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Analysis of Cellular Factors Involved in Adeno-Associated Virus Type 2 Entry

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The study of virus-host interactions has not only produced insights relevant to both preventative drug design and the emerging field of gene therapy, but it has also served as a valuable tool to the field of cell biology. The biological roles of many pathogen recognized cell-surface receptors have yet to be determined, yet are being exploited by pathogens to facilitate their entry and infection. Specifically a small family of sugar modified membrane protein known as heparan sulfate proteoglycans, have been shown to be a critical determinant in the efficiency of infection of many important pathogens. Adeno-associated virus type 2 (AAV) is a small (25nm) single stranded DNA, non-enveloped parvovirus that is currently being used as a vector for gene therapy. Valued for its lack of pathogenicity and ability to stably transduce a variety of terminal differentiated and quiescent cell types, the use of AAV during clinical trails has faced many complications. Consequently, the search for knowledge about this vector is more critical now then ever. Although extensive work on the safety of the *in vivo* application of this vector has been established, the cell-biological basis of AAV's life cycle remains to be fully elucidated.

Binding and entry of AAV requires heparan sulfate proteoglycan (HSPG) as well as two secondary receptors, fibroblast growth factor receptor and $\alpha_{V/5}$ $\beta_{5/1}$ integrins. In this study, we follow the cellular modifications induced upon AAV binding and subsequent

internalization into host cells. Earlier reports implicated receptor-mediated endocytosis as the sole pathway for AAV entry. We show that AAV's primary receptor, HSPG, mediates an alternate entry pathway that is clathrin-independent and caveolar-independent. We show that inhibitors of macropinocytosis, which did not inhibit the clathrin-dependent entry pathways, blocked the entry of AAV and HSPG as well as blocked AAV transduction. In cells that are deficient in cell surface HSPG, transduction by AAV was reduced but unaffected by drugs that block macropinocytosis. Membrane ruffles induced upon AAV binding were enriched in activated PKC alpha, actin and dynamin. Treatment with dynosore, expression of dominant negative dynamin 2, or an HSPG construct lacking the PKC recruitment domain greatly diminished the size of macropinosomes and AAV internalization. Finally, silencing of clathrin heavy chain by siRNA did not affect AAV infection. Our data shows that HSPGs play a critical role in chaperoning AAV into a macropinocytic pathway, which leads to productive infection.

I would like to dedicate this thesis to my family. I will be the first Ph.D. in our family's history and they have been my greatest fans and inspiration.

Acknowledgments

To the biggest little virus! Studying the endocytosis of Adeno-associated Virus Type 2 started out, like many scientific experiments, with trying to reproduce the literature. Several dead-ends, a brick wall or two, and lots of lessons later, we have a cute little story to tell.

For their constant assistance and companionship I'd like to thank members of the Simon Lab, past and present. Sandy was the best mentor I could have asked for. He pushed me to be creative, and let me shine in and outside of lab. He made the lab, its members and me feel like part of his family. Thank you to my committee Charlie Rice, Tom Muir and Phil Leopold for their advice and insight. And I'd like to acknowledge my many scientific mentors both past and present; you've shown me what it means to be a good person and how to aspire to be a great scientist (one day). Specifically I'd like to acknowledge my undergraduate thesis mentor Dr. Grant A. Hartzog. He showed me that hard work doesn't have to make a hardened scientist. And to my best friend and editor Andres Miguel Roden, he might not know DNA from DKNY, but he can string together prepositions, predicates and adverbs like a professional!

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List of Abbreviations

- AAV — Adeno-associated Virus Type 2
- C1/2/3 — Conserved Domain 1/2/3 of syndecan 1-4
- Cap — Genetic element encoding Coat Proteins VP1, VP2, VP3
- EEA1 — Early Endosomal Autoantigen 1 Protein
- ECM — Extracellular Matrix
- EGF — Epidermal Growth Factor
- EGFR — Epidermal Growth Factor Receptor
- Eps15EH29 (Δ95/295) — Epidermal growth factor receptor pathway substrate 15
- FAK — Focal Adhesion Kinase
- FGF — Fibroblast Growth Factor
- FGFR — Fibroblast Growth Factor Receptor
- GAG — Glycosamino Glycan
- GFP — Green Fluorescent Protein
- HEK 293 — Human embryonic kidney 293 (HEK 293) cells
- HRP — Horseradish Peroxidase
- HSPG — Heparan-Sulfate Proteoglycan
- ITR — Inverted Terminal Repeat
- MDCK — Madin Darby Canine Kideny cells
- MOI — Multiplicities of Infections
- NGR — Asparagine-Glycine-Arginine
- PDZ — Postsynaptic Density-95/Disc Large Protein/Zonula Occludens-1
- PIP2 — Phosphatidylinositol 4,5-bisphosphate
- PIP3 — Phosphatidylinositol 3,4,5-bisphosphate
- PIP3K — Phosphoinositide-3 kinase
- Rep — Genetic element encoding Replication proteins Rep78,68,52,40

- RGD Arginine Glycine Asparagine
- S4 Syndecan 4
- SNX Sortin Nexin
- SH Src Homology Domain
- SV 40 Simian Virus 40
- V Variable Domain of syndecan 1-4

Chapter 1

Introduction

Gene Therapy

While initially conceived as a means for complementing or even replacing defective genes (in monogenetic diseases) like cystic fibrosis or hemophilia, the scope of pathologies potentially curable by gene complementation by gene therapy has expanded over the past decade. The success of gene therapy requires circumvention of specific problems; the delivery system must be safe, work in a wide variety of tissues and maintain expression for the lifetime of the individual being treated. Attempts to use Cationic liposomes have proven inefficient due to the large amount of DNA-liposome complexes required, the low efficiency of internalization and the brief time of gene expression (several days)[20]. It has long been observed that viruses, through evolution, have gained the ability to readily infect cells and efficiently express their genes. Development of viruses for gene therapy is complicated by the fact that most viruses are human pathogens and result in unwanted pathologies. The stable integration of virus genomes into human chromosomes would have the benefit of prolonged transgene expression. Infection with a retrovirus would have this advantage, but also the disadvantage of lack of site specificity. Undesirable integrations could lead to disruption of essential genes [135]. In addition, the immune response to the large concentrations of virus required for stable transduction has been linked to the death of a patient undergoing Adenovirus gene therapy in 1999[104]. The discovery of Adeno-associated virus as a contamination of an Adenovirus preparation has led to renewed hope for the field of gene therapy. This virus has the unique advantage of causing no known pathology in infected

individuals [21]. The biological understanding of AAV has grown substantially over the past 10 years with most of AAV's lifecycle and host-pathogen interactions being determined by *in vitro* experimentations. Recent observations from *in vivo* data and clinical trails reveal continued complications in the efficiency of transduction of target tissues and unfavorable immune reactions [22].

Adeno-Associated Virus Type 2 (AAV) Overview

Viruses are studied to gain insight into their pathology, but also to gain insight into the workings of the cell. Understanding how viruses enter and infect cells can reveal internalization routes that might be poorly characterized or even novel [34,126,129,130]. The ~4.7KB single stranded DNA non-enveloped parvovirus adeno-associated virus Type 2 (AAV) has been in development as a vector for gene therapy. This is owing to its lack of pathogenicity, ability to infect and express in quiescent cell types, diverse tissue tropism and its ability to integrate site-specifically [112]. AAV is part of the parvoviridae family, which includes some of the smallest known viruses. The 25nm virus's genome has two open reading frames, flanked by 145 nucleotide inverted terminal repeats (ITRs), that code for genes required for its replication (Rep) and for formation of its capsid (Cap) [43]. The main structure of the virus is constructed of three capsid proteins: VP1 (87kDA), VP2 (73kDA) and VP3 (62kDA) that assemble into a 1:1:20 ratio, 60-subunit, icosahedral ball[13,88]. Since transcription of the Cap genes occurs on over-lapping reading frames, the capsid proteins share a common C-terminal sequence; but VP1 has a N-terminal domain with phospholipase A2 (PLA2) activity that is required for efficient

infection[56]. VP2 is expendable and has even been completely replaced with GFP for live cell microscopy studies[101,156].

AAV evolved a small genome incapable of independently completing its life cycle, making it part of a family known as dependoviruses. Independent of co-infection with a helper virus (such as Adenovirus or Herpes Virus) tissue culture cells become latently infected with wild type AAV. After its single stranded genome is converted to double-stranded DNA, AAV's genome integrates sites specifically into chromosome 19 at a site called AAVS1[73,194]. For clinical safety purposes, the Rep genes (Rep78, Rep68, Rep52, Rep40) have been removed from recombinant AAV (hence preventing the assembly of new viral particles *in vivo*), and in doing so the site specificity of genome integration is lost. In the latter case, the viral DNA may circularize into a stable episomal plasmid[10]. AAV's unique ability to integrate site specifically, combined with its lack of pathogenicity has prompted scientist to implement this vector in gene therapy trials. In order to create recombinant AAV particles *in vitro*, cells must be co-infected with a helper virus, or transfected with plasmids containing the helper genes alone. To avoid contamination of clinical samples, specific helper vectors-plasmids have been created that contain the minimal E2A, E4 and VA RNA helper virus genes from Adenovirus, which when combined with a plasmid carrying one's gene of interest (flanked by ITRs) and Cap genes, generate intact, infectious and helper virus free, particles incapable of initiating a lytic life-cycle[60].

The implementation of AAV has been hampered by its inability to transduce important organs such as lung tissue, the requirement for post entry conversion of single stranded DNA into the transcriptionally active double stranded DNA, the existence of neutralizing antibodies present in the general population, and the high multiplicities of infections (MOIs) needed to stably transduce permissive cell types [37,48,50,163]. Some immediate blockades to AAV transduction may lie in the lack of cell-surface expressed AAV receptors. Effort has been placed into modifying AAV and other serotypes to augment their cellular tropism. A few methods, such as mutational ablation of receptor binding sites, adding integrin binding motifs (RGD) into the capsid sequence, as well as screening random mutations in the capsid proteins for increased transduction have had some success [7,42,57,85,103,145,172,226].

Overview of AAV's Receptors

In order to initiate the first stages of the viral life cycle, internalization into host cells, viruses and other pathogens must bind to specific cell-surface proteins known as their receptors. Cells or tissues lacking some or all of these pathogen receptors are poorly infected or even refractory to infection. The presence of AAV's primary receptor, heparan sulfate proteoglycan (HSPG) and co-receptors $\alpha_V\beta_5$ and/or $\alpha_5\beta_1$ integrin and Fibroblast Growth Factor Receptor (FGFR) define a major part of the host-cell range[141,189,190]. A positively charged binding clef on the capsid of AAV has been shown to be required for AAV's HSPG binding ability whereby mutation of this site abolishes their interaction and diminishes AAV infection[79,121]. Contrary to the well-defined

interaction with AAV and its primary receptor, AAV's secondary receptors are mired in ambiguity. There are reports that AAV does not bind to $\alpha_V\beta_5$[142], whereas there is a proposed $\alpha_5\beta_1$ integrin-fibronectin like binding motif adjacent to the HSPG interaction domain in the capsid of AAV[5]. Ablation of this asparagines-glycine-arginine (NGR) motif in VP3 decreases AAV binding and transduction by an order of magnitude [in human embryonic kidney 293 (HEK 293) cells that do not express $\alpha_V\beta_5$] [5]. One caveat to the $\alpha_5\beta_1$ integrin work is that there is a decreased transducability of NGR mutant AAV in cell lines not expressing α5 integrin when compared to wild type virus, implying a potential complication in capsid integrity or downstream processing caused by this mutation[5].

There is conflicting data concerning the necessity of FGFR's presence as well[139,143,160]. In the search for co-receptors, researchers use cell lines devoid in the expression of certain receptors, or perform crude protein overlay assays to see what membrane proteins bind intact AAV particles. Inherent differences in myriad downstream effectors, receptor complexes and compensatory mutations that occur in cancer cell-lines can create confusion and ambiguity. In fact, many of AAV's putative coreceptors exist in stable complexes with each other in unstimulated cells [12,155]. Since several labs have come to contradictory results about AAV coreceptors, a clearer understanding of the early events of AAV attachment to these receptors and subsequent entry may shed light on these differences. Are receptors just attachment factors, signaling to the virus that it has reached its target tissue, or are there signaling capabilities inherent to these receptors and required for efficient transduction of permissive cells? For instance, there was no effect

on viral-transduction when the Tyrosine autophosphorylation of FGFR was inhibited using Tryphostin 1; implying that FGFR activation is not required for AAV entry[102]. And although FGF2 can compete with AAV for binding to HSPG, only one cell-line has been reported that requires both HSPG and FGFR for efficient transduction [140]. Interestingly, treatment of cells with a pan-kinase inhibitor, genistein, has no effect on the entry of AAV and may actually increase transduction in some cell types[102,140]. Hence, the identity of AAV-interacting receptors may not shed as much light on their necessity as linking their presence to the greater life cycle of the virus.

Receptor-mediated Endocytosis of AAV

The study of clathrin-mediated endocytosis (CME) has been advanced by the creation and implementation of adaptor and accessory proteins made dominate negative by point mutations and truncations or fused to fluorescent proteins and followed in live cells [150]. Marked by the recruitment of receptor- and membrane-binding proteins, CME is defined by the generation of a clathrin-coat around membrane curved pits at sites of internalizing ligand-bound receptors (during outside-in signaling), constituent receptor recycling and receptor clearance[146]. Viruses as well as other microbes have gained, through evolution, machinery and cell-interacting capabilities that permit them to exploit endogenous internalization routes such as CME for productive entry and infection[130]. A single fluorescently labeled AAV was tracked in live time as it interacted with a HeLa cell. Investigators saw that the particle touched multiple times (mean of 5) over 2 minutes before pausing and entering the cell in approximately 64 milliseconds[171]. Further, only 13% of the viruses that touched the surface went on to be adsorbed implying that the

membrane constitutes the major barier for AAV transduction. It was the over expression of a dominant negative mutant of the "pinchase" dynamin 1 that led to the assertion that after binding to its cellular receptors, AAV enters via receptor mediated endocytosis[41]. Although not affecting AAV binding, over 90% of bound virus was readily trypsonized off cells over expressing the dynamin1K44A mutation, compared to control cells. It was also observed that half of radioactively labeled AAV could be cleared from the surface of cells (internalized) in 10 min[8]. At the time this work was done, the cellular role of dynamin was believed to act primarily on the clathrin-mediated pathway. It has become clear in subsequent years that dynamin has a plethora of roles, including facilitating the internalization of ligands through clathrin-independent routes of interanalization[137,117]. In fact, several viruses have been shown to enter through dynamin-dependent caveolar endocytosis[1274].

The rearrangement of the actin cytoskeleton as well as the recruitment and polymerization of actin monomers is believed to be required for CME, as well as several other forms of endocytosis [78,198]. Actin bursts and actin tails have been associated with the internalization and movement of other viruses [128]. Expression of a dominant negative form of a Rho GTPase family member Rac blocks the recruitment of actin, required for the membrane deformation and internalization of AAV[41,161]. AAV internalization leads to the transient activation of Phosphoinositide 3-Kinase (PI3K), and inhibition of this kinase with the toxin Wortmannin prevents subsequent nuclear trafficking without affecting internalization[41,161]. The PI3K enzyme family interacts with small signaling molecules called phosphatidylinositols (Ptdlns), specifically PI3K phosphorylate

phosphatidylinositol (4,5)-bisphosphate (PIP2) at the 3rd position hydroxyl group of the inositol ring to make PI (3,4,5)P3 (PIP3)[186]. PIP2 acts at the plasma membrane to recruit effector molecules required for several types of endocytosis, and its generation at the membrane has been linked to integrin activation [106 193,229]. Phosphorylation of PIP2 acts to regulate trafficking of internalized plasma membrane containing endosomes in the cell, and might be dependent on the components found in the nascent endosomes. Finally, depolymerizing the actin or microtubule network before infection decreased viral transduction by up to 70%, but had little effect on transduction post-internalization, underscoring the role that the cellular cytoskeleton plays in the early events of AAV infection [161].

Trafficking, Uncoating and Nuclear Entry

The remaining steps of AAV's life cycle remain elusive. Reports differ as to the intracellular location of AAV before its entry into the nucleus, as well as the integrity of the viral capsid during and after nuclear entry [37,101,210]. As this is not the main focus of my work, a brief overview is as follows. A reoccurring theme that complicates the progress in the trafficking field stems from inherent differences in the cell types studied. Most studies on AAV trafficking have worked under the assumption that the virus follows the most well characterized path for ligands internalized via CME. All attempts to colocalize AAV with the classic markers for early endosomes (Rab5 and EEA1), late endosomes (Rab7) and lysosomes (Lamp1) have struggled to find extensive colocalization and a cohesive maturation timeline[225,27,9]. Lysomotropic drugs such as bafilomycin A1 have inhibitory effects on transduction, be them cell type specific, whereas chloroquine has a

slight stimulatory effect at earlier time points during trafficking in HeLa cells [183]. Pretreatment of several cell lines with Brefeldin A, a fungal toxin known to inhibit Arf GTPase activity, lead to a dose dependent decrease in AAV transduction [39]. It was belived that brefeldin A treatment leads to fusion of early and late endosomes, and the authors of the aforementioned paper interpreted that decrease in transduction as evidence that AAV needed to pass through late endosomes for proper transduction. It is now clear that proper ER to Golgi trafficking is disrupted by brefeldin A treatment [181,207]. The authors did not test if AAV attachment or cell-surface presentation of functional HSPG was effected. The accumulation of previous data has lead to the consensus that AAV requires passage through an acidic compartment for efficient infection. Conformational changes, be them acid induced or by another yet to be characterized interaction, lead to the exposure of previously buried residues in VP1 and VP2 (known as externalization)[184]. Inhibition of externalization by blocking antibodies reduces infectivity[184]. Recently, a two-hybrid screen against AAV capsid proteins and a liver complementary DNA library discovered the endosomal cysteine proteases cathepsins B and L directly interact with and cleave AAV *in vitro*[1]. These conformational changes and cleavage events are believed to aid in AAV nuclear trafficking, uncoating, and nuclear entry. Direct injection of viral particles into the cytoplasm did not lead to transduction, implying passage through, or conformational changes/modifications are acquired during viral trafficking [37,181]. Pretreatment of canine parvovirus (also a member of the dependovirus family) before microinjection also failed to lead to a successful infection (although perinuclear accumulation was observed [211]. The intracellular modifications needed for AAV infection and the precise trafficking routes taken are still being uncovered.

An interesting twist to the trafficking field was the observation that the minus-end-directed microtubule motor protein dynein (believed to aid in the perinuclear trafficking of nascent endosomes) is not required for AAV transduction [71]. Ubiquitination is believed to occur after viral-escape and externalization[39,44]. Inhibiting proteosome activity has been shown to increase viral transduction in several cell types [39,44]. The c-terminal sequence of all three Cap proteins contain nuclear localization capabilities, which are required for efficient transduction[182]. The exact mechanism by which viral particles enter the nucleus and begin the integration steps is still under investigation.

After capsid uncoating and highlighting the importance of linking early signaling events and cell-physiology to viral-transduction, the single-stranded D-sequence-binding protein (ssD-BP) in its phosphorylated state, binds to and inhibits AAV second strand DNA synthesis. ssD-BP phosphorylation is augmented by the activation of the epidermal growth factor receptor (EGFR), so that inhibiting the tyrosine kinase ability of EGFR by genistein increases transduction[102]. In the case of AAVS1 integration, a Rep68/78 complex is believed to bind to an ITR proximal recognition site and an AAVS1 recognition site before nicking the DNA strands to aid in strand invasion and genome integration[17,133,220]. The final viral assembly step is believed to occur mainly in the nucleus, with the Rep proteins shuttling ssDNA to nascent, self assembling viral particles[187,218,219].

Heparan Sulfate Proteoglycans (HSPG)

Although cells are poorly transduced in the absence of cell surface expressed HSPG, there has been little work done elucidating the role of HSPG as the agonist for receptor mediated endocytosis of AAV [16]. HSPGs are involved in the regulation of growth factor binding as well as stabilizing cytoskeletal elements at cell-cell junctions [30,91,118,165,223]. The list of pathogens known to require HSPG for productive infections continues to grow and includes: adenovirus, herpes simplex virus, HIV and some bacteria, underscoring this receptor's importance in infection [23,67,95,124]. A positively charged surface structure or binding pocket facilitates attachment of pathogens and cellular ligands to the highly sulfated and negatively charged glycosaminoglycan (GAG) chain. In short, a family of cell membrane proteins called syndecans (transmembrane) and glypicans [glycosylphosphatidylinositol (GPI) anchored] are modified with long chains of disaccharide molecules through a series of biosynthetic enzymes (glycosyltransferases, sulfotransferases, and an epimerase)[98]. Attachment of the sugar moieties to a serine residue on the core protein begins in the Golgi network. In the case of heparan, a glucuronic acid-galactose-gactose-xylose tetrasaccharide linkage is formed, and will act as the base for the polymerization of the rest of the sugar chain. (Haparin has an iduronic acid instead of glucuronic acid and chondroitin sulfate begins with uronic acid.)[98] The GAG backbone is then elongated by 20-80 disaccharides on average before sulfation and epimerization to HSPG[98]. These modifications and subsequent trafficking of GAGs can be inhibited by the fungal toxin brefeldin A (inhibitor of Arf 1 leading to a block in ER to Golgi trafficking and release of unsulfated or under modified proteoglycans) [86,207]. Several growth factors and extracellular matrix proteins bind to subdomains on HSPG

chains including: fibroblast growth factors, vascular endothelial growth factors, platelet-derived growth factors, fibronectin, antithrombin-1 and transforming growth factor –ß[203]. Interestingly, inhibition of CME by expression of dominant negative dynamin 1 or silencing of clathrin heavy chain by siRNA leads to increased expression of syndecans (as assayed by increased incorporation of radioactively labeled sulfate into GAGs)[99].

While previously understated, after the original cloning of syndecans in 1989, the importance of the roles these matrix binding proteins play in cellular physiology continues to gain appreciation [166]. Specifically, it was known that a GAG containing membrane anchored protein played an integral role in integrin-matrix binding that linked the cytoskeleton to the interstitial matrix at focal adhesion sites. Because of the observation of this "aggregation" of integrins and growth factor molecules with HSPGs, Saunders et al termed their protein syndecan (from the Greek word *syndein*, to bind together) [166]. In vertebrate species, the syndecan family is made up of four members (Syndecan 1-4), each with an extracellular domain, type 1 single pass transmembrane domain, two conserved domains (C1 and C2) and a variable domain (V)[12]. The extracellular domain is inconsistent between the syndecans, and although short, the cytoplasmic domain (specifically C1) contains residues required for dimerization, as well as the platform for the recruitment of intracellular proteins[12]. C2 contains the conserved postsynaptic density-95/disc large protein/zonula occludens-1 (PDZ)-binding site that is required for the binding of PDZ domain interacting proteins: synbinding, calcium/calmodulin-dependent serine protein kinase, syntenin, and synectin[203]. The PDZ domain name is derived from three proteins that contain this protein-protein interacting

domain: mammalian postsynaptic density protein PSD-95, Drosophila disc large tumor suppressor Dlg, and tight junction protein ZO1[203]. Most syndecan proteins are expressed in a tissue specific manner with syndecan 1 almost exclusively in mesenchymal tissues, syndecan 2 in neuronal and epithelial tissue, syndecan 3 primarily in neuronal and musculoskeletal tissue and syndecan 4 in nearly all cell types [12]. This broad tissue type expression of syndecan 4 as well as several other properties make it of special interest to the work done herein.

