80 from a cell extract. S denotes the supernatant of the precipitation, P stands for the pellet and T for total extract. Cells were infected for 8 hours. The blot was first probed with anti-phosphotyrosine antibodies and after stripping reprobed with anti-cortactin and anti-Ezrin antibodies (bottom).

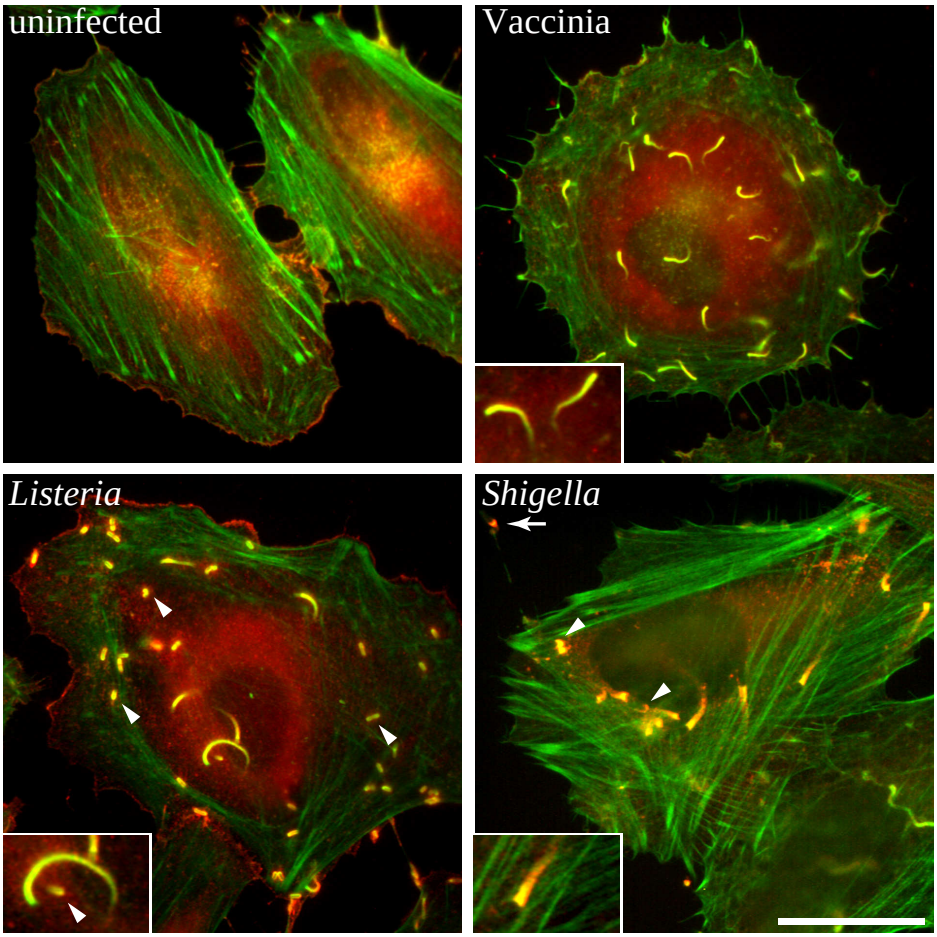
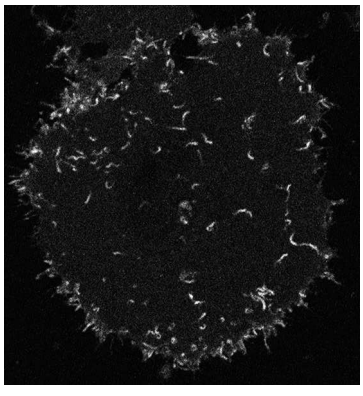


Figure 38: Localization of cortactin to actin tails of intracellular pathogens and to actin clouds around bacteria (arrowheads). In uninfected cells cortactin localizes to the cortical actin of the cell periphery. Co-localization of cortactin with F-actin is shown in yellow. The arrow points to a projecting *Shigella*. Scale bar: 20 μm.



