The Role of Early Endosomal Sorting in Integrin Function

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The Role of Early Endosomal Sorting in Integrin Function began in Fall of 2008 and was performed by the protagonist, Nicole Waxmonsky, under the tutelage of her advisor, Dr. Sean D. Conner. Initial progress was presented to the GCD department in 2009, at which a simple inquiry by Dr. Tom Hays spurred an additional approach to the project. Research progress culminated in 2013 with the final production of a first-author publication in *Journal of Cell Science*.

ACT I. -- Scientific building blocks and pursuit of initial graduate work
ACT II. -- Chaos, confusion and a new beginning
ACT III. --- Scientific discoveries and professional development

Dramatis personæ

Nicole Waxmonsky	*Main protagonist; Naive, curious graduate student*
Sean Conner	*Advisor of the protagonist; Provider of encouragement, wisdom and constructive criticism; Financer*
Conner Lab	*Providers of critical analyses; Comic relief*
Haink Tu	*Best friend, confidant and life partner of the protagonist*
Thesis Committee: Robert Brambl Robert Brooker Tim Griffin David Zarkower	*Providers of scientific advice and support during the protagonist's graduate career*
Waxmonsky - Tu Clan	*Prominent cheerleaders and eternal optimists*
Kathleen Conklin, Robert Elde, Tom Hays and numerous CBS associates	*Creators of a positive environment for graduate students to succeed; Enablers of schemes plotted by overzealous graduate student representatives*
Sue Knoblauch	*Navigator of bureaucracy and Feline Rescuer-in-arms*
H/R Lab Group	*Comic relief; Prominent cheerleaders*
Jocelyn Shaw, Tom Neufeld	*Ensurers of Acts II & III*
RLP, DD, LR, BK, PJ, VD, EL, VC + more	*Mentors; Confidants; Enablers of adventures*
Lihsia Chen	*Provider of last minute heroics for thesis defense*

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Dedication

To those that can – *try*.

Do your best;

Find your place;

Then advance the human race.

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Chapter I: Integrin history

Section A: Description and discovery

Even before there was a receptor formally known as ‘integrin’, cell adhesion was recognized as having an important role in the maintenance of proper cellular function. An interest existed in trying to understand the mechanism behind how a cell adhered to its environment. It was observed that cells that underwent oncogenic transformation had changes at its cell surface as well as changes in morphology, from a flat to a rounded shape. While it was unclear to what extent this impacted cellular function, one phenotypic change was a loss of adhesion to a substrate (Gail & Boone, 1972).

Several observations led to the idea that a physical connection existed between the ECM (**e**xtra**c**ellular **m**atrix) and the cell cytoskeleton. Through the use of immunofluorescence to label fibronectin and cytoskeletal proteins, overlapping staining and a similar patterning were seen between fibronectin and actin, but not fibronectin and intermediate filaments (Hynes & Destree, 1978; Heggeness et al., 1978). Addition of fibronectin to transformed cells, which normally lack fibronectin and actin cables, altered morphology and adhesion to look more like normal cells and also had organized actin bundle structures (Ali et al., 1977). Disruption of actin filaments with cytochalasin B also led to a decrease in fibronectin at the cell surface; however, use of a microtubule disruption agent, colchicine, did not affect cell surface fibronectin amounts (Ali & Hynes, 1977).

In order to identify the transmembrane linkage, antibodies against surface membrane preparations were developed. Sera were incubated with tissue culture cells and the antisera that caused cells to form a rounded phenotype and detach was likely to be against a molecule at the cell surface involved in adhesion. These antisera were also used to immunoprecipitate a glycoprotein of 140 kD (Wylie et al., 1979, Knudsen et al., 1981). Some antibody preparations would specifically detach cells from a particular ECM, but not others (Brown & Juliano, 1985) which originally led to the idea that there was a certain receptor for a certain ECM component. It was not until a few years later that this binding ability could be attributed to a specific sequence: Arg-Gly-Asp or RGD (Pierschbacher & Ruoslahti, 1984). This sequence was found to be present in multiple extracellular matrix proteins, including fibronectin and vitronectin. This was initially thought to be a universal adhesion motif, however; not all integrins recognized RGD for adhesion and eventually other motifs were defined (Hynes, 1992).

Prior to 1986, integrins had been referred to by names of historical origin (140kd glycoprotein), general observation (CSAT - **c**ell-**s**ubstrate **at**tachment) or functionality (fibronectin receptor). ‘Integrin’ was formally proposed in 1986 to “denote their integral membrane nature and probable role in the integrity of both the extracellular matrix and the cytoskeleton” (Tamkun et al., 1986) and it ended up being an insightful choice.

Section B: Structure and function

Integrins are heterodimeric, transmembrane proteins that consist of one α subunit

and one β subunit (Hynes, 1992). Currently, there are known to be 18 α and 8 β subunits which combine to form 24 different heterodimers (Fig. 1). Many integrins are not specific for a particular ligand and ligands are not typically recognized by only a single integrin (Hynes, 1992). Integrins have been described in numerous organisms, from vertebrates, like humans, to invertebrates like *Drosophilia* or *C. elegans*, and to even simpler invertebrates like sponges (Burke, 1999; Hynes & Zhao, 2000). While integrin homologs have not been identified in non-metazoans, integrin-like proteins have been slowly uncovered in plants, which may even use a similar RGD motif recognition system and may have possible involvement in mechanosensing (Sun et al., 2000; Monshausen & Gilroy, 2009).

Integrins consist of three main domains that 1) bind external ligands, 2) traverse the plasma membrane and 3) interact with cytoplasmic factors (Fig. 2). Integrins interact, generally, with an extensive number of ECM components via divalent-cation binding, extracellular domains (van der Flier & Sonnenberg, 2001). Transmembrane helices span the plasma membrane, linking the internal tail and external ligand interaction domain. The short, internal tail of integrins interacts with cytoplasmic proteins that mediate activation, signaling and connections to the cytoskeleton and specific lipids in the plasma membrane. Most integrins act as linkages to the actin cytoskeleton; however, one integrin, α6β4, connects to intermediate filaments through its anomalously longer cytoplasmic tail (Hynes, 2002; Wehrle-Haller, 2012).

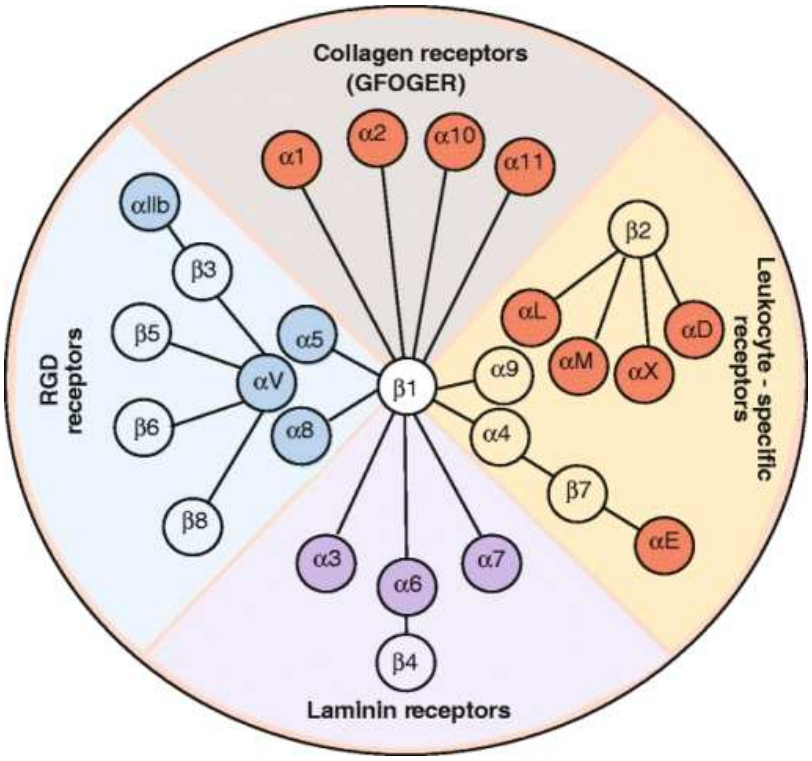

Figure 1: Vertebrate integrin family. 24 different heterodimeric pairs have been identified to assemble from the 18 α and 8 β subunits. Integrins are grouped according to their ligand interaction or on heterodimeric composition. Adapted from Barczyk et al., 2010.

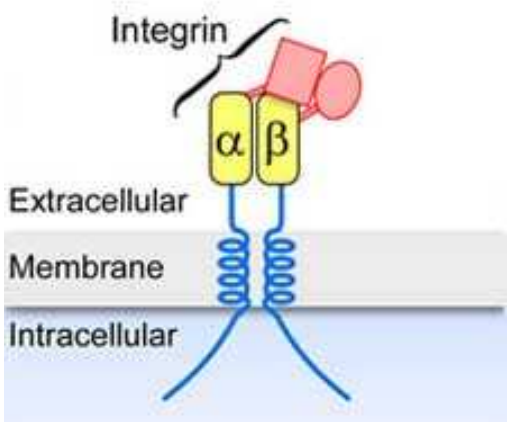

Figure 2: Basic integrin structure. External ligands, like the ECM, are bound by the large extracellular domain of integrins. The transmembrane domain crosses the plasma membrane, linking the extra- and intra-cellular domains. The intracellular domain of integrins is a short cytoplasmic tail that interacts with cytoplasmic factors, some of which link the integrin to the cytoskeleton. Adapted from Wang, 2012.

Studying the role of integrins via knockout mice has revealed that despite different integrins recognizing the same ligand, their functions appear to not overlap. Additionally, the mouse knockouts have revealed the myriad roles of integrins in development, varied cell processes and in human disease (Sheppard, 2000; Hynes, 2002). While some integrin knockouts appear to have no to mild phenotypes (β5 - Huang et al., 2000; α1 - Gardner et al., 1996), many lead to severe developmental, vascular or immunological defects, or even lethality (Hynes, 2002).

Section C: Activation, signaling and promotion of cell function

As transmembrane linkages, integrins must integrate intracellular molecular interactions (inside-out signaling) along with extracellular force cues (outside-in signaling). Resulting from this, many cell processes are regulated such as motility, polarity, proliferation, adhesion and survival. (Hynes, 2002; Harburger & Calderwood, 2009).

The conformational state of the integrin is important in modulating its response to extracellular environmental information and intracellular molecular signals (Fig. 3). Careful electron microscopy, crystallography and other structural analyses have exposed the highly dynamic conformations in which integrins can exist. These conformations change through a ‘breathing’ movement and exist in equilibrium with each other (Beglova et al., 2002; Takagi et al., 2002). The generally accepted model of integrin conformations is the ‘switchblade’ model (Beglova et al., 2002) which details three main

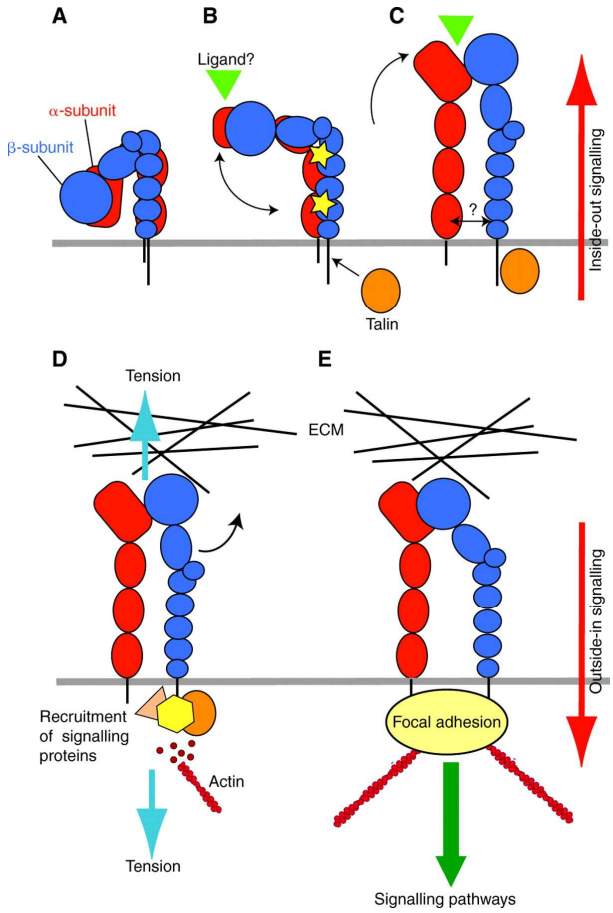

Figure 3: Integrin conformations. Integrins exist in three primary states: A) inactive or bent; B) primed; C) active or ligand-bound. Talin binding of the cytoplasmic tail leads to recruitment of other cytoplasmic factors that initiate cytoskeletal interactions and formation of focal adhesions (D & E). Adapted from Askari et al., 2009.

states of integrin conformations: ‘off’, ‘inactive’ or ‘low-affinity’ (bent), ‘primed’ or ‘high-affinity’ (more extended), ligand-bound (full extension; Fig. 3) It is still unclear as to what functions modulated by certain combinations of ligands, binding partners, and presence of specific divalent cations correspond to specific conformations or to any functional intermediates that may exist between the known integrin states (Hynes et al., 2002; Takagi et al., 2002; Nishida et al., 2006; Askari et al., 2009).