Syndecan 4

Besides being the most extensively studied of the four members, syndecan 4 contains the unique ability to bind the membrane-signaling molecule, PIP2, via the V domain. This binding leads to the dimerization and conformational change of two syndecan 4 molecules and brings their PDZ domains closer to the plasma membrane[174]. This dimerization also leads to the recruitment and activation of protein kinase Cα which displaces α-actinin from syndecan 4 at focal adhesion sites[58]. PKCα kinase activity is similar to that of integrin-linked kinases and can lead to the activation of focal adhesion kinase (FAK)[81]. The synergy between PIP2 and the actin cytoskeleton nucleating factor α-actinin places syndecan 4 in a regulatory role, controlling local cytoskeletal organization [58]. Syndesmos represents another syndecan 4 specific interacting protein and interacts with the focal adhesion adaptor protein paxillin and Hic-5[6,36]. The expression of syndecans is tightly regulated, with growth factors and mechanical stress, wound healing and tissue differentiation leading to increased expression[203]. Syndecan 4 null mice have several subtle but enlightening phenotypes. The most pertinent are the increased mortality

after injecting the immune system-stimulating molecule lipopolysaccharide, impaired skin wound healing, and impaired fibroblast growth factor-2 (FGF2) and hepatocyte growth factor-induced activation of extracellular signal-regulated kinase (Erk) 1/2 [28,47,74]. Syndecan null fibroblasts that were plated on fibronectin showed increased lamellipodia formation as well as increased Rac 1 activation and decreased Rho activation[164,216]. Taken together, it is believed that syndecans act as modulators of outside in signaling.

Syndecan 4 has been shown to be more then just a platform for growth factor binding and presentation to growth factor receptors; they have also been shown to directly modulate several signaling pathways. In the absence of syndecan 4's cytoplasmic tail (expression of a GPI-anchored extracellular domain), FGF2 addition did not lead to ERK activation [212]. Furthermore, transient ERK activation upon FGF2 addition was seen in a smooth muscle cell line expressing a dominant-negative FGFR, which was abolished upon heparinase treatment[24]. FGF has also been seen entering with HSPG independent of FGFR [203]. Focal Adhesion Kinase (FAK) can be directly activated by syndecan 4 clustering[216]. The c-terminus of syndecans acts as cytoplasmic scaffold for the binding of not only the aforementioned signal transducing proteins, but for linker proteins that connect syndecans to other membrane receptors. Specifically, beta 1 integrin associates with syndecan 4 via cytoskeletal attachments at focal adhesion sites[30,49]. These associations can modulate Rho signaling during migration and focal adhesion formation[9,165]. Syndecan 4 clustering, either by cross-linking antibodies or FGF addition, leads to a shift in plasma membrane localization to lipid rafts that do not colocalize with caveolae [205]. It has been shown that inhibiting CME by expression of dominant negative

genetic constructs has no effect on syndecan 4 entry, whereas expression of dominant negative dynamin or inhibition of macropinocytosis by the drug amiloride can inhibit syndecan 4 uptake [202]. Indeed, syndecan 4 can bind directly to dynamin 2[228]. Blocking Rac 1 or cdc42 activation or cholesterol sequestration also inhibits syndecan 4 as well as FGF uptake [202]. This makes syndecan 4 part of a small family of receptors known to antagonize macropinocytosis in non-professional phagocytic cells [198].

Macropinocytosis

Macropinocytosis is associated with clathrin- and caveolin-independent but actin-dependent plasma membrane ruffling that can lead to the uptake of bulk solutes from the extracellular space [53,198]. Macropinocytosis constitutes a major endocytic route in epithelial as well as fibroblast cells [199]. It is involved in the maintenance of adhesion junctions as well as the clearance of membrane domains and alterations in the connectivity and communication between cells [199]. Observed in human epidermoid carcinoma cells as an EGF stimulated increase in horseradish peroxidase uptake (HRP) (fluid phase marker), macropinocytosis was believed to be a rare event in non-phagocytic cells [63]. Up until the late 20th century, it was believed that coated pits could account for all forms of internalization. Indeed, techniques that were used to block clathrin-mediated endocytosis, such as the acidification of the cytoplasm, could halt the uptake of fluid phase markers as well [29]. A slight rise in cytosolic pH (0.1-0.2 units) was associated with EGF induced membrane ruffling[159]. Careful work by West *et al* showed that EGF induced membrane ruffles would lead to a 10 fold increase in HRP uptake without

changing the uptake of EGF or transferrin[214]. They further showed that depriving cells of external sodium ions could block HRP uptake[214]. This fortuitous observation lead to the implementation of a drug called amiloride which specifically inhibits the Na^+/H^+ proton exchanger[84]. West *et al* showed that amiloride treatment specifically inhibited EGF induced HRP uptake without affecting EGF uptake or transferrin uptake[214]. Amiloride has since become one of the only know macropinocytosis "specific" inhibitors, although the correlation between cytosolic pH and macropinosome formation is not yet understood.

Most information on macropinocytosis in mammalian cells comes from the study of phorbol ester stimulated or growth factor stimulated membrane ruffling, or the related process of phagocytosis. The generation and maintenance of membrane ruffles requires PIP2 and actin filament nucleation caused by activated Rac[72,197,198,198]. Membrane protrusions can grow to be as large as 0.2-10μm in diameter and can fuse end to end with another protrusion or directly back onto the plasma membrane[198]. The resulting endosome is believed to be unique from other nascent endosomal structures and mature through trafficking routes distinct from ligands internalized via CME or caveolae[196,198]. Maturation of macropinosomes has also been connected to the modifications of membrane bound phosphoinositides where by PIP2 is converted to PIP3 by PI3K to promote proper closing or fusing of nascent macropinosomes and their subsequent trafficking [4,97,206]. Interestingly, the use of PI3K drugs have been studied in relationship to AAV internalization to show PI3K's necessity in nuclear trafficking of the virus [161]. In these essays, it was reported that the majority of AAV remained at the cell surface or at a

location proximal to the plasma membrane in PI3K inhibited cells, while by that same time point, control cells showed nuclear accumulation of the virus[161]. It was not determined if the plasma membrane proximal viruses were actual internalized or not (i.e. by trypsonizing uninternalized particles).

Some pathogens have evolved mechanisms for triggering membrane ruffling in order to gain access to cells. Disruption of cell-cell contacts by pathogen-induced macropinocytosis may serve to activate advantageous signaling pathways for the virus to hitchhike this internalization route while leading to tissue breaches and the exposure of baso-lateral receptors to which the pathogen then binds.

Herein we explored the role of HSPG in the productive internalization of AAV(Fig A).

Other Clathrin Independent routes of entry

The cell must internalize a variety of different molecules to maintain a healthy pathology. Regulation of portals of entry requires selectively recognizing cell surface attachment of cargo and potential enrichment by discrimination of ligand. The internalization of the ligand requires the plasma membrane to distort and then fuse with itself, leading to a nascent endosome. The clathrin-mediated pathway is the most well characterized method for internalizing molecules, but in recent years it has become clear that cells have several other methods to endocytose extracellular cargo. These pathways, known as clathrin-independent pathways, are associated with lipid domains that are enriched in cholesterol and are regulated by the Rho family of small GTPases[105]. The membrane scission

machinery for some of these routes also use dynamin, but there is recent evidence that a subset of clathrin-independent pathways are also dynamin-independent[105].

The most well characterized clathrin-independent pathway is caveolar-endocytosis. Described as early as the 1950s as cave like smooth invaginations of the plasma membrane, caveolae are small (50-100nm) cholesterol enriched micro-domains which are coordinated by the protein caveolin [90,113,158]. Caveolae are found in a diverse set of tissue types and are highly immobile/static structures at the plasma membrane[200]. When cells are treated with several ligands (viruses, bacterial toxins, cross-linking antibodies to GPI anchored proteins), caveolae are internalized from the cell surface in a dynamin dependent process[66,120,122]. Once internalized, caveolae traffic to pH neutral compartments known as caveosomes[153]. From there, different pathogens and ligands follow a diverse set of trafficking pathways leading viruses to the ER, toxins to the Golgi, transcyotis of HIV particles from the apical to the basal surface of cells and recycling some caveolae back to the cell surface[174]. Caveolae also serve as a docking point for several kinases like Src, Fyn, H-Ras and have been associated with modulating growth and transformation in tumor models[153]. Two viruses, Simian Virus 40 (SV 40) and Polyoma virus, have been shown to enter cells via caveolae[181]. In an interesting twist, cells from knock out mice lacking the expression of caveolin –1, and having no surface caveolae, are still infected by SV 40[35]. In the later case, it is believed that viral attachment and clustering of SV 40 receptors leads to the formation of lipid microdomains and a clathrin- and caveolin-1-independent endocytic route.

Lipid rafts are believed to be small (tens of nanometers), transient, cholesterol enriched microdomains that can be formed by clustering of cell surface receptors[136]. Many receptors preferentially partition into rafts, while others, like syndecan 4, transition into rafts after activation or clustering at the cell surface[136]. In a process that is still under investigation, some of these microdomains can be internalized into cells in smooth and sometimes tube like structures that are clathrin- and caveolin-1-independent. For instance, there was no change in the rate of internalization of IL-2R-ß when clathrin assembly was blocked in cells expressing dominant negative EPS15 or AP180[93]. This receptor was shown to relocate to caveolin-1 independent raft domains and its internalization was sensitive to cholesterol sequestering drugs[92,93]. Like caveolae, it is believed that rafts can act as signal platforms for lipid anchored kinases and active sites of actin modulating GTPases like CDC42 and RhoA[105]. Many techniques, such as sensitivity to detergents or cholesterol depletion, affect the organization and stability of both lipid rafts and caveolae and in some cases make it difficult to determine a ligand's endogenous pathway of entry. Indeed, many raft dependent pathways share similar regulatory GTPases and effector proteins[105]. It may be possible to separate clathrin-independent pathways by observing colocalization of proteins with known ligands that enter through these pathways (i.e. colocalization with caveolin-1 protein in caveolae). Attempts to purify rafts or image raft formation have come with mixed results, mainly due the fact that methods for fixing and staining cells can disrupt or change microdomains. Tagging receptors or lipid modified fluorescent proteins have eluded the dynamic nature of microdomains, and markers believed to enter solely through lipid rafts have also been seen entering via caveolae[105,136]. There has been data from electron

microscopy (EM) showing smooth invagination associated with ubiquitinated epidermal growth factor receptor (EGFR) when added at high concentrations (100 ng/ml) in the cold[177]. The trafficking of molecules internalized via rafts is also not well understood.

Specific Aims

Binding and internalization of AAV represent the first requirements for the success of delivering transgenes for gene complementation via gene therapy. Herein we investigate endocytosis of AAV via macropinocysosis. This assertion is based on AAV's principle requirement for cell surface proteoglycans and the ability for syndecans to recruit components known to be required for AAV entry.

AAV can enter and infect cells in the absence of the clathrin-mediated pathway.

Using ligands that mark different routes of entry, we will see which routes of internalization colocalize best with AAV. Furthermore, we can use the expression of dominant negative genetic constructs or chemical treatments that inhibit the CME and a knock down of clathrin heavy chain protein by siRNA to study the effects on AAV entry and transduction.

AAV enters cells in an amiloride sensitive pathway that is dependent on cytoskeletal rearrangements.

Inhibition of filipodia formation by chemical inhibitors of cytoskeletal rearrangements inhibit fluid phase marker uptake. The Na^+/H^+ proton exchanger specific inhibitor, amiloride, has been show to inhibit the macropinocytic uptake pathway. We will study the effects of macropinocytosis inhibition on AAV uptake. We will also observe structural changes induced by AAV binding and subsequent internalization by looking at markers known to be enriched in macropinosomal structures.

AAV endocytosis is dynamin dependent.

Dynamin has been shown to regulate the scission and internalization of several endocytic pathways. Inhibition of dynamin 1 has been linked to blocking AAV uptake, and dynamin 2 has been shown to interact directly with AAV's primary receptor syndecan 4. We will explore the possibility that dynamin is enriched in AAV stimulated macropinosomes, and its inhibition with chemical as well as genetic inhibitors abolishes these structures and blocks infectivity.

Chapter 2

Materials and Methods

Viruses and Tissue Culture:

AAV CMV Luc was purchased from the Gene Therapy Program Vector Core at the University of Pennsylvania, pre-purified, tested and tittered in the form of genome copies (GC) (encapsulated copy of luciferase trans-gene).

HeLa and pgsA745 CHO cells were purchased from ATCC (Manassas, VA) and maintained in DMEM and DMEM-F12 (Gibco, Carlsbad, CA), respectively, containing 10% fetal bovine serum and 1% Pen/Strep (Gibco 15140-122). Cells were maintained in a humidified 37°C incubator with 5% pCO_2. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to manufacture's directions, with 2 μg of DNA for single transfections and 1.5 μg DNA each for double transfections, 24 hours before experiments. Clathrin heavy chain siRNA was performed as described [70]. Briefly, 24 hours before transformation HeLa cells were seeded at 30% confluence in 6 well dishes (Falcon BD Biosciences, San Jose, CA) in DMEM containing 10% fetal bovine serum but no Pen/Strep. Two rounds of siRNA treatment were performed with 80 pmol of RNA duplex per well, or 2ug of empty plasmid control (Dharmacon, Lafayette, CO). Oligofectamin (Invitrogen) was used to transfect cells with a final transfection volume of 800ul on day one, then again 48 hours later. Efficiency of knockdown was assessed by

western blot and transferrin (Tfn) uptake. All viral infection assays were done at 72 hours (24 hours after the second knock down transfection).

Western Blotting:

To detect the extent to which the protein levels were reduced by treatment by siRNA, cells were removed with Cellstripper (Mediatech, Herndon, VA) and lysed in 200ul of SDS. Protein lysates were separated on a 4-15% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA) before electroblotting onto Trans-Blot nitrocellulose membranes (Bio-Rad). Clathrin heavy chain was detected using a mouse monoclonal antibody (BD Biosciences, San Jose, CA) and visualized with horseradish-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO) and chemiluminescence reagents (Amersham, GE Health Care, Piscataway, NJ). Quantification of Western Blot signal was done using Meta Morph Imaging Software (Universal Imaging, Downington, PA). For visualization of Focal Adhesion Kinse phosphorylation, 20ug of protein were added (concentration calculated using BioRad DcProtein Assay (BioRad) according to manufacturers instructions). Rabbit anti pFAK tyrosine 397 antibody (Biosource, Camarillo, CA) was used at 1:4000.

Plasmids:

Caveolin 1-mRFP was a kind gift from Dr. Ari Helenius, Eps15E29H-eGFP from Dr. Alexandre Benmerah, Syndecan 4-HA and PDZ- HA from Dr. John M. Rhodes,

AP180C-eGFP and Clathrin-eGFP from Dr. Harvey McMahon, Dynamin 2K44A was from Dr. Sandra L. Schmidt.

Luciferase Assay:

Cells were seeded at ~2 X 10^5 cells per well in 12 well dishes (Falcon BD Biosciences, San Jose, CA) overnight before infection with 10,000 or 10 GC per cell of AAV. Cells were treated 30 min prior to or for 30 min after infection with: (from Sigma) 3mM amiloride, 10mM methyl-β-cyclodextrin (mβCD), DMSO, 5μM cytochalasin D, or 45mM sucrose (hypertonic solution in DMEM) (Fisher, Loughborough, UK). After drug incubation, viruses were added in DMEM without serum media to cells and allowed to incubate at 37°C for 40min before removal. Virus was removed from cells using heparinase treatment as previously described [191]. Cells were then incubated with DMEM for 48 hours before luciferase assay or treated for 30min with drugs to assess the impact of these drugs on later stages of the life cycle AAV. After the subsequent 30-minute incubation, these cells were washed 3 times with PBS before drug free media was added and infection allowed to continue for 48 hours. Neither drug treatment, nor heparinase treatment caused cells to round up or lift off the plate during the time of treatment. To gauge the level of AAV transduction, the Bright Glow Luciferase Assay Kit (Promega, Madison, WI) was used as directed by manufacturer's instructions. The cells were collected in 100μl 1X Reporter Lysis Buffer (Promega). Cells were lysed by freezing at –20°C overnight and clarified by centrifugation at 15,000rpm (Eppendorf Centrifuge 5415D) for 15min. 100μl of lysate was used in 96 well plates with white walls (Fisher)

for luminescence assay. Photons were counted on a Berthold Centro-LB 960 Luminometer (Berthold Tech, Oak Ridge, TN) with an integration time of 1 second. All values were normalized by protein concentration. Protein concentrations were determined using the BioRad D_c Protein Assay kit (BioRad, Hercules, CA) according to manufacturer's directions.

Immunofluorescence and Microscopy:

For immunofluorescence studies, cells were seeded sparsely on 35mm MatTek dishes (MatTek, Ashland, MA) 24 hours before transfection or endocytosis observations. Optimal immunofluorescence was gained by fixing cells in warm PBS (Gibco, Carlsbad, California) containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min. Cells were then permeabilized with 0.1% Triton X-100 (Sigma) for 5min before being washed 3 time (5min each) with PBS and incubated for 1 hour at room temperature with PBS containing 10% fetal bovine serum (Sigma). AAV was detected using the A20 intact capsid antibody (Progen, Heidelberg, Germany) at a dilution of 1:40, and activated PKCα, phosphoserine 657 PKCα sc12356 (Santa Cruz Biotech, Santa Cruz, CA) at a dilution of 1:100, integrin αV (Santa Cruz Biotech, Santa Cruz, CA) at a dilution of 1:500, integrin alpha 5 (abcam, Cambridge, MA) at a dilution of 1:500. in PBS containing 1% fetal bovine serum Cells were incubated with A20 overnight at 4°C for optimal detection of AAV. Syndecan-HA was detected using a rabbit HA antibody at a dilution factor of 1:100 (Sigma H6908) and allowed to incubate overnight at 4°C. Endogenous dynamin 2 was visualized using a goat polyclonal antibody at a dilution of

1:400 (Santa Cruz Biotech, Santa Cruz, CA) over night at 4°C. Primary antibodies were washed out with 3 exchanges (5min each) of PBS at room temperature. All appropriate secondary antibodies [labeled with Alexa488, Alexa546, and Alexa633 (Invitrogen)] were used at a dilution factor of 1:400 in PBS containing 1% fetal bovine serum, and incubated at room temperature for 1 hour for optimal labeling. Avidin-PE (Invitrogen) was used at a dilution factor of 1:50 in PBS. Actin was labeled with phalloidin coupled to Alexa Fluor 546 (Molecular Probes, Eugene, Oregon) at a dilution of 1:400 in 1% BSA in PBS for 1 hour at room temperature.

Cells were labeled by biotin-EGF and Tfn Alexa488 and Alexa546 (Invitrogen) by first incubating cells at 4°C in serum free DMEM for 30 min. 2μg of Tfn and 100ng EGF were added and allowed to bind at 4°C for another 30 min before rinsing once with PBS. 37°C, pre-warmed DMEM was then added for time course analysis and immunofluorescence.

Images were acquired on an inverted Zeiss Axiovert 200 LSM 510 laser scanning confocal microscope with a 63X oil immersion objective (NA 1.4) using LSM 510 v. 3.2 confocal software (Zeiss, Thornwood, NY). An argon laser, with a 488nm line, was used to excite GFP/Alexa488. HeNe lasers were used for red (HeNe 543nm for Alexa546) and far-red dyes (HeNe 633nm for Alexa633). Whole cell scans were acquired using a 200nm step size. Images were deconvolved using Huygens Imaging Software (Scientific Volume Imaging, Hilversum, Netherlands) and prepared for presentation using MetaMorph imaging software (Universal Imaging, Downington, PA). Wide field images were

acquired on an IX-70 Olympus microscope (Olympus America Inc., Melville, NY) using an Apo 60X NA 1.4 objective (Olympus). Alexa 543 labeled Tfn or AAV were excited with 540nm light and collected with a 560lp filter [Chroma Technologies Corp. (Brattleboro, VT, USA)]. Individual cells were outlined and their average intensity calculated using MetaMorph. Correlation coefficients were calculated from deconvolved images using Imaris image analysis software (Bitplane, Saint Paul, MN), and represent correlations of all voxels in the volume of the cell.

Dynasore and Amiloride Uptake Concentration Curves:

HeLa cells were seeded on MaTek dishes at 70% confluence 24 hours before drug exposure. Dynasore (EMD Biosceinces, Calbiochem, Darmstadt, Germany) or amiloride was added at different concentrations to DMEM at 37 degrees, 30 min before the addition of 10,000 GC AAV (continuous) or 2ug Tfn as described above. Cells remained in the presence of Dynasore for the entirety of the endocytosis assay (40min). In order to test the off target and toxicity effects of drug treatment, cells were exposed to various concentrations of amiloride for 1 hour before washing drug out (wash out) with 3 exchanges warm DMEM prior to infecting cells with AAV for 30min (as described above). Ligands that were not internalized were washed off the cell surface before staining (for AAV) and quantifying internal fluorescence intensity in arbitrary units (A.U.) or allowing infected cells to incubate for 48 hrs at 37 degrees (for amiloride experiment).

Fluorescence Activated Cell Sorting:

80% confluent HeLa cells in 6 well dishes were cold bound in the presence of AAV in serum free media (virus added) or in serum free media alone (no virus) at 4°C for 30 min. To measure dextran uptake cells were warmed up in 37°C DMEM for 0, 5, 10, 20, 30 or 60 min before pulsing for 5 minutes with 4mg/ml Alexa 488 lysine fixable 10 KDa dextran (Molecular Probes, Eugene, Oregon). Dextran that was not internalized was washed off with 3 changes of ice cold PBS. Cells were detached by trypsin-EDTA treatment (Sigma) and pelleted in DMEM. Tfn uptake was measured by cold-binding 2mg/ml Alexa488 (Molecular Probes, Eugene, Oregon) to cells before the addition of 37°C DMEM for the indicated time points. Uninternalized tfn was washed and removed and cells prepared as previously stated with dextran. Cell pellets were resuspended in 4% paraformaldehyde in 1% BSA for FACS analysis (FACSCalibur System BD Biosciences, San Jose, CA). Each point represents the average intensity of 20,000 cells.

Quantification of Perimeter of Maximum Macropinosome Projection:

Non-transfected cells (Wild Type), cells transfected 24 hours prior with GFP-Dyn2 K44A or cells transfected with Syndecan 4 PDZ- (2ug) were incubated with 10,000GC AAV in the cold, and then warmed for 10 min before fixation with 4% paraformaldehyde and immuno-staining for AAV with Alexa 546. Image stacks (20 nm) were taken as described above with GFP excitation at 488nm and emission collected with a 520/50 bp filter. Alexa 546 was excited with 540nm light and collected with a 560lp filter (Chroma). Images were analyzed using MetaMorph. The intensities of all stacks were summed and

collapsed onto a single frame. The maximum macropinosome outline was traced and perimeter of the projection was measured in pixels.