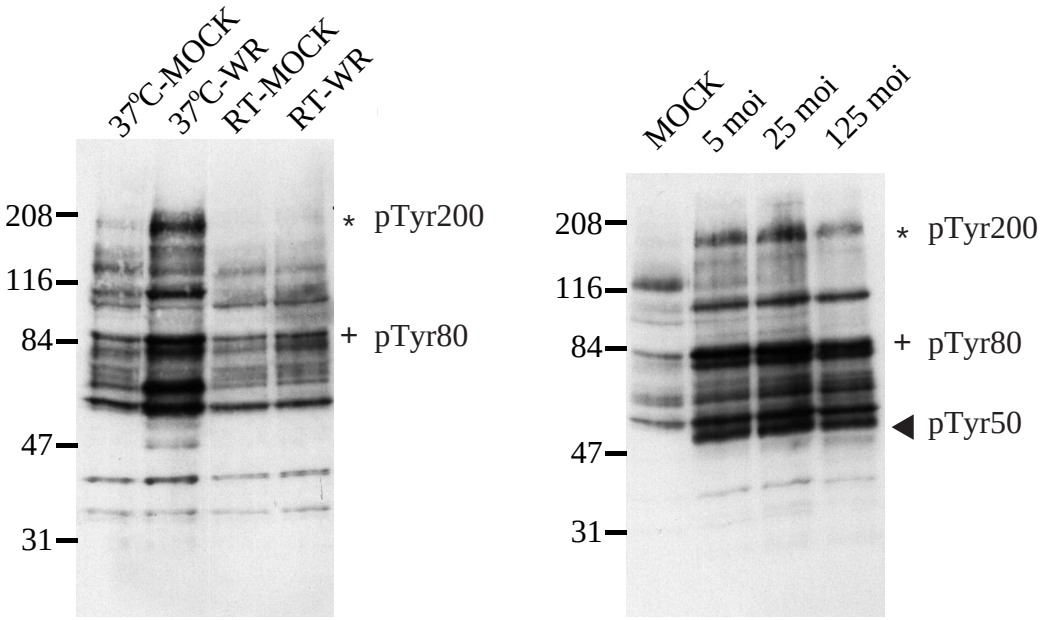


Figure 39: Induction of tyrosine phosphorylation is dependent on viral entry but independent of the multiplicity of infection. Left: Vaccinia does not enter cells at room temperature (RT) and fails to induce tyrosine phosphorylation. Extracts were prepared from cells 2 hpi. Right: Increasing numbers of infecting viral particles does not increase the level of phosphorylation. Extracts were prepared from cells 8 hpi. Equal amounts of cell extracts were loaded. moi stands for multiplicity of infection meaning the average number of infecting viruses per cell.

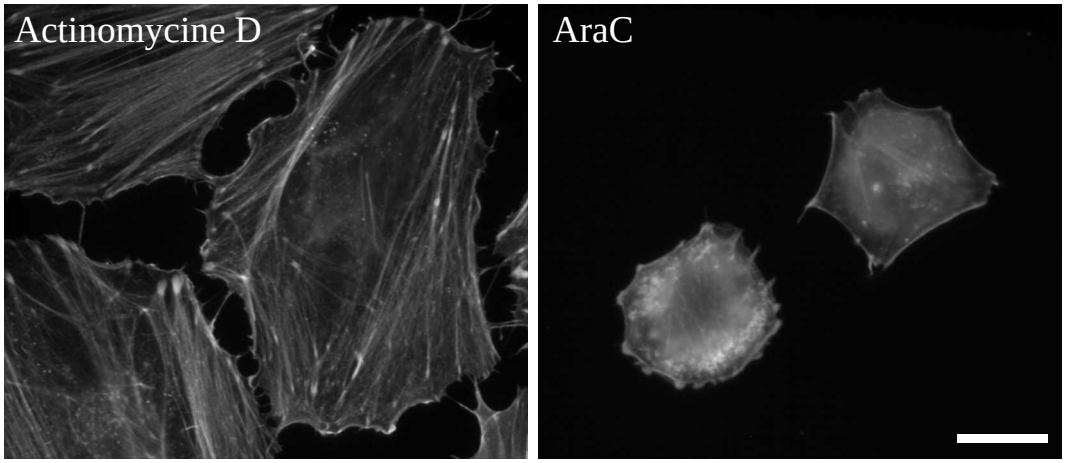
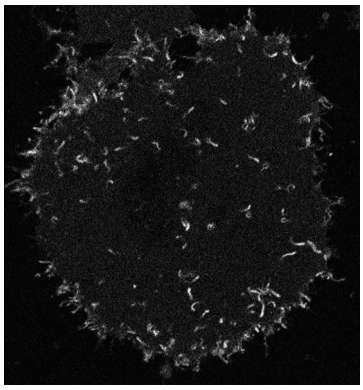


Figure 40: Blocking early but not late gene expression inhibits vaccinia induced loss of actin stress fibers and cell rounding. Cells were infected for 4 h in the precence of either Actinomycine D or AraC. Actinomycine D but not AraC also blocks the induction of viral induced phosphorylation (data not shown). Uninfected or WR infected control cells showed similar cell shapes as the cells infected with the respective drugs (data not shown). Scale bar: 20 μm.