Inside-out activation

The maintenance of a bent or inactive conformation of an integrin heterodimer is maintained through the interactions of the α and β integrin tails subjacent to the plasma membrane (Lu et al., 2001). Destabilizing this interaction induces the integrin to enter a more extended conformation, which is of importance in non-adherent cells, like platelets, which need integrins to be activated during specific events. The main molecular factor that separates this interaction and induces integrin activation is talin. Talin mediates binding to a NPXY motif in β integrin tails through its **p**hospho**t**yrosine-**b**inding (PTB) domain. While many PTB-containing proteins can also bind to β integrin tails, only talin can induce integrin activation (Takagi et al., 2002; Wegener et al., 2007; Askari et al., 2009). Talin contains a loop in its F3 subdomain within a N-terminal FERM (**4**.1 protein, **e**zrin, **r**adixin, and **m**oesin) domain which is critical for its interaction with the MP (**m**embrane-**p**roximal) region of the β integrin tail. Other proteins that have PTB domains lack this loop structure and thus, do not additionally interact with the β integrin tail (Wegener et al., 2007). Additionally, talin binds to cytoskeletal-associated proteins (e.g.

vinculin) and actin filaments, providing an important link between the cytoskeleton and integrins (Critchley & Gingras, 2008; Harburger & Calderwood, 2009). Talin promotion of integrin activation can be modulated through competitive binding with other PTB domain-containing proteins and can synergize activation with additional cytoplasmic factors (Kiema et al., 2006; Bouaouina et al., 2008; Ma et al., 2008; Harburger & Calderwood, 2009).

Outside-in activation

The interaction of integrins with ligand, integrin clustering or tension cues can induce a wide array of functional outcomes in adherent cells such as the initiation of downstream signaling events that lead to alterations in gene expression to cell migration and spreading (Hynes, 2002; Margadant et al., 2011). Inside-out activation, where integrin tail separation in the heterodimer induces an extended conformation of the integrin extracellular domain, increases integrin affinity to extracellular ligands. These increased interactions with the ECM induce clustering of ligand-bound integrins which then leads to the formation of adhesion structures, recruitment of cytoskeletal-binding factors and engagement with the cytoskeleton. The understanding of the involved mechanisms that modulate integrin binding affinities is still poorly understood (Hynes, 2002; Takagi et al., 2002; Qin et al., 2004; Legate et al., 2009).

Signaling from activation

The use of integrin function-blocking antibodies or cell-ECM adherence promotes

gene expression and affects cell function (Haskill et al., 1988; Werb et al., 1989; Miranti & Brugge, 2002). These observations spurred investigations into identifying cytoplasmic factors that were involved in mediating the signaling transduction events from integrin status. Today, the number of molecules that associate with adhesion structures (called the 'adhesome') is estimated to be 180 different factors, which are sorted into two main functional subnetworks: structural and regulatory. While these factors have been placed into different subnets, there is considerable cross-talk between the subnets. The structural subnet consists of molecules that form the linkage between integrins and the actin cytoskeleton, while the regulatory subnet can be further divided into several subnets: serine/threonine kinases and phosphatases, tyrosine kinases and phosphatases, Rho GTPases, lipids and molecules involved in proteolysis (Fig. 4; Zaidel-Bar et al., 2007; Zaidel-Bar & Geiger, 2010; Geiger & Yamada, 2011). The interplay between these molecules, the actin cytoskeleton and the integrin receptors can both initiate and regulate most known signaling pathways (Hynes, 2002; Legate et al., 2009; Geiger & Yamada, 2011).

Section D: Focal adhesions

Focal **a**dhesions (FAs) are the common unit of adhesion and the understanding of the structure and function of FAs has evolved considerably from their discovery in the early 1970s. FAs now involve upwards of 100 different proteins involved in various functions, from scaffolding to signaling, that complex with an integrin adhesion receptor and link primarily to the actin cytoskeleton (Dubash et al., 2009; Zaidel-Bar & Geiger,

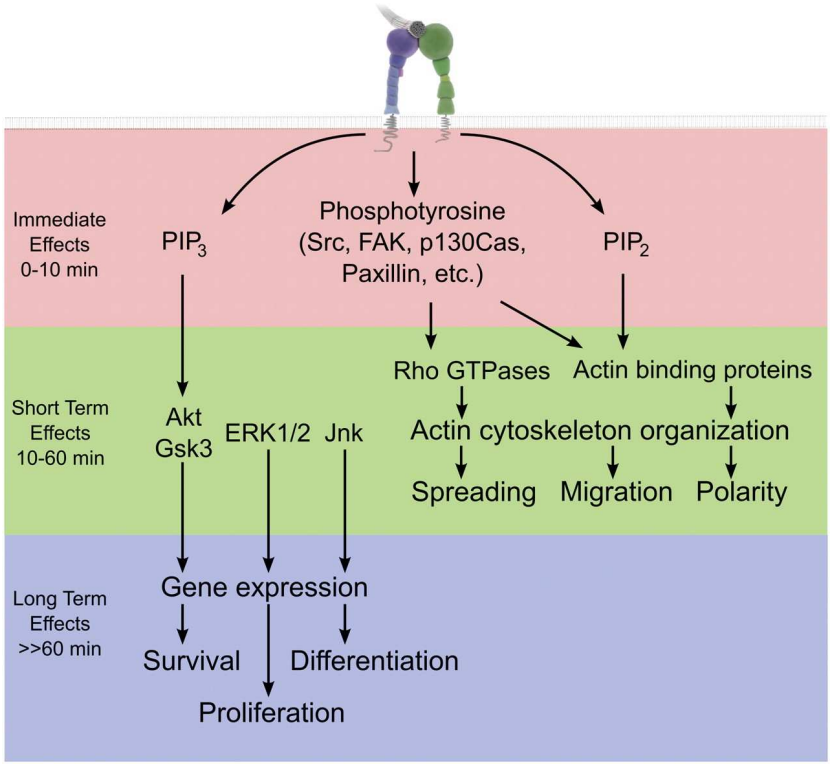

Figure 4: Integrin post-activation downstream signaling events. Integrin activation leads to many alterations in cell function that can be divided into three time periods. Within minutes of integrin activation, phosphorylation occurs of cytoplasmic factors, many of which are at the focal complex. Next, signaling pathways are induced leading to cytoskeletal re-organization. Finally, signaling outcomes influence cell viability, growth and changes in morphology. Adapted from Legate et al., 2009.

2010). The formation of a focal adhesion is a highly dynamic process which is initiated through a transient and rapid attachment process that is hyaluronan-dependent (Zaidel-Bar et al., 2004). Shortly after an initial attachment, integrin-positive adhesions begin to form near the leading edge of the lamellipodium that are called **f**ocal **c**omple**x**es (FCXs). These dot-like small adhesions can form and persist and eventually become FAs. They may also dissociate and disappear, or persist until new FCXs are formed in front of them, advancing with the leading edge (Fig. **5**; Nobes & Hall, 1995; Zaidel-Bar et al., 2004). The assembly of FCXs is hierarchical and the cytoplasmic protein composition present at the adhesion site determines the 'age' of the FCXs and also when FCXs become FAs (Zaidel-Bar et al., 2003). For example, paxillin, an actin-integrin linkage protein, is found early in FCXs and its phosphorylation state modulates its interactions with other adaptors (Laukaitis et al., 2001; Zaidel-Bar et al., 2003; Legate et al., 2009). Vinculin is another cytoplasmic protein that scaffolds between the actin cytoskeleton and integrins. Vinculin enters the focal complex later than paxillin and strengthens this linkage. Absence of vinculin reduces the actin-integrin linkage stability and prevents the FCXs from maturing into FAs (Zaidel-Bar et al., 2003; Gallant et al., 2005; Humphries et al., 2007; Legate et al., 2009).

The transition into FAs from FCXs results from several changes, which stem from the activation of the small GTPase, RhoA, induced through tension-dependent mechanisms. The adhesion contact will enlarge, elongate, localize near the ends of actin filament bundles and become zyxin-positive (Ballestrem et al., 2001; Zaidel-Bar et al.,

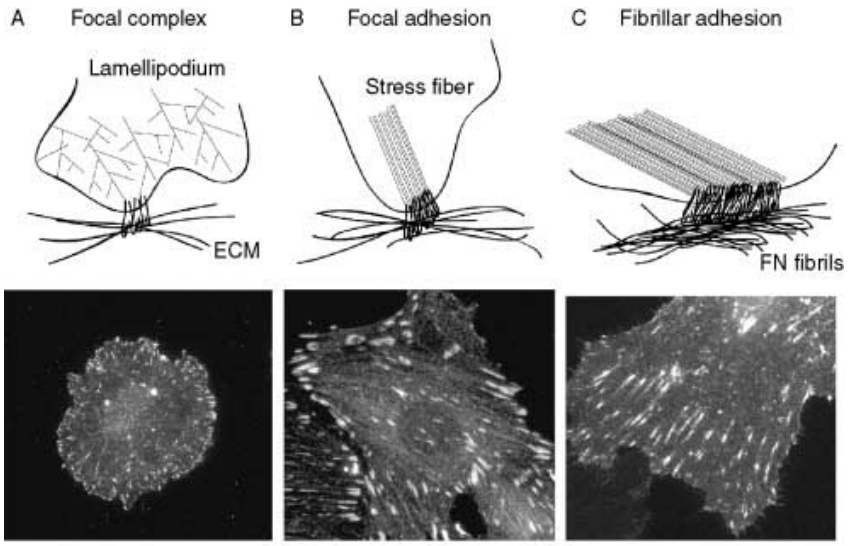

Figure 5: ECM adhesion structures. A) Focal complexes are small adhesion structures that primarily form at the cell periphery. These structures may exist transiently or mature into focal adhesions (B). Focal adhesions structures are where integrin-enriched plaques, at the plasma membrane, anchor to actin filament bundles. C) Fibrillar adhesions mature from focal adhesions and align along extracellular fibronectin. These structures have also been implicated in ECM assembly and remodeling. Adapted from Dubash et al., 2009.

2003; Zaidel-Bar et al., 2004; Dubash et al., 2009). Two RhoA targets promote FCX maturation into FAs: ROCK (Rho-associated kinase) and mDia1 (mammalian Diaphanous; formin homology protein). While both targets of RhoA each themselves have numerous downstream targets, in general, ROCK stimulates myosin II activity and cell contractility and mDia1 regulates actin polymerization and some microtubule targeting. In addition to biochemical stimulation, local FA growth can be promoted directly via a force-induced, mechanosensory mechanism that is not well understood (Amano et al., 2000; Fukata et al., 2001; Geiger & Bershadsky, 2001; Ishizaki et al., 2001; Bershadsky et al., 2006; Dubash et al., 2009).