Statistical Analysis:

P-values were calculated using the student t-test for experiments done three or four separate times.

Chapter 3

Clathrin-independent internalization of AAV

Introduction

First described by Roth and Porter in 1964 as "bristle-coated pits", clathrin mediated endocytosis, as a concept, has been fleshed out into a large and dynamic field in cell biology[157]. Even earlier in the 20th century, Mitchnikoff saw that after ingesting litmus particles, cells turned internalized structures from blue to red, evidence that an increase in pH had occurred[96]. Further observations of professional phagocytic cells by cinematographic microcopy showed small vesicles, filled with extracellular fluid, entering the cell in a process termed pinocytosis (Latin for "cell drinking")[96]. Cell biologists speculated that the process, to be termed endocytosis, was dynamic, rapid, and required for proper cell viability. Roth and Porter relied on the timing of events after mosquitoes ingest their blood meal and deposit yolk into the developing oocytes to catch endocytic events by EM; but even by this static method, they were able to catch and characterize the dynamics of clathrin-mediated endocytosis (CME) and early trafficking[157].

Advancements in molecular biology lead to the cloning and expression of chemeric proteins with fluorescent tags involved in CME, which allowed for more detailed tracking of ligands being internalized through this pathway. Interactions with ligand and receptor translate changes in the conformation of the receptor into the cytoplasm, leading to recruitment of CME adaptor AP2 and accessory proteins (i.e. EPS15 and AP180) and

the polymerization of the clathrin coat around nascent vesicles [32,125,149]. After internalization, the clathrin coat is rapidly lost[15]. The destination of ligands and receptors towards an acidic compartment was clarified and augmented by the discovery of tubular compartments that fused, concentrated and subsequently sorted ligands [such as low-density lipoproteins (LDL), and asiloglycoprotein] away from the receptors in a process first called CURL (compartment of uncoupling ligand from receptor)[55]. These organelles, later termed recycling endosomes, were subsequently shown to traffic ligands bound for digestion and processing from their receptors that shuttled back to the cell surface (recycled)[45,46]. The classic ligand used to study CME and receptor recycling is the transferrin (Tfn) receptor (TfnR). The TfnR is constitutively internalized, and Tfn marks sites of CME and early and late endosomes a[76]. Although ligands internalized by CME can follow different trafficking pathways once in the cytoplasm, many internalized ligands such as Tfn and AAV have been shown to mature into acidic compartments believed to be late endosomes or lysosomes[8]. In the subsequent studies, we use markers for clathrin dependent entry, caveolin-1 dependent entry and clathrin- and caveolin-independent entry to follow the internalization of AAV in HeLa cells.

Results

AAV association with endocytic machinery

To visualize the dynamics of the early interactions of AAV with the endocytic machinery, HeLa cells were transfected with fluorescently tagged versions of two proteins, clathrin-eGFP and caveolin-1-mRFP, markers for two different pathways of

internalization. After cells were incubated with virus for 30 mins, the cells were fixed and probed with an antibody to AAV. From examining individual viruses, there was no obvious overlap observed between AAV with either caveolae at the cell surface or caveosomes internally. Similarly, there was no detectable overlap between AAV and clathrin inside of the cell, nor, surprisingly, at the cell surface (Fig 1a).

Clathrin is known to dissociate from endosomes containing newly internalized cargo [217]. Thus, it was possible that as a consequence of this rapid dissociation, this approach could not detect transient co-localization of clathrin and internalized cargo. Thus, as a positive control for the sensitivity of this technique to detect cargo internalized through the clathrin-mediated pathway, we used labeled Tfn. Tfn and the Tfn receptor rapidly shuttle to recycling-endosome compartments (t< 3min) and are known to rapidly separate from clathrin after endocytosis [25]. Thus, we tested for co-localization of AAV and Tfn. HeLa cells were incubated with Tfn labeled with Alexa488, AAV and FM4-64 (a lipophilic dye which marks all pathways of membrane internalization). The cells were then warmed to 37°C for 30min to allow rapid internalization of AAV and Tfn, as well as FM dye. The cells were then fixed and stained with antibodies used to visualize AAV. Surprisingly, Tfn and AAV occupied different endosomal compartments at all stages seen, from endosomes close to the cell surface through endosomes at peri-nuclear regions (Fig 1c). The degree of co-localization was quantified between each ligand. The lipid dye FM4-64 co-localized strongly with both AAV (0.45) and Tfn (0.35). In contrast, the co-localization of Tfn with AAV was not correlated (-0.01), indicating that one is less likely to find AAV at Tfn sites (Table 1). To better understand the dynamics of AAV entry,

AAV was allowed bind to cells in the cold, and then warmed for 0, 5, 10 and 30 minutes to initiate internalization prior to fixation and staining. Membrane ruffles and diffuse membrane staining of AAV alone are seen immediately upon warming up at as early as time 0 (cells are transiently pulsed with 37°C and immediately washed at 0 minutes) and 5 minutes. Discreet punctuated membrane AAV staining is seen at 10 and 30 minutes as membrane ruffling begins to abate and AAV enters the cell and traffics towards a peri-nuclear compartment (20- 30 minutes) (Fig 1b).

HeLa cells over-expressing clathrin and caveolin1 constructs were incubated with AAV at 4°C and fixed 0, 5, 10 or 30 min after being shifted to 37°C. Correlation coefficients for co-localization were calculated from deconvolved confocal images and plotted as a function of time (Fig 2b). As a positive control for co-localization within the clathrin-mediated endocytic pathway, cells were simultaneously incubated with Tfn-Alexa488 and Tfn-Alexa546 (correlation coefficient= 0.61). AAV and clathrin and AAV and caveolin1 correlation coefficient increased slightly from a correlation of –0.01 to 0.045 (Fig 2b, 5 min). In both cases, the slight increase of correlation was transient and was lost by 7 min for clathrin and AAV or by 15 min for caveolin1 and AAV (Fig 2b). Since ligands that enter through caveolin-dependent mechanisms shuttle rapidly to caveosomes, and AAV was not seen in caveosomes at later time points (Fig 1a and 2b), the early, slight transient increase in co-localization between AAV and caveolin may represent background noise created by AAV-induced increased membrane ruffling. Similar to this behavior, the co-localization between clathrin and caveolin1 (which are believed not to mix or functionally interact at the cell surface) showed a 3.5 times greater correlation

value (0.16) at 5 min and remained above zero for all time points thereafter. In contrast, Tfn-Alexa488 and Tfn-Alexa546 showed a seven-fold greater co-localization (0.33) at all time points, as expected (Fig 2b).

Clathrin, caveolin-independent entry

To distinguish between clathrin-dependent and independent pathways, a cellular ligand was used that enters primarily in the absence of the clathrin-mediated endocytic machinery. It has been previously shown that EGF enters through a clathrin-independent pathway [177] when added at concentrations greater than 20ng/ml to cooled cells. To determine if AAV uses a similar pathway, HeLa cells were incubated with 100ng/ml EGF-biotin, AAV and Alexa labeled Tfn in the cold. The samples were visualized 30 min after shifting to 37°C to follow the transition of AAV from membrane ruffles to internalized spots. EGF incubated under these conditions showed a strong co-localization with AAV (co-localization = 0.86) and once again, the remaining AAV (that was not associated with EGF) was absent in Tfn-positive endosomes (Fig 2a) (Table 1). It should be noted that different pathways, receptors and ligands may have different max values for colocalization. This is a function of the amount of ligand present in a specific voxel, the strength of the particular fluorophore used, the strength of the laser for excitation and the efficiency of the filters for collection. Thus, the relative colocalization and correlation are always relative to something (the value 1 represents two molecules with the same fluorescence intensity in the same pixel; this is a theoretical number). For example, when Tfn was labeled with the fluorophores Alexa 488 and Alex 546, the highest correlation

value observed was 0.7. If the two fluorophores are not exactly matched in efficiency of excitation, emission and collection, a correlation value of 1 will not be observed.

Effects of genetic inhibitors of clathrin mediated endocytosis

As an alternative test for the requirement of specific pathways for AAV entry, HeLa cells were transfected with GFP-fused constructs that are dominant negative inhibitors of the clathrin-endocytic machinery. These constructs, Eps15EH29 and AP180C [11,51], successfully block the uptake of Tfn (Fig 4a) and are believed to act solely upon the clathrin-dependent pathway. Cells were transfected 24 hours before infection with AAV for 30min at 37^{o}C, then washed and stained for visualization. Wide-field fluorescence images were quantified for AAV uptake. Although hypertonic treatment showed a 70% decrease in viral entry (Fig 4b), the genetic inhibitors that are believed to be more specific for effects on clathrin-mediated entry, showed more subtle decreases in entry. AP180C showed the greatest decrease of 30% compared to non-transfected cells (Fig 4c) which Eps15EH29 positive cells showed a less than 15% decrease in AAV internalization (Fig 4c). Although prolonged expression of these inhibitors might have other off-target effects, these results indicate that some of the other treatments, such as hypertonicity, may not be as specific.

Clathrin heavy chain knock down by siRNA

To test for the role of clathrin on the transduction of cells by AAV, clathrin heavy chain specific RNA sequences were used to reduce cellular levels of clathrin heavy chain protein by >79% after 2 rounds of transfection (as assayed by western blot analysis) (Fig

11a and b) and reduce clathrin-based activity by greater than 50% as assayed by Tfn uptake (not presented). Reducing clathrin protein and activity had no statistically significant effect on AAV transduction (Fig 11c). We therefore conclude that the primary route of entry by AAV that leads to infection is via macropinocytosis that is dynamin dependent, and requires the signaling capabilities of the HSPG modified protein family syndecans and is not dependent upon clathrin. This underscores the complex cytoplasmic signaling required for macropinosome formation and maintenance and elucidates an important and novel route of infection by this HSPG binding pathogen that is clathrin-independent.

Chapter 4

HSPGs, receptors for macropinocytic uptake

Introduction

Heparan-sulfate proteoglycans are the primary and minimally required cell surface receptor for AAV. The syndecan family of heparan-sulfate (HSPG) and chondroitin-sulfate proteoglycans constitute an evolutionarily conserved family of single pass transmembrane proteins. The expression patterns of the 4 members show characteristic tissue specificity, with syndecan 1 and 3 being expressed in epithelial and neuronal cells, syndecan 2 in mesenchymal cells, parenchyma and neuritis and syndecan 4 being expressed in a wide range of cells[82]. The four syndecans have been placed into two families, with syndecans 1 and 3 being in one family and 2 and 4 in the other. This is due to the conservation of their primary amino acid sequence, signaling capabilities and tissues specificity[203]. Tissues that are refractory and permissive to transduction by AAV are closely linked to the two syndecan subgroups with refractory tissues resembling syndecan 1 and 3 group and permissive cells resembling the syndecan 2 and 4 group[18,21,22]. This syndecan specific transduction phenotype may be due to the associations of the variable cytoplasmic and extracellular domains with unique binding proteins. Specifically, syndecans 2 and 4 have been linked to efficient fibronectin induced cell spreading and focal adhesion formation, and syndecan 4 has been shown to colocalize with AAV co-receptors $\alpha 5\beta 1$ and $\alpha V\beta 3$ in fibroblasts[2,30,49,221]. Furthermore, syndecan 4 expression has been linked to filipodia induced cell movement during wound healing[26].

For the aforementioned reasons and others, we decided to look at the relationship between AAV binding to HSPG and endocytosis. We used the syndecan 4 construct, supplied by Dr. Michael Simons (Dartmouth Medical School), studied extensively for its ability to dimerize in the presence of PIP2 and activate PKCα (syndecan 2 does not activate PKCα)[119]. PIP2 generation at the membrane is linked to actin polymerization and Rac activation[206]. It has been further shown that syndecan 4 internalization is not inhibited by expression of clathrin and caveolar-endocytosis blocking, dominant-negative constructs; and that this pathway is Rac dependent[100]. Syndecan 4 shifts into detergent resistant lipid rafts after antibody clustering or addition of fibroblast growth factor (FGF); whereby disruption of these rafts decreases FGF mediated Erk activation and syndecan internalization [204]. Finally, Syndecan mediated activation of Rac, and FGF mediated activation of ERK, occur on a time scale similar to that seen during Rac mediated AAV internalization and AAV dependent PI3K activation (between 5 and 15 minutes)[24,161,202]. Taken together, it is plausible that some of the GTPase and kinase inhibition studies shown to inhibit AAV internalization and trafficking may be working by inhibiting syndecan activation and trafficking (Fig B).

In order to further elucidate the contribution syndecans have in the internalization of AAV, we decided to address the localization and internalization of AAV using known inhibitors of syndecan activation and internalization. Mainly, it has been shown that syndecan-4 enters cells via macropinocytosis[202]. There is a dearth of information in the scientific literature about the process of macropinocytosis. Defined as the bulk uptake of soluble extracellular fluids, macropinocytosis shares many characteristics with

phagocytosis. Several ligands such as arginine-rich peptides and TAT-HA fusogenic peptides have been shown to enter via lipid-raft enriched, macropinosomes[114,213]. Some bacterial species including *Salmonella* have been shown to induce membrane ruffling and internalization, but until recently only one virus has been seen to induce macropinosomal membrane ruffles[52]. Adenovirus has been shown to induce macropinosomes, but the report emphasizes that Adenovirus does not require productive macropinocytosis for infection (Adenovirus enters through CME as determined by sensitivity to CME inhibitory dominant negative constructs)[109]. Assayed by uptake of fluorescent dextran (fluid phase marker), Adenovirus induced macropinocytosis was dependent on PKCα activity, as well as actin cytoskeletal reorganization and the presence of Adenovirus's integrin receptors (it was not shown if this was due to a lack of Adenovirus binding, or receptor activation in cells lacking endogenous αV integrin) [108]. Although believed to enter cells primarily via CME, rubella virus infection is sensitive to macropinocytosis inhibitor amiloride. Recently, Ari Helenius' group identified macropinocytosis as the dominant route of entry for vaccinia virus, and Urs Greber's group characterized Adenovirus type 3's entry through this route as well. [3,75]. In all of the aforementioned cases, the signaling processes (i.e. outside-in) underlying the induction of membrane ruffling has not been described.

The structures of membrane ruffles seem to vary depending on the cell type, species, or method of antagonizing macropinocytosis. For instance, in the original documentation of macropinocytosis, "waving sheets" closed in to circular structures, and in growth factor stimulated cells and in *Dictyostelium*, macropinosomes appear as round ruffles [40,62,63].

These differ from cells in which macropinocytosis is generated by over expression of kinases such as v-Src which resemble filipodial projects that subsequently close into macropinosomes[209]. The percentage of ruffles that close into macropinosomes, the process of membrane-membrane fusion during endosome formation as well as the subsequent trafficking steps post internalization are all poorly understood mechanisms. Of the approximately 12 Rab (small GTP-ases that control membrane trafficking inside cells) only one (Rab34) has been associated with membrane ruffles[192]. A dominant negative Rab5 construct, generally associated with early endosome tubule formation and trafficking (used as a marker for early endosomes), leads to diminished circular ruffling but not fillipodial ruffling [94]. It is difficult to uncouple the effects of membrane trafficking from membrane ruffling since dominate negative Rab21 and 22a, also associated with early endosomes, lead to diminished membrane ruffling and early endosome formation [179]. The ADP-ribosylation factor (ARF)-6 has been shown to associated with the plasma membrane and modulate actin dependent clathrin and caveolin-independent endocytosis [38].

Finally, the trafficking of large macropinosomal structures is ambiguous at best. In EGF stimulated A431 cells, macropinosomes transiently associated with early endosomal autoantigen 1 protein (EEA1) (t<5min) but did not acquire Rab 5 as a marker[170]. Further examination by other labs showed that over a 25min period, macropinosomes became EEA1 positive but did not show markers for lysosomal maturation [64]. Work done in dendritic cells showed macropinosomal cargo colocalizing transiently with transferrin receptor, but not progressing on to acquire markers for late endosomes or lysosomes [80].

The complicated results of these studies might underline inherent differences in macropinosome identity depending on the treatment used to induce them or by cell type differences. Members of the sorting nexin (SNX) family of proteins has recently been associated with the tubulation and trafficking of membranes associated with nascent macropinosomes. The 30 member cytoplasmic membrane binding family is defined by their SNX phox homology (PX) domain that interacts with PIP3 containing structures [19]. 7 of the SNX proteins have a Bin/Amphiphysin/Rvs (BAR) domain that have been reported to bind and stabilize curved membrane structures [134,224]. SNX5 has been associated with transient EEA1 positive macropinosomes microdomains, where as SNX9 has been associated with dynamin activation in clathrin mediated endocytosis as well as tubular invaginations that are cytoskeleton dependent [134,175,185,227]. As markers for macropinosomal trafficking continue to be discovered, a more detailed analysis of membrane-associated signals (potentially cell-surface ligands, or internalized receptors) that promote endosome maturation can be uncovered.

Herein we illustrate the requirement for the signaling capabilities of HSPG in the induction of membrane ruffling by AAV. Many of the ligands described to enter or induce macropinocytosis also bind to cell-surface proteoglycans [77](see previous references). Although we do not help further the understanding of the trafficking of macropinosomes, we highlight the utility of AAV as a tool for understanding these cellular processes. We further speculate that binding and clustering of HSPG by

positively charged macromolecules and induction of macropinocytosis may constitute a ubiquitous mechanism for pathogen and toxin entry.

Results

AAV is interanalized with its primary receptor HSPG in a process related to HSPG endocytosis

During entry of AAV, its co-localization with either caveolin1 or markers of the clathrin-mediated pathway was not statistically different from background. Thus, we examined AAV's co-localization with HSPG, the protein implicated as the primary receptor for AAV. HSPGs are typified by a N-terminal sugar modification added to a small family of proteoglycans known as the glypicans and syndecans [14]. Syndecans have been shown to enter through clathrin-independent mechanisms that are blocked by inhibitors of macropinocytosis [202]. Tfn and AAV were bound in the cold for 30 min to HeLa cells over-expressing HA-tagged syndecan 4 (syndecan). The cells were then warmed to 37°C for 30 min to allow for internalization of Tfn and AAV, then fixed and visualized by confocal microscopy. Tfn rapidly entered cells but did not demonstrate detectable co-localization with syndecans (correlation = 0.03, Table 1). In confocal slices through the center of cells, AAV was observed within endosomes that demonstrated a strong co-localization with syndecans (correlation coefficient of 0.62) (Table 1). AAV and syndecans were observed to traffic to a peri-nuclear compartment 30 min post entry (Fig 3). It should be noted that cells expressing HA-tagged syndecan4 were highly enriched in bound AAV compared to adjacent cells. This is consistent with HSPG being the primary binding receptor of AAV.

To further characterize the entry pathway(s) of AAV, we visualized the effects of various treatments on AAV and Tfn entry including 1) inhibitors of macropinocytosis such as amiloride and cytochalasin D [202]; 2) disrupting clathrin lattice formation by hypertonic treatment (45mM sucrose) [69]; and 3) cholesterol sequestration by the addition of methyl-β-cyclodextrin (mβCD), which has been reported to affect clathrin coat formation and entry [61,154] and lipid raft dependent (i.e. caveolar and clathrin-independent) endocytosis [178]. To observe the early steps of entry, the pathways were disrupted 30min prior to the addition of AAV and Tfn. When cells were treated with amiloride to block macropinocytosis, there was no measurable effect on the internalization of transferrin (Fig 3 first column, Figure 4B black bar). In contrast with amiloride treatment, there was a 55% reduction of entry of AAV (Figure 3 second column and Fig 4B white bars), and syndecan4 (Figure 3 third column). Hypertonic treatment, which should inhibit clathrin lattice formation, decreased the entry of Tfn (down to 37%), as well as AAV (down to 26%) compared to untreated cells (Fig 3, Fig 4B). Syndecan4 and AAV that did enter the cell were dispersed through the cytosol in hypertonic treated cells compared to the control (Fig 3). Clathrin-independent endocytic mechanisms have been associated with cholesterol-enriched domains, whereby cholesterol sequestration via mβCD treatment can inhibit entry via these pathways. It has also been shown that clathrin lattice formation is perturbed in the absence of membrane cholesterol [61,154]. In agreement with these findings, we see a slight decrease in both AAV and Tfn uptake with cholesterol depletion treatment (Fig 4b).

These results indicated that the hypertonicity and □-cyclodextran affected to various degrees the entry of Tfn, AAV and Syndecan. To assess the potential nonspecific effects of these treatments, the entry of the fluid-phase endocytic marker dextran was also tested under these conditions. Dextran entry was perturbed by amiloride (31% decrease), cytochalasin D (39% decrease) and cholesterol sequestration (72% decrease). Surprisingly, hypertonic treatment also resulted in a 75% decrease in entry. Thus, the effects of hypertonic treatment on AAV entry may reflect effects on targets other than clathrin-mediated endocytosis (Fig 4b).

Transduction efficiency in multiple pathways

The next goal was to distinguish between treatments that blocked the entry of AAV into a particular pathway, as assayed by microscopy, and those treatments that affected the degree to which AAV could enter as well as productively transduce cells. The efficacy of transduction was assayed 48 hours after infection with AAV carrying the luciferase trans-gene. Cells were pulsed with inhibitors either before AAV [MOI of 10,000 genome copies (GC) per cell] was allowed to attach, or after infection and removal of unbound virus. Pre-treating cells with drugs that reduce macropinocytosis and which had no effect on the entry of transferrin reduced the transduction of cells by approximately 40% (amiloride) and 20% (cytochalasin D) (Fig 5a). Hypertonic treatment prior to the addition of virus reduced transduction by 90% (Fig 5a). Sequestration of plasma membrane cholesterol by mβCD treatment also dramatically decreased transduction of cells (approximately 80%) (Fig 5a).

To discriminate direct effects of these treatments on the entry of AAV from potential downstream effects on subsequent steps of viral infectivity such as endocytic transport, entry into the cytosol, transcription or translation, the cells were pulsed with inhibitors after viral entry. Treatment with amiloride or cytochalasin D had minimal effects when added after viral entry (Fig 5a), whereas sequestration of cholesterol and hypertonic treatment reduced transduction by approximately 20% (Fig 5a). To test if the transduction phenotypes were a function of the 10,000 GC MOI per cell, the experiments were repeated at a MOI of 10 GC per cell. At the lower MOI, the effect of blocking macropinocytosis on AAV transduction was less than observed for the higher MOI (approximately a 20% decrease for both amiloride and cytochalasin D treatments). Similarly, the effects of cholesterol sequestration and hypertonic treatment were less dramatic on the entry of AAV, an approximate 40% decrease for both (Fig 5b). Additionally at an MOI of 10 GC per cell, there was minimal to no effect on transduction when any treatment was given after viral entry (Fig 5b).

To further probe the effects of syndecan4-induced macropinocytosis and clustering on AAV entry, we used a cell line, pgsA745 CHO, that is unable to express cell surface HSPG. These cells were previously used to demonstrate the dependence of AAV on cell surface HSPG for entry [16]. Although generally refractory to transduction, pgsA745 cells can be transduced by AAV, albeit at a low levels [7 fold reduction in binding and greater then 10 fold transduction when compared to CHO wt [188]]. Transduction is readily detectable even at low levels of luminescence, and hence should be insensitive to HSPG-specific entry blockers. Treating these cells with inhibitors of macropinocytosis had no

effect on AAV transduction (Fig 5c). Furthermore, neither hypertonic treatment (45mM sucrose) nor cholesterol sequestration (mβCD) affected transduction in this cell type (Fig 5c). The nature of AAV endocytosis in pgsA745 cells and other cell types lacking cell surface HSPG is still not clearly understood, but may reflect AAV's ability to bind to a lower affinity heparin sulfate-like surface molecule, enter through a third alternate pathway or to enter through binding co-receptors.