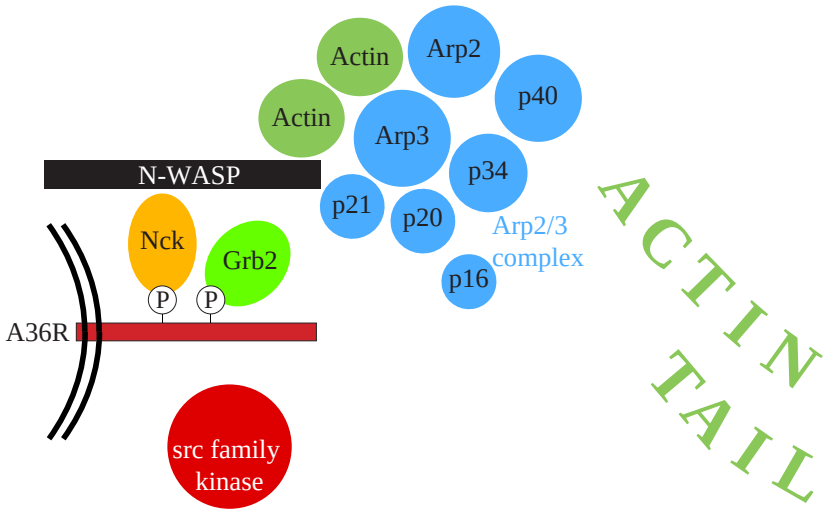


Figure 41: Cartoon of the possible interactions of proteins involved in vaccinia actin tail formation. Please compare with the models for *Listeria* and *Shigella* in figure 7.

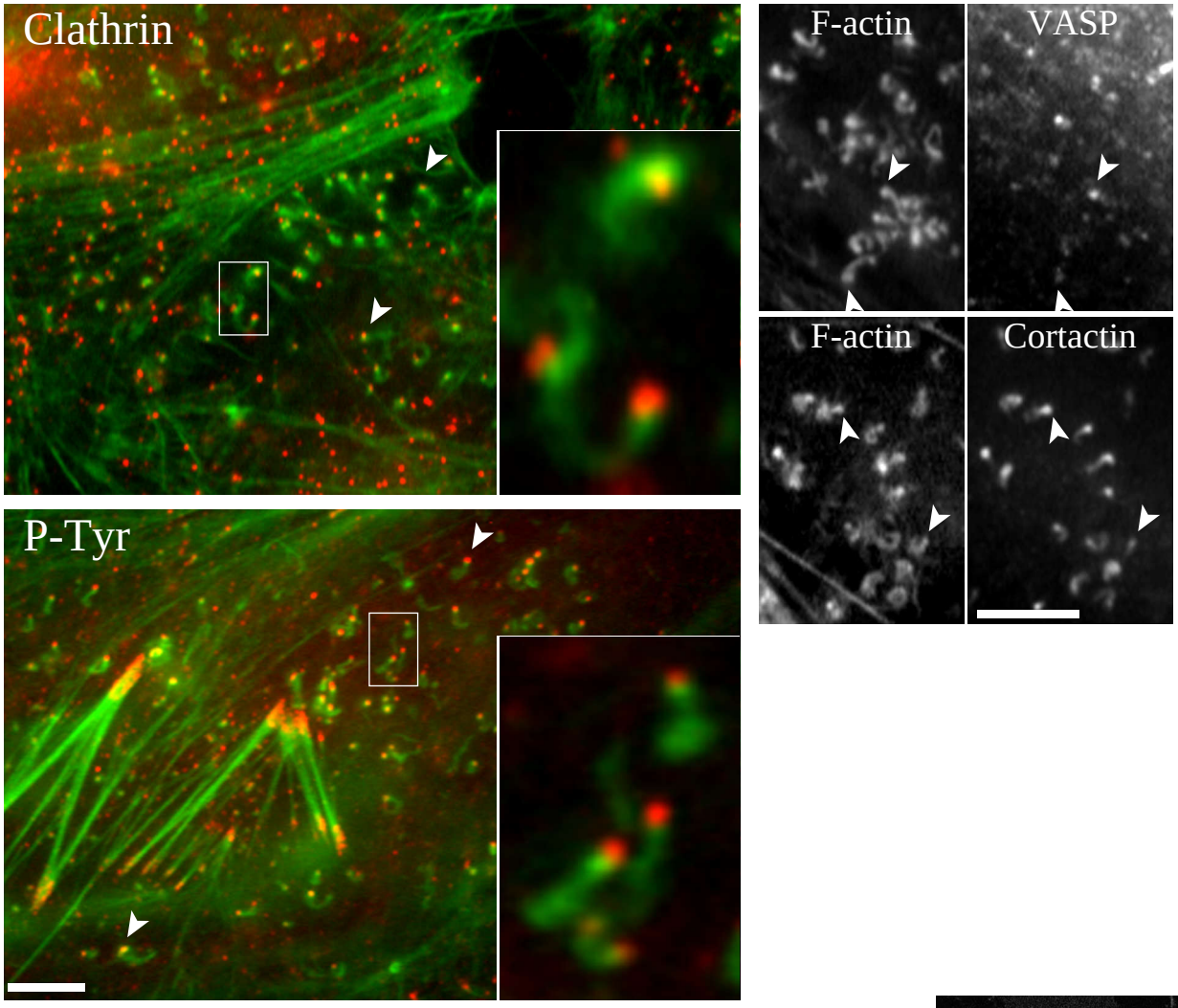


Figure 42: Actin tails in the absence of infection. Clathrin coated vesicles can nucleate little actin tail like structures (LATs) that also have an associated phosphotyrosine signal. The same complement of proteins is found in LATs as in vaccinia induced actin tails. Arrowheads point to clathrin coated vesicles with associated little actin tails. Scale bars: 5 μm.