Section E: Trafficking of adhesion receptors

The ability of the cell to traffic integrins – to internalize, sort and re-direct integrins – is imperative for maintenance of cellular functions, such as motility, adhesion and survival. Our understanding of the mechanisms governing integrin function, specifically of integrin trafficking, has been growing steadily since the late 1980s when Bretscher showed that the integrin α5β1 (referred to as fibronectin receptor) was able to internalize and recycle back to the cell surface, participating in an endocytic-exocytic cycle as previously described for the receptors for transferrin and LDL (Bretscher, 1989). This result provided an understanding for how a cell could detach and transport integrins from the rear of the cell to a leading edge in order to achieve forward motion. More intriguingly, it linked an important cellular function, motility, to trafficking and implied that regulation of trafficking could control the cell's interactions with its environment

(Bretscher, 1989; Bretscher, 1992; Caswell & Norman, 2006).

Endocytosis

Integrin endocytosis is important for polarized cell migration and disruption of integrin uptake leads to defective focal adhesion disassembly (Nishimura & Kaibuchi, 2007; Ezratty et al., 2009). Integrin heterodimers are capable of following multiple internalization routes into the cell which include clathrin-dependent, caveolin-dependent and clathrin- and caveolin-independent pathways. The route that an integrin is endocytosed is heterodimer-specific, as some integrins are dependent on one pathway while others are promiscuous (De Deyne et al., 1998; Pellinen et al., 2008; Shi & Sottile, 2008; Caswell et al., 2009).

Clathrin-dependent internalization

Most integrin β-subunits contain conserved NXXY (X=any amino acid) motifs in their cytoplasmic tails. Mutation of these motifs can block endocytosis (Pellinen et al., 2008). These motifs often recruit proteins that contain phosphotyrosine-binding (PTB) domains, many of which are clathrin adaptors, like Disabled (Dab)-2, Numb, and autosomal recessive hypercholesterolemia (ARH) protein (Calderwood et al., 2003; Nishimura & Kaibuchi, 2007; Caswell & Norman, 2008). Knockdown of clathrin, clathrin adaptors, like AP-2 or Dab-2, or dynamin 2 leads to defects in cell migration and focal adhesion disassembly (Chao & Kunz, 2009; Ezratty et al., 2009).

Clathrin-independent internalization

Several internalization pathways that are not clathrin-dependent are constitutive uptake, phagocytosis, macropinocytosis and caveolin-dependent uptake (Nichols & Lippincott-Schwartz, 2001; Ramsay et al., 2007). These divisions are further broken down into categories that reflect dynamin-dependence and by which GTPase is commonly associated with the particular pathway (Mayor & Pagano, 2007). Similarly to clathrin-dependent endocytosis, integrin internalization by clathrin-independent mechanisms can be induced by growth factor stimulation, can utilize both the actin and microtubule cytoskeleton for vesicle transport and are regulated through kinase activity. One proposed role for clathrin-independent internalization is in ECM remodeling/turnover and cell migration (Mundy et al., 2002; Upla et al., 2004; Pellinen & Ivaska, 2006; Shi & Sottile, 2008; Gu et al., 2011; Margadant et al., 2011).

Following internalization, integrins enter into a compartment called the sorting or early endosome. From this endosome, the integrin can be returned to the plasma membrane via short- or long-loop recycling or trafficked to lysosomes for degradation.

Degradation

Degradation of integrins is important for efficient cell migration and is proposed to regulate the amount of functional integrin at the cell surface by allowing the cell to sort defective integrins to the lysosome (Caswell & Norman, 2006; Lobert et al., 2010). Much of the integrin that is internalized is recycled and sent back to the cell surface rather than

degraded (Bretscher, 1989; Sczekan & Juliano, 1990; Bretscher, 1992; Pellinen & Ivaska, 2006; Lobert et al., 2010). Relatively recently, integrin targeting to the lysosome was shown to be dependent on ubiquitination, similar to how other receptors are marked for degradation. In migrating fibroblasts, α5 integrin (in the heterodimer α5β1) was ubiquitinated and this was found to be in response to fibronectin binding. Ubiquitination of α5β1 integrin was required for its sorting to the degradative pathway and the integrin directed to the lysosome was also found to be complexed with its bound ECM ligand, fibronectin. It is proposed that directing integrin-ECM complexes to the lysosome prevents accumulation of these bound complexes in the cell, which can avert improper signaling events (Lobert et al., 2010). These findings contrast earlier work which showed that the αvβ5 integrin and its ECM ligand, vitronectin (VN), internalized together, but the integrin was found to be sorted into a different endosomal compartment from VN, which was directed to lysosomes (Memmo & McKeown-Longo, 1998). Additional research will need to clarify whether other integrins are sorted to the lysosome similarly to α5β1 and if these sorting mechanisms are cell-type specific.

Recycling

Proper adhesion receptor recycling is important for many cell processes, including cell division, maintenance of cell polarity, neuronal remodelling and migration (Grant & Donaldson, 2009). As integrins are primarily returned to the cell surface post-internalization, understanding mechanisms of recycling is imperative to understand cellular function (Bretscher, 1989; Sczekan & Juliano, 1990; Bretscher, 1992; Pellinen &

Ivaska, 2006; Lobert et al., 2010). Recycling maintains the distinctiveness and separation of specific functions between compartments and organelles. How receptors traffic through the endosome is a combination of the properties of the receptor itself, the factors responsible for receptor sorting at the particular endosomal compartment and how the various trafficking routes are linked (Maxfield & McGraw, 2004). While the primary trafficking routes and associated molecules are common between different receptor types in recycling, the mechanisms and molecules associated with the trafficking of integrin adhesion receptors and their functional relevance will be specifically discussed below, particularly for αvβ3 and α5β1 integrin, where the majority of work has been focused. Integrin function has been increasingly demonstrated to be influenced by the route the integrin traffics through the cell, and these routes are influenced by integrin association with small GTPases, kinases and motor proteins, and growth factors and their receptors. (Pellinen & Ivaska, 2006).

Long-loop or slow recycling

Receptors aggregate in a perinuclear compartment, identified as the ERC or PNRC, typically 30 minutes post-internalization. Varied factors have been implicated in regulating the exit of integrins from this compartment. The kinase, PKC, regulates β1 integrin recycling through targeted phosphorylation of an intermediate filament protein, vimentin, and disruption of transport leads to accumulation in a perinuclear compartment and inefficient directional cell motility. However, PKC-positive vesicles did not colocalize with endocytosed transferrin, EEA1 or Rab11 so it is unclear whether this is a

separate step of endocytic regulation, or a pathway that is particular to fibroblasts (Ivaska et al., 2002; Ivaska et al., 2005). Use of dominant negative Protein Kinase B (PKB)/Akt inhibited recycling of both αvβ3 and α5β1 integrins from the ERC. This inhibition led to cell spreading defects of fibroblasts on both fibronectin and vitronectin (Roberts et al., 2004). Additionally, expression of dominant negative forms of Rab11 and ARF6 interfere with β1 integrin recycling (Powelka et al., 2003). EHD1, a member of the EH-domain containing family of endocytic transport factors, colocalizes with ARF6, is linked to long, tubular endosomes and RNAi knockdown disrupts β1 integrin recycling, cell spreading and migration (Jovic et al., 2007; Sharma et al., 2009).

Short-loop or rapid recycling

Under basal conditions, β3 and β1 integrin both rapidly recycle and pass through a Rab4-positive compartment (Roberts et al., 2001). Growth factors also promote the rapid recycling of integrins and appear to differentially affect trafficking of particular integrin heterodimers. Disruption of integrin rapid recycling events leads to slower cell migration, decreased delivery of integrins to nascent focal adhesions and reductions in cell spreading and adhesion (Roberts et al., 2001; Woods et al., 2004; Fang et al., 2010). Platelet-derived growth factor (PDGF) promotes short-loop recycling of αvβ3, but not α5β1, integrin via an interaction with protein kinase D1 (PKD1) in a Rab4-positive compartment. However, PKD1 does not colocalize with common EE/SE markers like transferrin or EEA1 (Roberts et al., 2001; Woods et al., 2004; White et al., 2007). Epidermal growth factor (EGF) and supervillin, a lipid-raft associated protein, promote

β3 and β1 integrin rapid recycling, but not recycling from the PNRC (Fang et al., 2010). It is not clear what factors are involved in the regulation of integrin sorting step at the EE/SE.

Receptors like transferrin and the β_2-adrenergic receptor traffic through actin-dependent rapid recycling pathways. The rapid recycling pathway for these receptors is regulated by a protein complex called CART (**c**ytoskeleton-**a**ssociated **r**ecycling and **t**ransport; Yan et al., 2005; Millman et al., 2008). Thus far, the only factor identified with a potential role in actin-dependent rapid integrin recycling has been supervillin, which promoted integrin recycling of endosomes near the periphery, localized with dynamic tubulovesicles and was actin-dependent (Fang et al., 2010).

Recycling and impact on activation state

Integrins can be in an activated/inactivated state similarly to the on/off (or densensitized/resensitized) state of other receptors. It is unclear whether integrins maintain their activated state as they traffic through the endosome and if so, whether this state renders the integrin capable of signaling from the endosome.

Protein **k**inase **Cα** (PKCα) associates with β1 integrin and promotes the return of the activated form of the integrin to the cell surface. Activated integrins can travel through the endocytic pathway in tubulovesicular structures and the activated form of β1 integrin colocalizes with endocytic markers and internalized fibronectin (Ng et al., 1999;

Arjonen et al., 2012). While both conformations internalize through a clathrin- and dynamin-dependent pathway and are detected in the same Rab4-positive early endosomal compartment, the inactive β1 integrin is more quickly returned to the plasma membrane and its transit is disrupted with the use of dominant negative Rab4a or an actin polymerization inhibitor. As inactive, but not active, β1 integrin also colocalized with ARF6-positive endosomal compartments, it suggests that β1 integrin traffics differently depending on its activation status (Arjonen et al., 2012).

To extend our understanding of how transport through the early endosome influences integrin function, I investigated the role of EE/SE-associated factors, AAK1L (**a**daptor **a**ssociated **k**inase **1** **l**ong form; Henderson & Conner, 2007) and EHD3, a member of the **E**ps15 **h**omology **d**omain (EHD) family which have key roles in endosomal sorting (Naslavsky et al., 2006; Naslavsky et al., 2009; Naslavsky & Caplan, 2011). A loss-of-function approach revealed that disruption of early endosome transport leads to adhesion loss, increased apoptosis and a retargeting of receptor to other trafficking pathways. Additionally, β3 integrin, not the more predominant β1 integrin, was discovered to be required for HeLa cell adherence and survival.

Chapter II: AAK1L depletion leads to β1 integrin trafficking defects

Section A: Summary

Integrin trafficking through the endosome is increasingly demonstrated to have an important role in ensuring proper cell migration and adhesion. Here, a siRNA-mediated depletion strategy is used to determine the role of an early endosomal factor, AAK1L, in integrin trafficking. We discover that AAK1L is important for mediating cell adhesion in HeLa cells. AAK1L depletion disrupts the distribution of the activated form of β1 integrin at the cell surface and leads to mis-sorting of β1 integrin to other endosomal compartments. The data demonstrate an important role for AAK1L in promoting β1 integrin recycling from the early endosome.

Section B: Introduction

Integrins are transmembrane heterodimers that are the major cell adhesion receptors in multicellular organisms (Hynes, 2002). Integrins consist of α and β subunits and are implicated in a wide range of cellular processes, including cell survival, growth, development and migration (Shattil et al., 2010). For adherent cells that are dependent on integrins, integrin trafficking through endocytic and recycling pathways is imperative for migration, ECM turnover and adhesion (Margadant et al., 2011).

Internalization mechanisms of integrins are clathrin-independent (using caveolin or bulk uptake) or clathrin-dependent and deliver integrins to the early/sorting endosome

(EE/SE). From the EE/SE, receptors can be routed to the lysosome for degradation, sent to the perinuclear endosomal recycling compartment (ERC) through which integrins recycle more slowly to the plasma membrane (long-loop pathway) or directly returned to the plasma membrane (short-loop pathway; Maxfield & McGraw, 2004; Caswell & Norman, 2006; Grant & Donaldson, 2009; Margadant et al., 2011).