The entry of AAV's and HSPG into cells, as assayed by microscopy was blocked by the addition of amiloride (Figure 3). We tested the effects of amiloride on AAV infection transduction when added 30min before and maintained with AAV (continuous) during entry. AAV transduction was completely inhibited at 22 mM amiloride. A potential concern is that this concentration of amiloride may compromise cell viability, which would also decrease transduction competence. This was tested by treating cells with identical concentrations of amiloride before infection, but washing out the amiloride just before addition of AAV. The amiloride, when washed out, had no effect on AAV transduction and there were no detectable effects on cell viability (Fig 10b).

HSPG activates membrane ruffling and Protein Kinase Cα (PKCα)

Large membrane ruffles and membrane protrusions characterize macropinocytosis. Activation of PKCs have also been implicated in macropinocytosis in general, and more specifically in the internalization and trafficking of β_1 integrins [115]. Clustering and activation of syndecans has also been described to be dependent on the recruitment and phosphorylation of PKCα due to a PKCα binding-domain in their C-terminus [202].

Therefore, we tested whether AAV induces a PKCα-dependent membrane ruffling through its interactions with syndecans. AAV was bound in the cold to HeLa cells and the cells then fixed at various times after warming to 37°C, then stained for AAV as well as with an antibody specific to the activated form of PKCα (phosphorylated PKCα) and visualized by confocal microscopy. Single deconvolved slices show that there is rapid (as early as 5 min post warm-up) and sustained (30min) membrane ruffling of the cells upon infection (Fig 6). Furthermore, AAV binding alone was not sufficient to cause PKCα phosphorylation since activated PKCα was only observed at sites of membrane ruffles (arrows) and not at all sites where AAV was bound (arrow heads) (Figure 6). Tfn did not cause activation or site-specific recruitment of pPKCα (Fig 10d). G418 selected cells expressing wild type Syndecan 4 (S4+), or syndecan 4 lacking the PDZ c-terminal domain (PDZ-) have nearly a 10-fold difference in their ability to be transduced by AAV (Figure 10a). This underscores the importance of HSPG signaling in the internalization and infection of AAV.

To probe if cytoplasmic signaling by HSPG was required for macropinosome formation, HeLa cells were transfected with Syndecan-4 lacking the PDZ domain (Synd4 PDZ-). In these cells, macropinosome formation and PKC alpha recruitment was decreased and delayed (Fig 9). Cells expressing dynaminK44A as well as Synd4 PDZ- had smaller macropinosomes than non-transfected cells (Fig 8c). We also observed that hypertonic treated cells failed to recruit activated PKCα, and failed to produce membrane ruffles (data not shown).

Chapter 5

Dynamin dependent uptake of AAV

Introduction

Although CME was discovered and heavily researched in the early 20th century, it was not until 1991 that a mechanism by which membranes could be fused to create the nascent endosome was described. Work performed in the fruit fly *Drosophila melanogaster* revealed a mutant fly that would become paralyzed in a temperature sensitive mechanism [59]. It was found that this paralysis was caused by a mutation in a single gene called *Shibire* (Japanese for paralyze) [195]. This locus would later be identified as dynamin, but the connection to dynamin (originally characterized as a microtubule interacting GTPase) would not be appreciated for several years [116,176]. Following a shift to the restrictive temperature, EM pictures showed that *shibire* mutants would accumulate membrane associated clathrin coated structures with elongated necks [87]. This neck elongation at synapses was believed to be due to the inability for the GTPase, dynamin 1, to hydrolyze GTP to GDP, thereby preventing the final scission step in CME and blocking signal transductions in neurons (leading to paralysis). While the actual mechanism by which dynamin fuses membranes at the neck of the nascent vesicle is still under debate ("pinchase" versus "popase") the *shibire* mutations [Lysine amino acid 44 mutated to Alanine (K44A)] has been used to study the process of CME in yeast and mammalian cells [138].

Expression of dynaminK44A acts as a dominant negative inhibitor of dynamin GTPase function when expressed in mammalian cells [208]. In the presence of this mutation,

dynamin can still oligomerize by complementary binding via its GTPase effector domain (GED), but cannot complete the membrane scission step [33,68]. Indeed, it was the use of the dynamin 1 mutant that lead to the assertion that AAV and other viruses enters cells via CME [181]. In recent years, researchers have come to appreciate dynamin's original role as a cytoskeletal associated protein as well as discovered dynamin playing some unexpected roles. As previously described, dynamin is associated with the budding of caveolae from the cell membrane [120,181]. Dynamin has also been discovered to play a role in clathrin- and caveolar-independent entry of γc cytokine receptor [167]. In the latter case, receptor uptake was found to require the F-actin nucleating protein cortactin [167]. In subsequent studies, it was resolved that dynamin 2 can directly interact with cortactin via the praline-rich Src homology (SH) domain of cortactin, and is enriched in growth factor induced membrane ruffles [107]. Oblation of the proline-rich domain (PRD) of dynamin 2 leads to increased stress fibers and diminished localization of dynamin to growth factor induced membrane ruffles [107]. Several other actin binding or actin nucleating factors have been shown to directly bind to dynamin 2 including Intersectin 1/s, Syndapins and Profilin 2 [168]. Although dynamin's role in the dynamic process of actin remodeling during cell movement and growth-factor induced membrane ruffling is still being elucidated, its role in macropinocytosis has yet to be described. It is clear that dynamin's association with actin modulating proteins highlights dynamin's role as more then a pinchase in clathrin mediated endocytosis.

Results

AAV induces macropinocytosis at sites enriched in actin and dynamin 2

Membrane ruffling is maintained by polymerization of the actin cytoskeleton beneath the plasma membrane. To further elucidate the composition of membrane ruffles induced upon AAV binding, macropinosomes were stained for enrichment of the actin cytoskeleton with the fungal toxin phalloidin. Alexa 546 labeled phalloidin staining was indeed enriched in AAV and activated PKCα pinosomes (Fig 7a). Although membrane ruffles can be detected at sites where no AAV staining is present, control cells not incubated with virus failed to show activated PKCα enriched macropinosomes (Fig 7b). When cells cold bound with AAV were warmed up for various periods before being pulsed with fluorescently labeled dextran molecules (a marker for fluid-phase endocytosis) for 5 min, there was a rapid but transient virus-specific increase in dextran uptake (Fig 7c). The time scale of increased macropinocytosis is during the time period of maximum sustained membrane ruffling seen in our previous time courses (Fig 1b 0, 5 and 10 min). AAV binding and membrane ruffling do not cause an increase in the uptake of the classic clathrin mediated endocytic ligand Tfn (Fig 7c).

Over expression of a dominant negative mutant of dynamin 1 abolishes the entry of AAV [162]. Indeed, it has been shown that dynamin is enriched in EGF induced membrane ruffling, and may in fact play a role in macropinocytosis [107,162]. The localization of dynamin2 was probed in HeLa cells that had been cold bound with 1000 GC AAV and then warmed for 10 min. Dynamin 2 was enriched at sites of AAV induced macropinosomes (Fig 8a). The experiment was repeated in HeLa cells that were

transfected 18 hours previously with the dominant negative construct dynamin 2K44A-GFP. There was a marked decrease in AAV induced membrane ruffles on transfected cells, with those ruffles no longer being enriched in dynamin 2 (Fig 8b). Thus, dynamin may play a direct role in the generation, maintenance or maturation of AAV induced macropinosomes. Since prolonged expression of a dominant negative dynamin constructs may not completely inhibit dynamin, or have off target effects, we tested the effects of Dynasore, a dynamin 1 and 2 specific inhibitor, on the uptake of Tfn and AAV (Fig 12). Blocking dynamin activity alone can inhibit AAV and Tfn entry (Fig 12b and c). The inhibition was not the result of cell death as assayed by Trypan Blue exclusion (data not shown).

Chapter 6

Role of Integrin signaling in AAV endocytosis

Introduction

Integrin receptors are composed of two single-pass transmembrane non-covalently linked subunits: an α chain and a β chain. 19 α subunits and 8 β subunits have been discovered in vertebrates leading to at least 25 different αβ heterodimers [173]. Known for integrating outside-in signaling, integrins bind to extracellular matrix (ECM) proteins such as fibronectin and transduce information about stretching, shearing and membrane stress[173]. Primarily studied in blood clotting and leukocyte movement, integrins have become important to the pathogen field due to their involvement in the pathology of several viruses. Consisting of a large extracellular domain and a relatively short intracellular domain, integrin binding to ECM leads to clustering and "activation" [83]. This activated state leads to conformational changes in the cytoplasmic tails of the α and β subunits that allow recruitment and activation of talin and the polymerization of cytoskeletal elements to form strong focal adhesions [83]. The immediate downstream effect of integrin ligation is the activation of phospholipases that cleave membrane bound phosphoinositides into signaling molecules capable of activating PKCα [65]. Another downstream target of integrin activation is the phosphorylation of focal adhesion kinase (FAK), a powerful modulator of cytoskeletal dynamics and cell shape determination [27]. The exact link between ligand attachment, outside-in signaling and integrin endocytosis is still not clear.

The link between integrin binding and cytoskeletal rearrangement may have been exploited by pathogens to antagonize their internalization. Several viruses and bacteria

have, as a necessary component to recognizing host-cells, an integrin coreceptor[131]. It is well documented that Adenovirus entry requires cell surface αVβ5 integrin, and that binding to integrins helps the virus enter through clathrin coated pits [109,189]. This same integrin pair was found to be the receptor for vitronectin endocytosis [108,110]. Hypertonic treatment was used to show that these receptors enter via CME although, interestingly, only antibodies to β5 integrin were used to confirm the colocalization of the heterodimer with vitronectin containing endosomes [108,110]. Does Adenovirus resemble vitronectin? Do the two ligands enter in the same mechanistic way? Adenovirus also binds HSPG [10]. It has been shown that syndecans can help modulate and in some cases activate integrin singaling[9,30,108,216,221]. In fact, when Adenovirus was modified to have high affinity HSPG binding sites on its capsid, transduction, in normally refractory tissues, doubled [215]. Given our data showing the principle necessity for HSPG in AAV infection, we wondered if AAV was also entering cells with its integrin coreceptors.

Results

AAV is believed to interact with αVβ3 integrin [188]. This data is based on some conflicting results. Studies showed that overlaying cell membrane extracts with AAV capsids revealed AAV's direct interaction with αV integrin [188]. Subsequent work attempted to repeat this work and failed to find this association [144]. They further showed that AAV capsids would not interact with soluble αV integrins using surface plasmon resonance [144]. Other labs have verified that blocking antibodies to αV integrin cannot reduce AAV binding and infection [5]. The most recent work performed on the structure of

AAV uncovered a fibronectin like peptide recognition sequence in the VP3 subunit [5]. This asparagine-glycine-arginine (NGR) motif is believed to be a substrate for α5β1 integrin, and the addition of soluble blocking antibodies to these integrins during AAV infection decreased viral transduction by half [5]. αV/5 integrins are part of a subfamily of heterodimer integrin pairs that can bind to fibronectin in the ECM [27,108]. αV can heterodimerize with several other β chains, including β3, 1 and 5 and α5 with β1 and 5[27,108]. It is not well understood how much cross-talk, clustering and colocalization occurs between integrin heterodimers that can bind the same substrate. Therefore, we looked by immunofluorescence for the presence of several αβ pairs at sites of AAV binding and membrane ruffling.

HeLa cells were cold bound with 10,00 GC/cell AAV before being warmed for 10 min at 37 degrees and fixed for immuno-staining. Figure 13 shows that both α5 (top) and αV (bottom) integrins co-label surface bound and membrane ruffle enriched AAV. It is also interesting to note that, unlike AAV and syndecans (table 1, Fig 3), some internalized AAV (bottom) no longer colocalizes with αV integrin (Fig 13). The antibodies used to label integrins recognize the c-terminal region. Failure to label AAV endosomes with integrins may be due to cleavage of the c-terminus of integrins, or due to sorting of AAV and integrins into separate and distinct endosomes after internalization. Are integrins acting as attachment Velcro for AAV, or is the ligation of AAV to this receptor leading to downstream signaling?

As discussed previously, AAV's primary receptor, HSPG modified syndecans, has the ability to activate PKCα and synergize with integrins at focal adhesion sites [36,221]. It has also been shown that syndecan c-terminal binding protein, syndesmos, can lead to cell spreading and focal adhesion formation independent of stimuli [6,36]. Indeed, it is now believed that syndecan activation can lead to recruitment and activation of focal adhesion kinase (FAK) independent of integrin signaling [203,216]. We decided to see if FAK activation was occurring during AAV binding and internalization. MDCK cells were seeded to 80% confluence in 6 well dishes overnight. Cells were serum starved in DMEM containing 1% serum for 2 hours at 37 degrees. Cells were subsequently washed and incubated in completely serum free media containing 0.5mM sodium vanadate (which prevents rapid dephosphorylation) and cold bound with AAV for 30min. Cells were immediately warmed with serum free media at 37 degrees for 0,5,10, 30 and 60min before cells were immediately lysed in 1X SDS for analysis by Western blot. AAV ligation leads to a rapid but transient increase in the activation (phosphorylation of Tyrosine 397) of FAK (Fig 14 a). FAK activation peaks between 5 and 10min after AAV ligation in a time scale resembling peak membrane ruffling (Fig 1b). Identical results were seen in A549 lung carcinoma cells (data not shown). HeLa cells showed constant FAK activation which did not appear to change even at time 0 min, as visualized by Western blot (data not shown).

In an attempt to differentiate between FAK activation caused by either integrin ligation or by AAV induced syndecan clustering, we looked for AAV induced FAK activation in cells stably transformed with dominant negative syndecan 4 lacking the PDZ domain

(PDZ-) and in CHO cells deficient in cell surface proteoglycans (pgsA745). Cells that were deficient in surface expression of HSPG and extremely refractory to transduction by AAV showed no increase in FAK activation upon AAV ligation (Fig 14 middle). In fact, it appears that there may be a decrease in endogenous FAK activity at 10min post warm up (no quantification). PDZ deficient stable HeLa cells (Fig 14 bottom), on the other hand, showed a delay in FAK activation that continued for 3 times as long (30min) and perhaps longer than activation seen in MDCK cells (Fig 14 top) or A549 cells (not shown). The abolishment of FAK activation at unstimulated time points in a PDZ deficient background (as compared to wild type) may imply that FAK activation by AAV ligation is modulated by syndecan activation and not integrin activation. If integrin ligation alone was sufficient to activate FAK, then pgsA745 cells [which express AAV integrin coreceptors by immunofluorescence (data not shown)] would show time dependent AAV induced FAK phosphorylation. Inhibiting syndecan mediated cytoplasmic signaling by ablation of the PDZ domain leads to abolished non-specific FAK activation and delayed AAV induced FAK activation (Fig 14). Integrin activation can also lead to the activation of other cellular factors not tested here. Further evaluation of the effects of integrin activation on AAV transduction may illuminate synergistic communication between HSPG and integrins during AAV binding and internalization.

Chapter 7

Conclusion and Future Directions

Viruses have evolved to package all the tools necessary to exploit their host's entry machinery into the smallest form possible. In the case of non-enveloped viruses, the viral coat's ability to bind a small set of host cell-surface proteins defines the virus-host tropism, as well as sets the constraints on the entry process, the earliest point in their life cycle. We show here that AAV's binding to HSPG does more than just anchor the virus to the target cell; it also induces macropinocytosis as a productive route of infection.

Where does AAV enter the cell? Immediately after internalization, virus particles reside in endosomes that did not co-localize with Tfn (Fig 1). However, a subset of AAV co-localized with EGF, which has been shown to enter through a clathrin-independent mechanism when incubated with cells in the cold at high concentrations. A number of experiments linking AAV's entry dependence to clathrin may be deduced from the effects of drugs, such as hypertonic treatment, which reduced AAV entry (Fig 4b) but also prevented PKC activation (data not shown). However, the genetic constructs that specifically inhibit the clathrin pathway had less of an effect on viral entry than drug treatment (Fig 4c) and depleting >70% of the clathrin heavy chain subunit had no effect on AAV transduction (Fig 11c). This suggests that some of the drug treatments that implicated a role for clathrin may have had significant off-target effects. Using a lower MOI revealed a transduction phenotype that was less dramatic than at higher MOIs. This might imply a crowding or saturation of productive AAV binding sites at higher MOIs

that lead to a higher percentage of non-productive endocytic events. Any association of AAV or its receptors with clathrin may be rapid and transient and hence faster than the resolution at which we acquired images. The clathrin-medicated endocytic pathway may have roles at other stages of AAV's life cycle not studied herein. For instance, downregulation of CME by prolonged expression of dominant negative inhibitors could lead to a clearance of AAV receptors from the cell surface over time. Since CME is required for normal processes in the cell, prolonged elimination of this pathway could make the cell a less hospitable environment for viral transduction. Advances in single particle tracking along with fluorescently tagged molecules required for endocytosis can be used to better probe the complex mechanisms during host-pathogen interactions. However, it is critical to resolve the relative contributions of different pathways to viral entry (which can be assayed by single particle tracking) from the relative contributions of different pathways to productive viral infection. Deletion of the PDZ domain of the HSPG modified protein Syndecan 4 alone leads to a nearly tenfold decrease in the transduction of cells once susceptible to AAV infection (Fig 10a). The ability for AAV's primary receptor to act as a signaling platform may therefore act as the major determinant of the macropinocytic machinery required for AAV infection (Fig 8c, 9).

Previous work on AAV utilized a mutant dynamin 1 to constitutively downregulate endocytosis [41,161]. It was once believed that dynamin's key role was in clathrin mediated endocytosis. However, the effects of dynamin may not be restricted to caveolin or clathrin mediated endocytosis. Dynamin mutants may also affect subsequent trafficking steps or the internalization of a co-factor necessary for AAV's life cycle. It has been

shown that inhibition of dynamin 2 function inhibits membrane ruffling, cell-movement and Rac localization [89,107,169]. We observed that dynamin 2 is indeed enriched at AAV induced membrane ruffles, and expression of a dynamin 2 mutant decreases the size and number of these ruffles in transfected cells (fig 8). Adenovirus binding induces macropinocytosis, presumably through a similar mechanism as AAV [108]. Additionally, expression of a dominant negative dynamin 2 construct decreased the size of adenovirus induced macropinosomes [108]. Further validating the necessity for this GTPase, using Dynasore, a dynamin specific inhibitor can completely abolish the entry of both Tfn (a clathrin-dependent ligand) and AAV (Fig 12). Inhibition of clathrin-mediated endocytosis has been shown to restrict the entry of adenovirus while the escape of adenovirus from endosomes is decreased in the absence of macropinocytosis [108]. Multiple receptors, including integrins and syndecans, have been implicated in the entry of both AAV and adenovirus. It is possible that when pathogens use multiple receptors, it may not be appropriate to classify them as being dependent versus independent of clathrin. Instead, a comprehensive analysis of endogenous host mechanisms must be considered, and how multiple pathways, internalizing both pathogen and host molecules, are required for a productive infection.

Studying the role AAV's integrin receptors play may also shed light on the activation, generation or maintenance of AAV induced macropinosomes. We have some early evidence that two putative AAV integrin receptors αV and α5 integrins colocalize at sites of AAV attachment and membrane ruffling (Fig 13). Interestingly, it seems that AAV and integrins rapidly separate at a site proximal to the membrane, and potentially

immediately after internalization, and since colocalization of internalized AAV and integrins are low (Fig 13), it is also possible that a cleavage event prevents antibody recognition of internalized integrins. We further analyzed the activation of FAK upon AAV internalization. We observed that cell surface expression of HSPG was a better indicator of FAK responsiveness. Cells devoid in HSPG (pgsA745) or cells unable to signal after HSPG clustering (PDZ-) failed to upregulate FAK activation efficiently (Fig14). HSPG deficient cells were unresponsive to AAV binding. These cells are highly refractory to AAV infection, but can be transduced at low levels (Fig 5) although they express α5 and V integrins (data not shown). It is possible that integrins or another HSPG like cell surface attachment factor is able to allow transduction on pgsA745 cells. When the PDZ domain was removed from syndecan 4, basal FAK activation was completely abolished compared to wild type cells (Fig 14 and data not shown). AAV binding was able to cause FAK phosphorylation, but the time to activation was delayed by 10 minutes (Fig 14). Furthermore, the activation of FAK was not transient (10-15 min) as seen in wild type cells. It lasted for 30+ minutes (Fig 14). This lack of FAK regulation in a PDZ background may help us understand the role HSPG and integrins play in the cellular signal cascade initiated after AAV binding. Indeed, it has been shown that syndecan 4 null fibroblasts have lower endogenous FAK activity when compared to wild type cells [216]. This difference could not be overcome by activating PKCα with phorbal esters, but could be augmented by activation of Rho GTPase [216]. My data also show that the addition of PMA does not increase FAK phosphorylation in the cell lines tested (Fig 14). It then seems that AAV induced activation of a RhoGTPase (presumably Rac) signals syndecan

induced up regulation of FAK phosphorylation. It still remains to be elucidated if other downstream effects of integrin binding affect AAV transduction.

We have shown that pharmacological inhibition of macropinocytosis blocks the entry of a specific HSPG (syndecan4) and AAV, but not the clathrin-dependent endocytic ligand Tfn. This block also leads to a decreased transduction efficiency that is not seen in cells that lack cell-surface HSPG. The clustering of syndecan4 by AAV leads to rapid membrane ruffling as well as the site-specific activation of PKC□. In light of the ability of HSPG negative CHO cell lines to be transduced by AAV, the question of AAV's dependence on other PKCs remains. Additional questions remain about what roles other glycans or heparan-sulfate modified membrane proteins and secondary receptors play in AAV entry. Pathogen-induced macropinocytosis, through binding to cell surface proteoglycans, may then represent a ubiquitous pathway exploited by many pathogens for cell entry or internalization of factors required for a productive life cycle. To date, only 3 viruses have been published citing macropinocytosis as their mode of entry, and all three of those were in the past year [3,31,111]. Live cell imaging and imaging of single particle endocytosis would be a powerful tool to help understand the complex cascades that occur at the cell membrane when the virus interacts with the host. Earlier work imaging single AAV particles in real time revealed that AAV touches the cell surface many times before pausing and rapidly entering cells [171]. The residence time at the membrane and interanalization was rapid (approximately 64ms), which is much faster than what others have measured for CME (10s of seconds to minutes) [147]. This observation gives more credence to our assertion that AAV does not enter through CME. Literature on

macropinocytosis is very limited. The mechanism by which non-phagocytic cells initiate, maintain and fuse membrane ruffles is still poorly understood [77]. Membrane ruffle-induced by pathogen receptor clustering as well as cross talk between host entry pathways are cell-biological questions that can be answered using viruses as tools (Fig C).