While disruptions in ERC sorting re-route integrins to other trafficking pathways and impact integrin function (Lobert et al., 2010; Zech et al., 2011; Steinberg et al., 2012), it is still relatively unknown what sorting defects occur when disruption of EE/SE sorting occurs (Tiwari et al., 2011). The connection between activation status of a receptor and what factors regulate its trafficking through the EE/SE is also poorly understood. Activation status may influence how the receptor travels through the EE/SE and traveling through the EE/SE may be critical for re-priming/re-sensitization of receptors (Hanyaloglu et al., 2005; Arjonen et al., 2012). Activated integrins have been detected in tubulovesicular structures and the activated form of β1 integrin colocalizes with early endosomal markers, like Rab4a, Rab5 and EEA1, and internalized fibronectin (Ng et al., 1999; Arjonen et al., 2012).

To extend our understanding of how transport through the early endosome influences integrin function, we investigated the role of an EE/SE-associated factor, AAK1L (adaptor associated kinase 1 long form, Henderson & Conner, 2007), given the growing importance of kinases in integrin recycling (Roberts et al., 2001; Woods et al.,

2004). We employed flow cytometry to assess the impact of AAK1L depletion on integrin transport in adherent cells and provide evidence demonstrating that β1 integrin recycling is maintained through an AAK1L-dependent recycling pathway. We also find evidence that disruption of EE/SE transit reroutes β1 integrin to the degradative and long-loop recycling pathway.

Section C: Materials & Methods

Cells and culture conditions: tTA HeLa cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 4.5 g/L glucose, and 100 U/mL penicillin streptomycin at 37°C with 5% CO2. tTA HeLa cells were maintained in the presence of 400 μg/mL G418.

Antibodies and constructs: AAK1 polyclonal antibody against the ΔAID region was previously generated and described (Conner & Schmid, 2003). mAb antibody E7 and TD.1 (Covance Research Products, Denver, PA) were used to identify beta tubulin and clathrin heavy chain, respectively. GAK mAb antibody was a gift from Lois Greene (NIH). Tsg101 mAb (5B7, Novus Biologicals), β1 integrin (MAB1981MI, Millipore) and mAb against the activated form of β1 integrin (MAB2247, Millipore) were acquired from the indicated companies.

siRNA knockdown: siRNA knockdowns were performed as described (Sorensen & Conner, 2010). Briefly, siRNA transfections were performed on successive days. Cells

were used in experiments on day 3 or 4, depending on the experiment. Silencer negative control #1 and β1 integrin siRNA were acquired from Ambion. Other siRNAs were acquired from either Invitrogen or Shanghai GenePharma: AAK1 (GCGCGAUUGACACGCAUAUUCCUAU); clathrin heavy chain (UAAUCCAAUUCGAAGACCAAUUU); Tsg101 (CCUCCAGUCUUCUCUCGUC); GAK (GUCCGUCGCUAAUUAUGCA). The extent of protein depletion was validated by immunoblot (Fig. S1).

β1 integrin immunofluorescence recycling assay: Protocol was followed as indicated in Jovic et al., 2007 with some modifications. Cells were plated on acid-washed coverslips in 35mm dishes and incubated in DMEM + 10% FBS. Cells were then knocked down with siRNAs as indicated above. On the 4th day, cells were serum starved for 2 hours and then pulsed for two hours with 5ug/mL β1 integrin mAb. After pulse, cells were acid stripped of surface antibody for 45s. Cells were stimulated to recycle antibody by the addition of media containing 10% FBS. Cells were then fixed in PBS + 4% paraformaldehyde, washed and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) for 1 hr. Images were acquired on a Zeiss Axio Imager M1 upright microscope with a 1.4 NA 63x objective, the Zeiss Axiovision software, and a ProgRes® MF (Jenoptik) camera. Image panels were generated using Adobe Photoshop® and Illustrator®.

Flow cytometry: To measure β1 integrin surface levels, cells were lifted from tissue

culture dishes with PBS/EDTA, washed with PBS and fixed with ice cold PBS containing 4% paraformaldehyde for 20 min. Cells were then blocked for 30 min at RT in PBS + 1% BSA, washed, and incubated with a non-conformation-specific antibody against β1 integrin or antibody against the activated form of β1 integrin for 1 hr at RT. Cells were washed again and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) for 1 hr. β1 integrin levels were quantified, gating on intact cells and median fluorescent intensity determined for 10,000 cells. To measure β1 integrin total levels, the above procedure was repeated with the inclusion of 0.05% saponin.

Section D: Results

AAK1L depletion leads to cell adhesion defects

Through a siRNA-mediated loss-of-function approach, AAK1L was implicated in the regulation of rapid recycling of the transferrin receptor through the EE/SE (Henderson & Conner, 2007). It was observed that AAK1L-depleted HeLa cells, after 48hrs of siRNA treatment, take on a stretched, rounded phenotype (Pelkmans et al., 2005; Henderson & Conner, 2007). After a 72hr knockdown, siRNA-mediated AAK1L-depletion resulted in an approximate 50% reduction in adhesion to tissue culture dishes (Fig. 1A). This raised the possibility that adhesion receptor transport through the EE/SE was important for the maintenance of cell adhesion. AAK1L has also been shown to have a role in regulating receptor internalization (Conner & Schmid, 2002). To test whether impaired internalization resulted in cell adhesion defects, clathrin heavy chain (CHC) depletion, a factor critical to endocytosis, was performed (Fig. 1A, Teckchandani et al.,

2009). No cell adhesion defect was observed in disruption of internalization; thus, we interpret both of these observations to suggest that adhesion receptor transit through the EE/SE is critical for maintaining cell adhesion in HeLa cells.

Loss of adhesion in AAK1L-depleted cells could indicate a deficiency in cell surface delivery of adhesion receptors. The major cell adhesion receptors in multicellular organisms are integrins and the most abundant integrin in HeLa cells have β1 integrin as a subunit (Hynes, 2002; Teckchandani et al., 2009). To test whether cell surface levels of β1 integrin were altered when receptor transport through the EE/SE was impaired, we depleted AAK1L from HeLa cells and measured the level of β1 integrin (non-conformation-specific antibody) at the plasma membrane by flow cytometry. Depletion of AAK1L resulted in reduced β1 integrin cell surface levels (Fig. 1B). We interpret these observations to suggest that integrin transport through the EE/SE is critical for maintaining β1 integrin cell surface levels in HeLa cells. In contrast, depletion of clathrin heavy chain (CHC), a factor critical to integrin internalization, showed an increase in β1 integrin surface levels, consistent with its role in endocytosis (Fig. 1B; Teckchandani et al., 2009).

Depletion of GAK does not affect β1 integrin surface levels

Another member of the Ark1/Prk1 kinase family, cyclin G-associated kinase (GAK), shares homology in the kinase domain with AAK1L and has also been implicated in endocytic regulation (Zhao et al., 2001; Conner & Schmid, 2002). Similar to AAK1L,

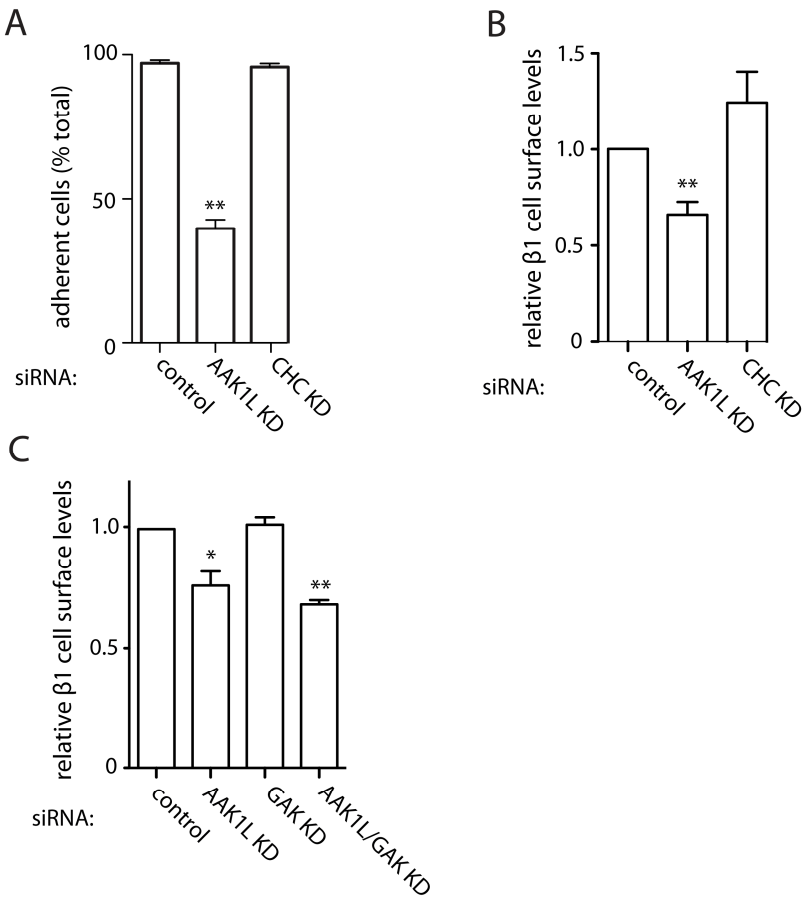

Figure 1: AAK1L promotes adhesion and maintains β1 integrin surface levels. A) Quantification of adherent tTA HeLa cells to tissue culture dishes following siRNA-mediated knock down (KD) for the indicated factors. B-C) β1 integrin cell surface levels were evaluated in AAK1L- , CHC- or GAK-depleted tTA HeLa cells using flow cytometry counting 10,000 cells and compared to cells treated with control siRNA or cells treated with secondary antibody alone. Error bars represent ± SD of three independent experiments. * and ** indicates a significance of $p<0.05$ and $p<0.005$ for a paired *t*-test.

GAK can interact with and phosphorylate the μ subunits of adaptor protein (AP) 1 and 2 (Umeda et al., 2000; Korolchuk & Banting, 2002; Conner & Schmid, 2002). However, GAK has been demonstrated to be required for efficient recruitment of AP1 to the trans-Golgi network (TGN) and certain β1 integrin heterodimers can traffic to the cell surface via the TGN (Lee et al., 2005; Riggs et al., 2012). To test whether reductions in β1 integrin delivery to the cell surface resulted from disrupted integrin transport through the TGN and if the AAK1L effect is specific, GAK was depleted in HeLa cells and the level of β1 integrin at the plasma membrane was measured by flow cytometry. Unlike in AAK1L depleted cells, GAK depletion did not affect β1 integrin cell surface levels (Fig. 1C). Depletion of both AAK1L and GAK had no additive effect and β1 integrin cell surface levels were similar to AAK1L depletion alone (Fig. 1C). This indicates that proper integrin trafficking through the EE/SE maintains β1 integrin surface levels.

AAK1L depletion alters activated β1 integrin trafficking

The adhesion defects observed in AAK1L-depleted cells raised the possibility of alterations in integrin transport. Rapid recycling from the EE/SE is increasingly being demonstrated to provide a form of regulation of signaling receptors by controlling their placement in time and space on the plasma membrane (Yudowski et al., 2009). It is unclear whether the activation state of integrins is altered during endocytosis and downstream endocytic trafficking. Recently, β1 integrin was shown to be sorted into different pathways and recycle at different rates, depending on its activation status (Arjonen et al., 2012).

Given the adhesion defects, we postulated that AAK1L depletion resulted in a loss of functional, activated β1 integrin at the plasma membrane. To test whether depletion of AAK1L affected activated β1 integrin transit, we depleted AAK1L from HeLa cells and measured the level of β1 integrin at the plasma membrane by flow cytometry using an antibody that specifically recognizes the activated conformation of β1 integrin. Depletion of AAK1L resulted in reduced activated β1 integrin cell surface levels (Fig. 2A). This indicates that delivery of the activated form of β1 integrin to the plasma membrane is altered when transit through the EE/SE is compromised. The reduction in activated β1 integrin levels may be the source of the reduction of total (non-conformation specific) β1 integrin cell surface levels which were also reduced (Fig. 1B). As β1 integrin can be internalized in an activated state (Lobert et al., 2010; Arjonen et al., 2012), recycling through the EE/SE may be imperative for efficient return to the cell surface.

To extend our analysis, we evaluated whether the localization of activated β1 integrin was altered when AAK1L function was perturbed. We hypothesized that if delivery from the EE/SE to the plasma membrane was compromised in AAK1L-depleted cells, β1 integrin may be mis-sorted to other trafficking pathways. Adherent HeLa cells were allowed to internalize and recycle antibody recognizing activated β1 integrin and then fixed for immunofluorescence. Control cells showed a speckled pattern on tubulo-vesicular structures throughout the cell (Fig. 2B). In contrast, activated β1 integrin in AAK1L-depleted cells was found to be concentrated in a perinuclear compartment (Fig.

2B). This localization to a perinuclear compartment is similar to previous observations of transferrin receptor in AAK1L-depleted cells (Henderson & Conner, 2007). Collectively, we interpret these observations to suggest that integrin transport through an AAK1L-positive compartment is important for maintaining appropriate levels of activated β1 integrin at the cell surface in HeLa cells.

Integrin degradation is enhanced following AAK1L depletion

An increase in β1 integrin in a perinuclear compartment in AAK1L-depleted cells suggested that AAK1L depletion led to receptor mis-sorting at the EE/SE. This raised the question of whether β1 integrin was being only re-directed to the long-loop recycling pathway or if β1 integrin was also being mis-sorted to the degradative pathway. To test this, flow cytometry was used to measure integrin levels with β1-specific antibodies in permeabilized cells. This revealed a marked decrease in β1 integrin levels in cells depleted of AAK1L relative to controls (Fig. 3), which suggested that when AAK1L-mediated sorting at the EE/SE is disrupted, β1 integrin is also targeted to the lysosome for degradation. Recent studies indicate that integrin targeting for degradation within lysosomes requires ESCRTs (endosomal sorting complex required for transport; Lobert et al., 2010). To test the idea that β1 integrin was being routed for degradation to the lysosome following AAK1L siRNA treatment, we next depleted cells of an ESCRT factor, Tsg101 (Tumor susceptibility gene 101), to impair degradation. We hypothesized

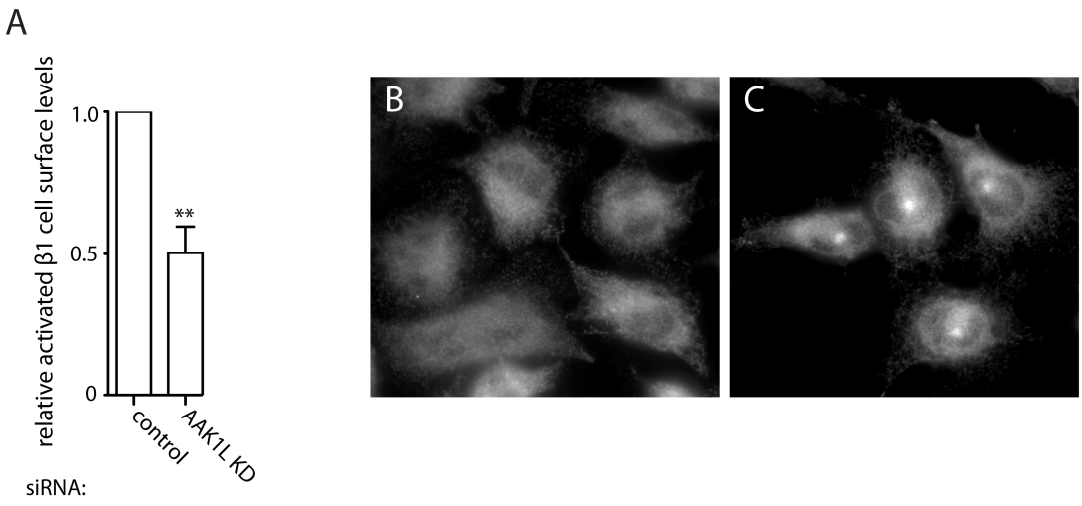

Figure 2: AAK1L maintains activated β1 integrin surface levels. A) Activated β1 integrin cell surface levels were evaluated in AAK1L-depleted tTA HeLa cells using flow cytometry counting 10,000 cells and compared to cells treated with control siRNA or cells treated with secondary antibody alone. Error bars represent ± SD of three independent experiments. ** indicates a significance of $p<0.005$ for a paired *t*-test. B-C) Cells were plated on acid-washed coverslips in 35mm dishes and incubated in DMEM + 10% FBS. Cells were then knocked down with control (B) or AAK1L (C) siRNAs. After 36 hrs, cells were serum starved for 2 hours and then pulsed for two hours with mAb against the activated β1 integrin. Cells were stripped of surface antibody and then stimulated to recycle antibody by the addition of media containing 10% FBS. Cells were then fixed and probed with secondary antibody.

that if the β1 integrin reduction in AAK1L-depleted cells was due to increased delivery to the lysosome, that blocking sorting to the lysosome would lead to an increase in β1 integrin levels. Simultaneous depletion of AAK1L and Tsg101 resulted in a partial restoration of β1 integrin levels, while depletion of Tsg101 alone did not significantly change the levels of β1 integrin (Fig. 3). Taken together, these observations demonstrate that when AAK1L-dependent transport is disrupted, β1 integrin is sorted toward the degradative pathway, similar to that observed for β1 integrin following syntaxin 6 inhibition (Tiwari et al., 2011).

HeLa cell adherence is independent of β1 integrin

It was unclear whether reductions in functional β1 integrin at the cell surface in AAK1L-depleted cells were directly mediating the observed loss of adherence on tissue culture dishes (Fig. 1A). As β1 integrin is the predominant integrin expressed in HeLa cells (Teckchandani et al., 2009), we anticipated that it would have a major role in mediating cell adhesion. To test this, we again used a siRNA-mediated knockdown approach and observed that depletion of β1 integrin did not lead to cell adhesion defects (Fig. 4). In contrast, depletion of β3 integrin, an integrin demonstrated to travel through a short-loop recycling pathway through EE/SEs (Roberts et al., 2001), resulted in a >50% reduction in cell adhesion on tissue culture dishes (Fig. 4). Similarly reductions in adherence were also observed for its primary binding partner, αv integrin (Fig. 4;

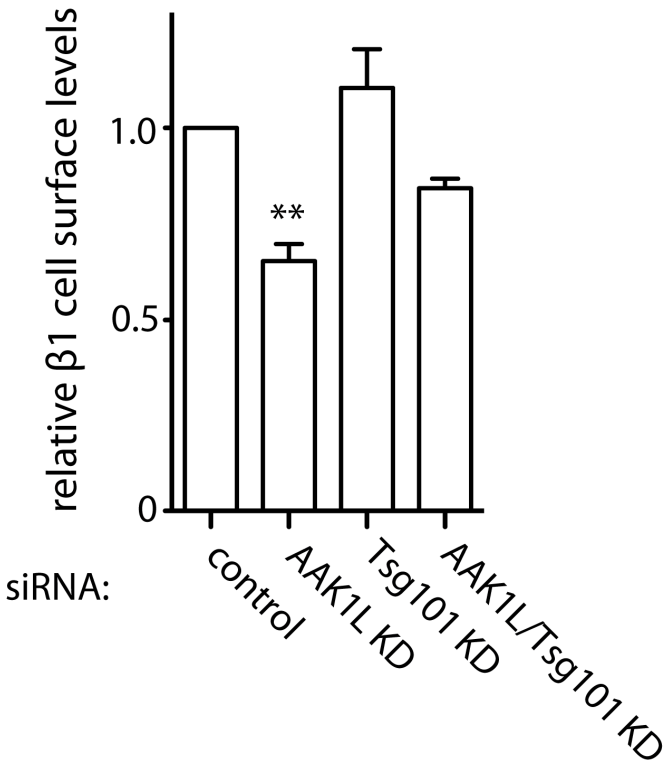

Figure 3: AAK1L depletion leads to increased β1 integrin sorting to the degradative pathway. β1 integrin cell surface levels were evaluated in AAK1L- and Tsg101-depleted tTA HeLa cells using flow cytometry counting 10,000 cells and compared to cells treated with control siRNA or cells treated with secondary antibody alone. Error bars represent ± SD of three independent experiments. ** indicates a significance of $p<0.005$ for a paired *t*-test.

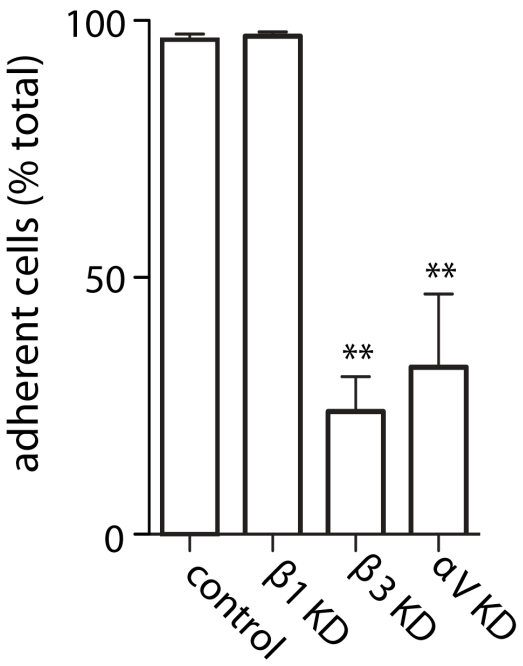

Figure 4: αv and β3 integrin promote adhesion in tTA HeLa cells. Quantification of adherent tTA HeLa cells to tissue culture dishes following siRNA-mediated knock down (KD) for the indicated factors. Error bars represent ± SD of three independent experiments. ** indicates a significance of $p<0.005$ for a paired *t*-test.

Margadant et al., 2011), These observations indicate that while β1 integrin transport through an AAK1L-mediated pathway is important for maintaining proper transport dynamics, the perturbations of β1 integrin at the cell surface are not responsible for the loss of adhesion observed in AAK1L-depleted HeLa cells. Certain integrin-ECM interactions have been demonstrated to promote cell survival, such as the ligation of αvβ3 integrin in CHO cells promoting transcription of the anti-apoptotic gene *bcl-2* (Matter & Ruoslahti, 2001; Stupack & Cheresh, 2002). Future work will focus on determining how β3 integrin transport through the endosome affects integrin function and cell survival.

Section E: Discussion

AAK1L-dependent sorting decisions from the EE/SE

Growing evidence suggests that the EE/SE has a critical role in the maintenance of receptor function as a sorting station, either by redistributing receptors to the proper spatial and temporal membranes, or in regulation of signalling and resensitization. Here we provide evidence demonstrating an EE/SE-associated factor, AAK1L, not only affects the redistribution and sorting of β1 integrin but also the activation state of the receptor at the plasma membrane.

Expression of a dominant-negative Rab4 prevents the recycling and resensitization of the β_2-adrenergic receptor (β_2AR) from the early endosome (Seachrist et al., 2000). The mechanism of resensitization of integrins for ligand has been proposed for the growth-factor promoted short-loop recycling of β3 integrin and the long-loop recycling of β1 integrin in fibroblasts to explain their contributions and influence on cell

migration (White et al., 2007). A recent study provided evidence that integrins can undergo endocytosis and recycling and are transported through Rab4-, 5- and 11-positive compartments in an activated state, which is contrary to what happens to β_2AR, which is desensitized through its internalization (Seachrist et al., 2000; Odley et al., 2004; Arjonen et al., 2012). After internalization, active and inactive β1 integrin returned to the plasma membrane through different routes and at different rates, with active β1 integrin returning more slowly. It is unclear whether this is due to ligand separation from β1 integrin or if there is more complexity in integrin activation/inactivation occurring in the endosomal compartments (Arjonen et al., 2012). Depletion of AAK1L led to a reduction in the cell surface numbers of total β1 integrin at the cell surface and also reductions in the surface levels of the activated form of β1 integrin. Further investigation is needed to determine whether the changes in total β1 integrin at the cell surface are due to the loss of the activated receptor only, or if there are disruptions in inactive receptor transport as well. Future experiments will better determine the mechanism of action of AAK1L.