Taken together, our results demonstrate that AAV has used multiple routes of entry, but HSPG-mediated macropinocytosis is the predominant entry path for productive infection. This pathway is independent of clathrin and leads to the internalization of AAV into syndecan4 containing endosomes that do not merge with Tfn endosomes or caveosomes. Inhibitors of clathrin-mediated endocytosis by siRNA had no effect on AAV transduction. Expression of genetic inhibitors and potential chemical inhibitors of clathrin may affect AAV entry either by; preventing AAV's direct association with the endocytic machinery or preventing activation, reorganization or proper trafficking of other factors in the viral life-cycle through immediate and/or downstream mechanisms. It may be that early endocytic events can make a cell more or less permissive to AAV transduction. In fact, it has already been shown that flux through the EGFR can lead to phosphorylation of the ssDBP, and ultimately reduction in AAV second strand synthesis [180]. It would then be rational to think the perturbation of cell surface growth factor receptors with internalization inhibitors would also lead to transduction phenotypes with AAV.

Future directions

An essential question in the field of pathogen entry is host cell selection, and more specifically, what determines that actual site of internalization. In the case of viruses circulating in the lung or blood stream, one might speculate that sites of tissue damage, that expose baso-lateral receptors, might make for interesting candidates. A large caveat to the aforementioned studies is that they are done *in vitro*. Caveats aside, there are some interesting observations that would be worth future illumination.

Preferred sites of AAV attachment:

When I have looked at macropinosome generation at low MOIs (between 10 and 100 GC/cell), I have observed that AAV do not bind uniformly to the cell surface. Macropinosomes form at lateral edges, and can have several (2-4) membrane ruffles emanating from a single site, or even off of a single ruffle. I have also seen (at MOIs of 10) several cells without a single (detectable by light microscopy) virus on their surface, while one cell in the field has several macropinosomes. It is clear from the previous studies done herein and by others that the integrity of receptor complexes is essential to their ability to function properly and transduce activation signals through the cell. Are the multiple touches seen when AAV was observed in live cell (and the 87% failure rate of successful entry) a product of finding a functional HSPG-integrin-FGFR cluster? Multicolor live-cell imaging with fluorescently tagged receptors and viral particles might begin to address these questions. Several laboratories have pioneered reporter constructs for looking at growth factor receptor and integrin activation [123,201]. A greater

understanding of receptor clustering, activation and endocytosis could be gained by studying these processes live using these constructs.

Receptor clustering selection and exclusion:

Another interesting cell biological question that could be addressed using AAV as a ligand is the question of receptor clustering and exclusion. It is believed that AAV has, at minimum, two receptor binding clefs on its capsid shell; one for HSPG and one NGR domain for α5 binding [5,132]. Asokan et al proposed a "click to fit" model, in which AAV binds at low affinity to any one receptor, but when it engages both receptors, a high affinity engagement is made, which allows for subsequent internalization [5]. Since AAV has icosahedral symmetry, there are several such binding sites on the virus. This would allow for multiple receptors to bind a single virion and create a mini-cluster. Clustering of syndecan 4 results in relocation of receptors into lipid raft domain [222]. It may now be possible to visualize the formation of microdomains by imaging single viral particle interactions with their receptors. Furthermore, how receptors are sorted at sites of macropinosome formation has not been characterized [77]. AAV internalization may be a good system for seeing if all receptors enter an early endosomal structure together, or if they are disengaged, sequestered or clustered away into domains that are left at the cell surface after internalization. While tagging of syndecans with fluorescent markers has not been successful (private correspondences, data not shown), integrins, AAV, membrane lipids and growth factor receptors can be followed by fluorescence microscopy.

A downstream question from analyzing which receptors end up in nascent macropinosomes is: does the presence of these receptors aid in the maturation and trafficking of AAV to an acidic compartment? Although the bulk of the work I have chosen to focus on is specific to events at the plasma membrane, several pieces of evidence would indicate that macropinosomal membranes are different from other endosomes. Arginine rich membrane fusing peptides, believed to enter through macropinocytosis, exit the macropinosome by endosome "leakiness" [54]. Adenovirus can also induce membrane "leakiness" of macropinosomes by a yet-to-be understood mechanism [77,109,203]. Finally, AAV itself contains a phospholipase A2 like domain that is believed to aid in endosome escape (presumably by cleaving endosomal phospholipids to rupture their endosome) [56]. Is the nature of receptors in these membranes, or in the lipid components of macropinosomes inherently unstable and hence prone to leakage; or is it possible that membrane binding cytoplasmic proteins selectively bind to receptors or components in macropinosomes to induce membrane breaks?

Accumulation of accessory and adaptor proteins at sites of macropinosome formation:

The cytoplasmic interactions that lead to the regulation of syndecans have been mapped, and continue to be elucidated [77,203]. Many of the proteins that bind the PDZ domain of syndecans regulate the actin cytoskeleton via activation of actin modulating proteins such as dynamin, paxillin, synectin and syndesomes [6,58,77,119,164,165,216,221,222]. A great deal of knowledge about CME was gained be analyzing the recruitment and departure of adaptor

and accessory proteins at sites of forming coated pits [148,151,152]. This same approach can be used to understand the dynamics of macropinosome formation in real time with a real cellular ligand (AAV).

Single particle or multiple particles, the physiologically relevant event:

A final question is partially a thought experiment and partially a chance to appreciate the true evolution of viral endocytosis. It is clear that I can see, in my system, the productive infection of tissue cultures cells by as few as 10 viral particles/cell (Fig 5). Since the origin of AAV infections in humans is still not understood, it is difficult to know what the true MOI of AAV is *in vivo*. To this end, is it rational to believe that this virus infects 1 particle at a time? In a lytic infection or during the manufacturing of recombinant AAV, a single dish of infected cells yields millions of particles. This means each cell is capable of creating thousands of AAV virions, which *in vivo* would be released into the body. Cooperative binding to preferred domains or receptor enriched sites on target cells may be advantageous to a particle that is only 25nm in diameter. Using single particle tracking, it would be interesting to see how the efficiency of endocytosis increases with MOI.

Support:

This work was supported by research grants from NSF (BES-0620813) and NIH (P20 GM072015 to SMS and Rockefeller University NIH Training Grant: Genetics & Cell Biology Training Grant - GM066699. to CDB and The Rockefeller University Anderson Cancer Fellowship.

Table (1) Correlation coefficient values for endocytic ligands: Deconvolved images were analyzed for co-localization between two channels using Imaris software and correlation coefficients are presented from fields containing 6-10 cells. All experiments were performed as double labeling and analyzed after fixation and staining by confocal microscopy. Data represent 3 independent experiments.

	Tfn	EGF	Synd	FM4-64
AAV	-0.0028 +/- 0.0036	0.6080 +/- 0.0140	0.6233 +/- 0.0186	0.4933 +/- 0.0296
Tfn	0.6108 +/- 0.0488	0.0103 +/- 0.0001	0.03117 +/- 0.00205	0.3147 +/- 0.0183

Table 1:

Fig 1:

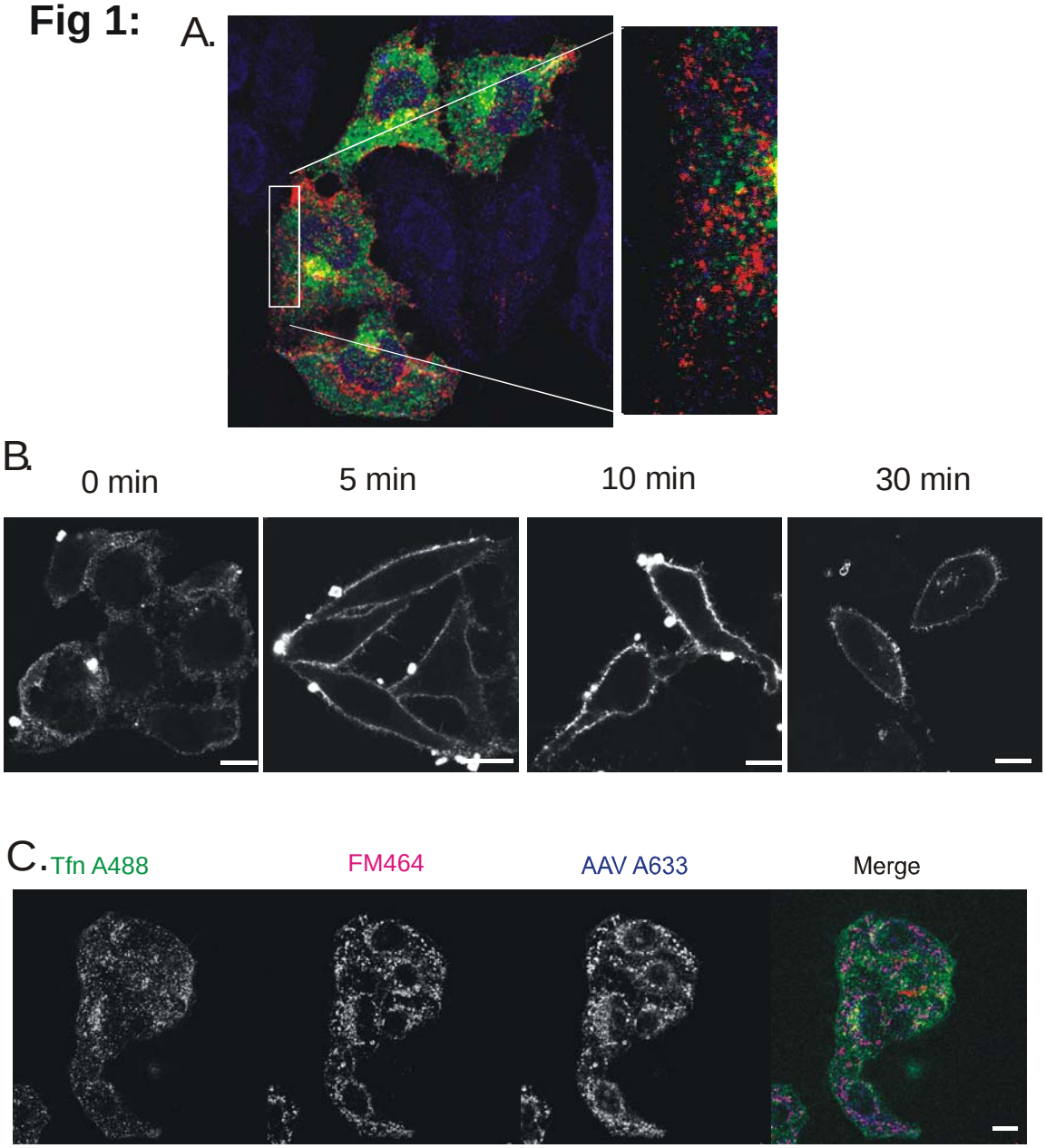


Figure (1) Clathrin independent entry of AAV: Confocal images showing HeLa cells incubated with (a)10,000 GC per cell AAV (blue) 24hrs after transfection with Caveolin-1mRFP (red) and Clathrin–eGFP (green) , or (c)10,000 GC per cell AAV, 2 µg Tfn-Alexa488 (green) with 4 µM FM4-64 (red) in SF media, on cells at 37°C for 30min. Red and blue images merge as purple, green and red merges as yellow, all three images merges as white. (b) Time course analysis of membrane ruffles induced upon 10,000 GC AAV binding (cold bound and warmed up for indicated times and stained for AAV). Images represent a 0.2 µm confocal slice. AAV was visualized with an antibody labelled with Alexa633, the scale bars represents 10µm.

Figure 2

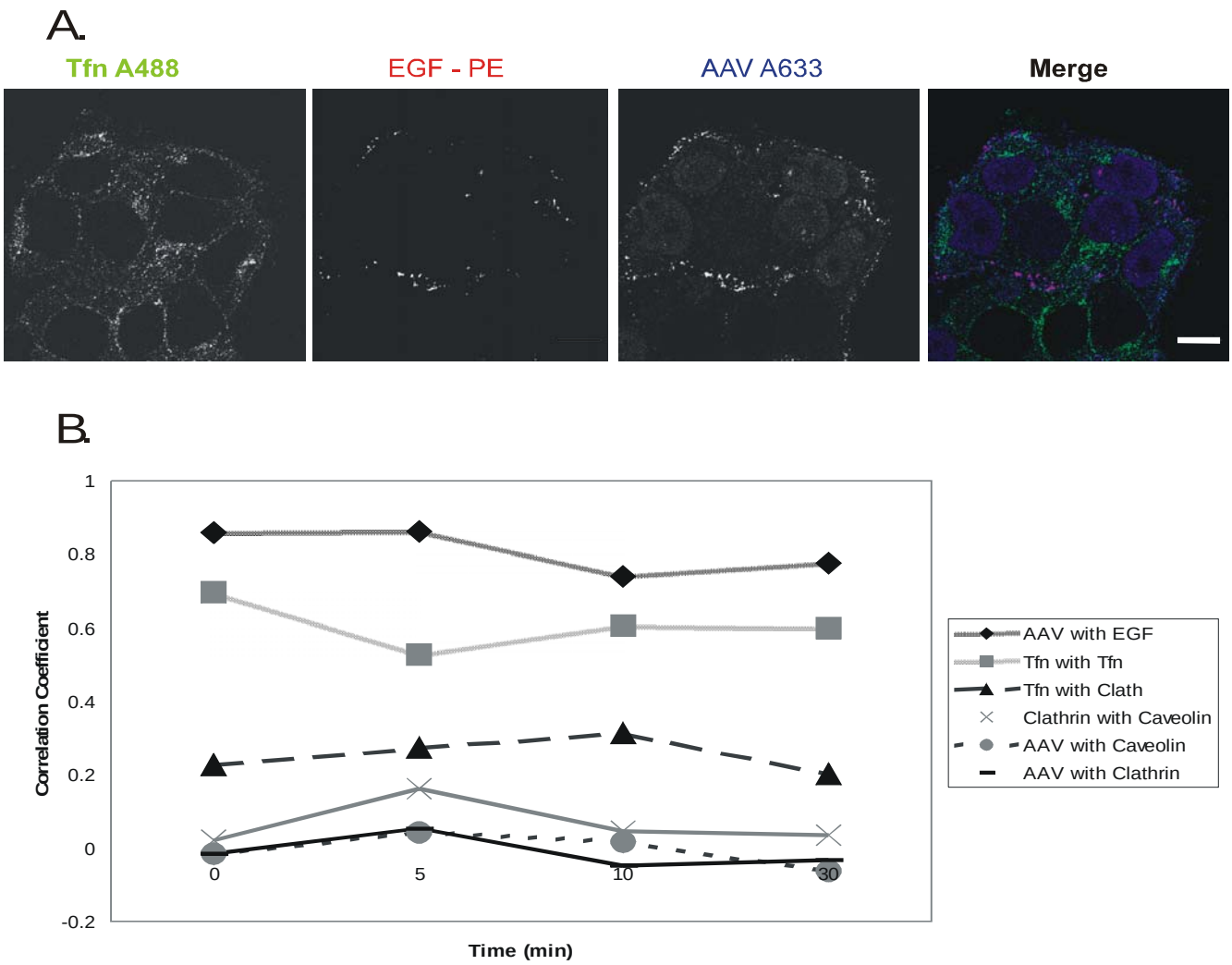


Figure (2) AAV correlates with markers for fluid phase entry: (a)100 nM EGF-PE, 10,000 GC per cell AAV and 2 µg Tfn-Alexa488 were bound in the cold to HeLa cells for 30 min and then cells were warmed to 37°C for 30min before fixation. AAV was visualized with Alexa633 labelled antibodies and images acquired by confocal microscopy. A 0.2µm slice is presented, the scale bars represents 10µm. (b) Confocal images of cells co-labelled with Tfn, AAV, EGF and/or co-transfected with Caveolin-1mRFP and Clathrin-eGFP were analyzed for co-localization at 0, 5, 10, and 30 min after the start of the incubation with the markers for fluid phase entry and presented as correlation of ligands over time.

Figure 3

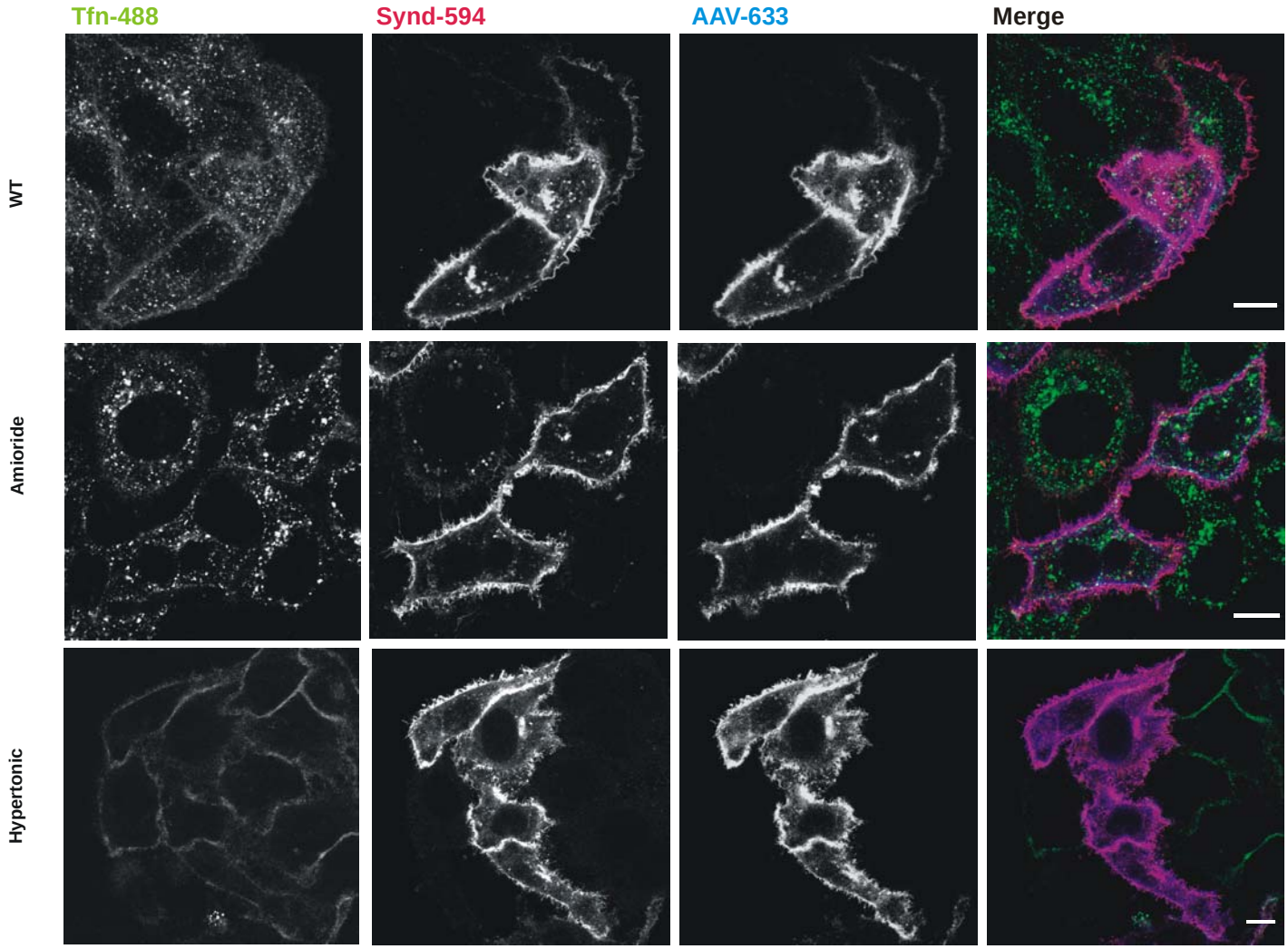
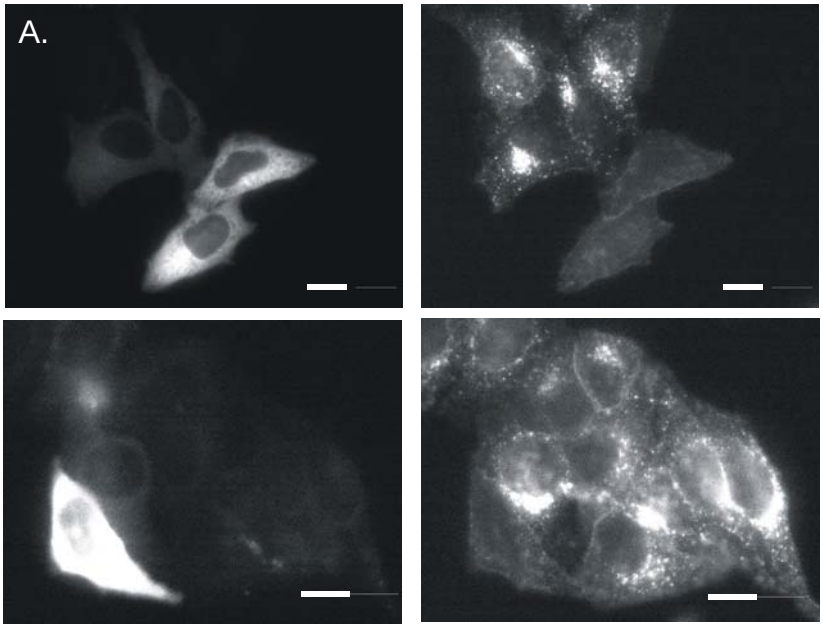
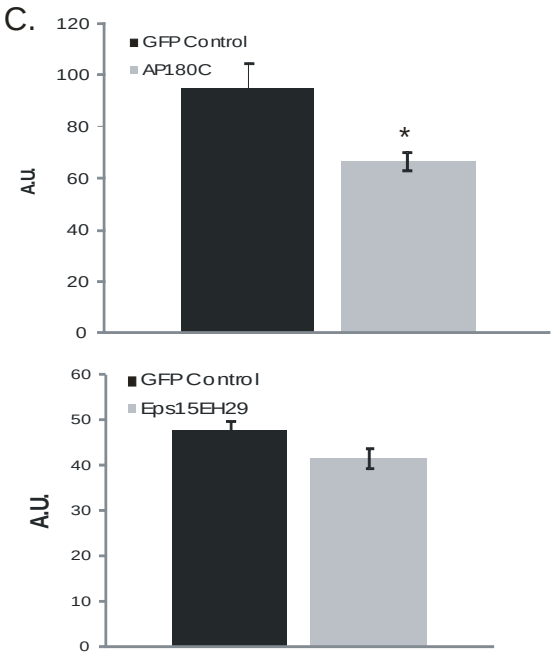


Figure (3) Inhibiting macropinocytosis blocks a subset of Syndecan4 and AAV entry, as does inhibiting clathrin mediated endocytosis (CME): HeLa cells were transfected with wtSynd 4-HA 24 hrs prior to treatment with DMSO (WT), 3 mM Amiloride or 45 mM sucrose (hypertonic solution). Cells were then incubated with 10,000 GC per cell AAV and 2µg Tfn-Alexa488 at 37°C for 20min. Images represent a 0.2 µm confocal section through upper center of cell, the scale bars represents 10µm.

Fig 4





B.