Abnormal ECM remodeling contributes to a variety of diseases, including cancer and arthritis. Internalization and lysosomal sorting of integrin and its ligand ECM is imperative for proper ECM turnover (Shi & Sottile, 2008). Most integrin that is internalized is recycled back to the plasma membrane. α5β1 integrin can traffic to the lysosome in a ligand-bound state with fibronectin. Blocking transit to the lysosome led to accumulation of integrin in an early endosomal compartment (Lobert et al., 2010). Similar to AAK1L depletion, an EE/SE-associated SNARE, syntaxin 6, was shown to

target β1 integrin to the plasma membrane and depletion of this factor, led to mis-sorting to the lysosome (Tiwari et al., 2011). AAK1L is known to target AP1, a factor that has a role in the formation of endosome-derived vesicles via a Rab4-mediated pathway (Pagano et al., 2004). Depletion of AP1 did reduce β1 integrin surface levels but did not affect cell adhesion to tissue culture dishes (data not shown) so future studies will focus on finding additional AAK1L targets in receptor trafficking.

While β1 integrin is the predominant integrin on the plasma membrane of HeLa cells (Teckchandani et al., 2009), depletion of β1 integrin did not lead to a loss of adhesion on tissue culture dishes. While this does not demonstrate that β1 integrin is not used by HeLa cells for adhesion, *per se*, it does demonstrate that β1 integrin engagement of ligands is not a requirement for survival in HeLa cells. Integrin heterodimers differ in their ability to promote cell survival and this varies considerably between cell types (Meredith & Schwartz, 1997). Depletion of β3 integrin in HeLa cells led to a considerable adhesion defect and further investigation will be done to determine whether AAK1L depletion is disrupting β3 integrin sorting and impacting adhesion and/or cell survival.

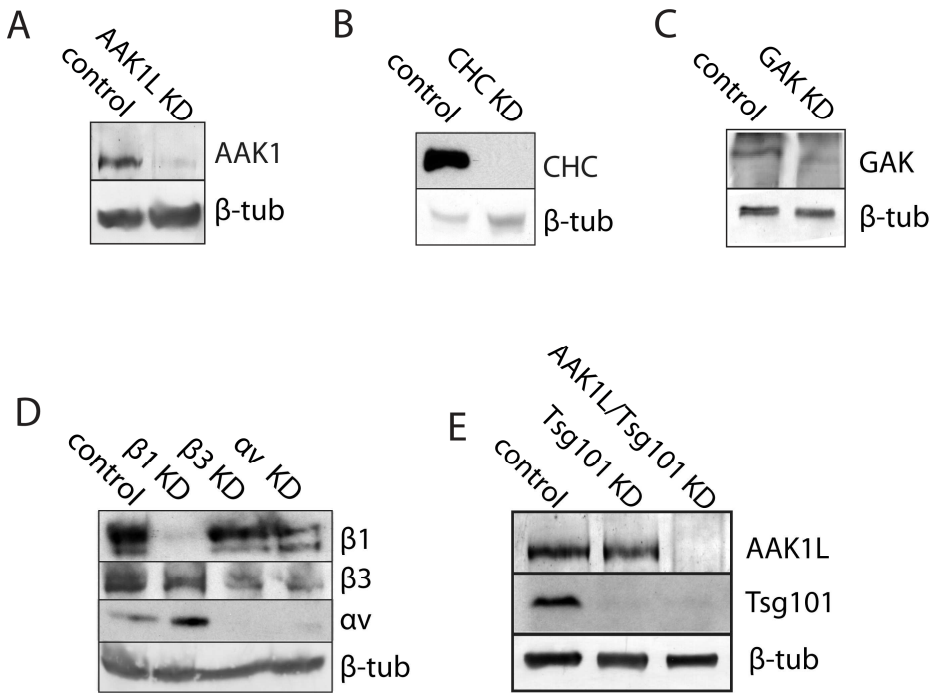

Figure S1: Western blot analysis of siRNA-depleted factors in tTA HeLa cell lysates. Blots were probed for beta tubulin as a protein loading control.

Chapter III: αvβ3 integrin-mediated adhesion is regulated through an AAK1L- and EHD3-dependent rapid recycling pathway

Section A: Summary

Protein transport through the endosome is critical for maintaining proper integrin cell surface integrin distribution to support cell adhesion, motility, and viability. Here we employ a live-cell imaging approach to evaluate the relationship between integrin function and transport through the early endosome. We discovered that two early endosome factors, AAK1L and EHD3 are critical for αvβ3 integrin-mediated cell adhesion in HeLa cells. siRNA-mediated depletion of either factor delays short-loop β3 integrin recycling from the early endosome back to the cell surface. TIRF-based colocalization analysis reveals that β3 integrin transits AAK1L- and EHD3-positive endosomes near the cell surface, a subcellular location consistent with a rapid-recycling role for both factors. Moreover, structure-function analysis reveals that AAK1L kinase activity, as well as its carboxy-terminal domain, is essential for cell adhesion maintenance. Taken together, these data reveal an important role for AAK1L and EHD3 in maintaining cell viability and adhesion by promoting αvβ3 integrin rapid-recycling from the early endosome.

Section B: Introduction

Integrins are heterodimeric αβ receptors that engage the extracellular matrix to mediate a variety of biological processes ranging from cell adhesion to proliferation

(Hynes, 2002). Integrin-mediated signalling must be tightly regulated given that perturbations in integrin activity can alter cell motility, promote changes in cell survival, and alter gene expression (Harburger & Calderwood, 2009). An important mechanism by which integrin activity is modulated is through the endocytic and recycling pathways that control integrin distribution within cells (Shin et al., 2012).

Integrins are predominantly internalized by either clathrin- or caveolin-dependent mechanisms that serve to deliver integrins to the early/sorting endosome (EE/SE). Within the EE/SE, integral membrane proteins are selectively packaged and targeted to the lysosome for degradation, rapidly recycled back to the plasma membrane for reuse (short-loop recycling), or directed to the perinuclear endosomal recycling compartment (ERC) through which integrin recycling to the plasma membrane occurs more slowly via a long-loop pathway (Maxfield & McGraw, 2004; Caswell & Norman, 2006; Grant & Donaldson, 2009).

Published studies reveal that rapid-recycling from the EE/SE occurs via a rab4-dependent mechanism, which can be stimulated by platelet derived growth factor and protein kinase D1 activity (Roberts et al., 2001; Woods et al., 2004; White et al., 2007). Rapid recycling from the EE/SE is also dependent on the actin cytoskeleton and myosin V (Yan et al., 2005; Millman et al., 2008). Actin-dependent recycling can be accelerated by the F-actin binding protein supervillin (Fang et al., 2010) and necessitates CART (cytoskeleton-associated recycling or transport), a complex thought to link constitutively

recycled cargo-containing endosomes to the actin cytoskeleton (Yan et al., 2005).

To extend our understanding of how transport through the early endosome influences integrin function, we investigated the role of two EE/SE-associated factors: 1) AAK1L (adaptor associated kinase 1 long form, Henderson & Conner, 2007), given the growing importance of kinases in integrin recycling (Roberts et al., 2004; Woods et al., 2004), and 2) EHD3, which belongs to the Eps15 homology domain (EHD) family of proteins, which serve key roles in endosomal sorting decisions (Naslavsky et al., 2006; Naslavsky et al., 2009; Naslavsky & Caplan, 2011). We employed a live cell imaging strategy to visualize the impact of AAK1L or EHD3 depletion on integrin transport in adherent cells and provide evidence demonstrating that αvβ3-mediated cell adhesion is maintained by integrin recycling through an AAK1L- and EHD3-dependent recycling pathway.

Section C: Materials and Methods

Cells and culture conditions: tTA HeLa cells and mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 4.5 g/L glucose, and 100 U/mL penicillin streptomycin at 37°C with 5% CO_2. tTA HeLa cells were maintained in the presence of 400 µg/mL G418.

Antibodies and constructs: AAK1 polyclonal antibody against the ΔAID region was previously generated and described (Conner and Schmid, 2003). mAb antibodies HA.11

(MMS-101P, Covance Research Products), 9E10, and E7 were used to identify the HA tag, myc tag, and beta-tubulin, respectively. Polyclonal antibodies against EHD1 and EHD3 were generous gifts from Dr. Steve Caplan (University of Nebraska, Naslavsky et al., 2004). Numb mAb antibody was from R&D Systems. mAb antibody against αv integrin was a gift from Sundaram Ramakrishnan (University of Minnesota). αv integrin-mCherry encoding plasmid (Case and Waterman, 2011) was a generous gift of Michael W. Davidson (Florida State University). Antisera against β3 integrin (4702, Cell Signaling Technology), β1 integrin (MAB1981MI, Millipore) were acquired from the indicated companies.

Paxillin in pCAG and actin in pEGFP were generous gifts from Drs. Lorene Lanier and Paul Letourneau (University of Minnesota), respectively. Paxillin was subcloned into the EcoRI/BamHI site of pAdTet7 containing mCherry to generate a fusion. The β3 integrin in pEGFP-N1 was a generous gift from Dr. Jonathan Jones (Northwestern University). AAK1L-Chr was generated by subcloning AAK1L into the EcoRI site of pAdCMV containing mCherry. Adenovirus encoding AAK1L, AAK1s, and CBD2 were generated and employed as previously described (Henderson and Conner, 2007). EHD3 in pcDNA3.1(-) was a gift from Dr. Steve Caplan (University of Nebraska). mChr-EHD3 was generated by subcloning mCherry into the XbaI site of pcDNA3.1(-) that contained EHD3.

siRNA knockdown: siRNA knockdowns were performed as described (Sorensen and

Conner, 2010). Briefly, siRNA transfections were performed on successive days. Cells were used in experiments on days 3 or 4, depending on the experiment. Silencer negative control #1 and β1 integrin siRNA were acquired from Ambion. Other siRNAs were acquired from either Invitrogen or Shanghai GenePharma: AAK1(GCGCGAUUGACACGCAUAUUCCUAU); clathrin heavy chain (UAAUCCAAUUCGAAGACCAAUUU); Numb (CCGGAAAAUGUAGCUUCCCUU); β3 integrin (CCUCCAGCUCAUUGUUGAUGCUUAU); αv integrin, EHD3 and EHD1 sequences were previously described (Ballana et al., 2009; Jovic et al., 2007; Naslavsky et al., 2009). The extent of protein depletion was validated by immunoblot (Sup. Fig. 8).

Adhesion/attachment assays: The number of cells that remained adherent to tissue culture dishes was determined by subtracting the percentage of cells in solution from the total cell number. For attachment assays, wells of a 96-well plate were pre-coated with 10 ng/mL vitronectin (Invitrogen) or 10 ng/mL fibronectin (Sigma) overnight at 37°C. The next day, wells were blocked for 1 hr at 37°C in 3 mg/mL BSA. tTA HeLa cells were then seeded at $1x10^4$ cells/well in triplicate in serum-free media and allowed to adhere for 15 min at 37°C (Humphries, 2001). Wells were then washed with serum free media and incubated in CellTiter 96 (Promega) to quantify adherence according to manufacturer protocols.

β3 integrin cell surface levels: To measure β3 integrin surface levels, cells were lifted

from tissue culture dishes with PBS/EDTA, washed with PBS and fixed with ice cold PBS containing 4% paraformaldehyde for 20 min. Cells were then blocked for 30 min at RT in PBS + 1% BSA, washed, and incubated with antibody against β3 integrin (#555752, BD Biosciences) for 1 hr at RT. Cells were washed again and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) for 1 hr. β3 integrin levels were quantified, gating on intact cells and median fluorescent intensity determined for 50,000 cells.

Rescue experiments: After the second siRNA transfection (see above), cells were infected with adenovirus encoding the tetracycline transactivator (tTA), AAK1L, K74A AAK1L, or AAK1s. Expression was controlled to near endogenous levels by the addition of 15 ng/mL tetracycline, as previously described (Henderson & Conner, 2007). Cell adhesion or attachment was then evaluated as described above.

Apoptosis assay: To measure apoptosis, cells were gently detached with PBS/EDTA, washed twice with cold PBS and then resuspended in 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated with Annexin V-APC and Propidium Iodide (BD Biosciences) for 15 min at RT and ten thousand cells were counted by flow cytometry. To impair apoptosis, cells were treated with 100 μM Z-VAD-FMK (R&D Systems) or DMSO as a control following the second siRNA transfection (see above).

TIRF Microscopy and image quantification: Cells were plated on acid-washed coverslips in 35 mm dishes with an appropriate sized hole and incubated in DMEM containing 10% FBS. Cells were then transfected with plasmid encoding the indicated fluorochrome-tagged construct. The following day, cells were imaged using a Zeiss Observer Z1 TIRF microscope equipped with a temperature-, humidity-, and CO_2-controlled incubator and 488 nm and 561 nm laser lines. Images were acquired using a 1.46 NA 100X objective, the Zeiss Axiovision software, and either a QuantEM CCD or an AxioCam camera. Image panels were generated using Adobe Photoshop® and Illustrator®. Time-lapse movies were generated using Apple Motion® Software.

For focal adhesion formation experiments, multiple images were captured of β3-GFP focal adhesions before photobleach in the evanescent field. To photobleach focal adhesions, the evanescent field was illuminated with laser light at 100% intensity for 25 sec. Images were then captured at the indicated time intervals for up to 130 minutes. To quantitate the recovery of fluorescence after photobleach, several focal adhesion-containing regions from each cell were selected and the time course was analyzed using ImageJ Software and the McMaster University Biophotonics Facility plugin for intensity vs. time. For each experimental condition, at least 7 cells were quantified. Raw intensity values were then normalized against the focal adhesion intensity immediately before photobleach.

Rapid recycling assay: Rapid β3 integrin recycling was performed as previously

described (Roberts et al., 2001; Fang et al., 2010) with minor modification. Recycling timepoints were performed in the presence of DMEM and 10% FBS at 37°C. Biotin removal was performed for 30 min at 4°C by incubating cells in 20 mM MesNa (Sigma) in 100 mM NaCl, 50 mM Tris, pH 8.6.

Section D: Results

AAK1L and EHD3 promote αvβ3 integrin-mediated cell adhesion

To investigate the relationship between cell adhesion and integrin transport through the EE/SE, we pursued a loss-of-function approach using a siRNA-mediated depletion strategy. We initiated our studies by targeting two early endosomal sorting factors: 1) AAK1L (Henderson & Conner, 2007) and 2) EHD3 (Naslavsky et al., 2006; Naslavsky et al., 2009). Depletion of either AAK1L or EHD3 in tTA HeLa cells resulted in an ~50% reduction in cell adhesion to tissue culture dishes after a 72 hr knockdown (Fig. 1A). In addition to regulating rapid recycling of the transferrin receptor (Henderson & Conner, 2007), AAK1L has also been implicated in regulating receptor internalization (Conner & Schmid, 2002). This raised the possibility that the observed cell adhesion defects might result from impaired integrin endocytosis. However, this was not the case since cell adhesion defects were not observed following siRNA-mediated depletion of either clathrin heavy chain (CHC) or Numb1, two factors critical to integrin endocytosis (Fig. 1A , Teckchandani et al., 2009; Nishimura & Kaibuchi, 2007). We interpret these observations to suggest that integrin transport through the EE/SE is critical for

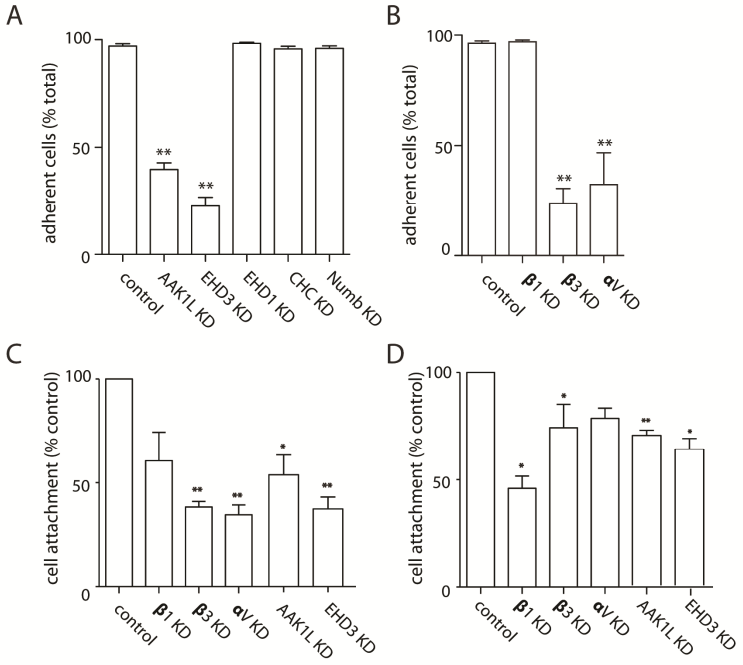

Figure 1: AAK1L and EHD3 promote αvβ3 integrin-mediated adhesion. Quantification of adherent HeLa cells to tissue culture dishes following siRNA-mediated knock down (KD) for the indicated factors (A, B). Quantification of HeLa cell adherence following depletion of the indicated integrin using an acute attachment assay (see Materials and Methods) for cell binding to either vitronectin (C) or fibronectin (D). Error bars represent ± SD of three independent experiments. * and ** indicates a significance of $p<0.05$ and $p<0.005$ for a paired *t*-test.

maintaining cell adhesion in HeLa cells. To test whether the adhesion defect was specific for integrin sorting at the level of the EE/SE, we next depleted EHD1, an EHD protein family member that is critical for integrin recycling from the perinuclear ERC (Jovic et al., 2007). Like that following CHC or Numb1 depletion, cell adhesion was unaffected by EHD1 loss (Fig. 1A). Taken together, these results suggest that when protein transport through the EE/SE is impaired, cell adhesion defects arise.To determine the integrin type responsible for mediating adhesion in HeLa cells, we again used a siRNA-mediated knockdown approach. We initially tested β3 integrin given that it is transported via a short-loop recycling pathway through EE/SEs (Roberts et al., 2001). Indeed, β3 integrin depletion, like that for its primary binding partner, αv integrin (Margadant et al., 2011), resulted in a >50% reduction in cell adhesion on tissue culture dishes (Fig. 1B). By contrast, β1 integrin, the major integrin expressed in HeLa cells (Teckchandani et al., 2009), did not lead to cell adhesion defects when depleted by siRNA. This later observation reinforces our EHD1 loss-of function results (Fig. 1A) since β1 integrin is thought to preferentially recycle via a long-loop pathway through the ERC (Roberts et al., 2001; Jovic et al., 2007).

Cell adhesion is a multistep process involving receptor-ligand binding, signaling, and downstream changes in the cytoskeletal network. Therefore, to more precisely resolve the nature of the adhesion defect observed, we performed acute, ligand-dependent attachment assays that test initial integrin-dependent binding using a defined extracellular matrix (Humphries, 2001). Given the importance of αvβ3 integrin in our previous adhesion

assays (Fig. 1B) and its ability to engage RGD-containing extracellular ligands (Margadant et al., 2011), we next tested cell attachment to vitronectin and fibronectin. Not surprisingly, αvβ3 depletion, like that for AAK1L and EHD3, resulted in significant reductions in cell binding to vitronectin (Fig. 1C). Similarly, a reduction in cell attachment to fibronectin was also observed following siRNA-mediated knockdown of αvβ3 integrin, AAK1L, or EHD3, although the extent of impairment was less substantial (Fig. 1D). We also observed reduced cell adhesion to vitronectin and fibronectin following β1 integrin knockdown in acute attachment assays, which possibly reflects a loss of αvβ1 and α5β1 integrin attachment to fibronectin and vitronectin (van der Flier & Sonnenberg, 2001). Collectively, we interpret these results to indicate that AAK1L and EHD3 depletion leads to reduced integrin attachment to the extracellular matrix with the most significant impact on αvβ3 integrin-mediated attachment.

To extend our analysis, we evaluated the consequence of AAK1L and EHD3 loss on apoptosis given that integrin-mediated adhesion to extracellular matrix ligands is required for survival in many cell types (Meredith & Schwartz, 1997). To test this, we measured annexin V binding, an indicator of cells undergoing apoptosis. As expected, AAK1L- and EHD3-depleted cells showed a significant increase in Annexin V binding relative to controls (Fig. S1A). As a complementary approach, we also evaluated cell adhesion on tissue culture dishes following AAK1L or EHD3 depletion in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) to impair apoptosis. In the presence of Z-VAD-FMK, we observed a significant recovery in cell adhesion relative to controls when either

early endosome factor was depleted (Fig. S1C). Collectively, we interpret these observations to indicate that αvβ3 integrin transport through AAK1L- and EHD3-positive endosomes is critical for maintaining αvβ3 integrin function in adhesion and signaling events critical for cell viability.

β3 integrin transits AAK1L and EHD3 positive endosomes

Given that AAK1L and EHD3 loss-of-function data suggested defects in αvβ3 integrin sorting within the endosome, we next asked if β3 integrin transits endosomes containing AAK1L or EHD3. To do so, we pursued a live-cell imaging approach with well-spread mouse embryonic fibroblasts (MEFs) to allow better visualization of endosomes by total internal reflection fluorescence (TIRF) microscopy. Following cotransfection with plasmid encoding β3 integrin-GFP (β3-GFP) and AAK1L or EHD3 fused to mCherry (AAK1L-mChr or mChr-EHD3), colocalization was readily observed in tubulovesicular structures located near the cell surface (Fig. 2, Sup. Mov. 1 and 2[1]), similar to that observed in HeLa cells (Fig. S2). In the case of AAK1L-mChr, we also observed colocalization with β3-GFP on tubular endosomes that extend into dynamic lamellar-like protrusions (Fig. 2C, Sup. Mov. 1). Colocalization analysis in HeLa cells expressing paxillin-mCherry (paxillin-mChr) and AAK1L-GFP or GFP-EHD3 also revealed that AAK1L- and EHD3-containing endosomes were present adjacent to paxillin-positive focal adhesions (Fig. S3).

[1] To view Supplemental Movies, please see the reference for Waxmonsky & Conner, 2013

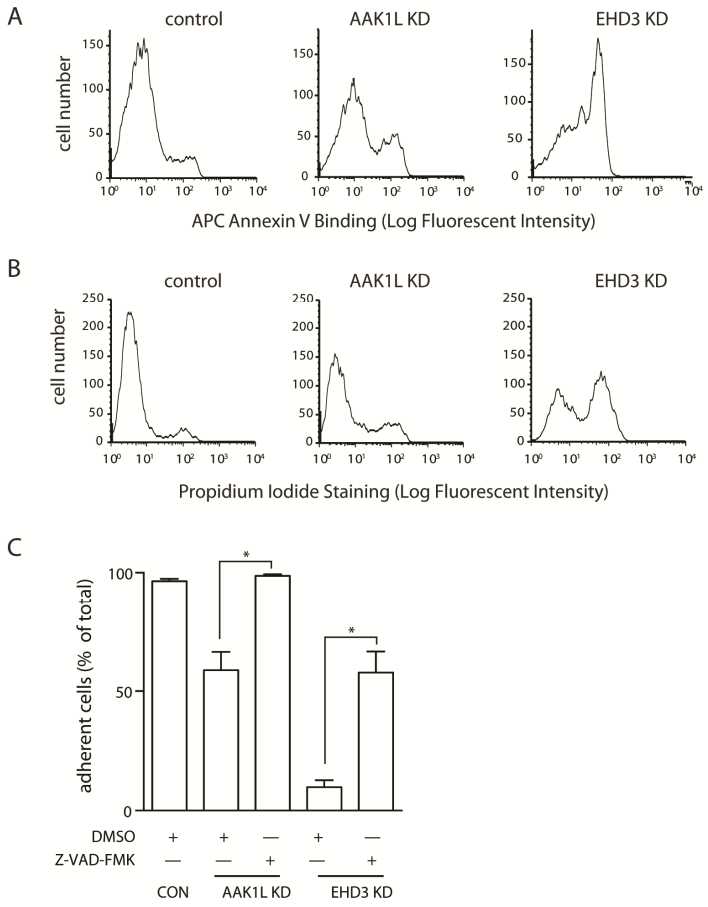

Figure S1: AAK1L and EHD3 depletion leads to apoptosis. Cells were depleted of AAK1L or EHD3 and evaluated for apoptosis using flow cytometry (see Materials and Methods). Representative flow cytometry plots from one of three independent experiments for ten thousand cells stained with APC (allophycocyanin)-conjugated Annexin V (A) and Propidium Iodide (B) are shown. C) AAK1L- or EHD3-depleted cells were incubated in the presence of DMSO or Z-VAD-FMK and adherent cells were quantitated. Error bars represent ± SD of three independent experiments. * and ** indicates a significance of $p<0.05$ and $p<0.005$ for a paired *t*-test.