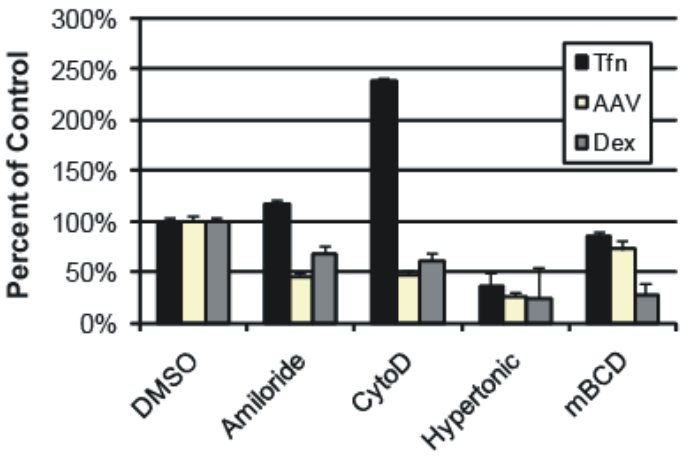
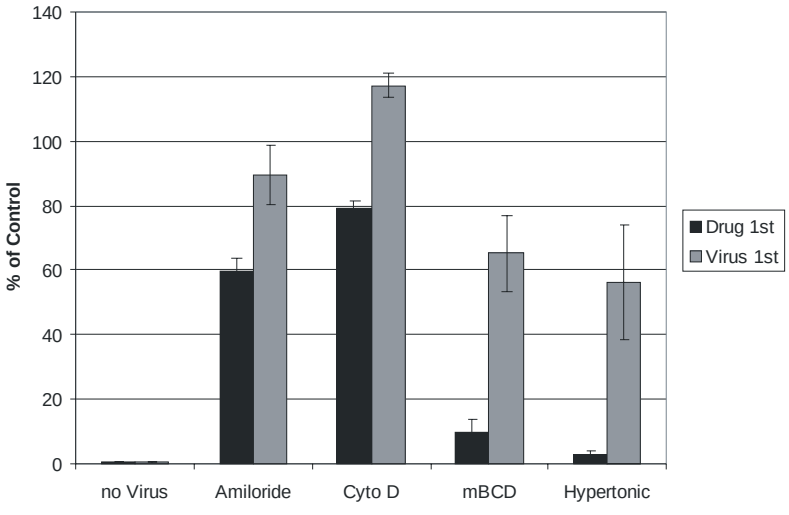


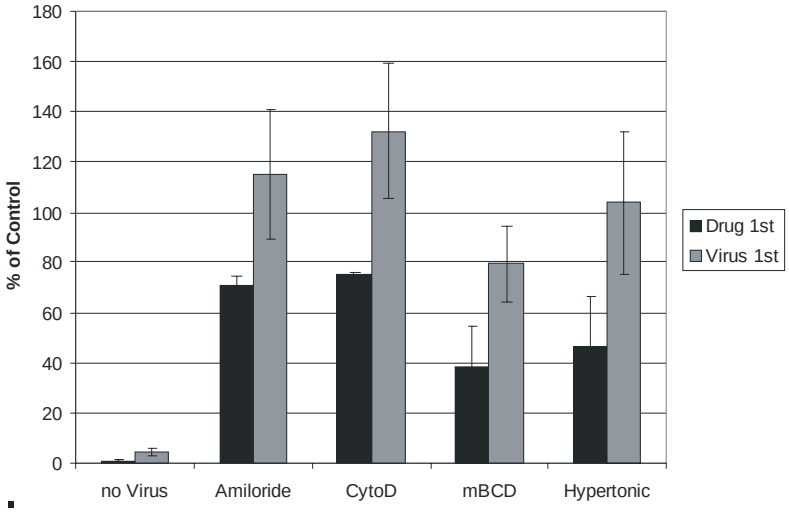
Figure (4) Alternative pathways of infection by AAV: Cells were transfected overnight with the eGFP tagged dominant-negative inhibitors of CME Eps15EH29 and AP180C (a, c), that were both shown to inhibit the entry of clathrin (a), the scale bars represents 10μm. The effect on Tfn, Dextran or AAV uptake in cells treated with drug inhibitors of CME or macropinocytosis for 30 min at 37°C was assessed (b). Substrates were allowed to enter cells for 30 min before non-internalized ligands were washed of and cells fixed and stained. Displayed values are in arbitrary fluorescence units from at least 100 cells for drug treatments or 50 cells for genetic inhibitors from at least 2 separate experiments, with effects of drugs on AAV entry shown (b) or effects of dominant-negative inhibitors shown (c). Camera background noise has been subtracted. (*) represents $p<0.001$ and (**) represents $p<0.05$ as compared to a non-transfected or DMSO treated control as assessed by the student t-test.

Figure 5

A.



B.



C.

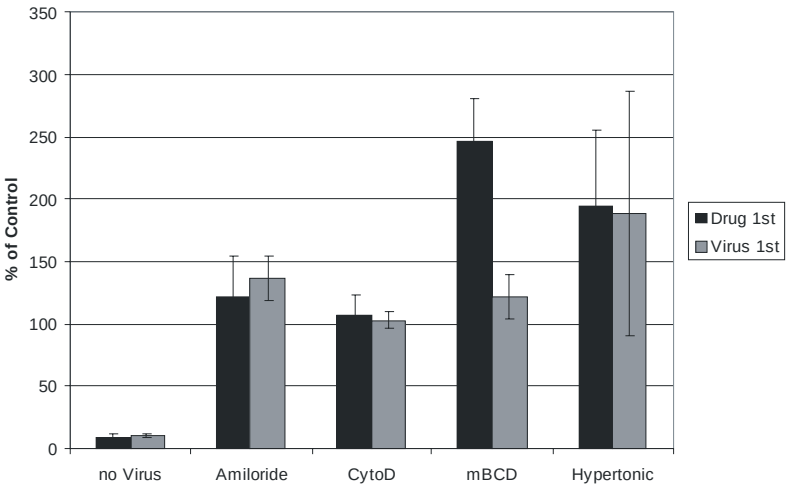


Figure (5) Inhibitors of internalization affect AAV transduction at high (10,000) and low (10) MOIs in HeLa but not HSPG-negative pgsA745 cells: Cells were pulsed with drugs 30min before (Drug 1st) or after (Virus 1st) addition of 10,000 GC (a) or 10 GC (b) of AAV per cell at 37°C. Surface bound virus was removed by heparinase treatment and transduction as assessed by luminescence was obtained after 2-day incubation at 37°C. The cell surface HSPG deficient CHO cell line pgsA745 was used to determine the effects of the later treatment on AAV entry (c).

Figure (6) PKCα is activated at sites of macropinosomes: HeLa cells where cooled to 4°C for 30min in the presence of 10,000 GC AAV per cell before warming to 37°C for the indicated times. Cells were fixed and stained for AAV as well as with an antibody specific for the phosphorylated form of PKCα. PKCα membrane staining is localized specifically to sites of AAV macropinosomes (arrows) and not just surface bound AAV (arrow heads). Macropinosomes seen primarily on lateral surfaces of cells (confocal slice through middle of cells are shown) a 0.2 μm slice shown, the scale bars represents 10μm.

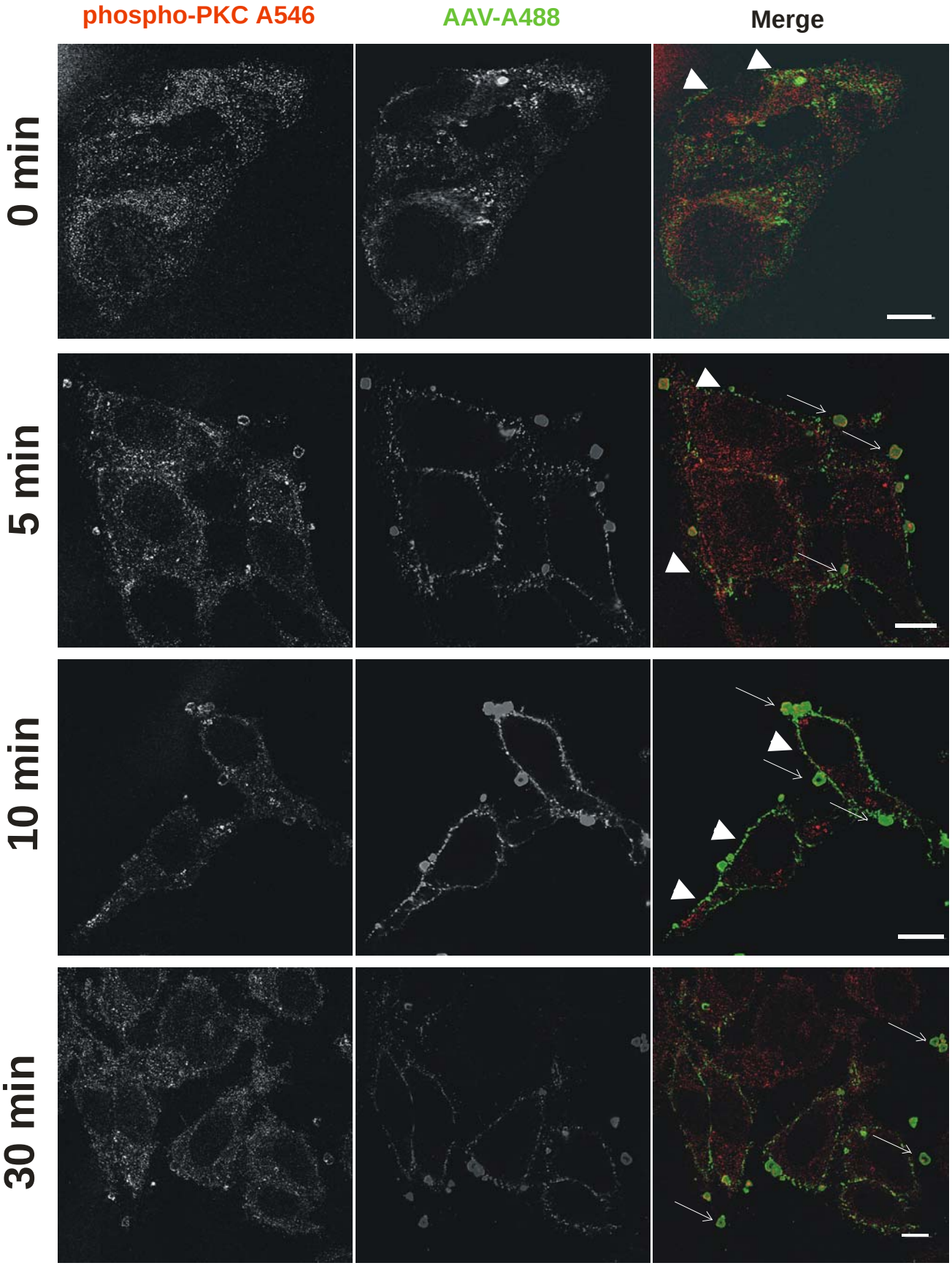


Figure 6

Figure (7) Actin is enriched in AAV induced macropinosomes which cause up-regulation of fluid-phase internalization but not CME: HeLa cells were cold bound with AAV (a) or serum free media (b) before warming up at 37 degrees for 10min, fixed and stained as indicated (scale bar represents 10um). (c) HeLa cells in 6 well dishes where cold bound with AAV for dextran-Alexa 488 uptake or with AAV and Tfn-Alexa 488 for Tfn uptake, before being warmed up and cells removed by trypsin (at indicated times. 2mg/ml dextran was pulsed onto cells for 5 min after the indicated times before being washed off. Each point represents 20,000 cells sorted via FACS.

Figure 7

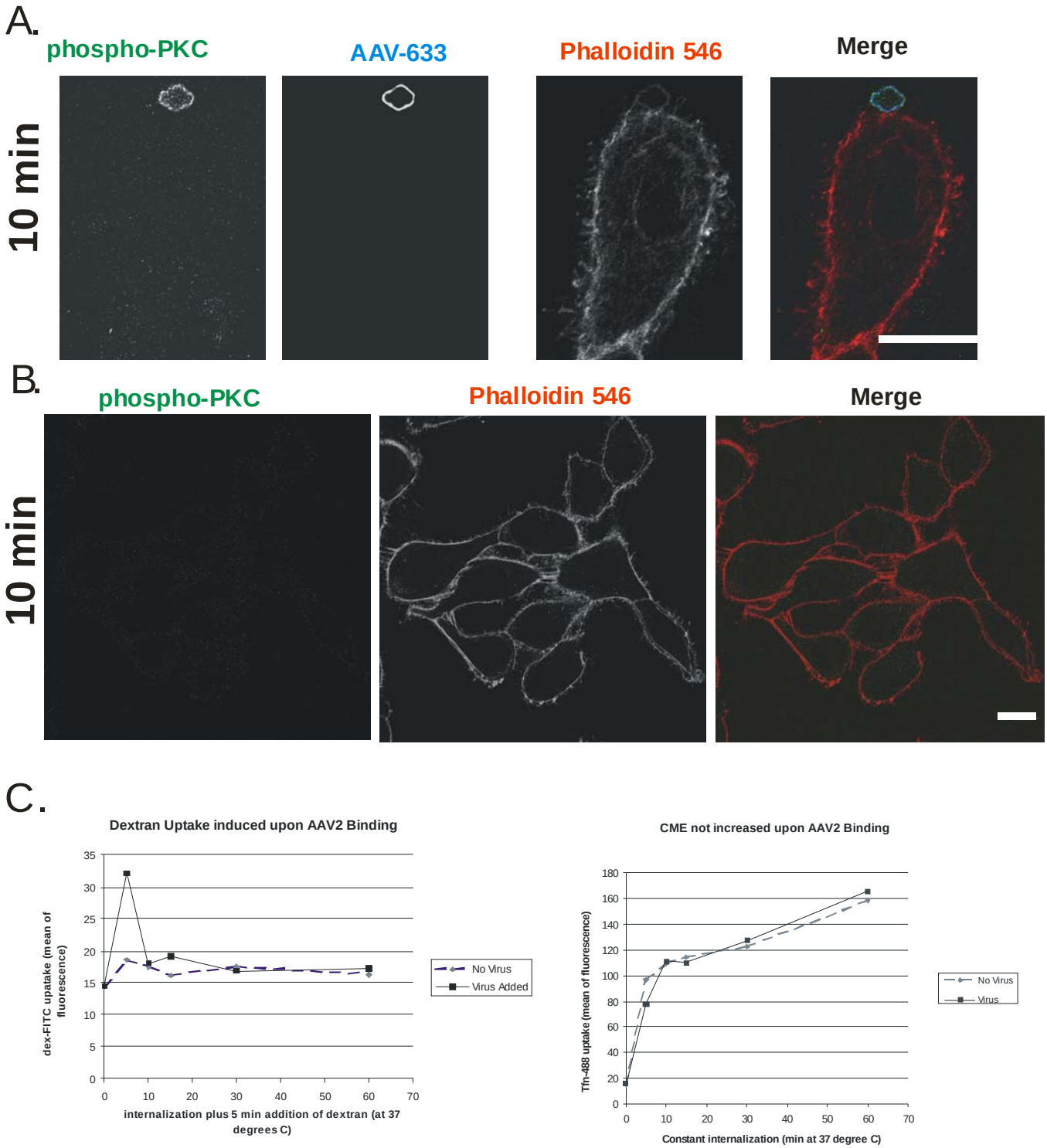
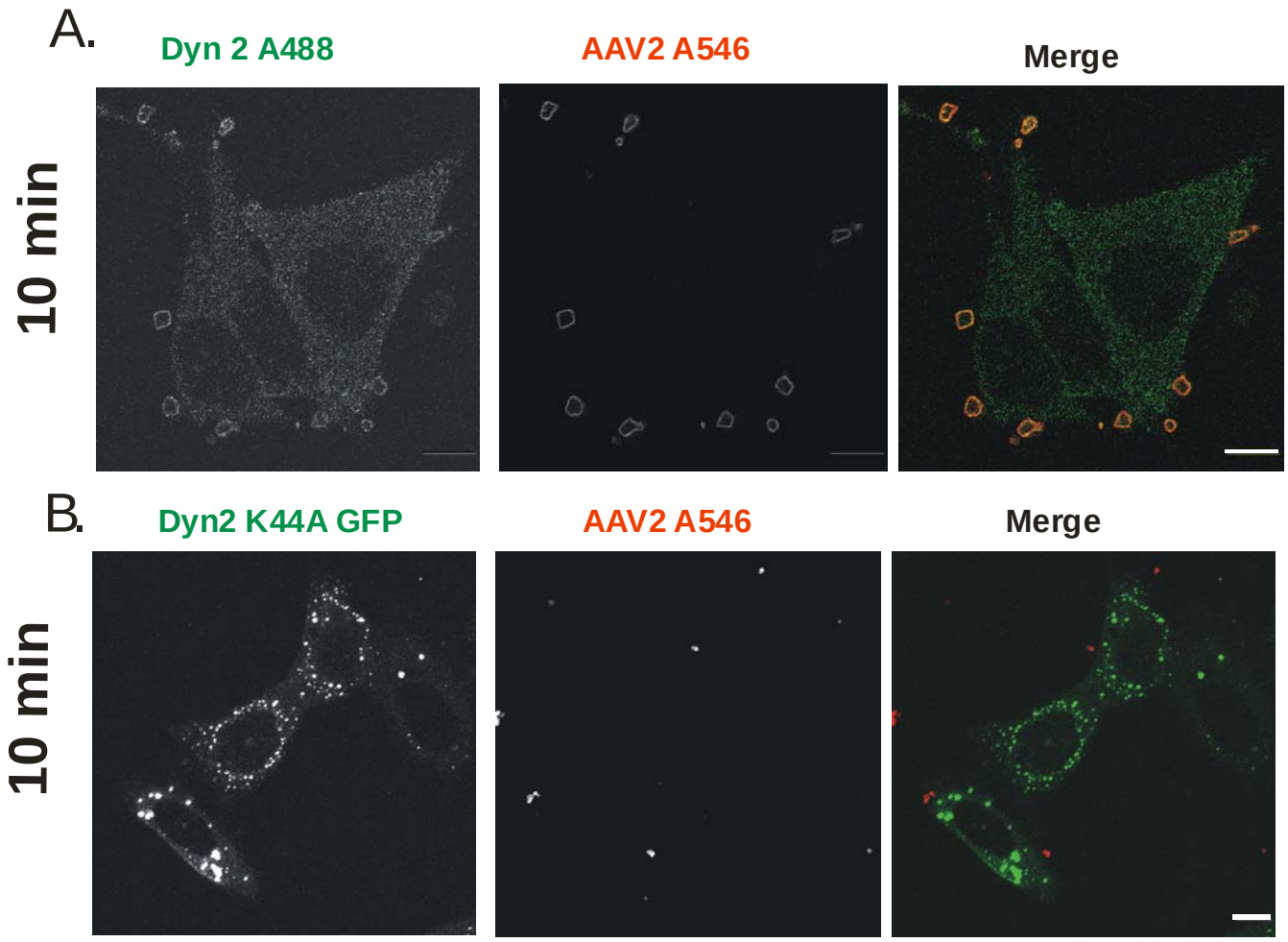

A.
phospho-PKC
AAV-633
Phalloidin 546
Merge
10 min
B.
phospho-PKC
Phalloidin 546
Merge
10 min
C.
Dextran Uptake induced upon AAV2 Binding
dex-FITC upatake (mean of fluorescence)
internalization plus 5 min addition of dextran (at 37 degrees C)
No Virus
Virus Added
CME not increased upon AAV2 Binding
Tfn-488 uptake (mean of fluorescence)
Constant internalization (min at 37 degree C)
No Virus
Virus

Figure (8) Dynamin is enriched in AAV induced macropinosomes: HeLa cells were stained for endogenous dynamin 2 (Alexa 488) (a) or transfected 18 hours over night with a dominant negative dynamin 2K44A-GFP construct (b) before cold binding with 1000 GC AAV and warming up at 37 degrees for 10 min. Cells were fixed and stained for visualization of AAV (Alexa 546). Image represents a 2um confocal slice, scale bar represents 10um. The maximum perimeter of macropinosomes were measured from confocal stacks of wild type HeLa cells or cells transfected with dynamin 2-GFP, syndecan 4 –PDZ- (c).

Figure 8


A.
Dyn 2 A488
AAV2 A546
Merge
10 min
B.
Dyn2 K44A GFP
AAV2 A546
Merge
10 min

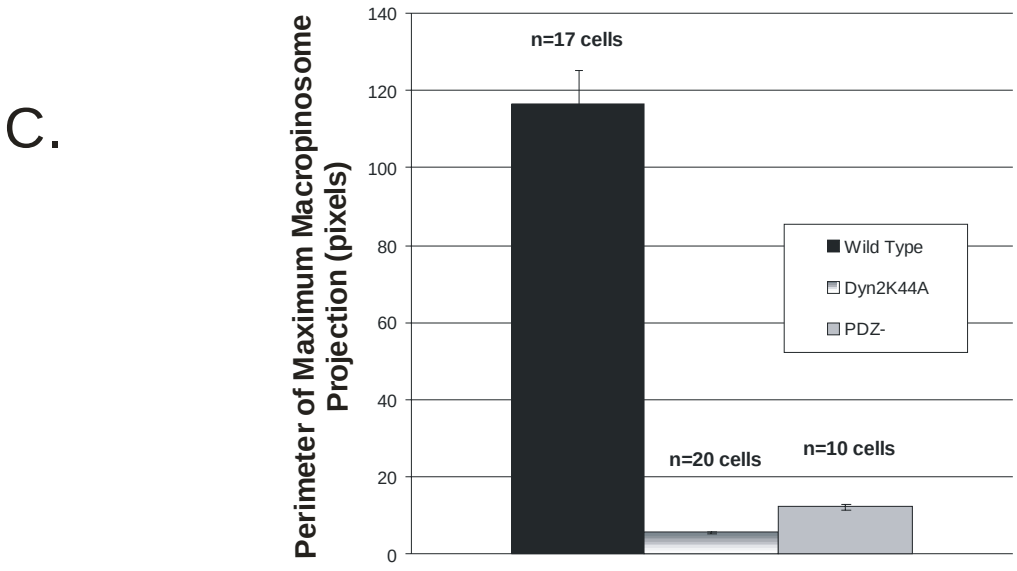

C.
Perimeter of Maximum Macropinosome Projection (pixels)
140
120
100
80
60
40
20
0
n=17 cells
n=20 cells
n=10 cells
Wild Type
Dyn2K44A
PDZ-

Figure (9) Syndecan 4-PDZ- stable transformants fail to produce large macropinosomes: G418 selected HeLa cells expression Syndecan 4 lacking the PDZ domain where cooled to 4°C for 30min in the presence of 10,000 GC AAV per cell before warming to 37°C for the indicated times. Cells were fixed and stained for AAV as well as with an antibody specific for the phosphorylated form of PKCα. PKCα membrane staining is localized specifically to sites of AAV macropinosomes (arrows) and not just surface bound AAV (arrow heads). Small macropinosomes seen primarily on lateral surfaces of cells (confocal slice through middle of cells are shown) a 0.2 μm slice shown, the scale bars represents 10μm.

Figure 9

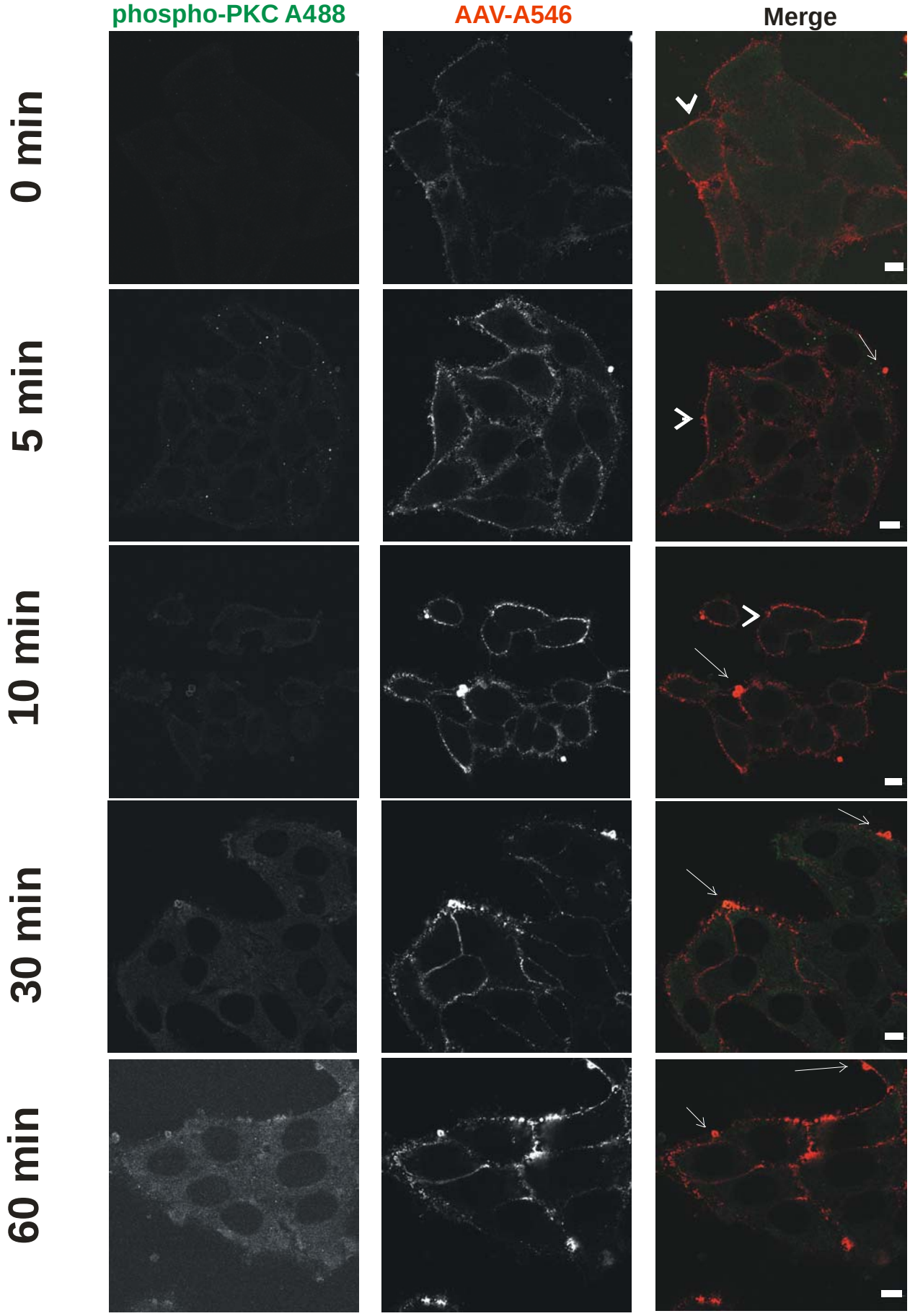
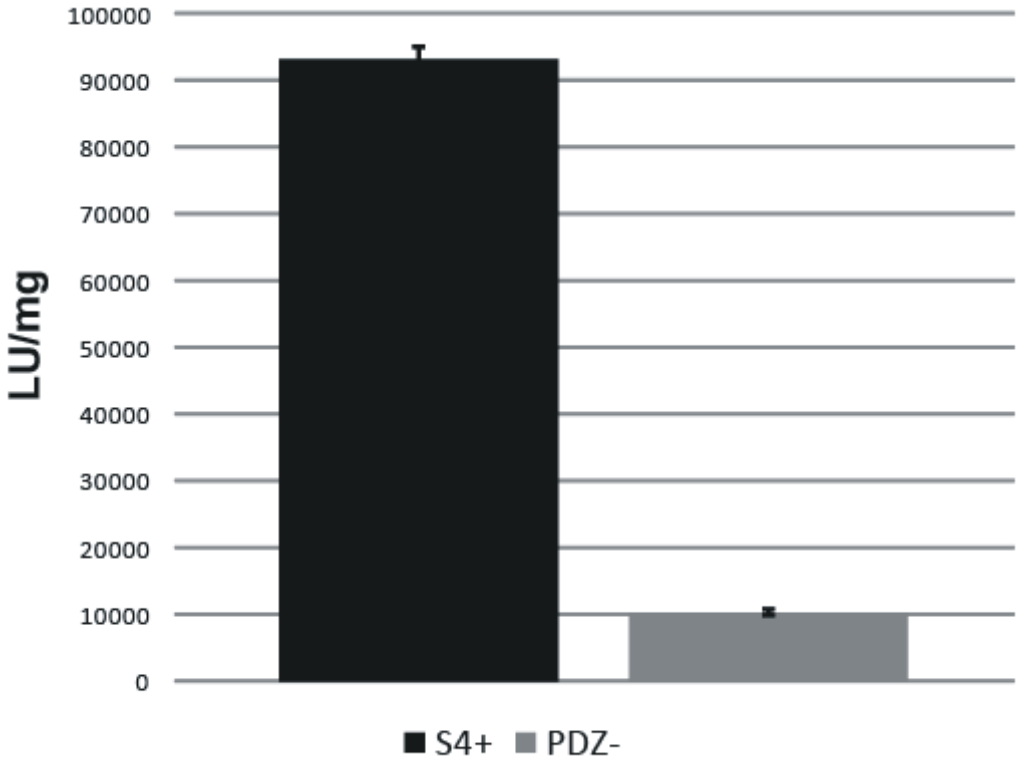


Figure (10) Amiloride or PDZ- mutants inhibit AAV infectivity: G418 selected HeLa cells expressing wild type Syndecan 4 (S4) or Syndecan 4 lacking the PDZ domain (PDZ-) were infected with AAV for 30min at 37 degrees before washing off un-internalized virus (a). (b) Increasing concentrations of Amiloride (7.5, 11.25, 15, 22.5, 30 mM) lead to complete inhibition of AAV transduction when present during viral binding and internalization (continuous); while exposure to these concentrations of drugs for 1 hour before but not during infection do not affect cells ability to be infected (Wash out). Data represents 3 separate experiments. Infectivity is expressed as luminescence units per milligram of cell lysate used. (c) PMA can rescue a PDZ domain deficiency. PDZ – stable HeLa (PDZ) cells and wild type HeLa cells (S4 NOT over expressing wild type syndecan) were seeded at 80% confluence in 12 well plates over night before treatment for 30min with DMSO or .1uM PMA for 30min before (Drug 1^{st}) or 30min 1 hour after (virus 1^{st}) AAV addition. Uninternalized virions were washed off 30min after addition and luminescence was assayed 72 hours later. Shown is the product of 3 independent experiments. (d) 2ug/mL Tfn A633 was cold bound to HeLa cells for 30min before warming up with DMEM at 37 degrees for 10min. Cells were fixed and stained for phosphorylated PKCα.

Figure 10

A.


100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
LU/mg
S4+
PDZ-

B.

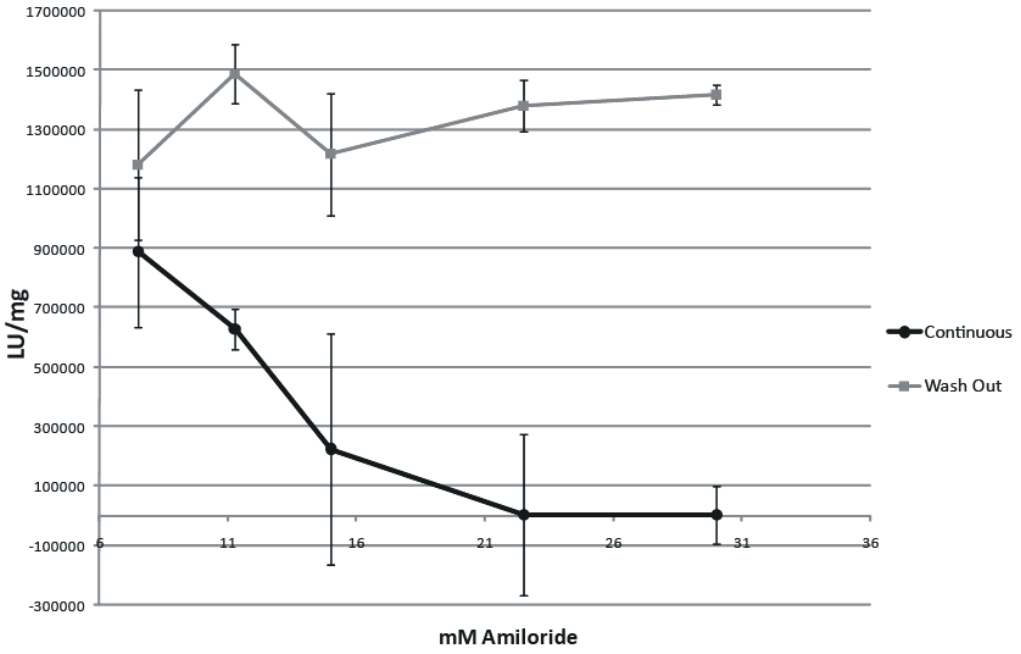
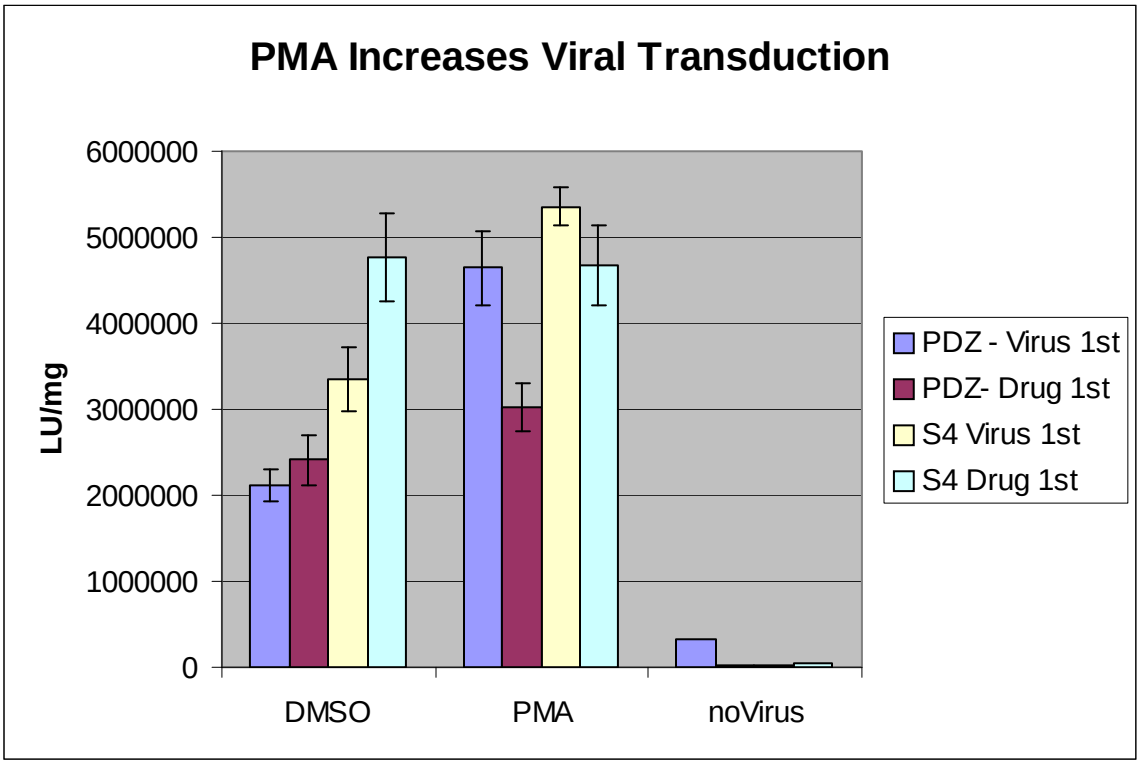

1700000
1500000
1300000
1100000
900000
700000
500000
300000
100000
-100000
-300000
LU/mg
6
11
16
21
26
31
36
mM Amiloride
Continuous
Wash Out

Figure 10 Continued

C.



D.

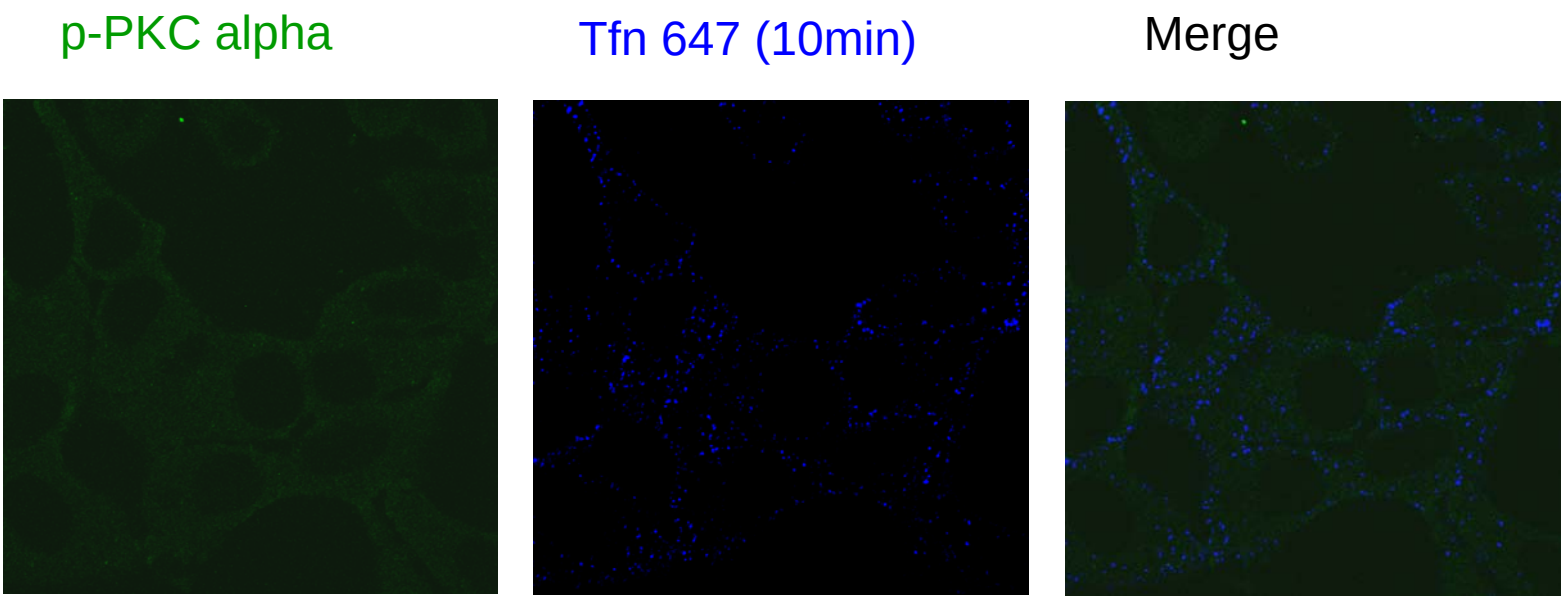
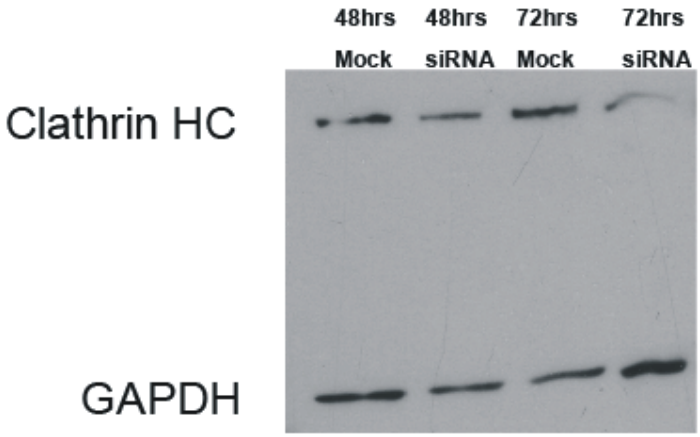


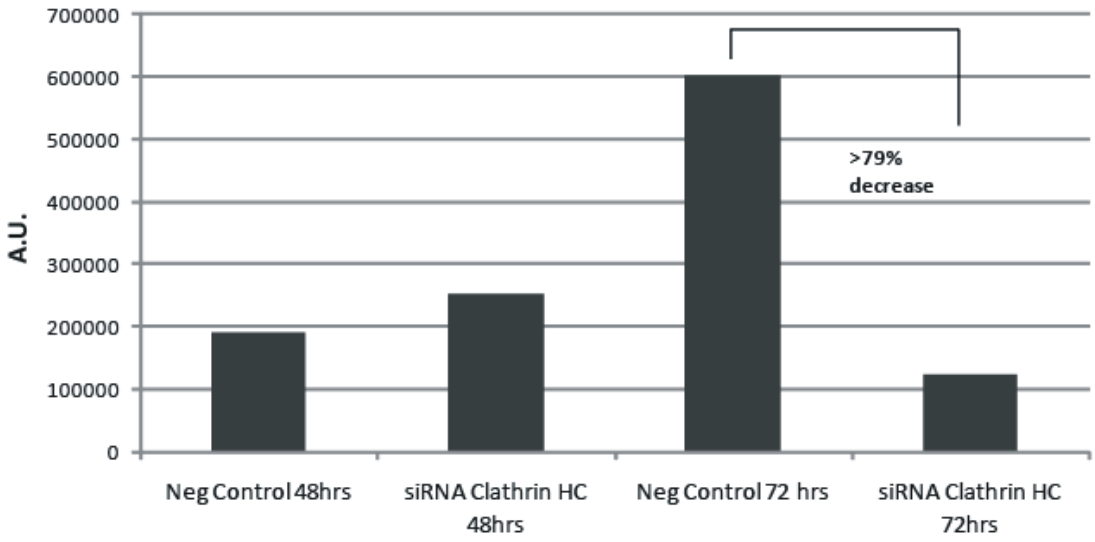
Figure (11) Inhibition of clathrin-mediated endocytosis by siRNA does not affect AAV transduction: To more clearly discern the exact role of the clathrin pathway in AAV entry, the heavy chain subunit of clathrin was knocked down by siRNA. (a) Western blot analysis of mock transfected cells (empty vector) or clathrin heavy chain specific RNA (siRNA) 48 hour or the first transfection (48hrs) or 24 hours after the second tranfection (72 hrs). Clathrin heavy chain protein and GAPDH (load control) were loaded on a 4-15% gradient SDS-PAGE. Protein levels were quantified using Meta Morph Imaging software (b). 72 hour control and siRNA knockdown cells were seeded in 12 well plates and infected with 10,000 GC AAV for 30min at 37 degrees before washing off uninternalized virus particles. Transduction of cells by AAV was measured 48 hours after infection in luminescence units per milligram of cell lysate (c).

Figure 11

A.



B.



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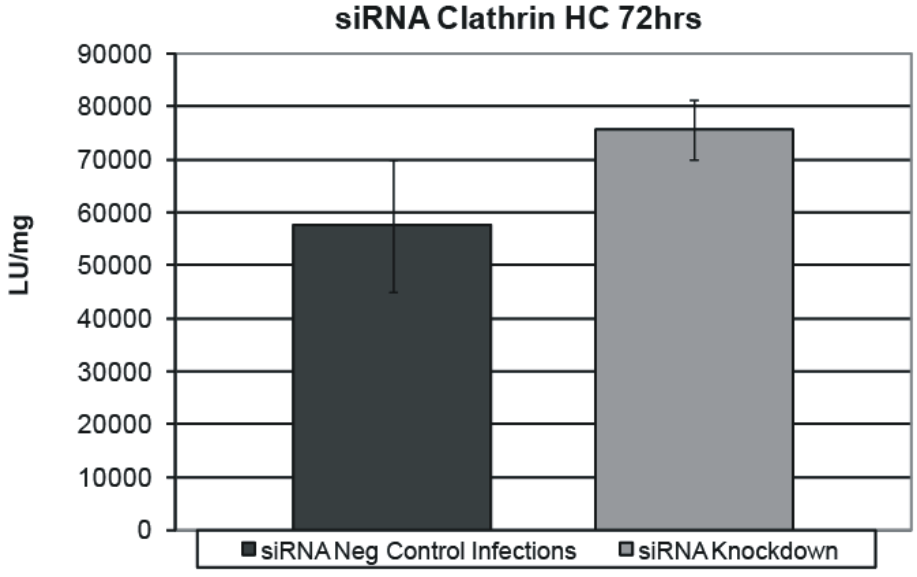
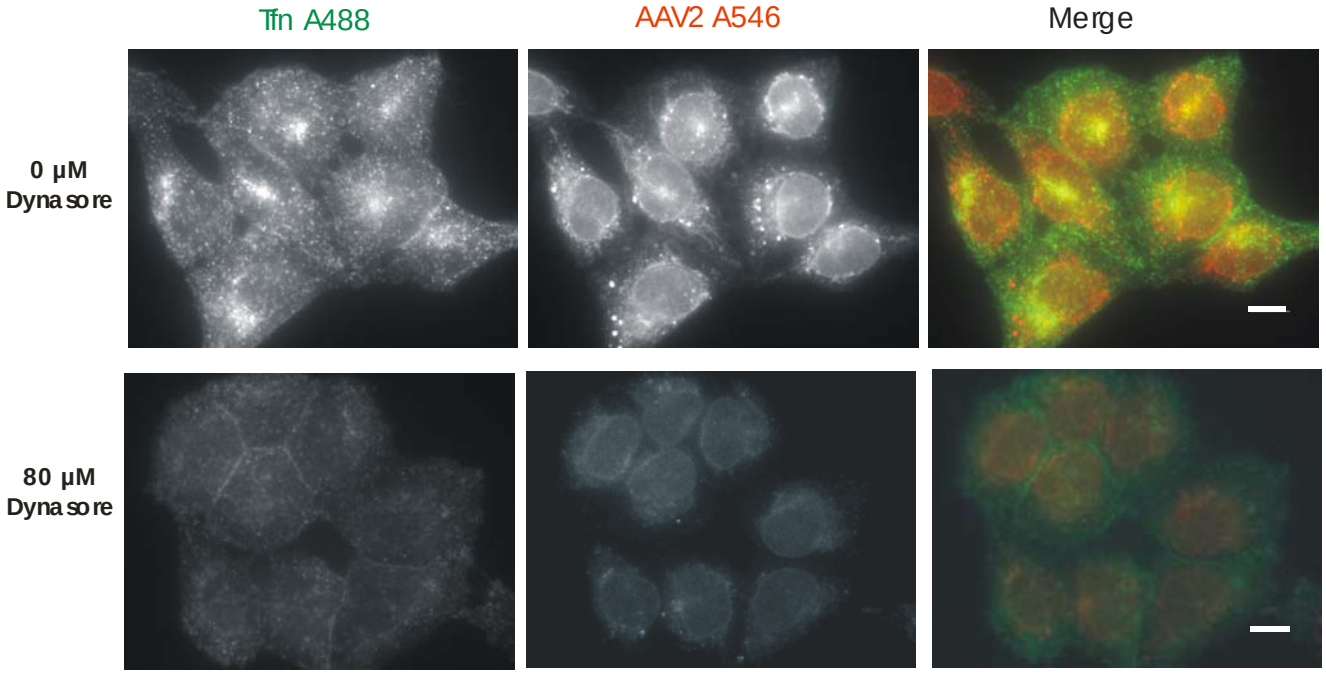


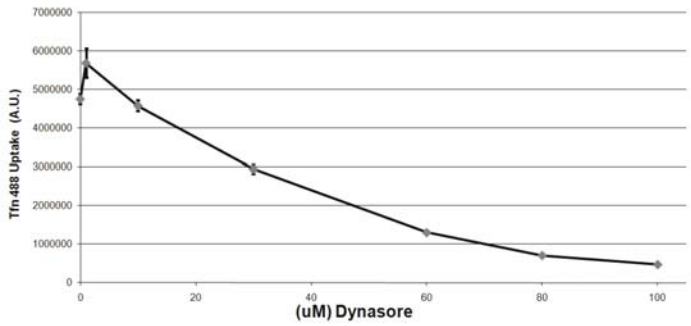
Figure (12): Dynasore inhibits clathrin-mediated endocytosis as well as AAV entry: HeLa cells were seeded at 30% confluence on MatTek dishes and 24 hours later, treated with Dynasore at 37 degrees (0, 1, 10, 30, 60, 80, 100uM in DMSO) for 30min. Cells were then incubated with 2ug Tfn Alexa 488 (b) or 1000 GC AAV (c) for 40min in the presence of Dynasore at 37 degrees. Uninternalized ligands were washed off and cells were fixed with 4% paraformaldehyde. Cells were stained for AAV (c) using Alexa 488 as the secondary antibody. Internalized ligands were visualized by wide field microscopy (a), quantified and analyzed using Meta Morp

Figure 12

A.



B.



C.

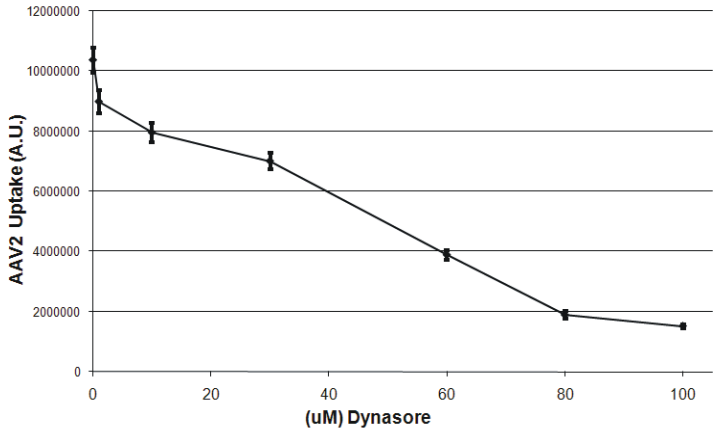


Figure 13

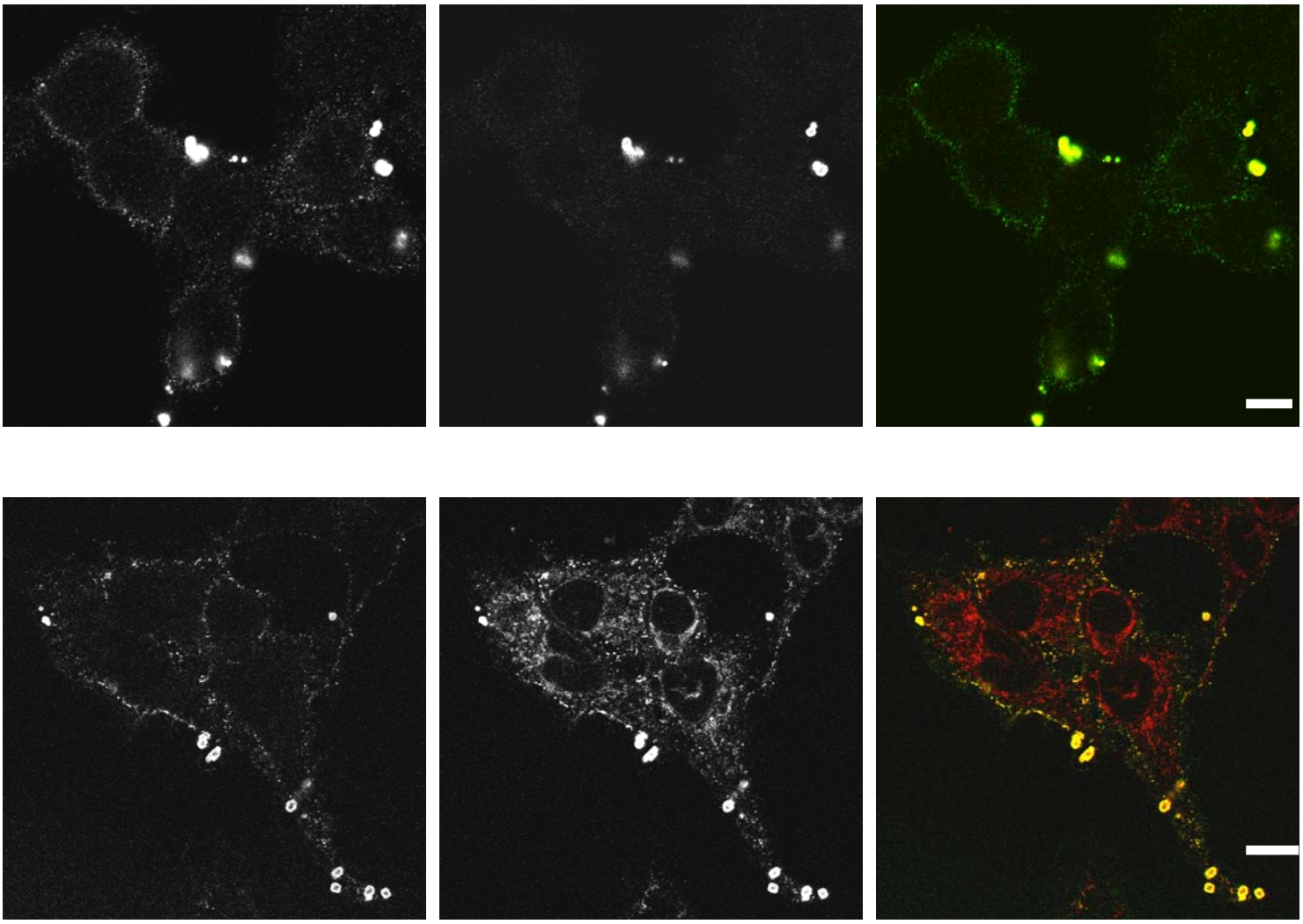


Fig 13: AAV colocalizes with both alpha 5 and V integrins at membrane ruffles: HeLa cells were cold bound with 1000 GC/cell AAV for 30min at 4 degree C before warming for 10min and visualizing with anti alpha 5 (top) or V (bottom) integrin. Merge shows colocalization as yellow. Scale bar is 10um.

Fig 14

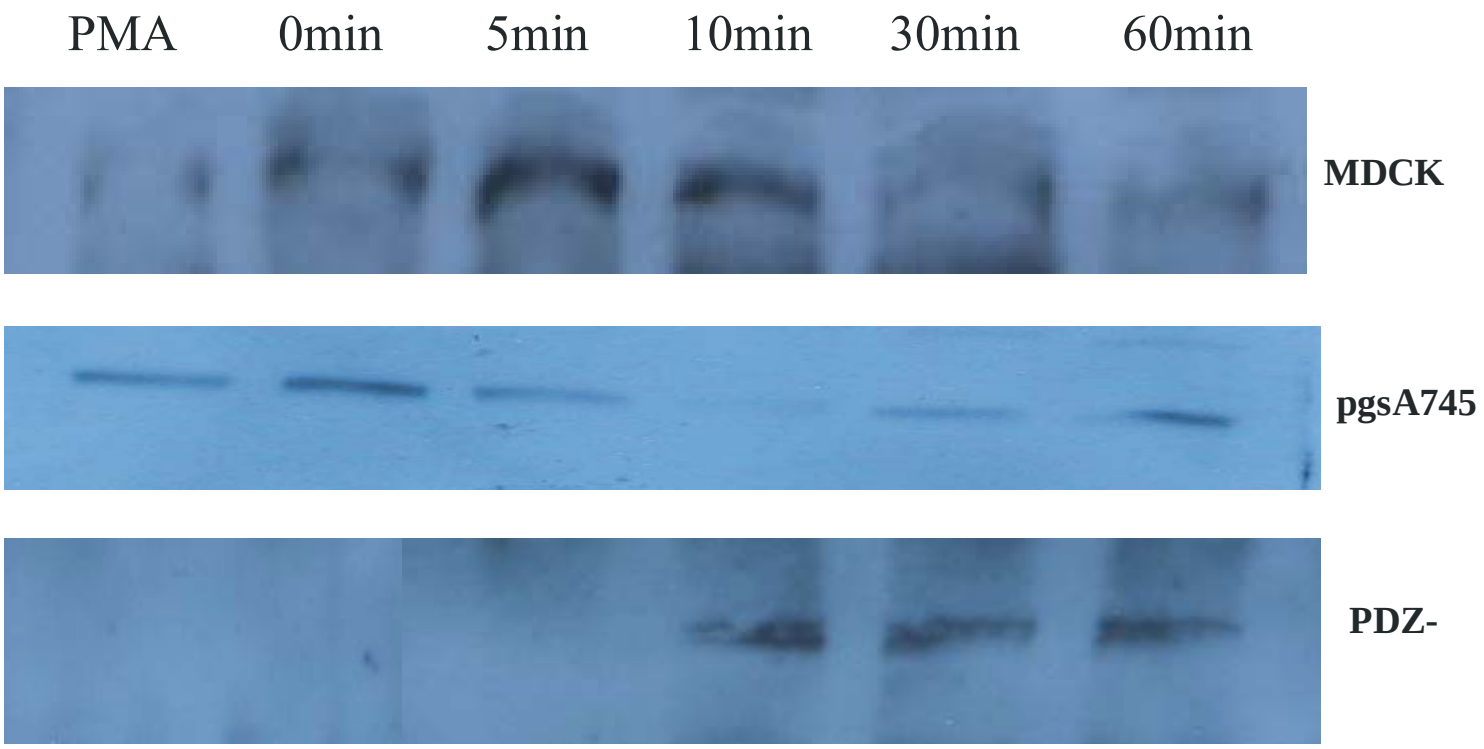


Fig 14 AAV induced FAK activation in various cell types: Cells were seeded at 80% confluence in 6 well dishes overnight. Cells were serum starved in DMEM containing 1% serum for 2 hours at 37 degrees. Cells were subsequently washed and incubated in completely serum free media containing 0.5mM sodium vanadate (prevents rapid dephosphorylation) and cold bound with AAV for 30min. Cells were immediately warmed with serum free media at 37 degrees for 0,5,10, 30 and 60min before cells were immediately lysed in 1X SDS for analysis by Western blot. 20ug of protein was loaded into each well and run on a 7% gel. FAK phospho-Tyrosine specific antibody was used.

Fig A: Focal Adhesion Site

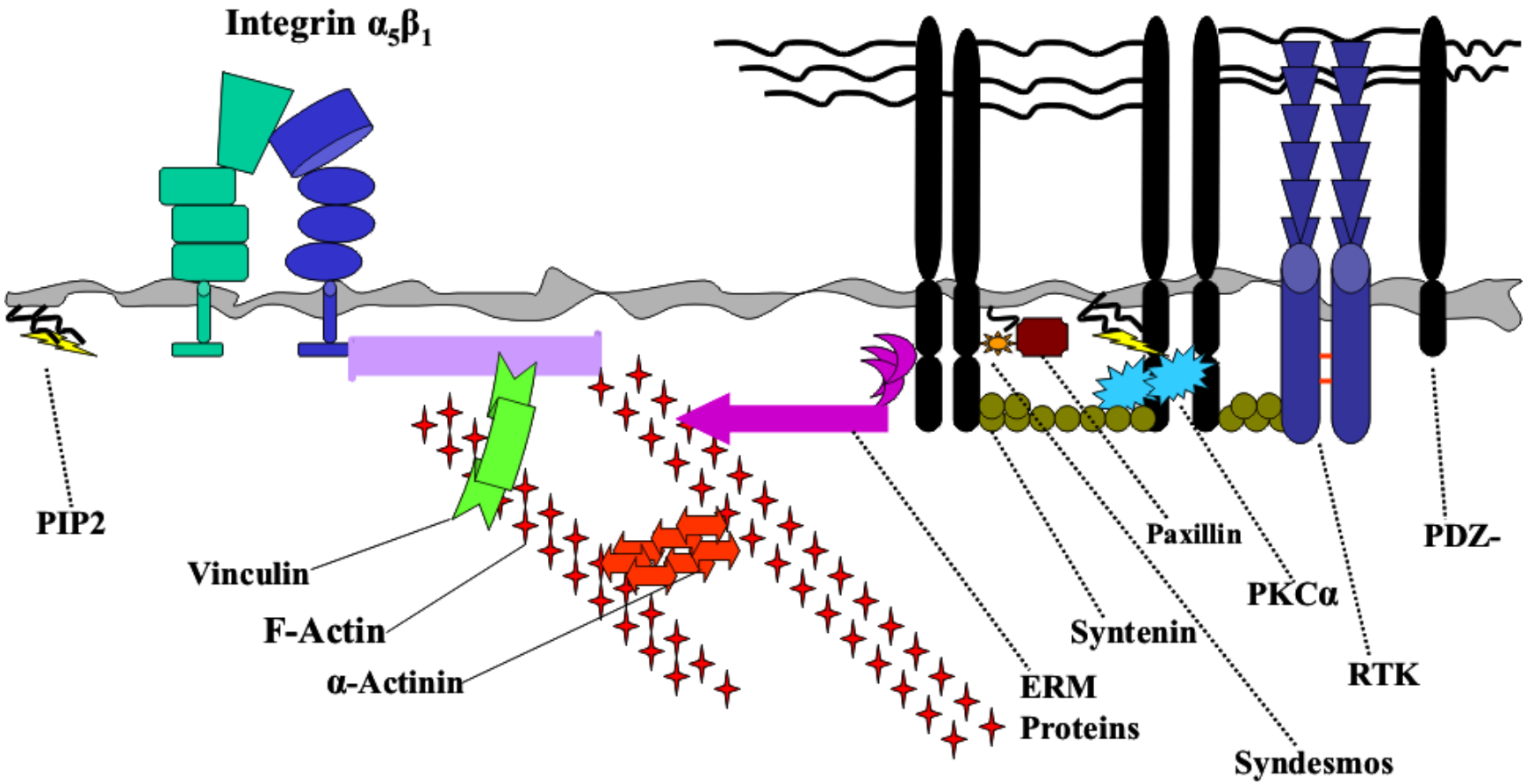


Fig A: Model for syndecan-integrin cooperation

Modified from *Bass, M. and Humphries, M. Biochem J. (2002) 368, 1-15*

Fig B: Syndecan 4, Integrins and FAK

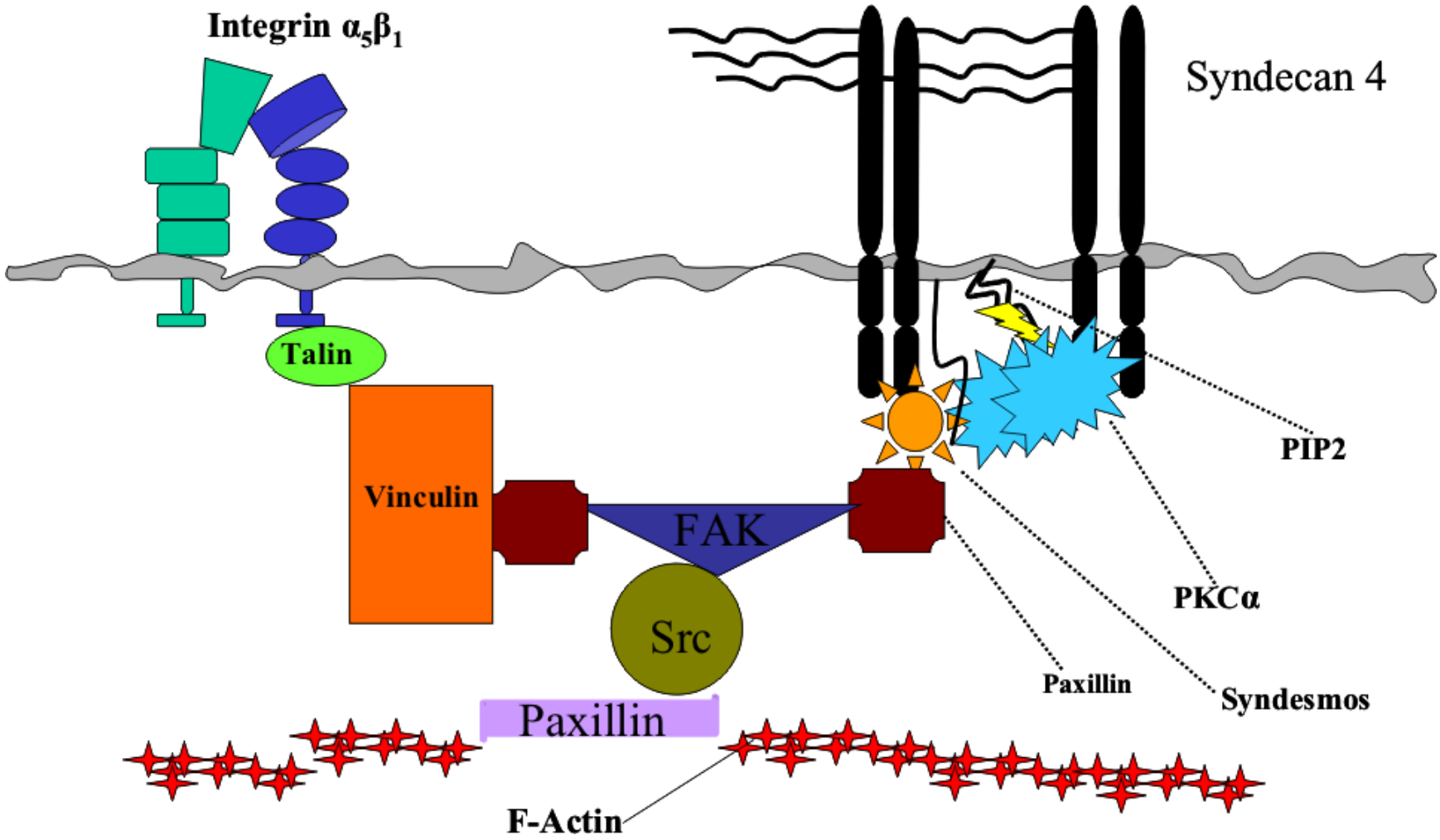
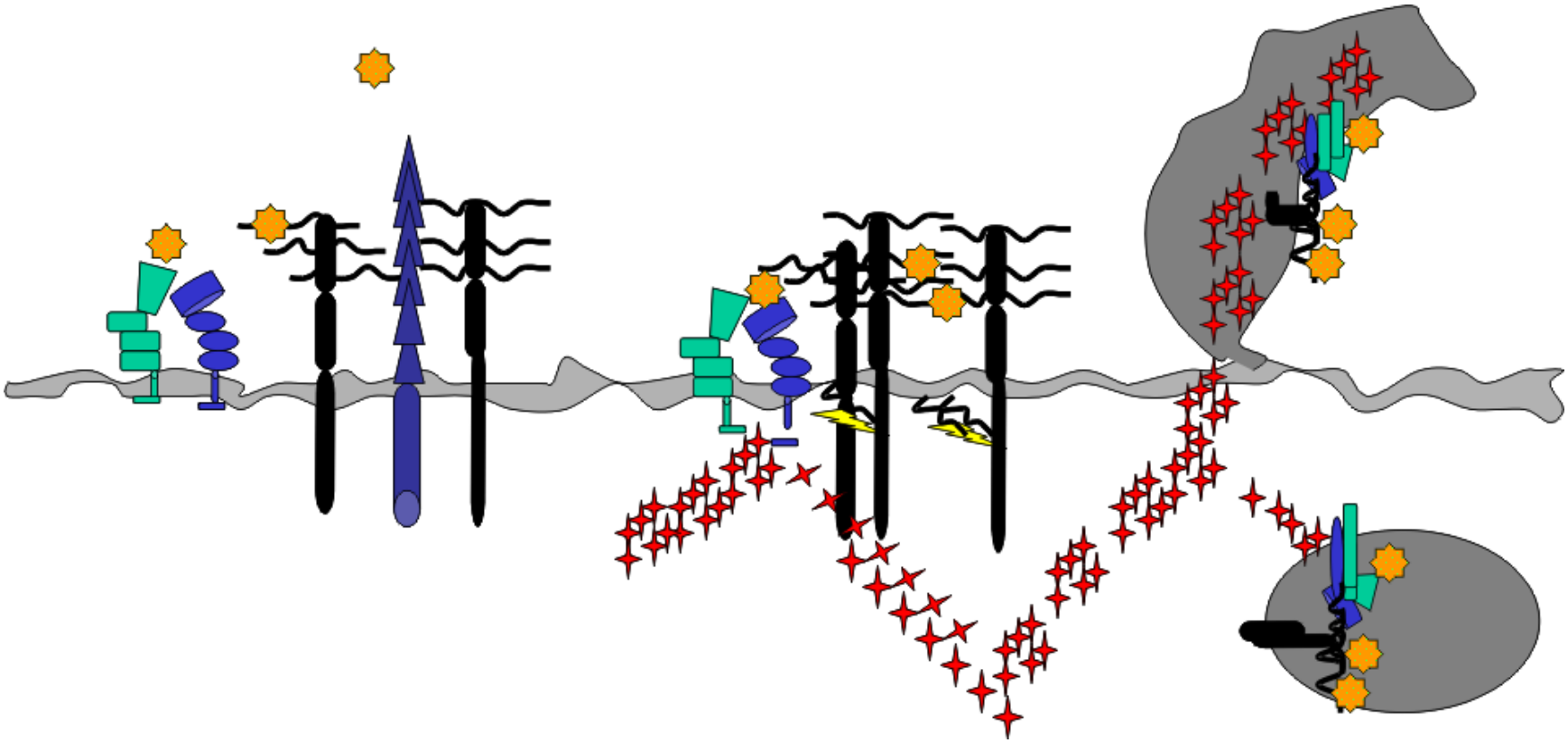


Fig B: Syndecans and integrins can individual upregulate Rho signaling and FAK activation
Modified from Denhez *et al*. JBC 277, 14, 12270-74 2002

Fig C: Model for AAV induced Macropinocytosis



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