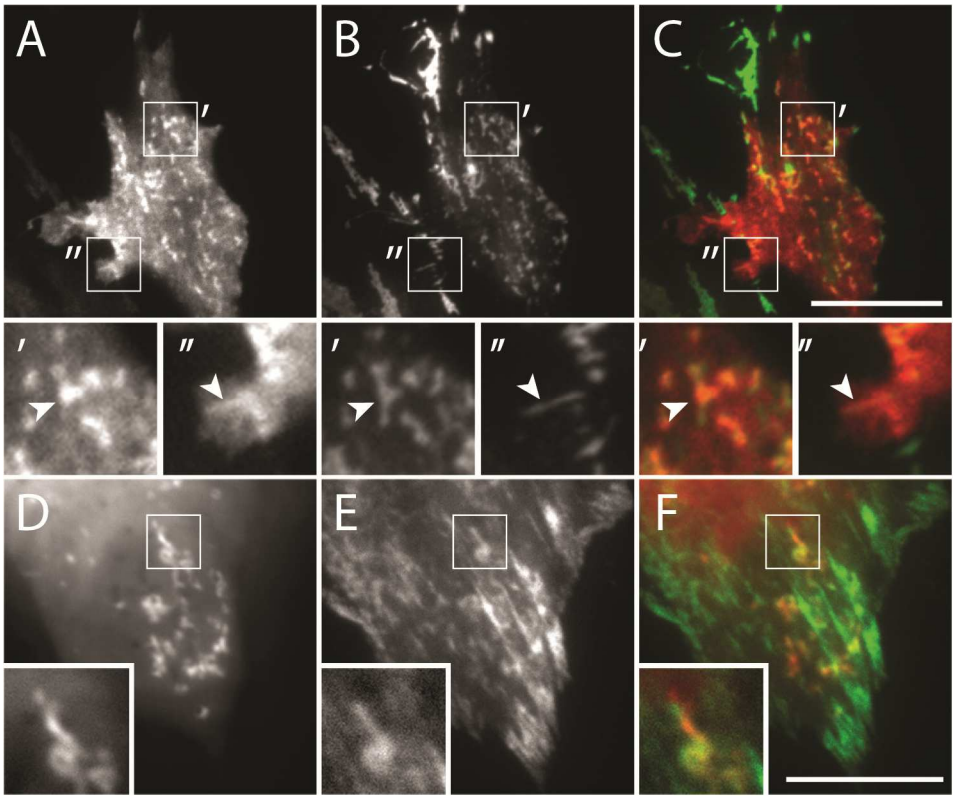

Figure 2: β3 integrin transits AAK1L- and EHD3-positive endosomes near the cell surface. MEFs were cotransfected with plasmid encoding β3 integrin-GFP (B, E) and AAK1L-mChr (A) or mChr-EHD3 (D). Live cells were visualized by TIRF microscopy and a time series was acquired (see Sup. Mov. 1 and 2). Colocalization is indicated in the merged panels (C, F). Boxed insets indicate regions of higher magnification. Bar = 20 µm.

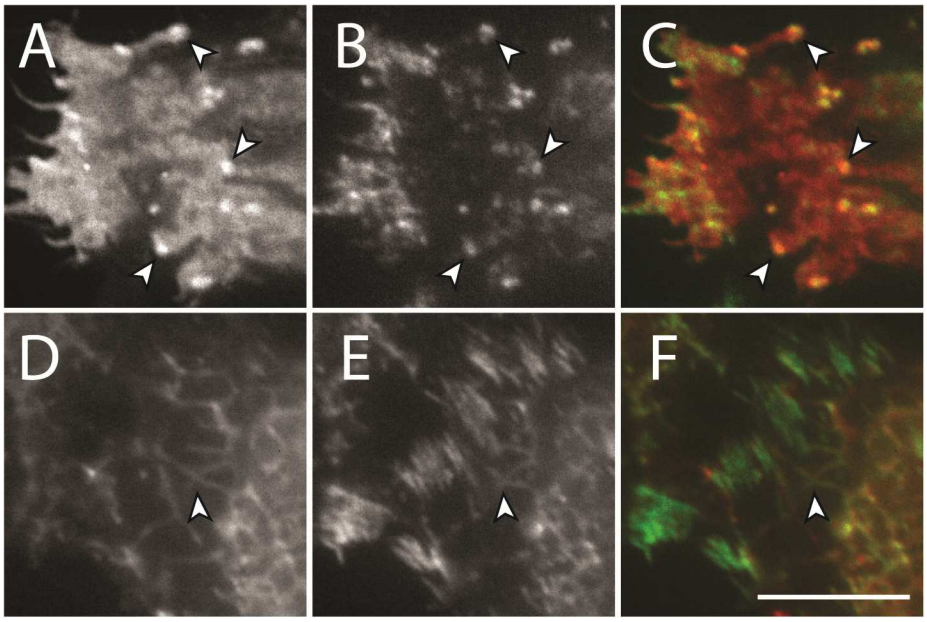

Figure S2: β3 integrin transits AAK1L- and EHD3-positive endosomes. HeLa were cotransfected with plasmid encoding β3 integrin-GFP (B, E) and AAK1L-mChr (A) or mChr-EHD3 (D). Live cells were visualized by TIRF microscopy. Colocalization is indicated in the merged panels (C, F). Arrow heads indicate examples of colocalization. Bar = 20 µm.

Previous studies indicate that the intracellular spatial distribution of EHD3-positive endosomes is dependent on microtubules (Galperin et al., 2002). By contrast, AAK1L endosomes near the cell surface appeared to partially align along actin stress fibers. To test this possibility, we cotransfected AAK1L-mChr-expressing cells with plasmid encoding actin fused to GFP and visualized cells by TIRF. Live cell imaging revealed that AAK1L-mChr positive endosomes partially aligned along actin filaments directly below the cell surface (Fig. 3C).

AAK1L and EHD3 colocalize on highly mobile endosomes

AAK1L and EHD3 both colocalize with β3-GFP (Fig. 2) and associate with EEA1-positive EE/SEs (Henderson & Conner, 2007; Naslavsky et al., 2006). However, near the cell surface, EHD3-positive endosomes did not appear to align with actin stress fibers like that of AAK1L (Fig. 3). This raised the possibility that AAK1L and EHD3 may function within different early endosome subcompartments to regulate integrin transport. To evaluate this possibility we cotransfected MEFs with plasmid encoding mChr-EHD3 and AAK1L-GFP and performed live cell imaging. In doing so, we discovered that EHD3 did not colocalize with AAK1L on the majority of endosomes near the cell surface (Fig. 4). However, colocalization was observed on highly dynamic vesicular endosomes (Fig. 4C, Sup. Mov. 3). We interpret these observations to suggest that AAK1L and EHD3 might function within different early endosome subcompartments to regulate integrin transport. Alternatively, it is possible that critical integrin sorting decisions may occur within the mobile vesicular endosomes where AAK1L and EHD3 colocalize.

β3 integrin recycling is dependent on AAK1L and EHD3

Colocalization analyses position AAK1L and EHD3 as key regulators of β3 integrin transport within the EE/SE (Fig. 2). Additionally, cell adhesion defects that result from AAK1L or EHD3 loss suggest that these factors are essential for proper cell surface integrin distribution of functional β3 integrin (Fig. 1). These observations strongly argue that AAK1L and EHD3 function to promote β3 recycling from the EE/SE via the short-loop pathway. To more rigorously test this idea, we developed a strategy to selectively visualize integrin recycling dynamics using TIRF to image focal adhesion formation following photobleaching in live cells expressing β3-GFP (Fig. 5A). β3-GFP recruitment to focal adhesions is readily observed by TIRF microscopy where it colocalizes with paxillin-mChr (Fig. S4C), a cytosolic focal adhesion marker, and αv integrin-mCherry (Fig. S5C). These focal adhesions are readily photobleached in TIRF by increasing laser strength. However, additional, photobleach-resistant β3-GFP signal was observed in dynamic tubular endosomes (Sup. Mov. 4), that also contain αv integrin-mCherry (Fig S5F). This population likely reflects the loading of vesiculotubular endosomes near the cell surface that results from construct expression, similar to that previously reported (Lampson et al., 2001).

To test the impact of AAK1L or EHD3 loss on focal adhesion formation, we transfected plasmid encoding β3-GFP into cells depleted of AAK1L or EHD3 after 72 hr

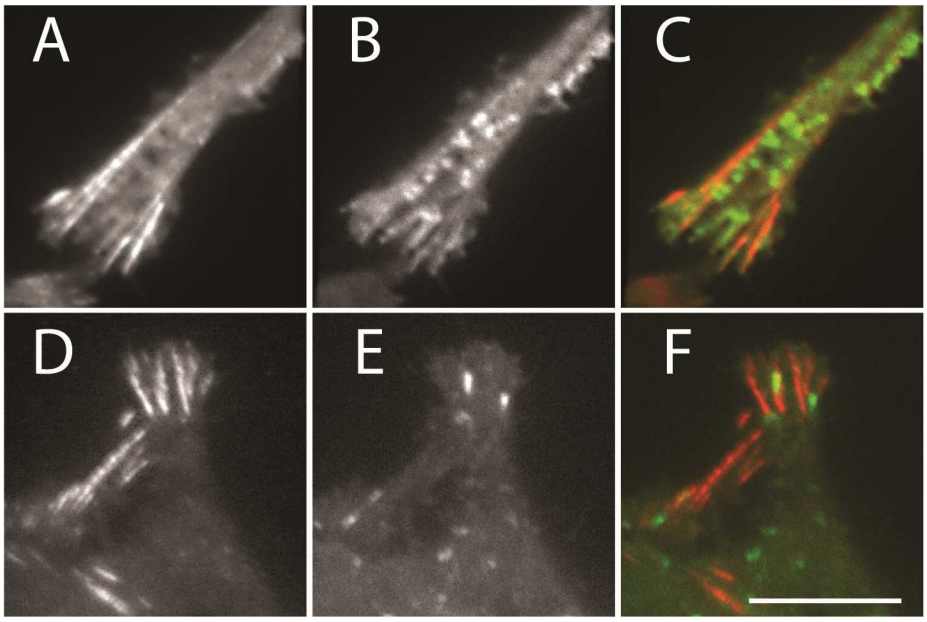

Figure S3: AAK1L- and EHD3-positive endosomes are present adjacent to focal adhesions. HeLa were cotransfected with plasmid encoding β3 integrin-GFP (B, E) and AAK1L-mChr (A) or mChr-EHD3 (D). Live cells were visualized by TIRF microscopy. Colocalization is indicated in the merged panels (C, F). Arrow heads indicate examples of colocalization. Bar = 20 µm.

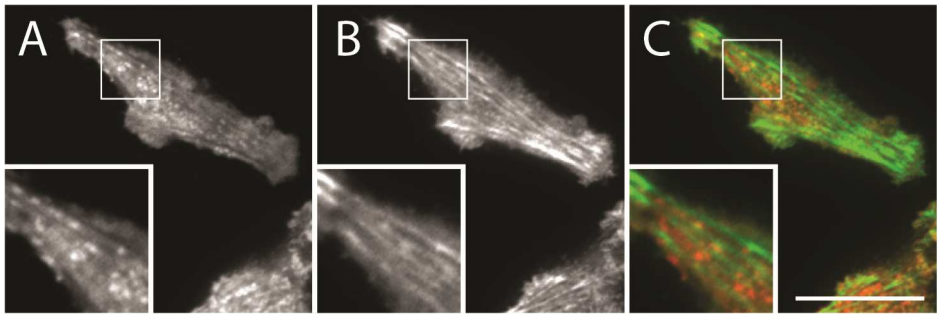

Figure 3: AAK1L-positive endosomes align with actin filaments near the cell surface. MEFs were cotransfected with plasmid encoding AAK1L-mChr (A) and actin-GFP (B) and live cells were imaged by TIRF microscopy. Merged images are shown in C. Boxed inset shows region of higher magnification. Bar = 20 µm.

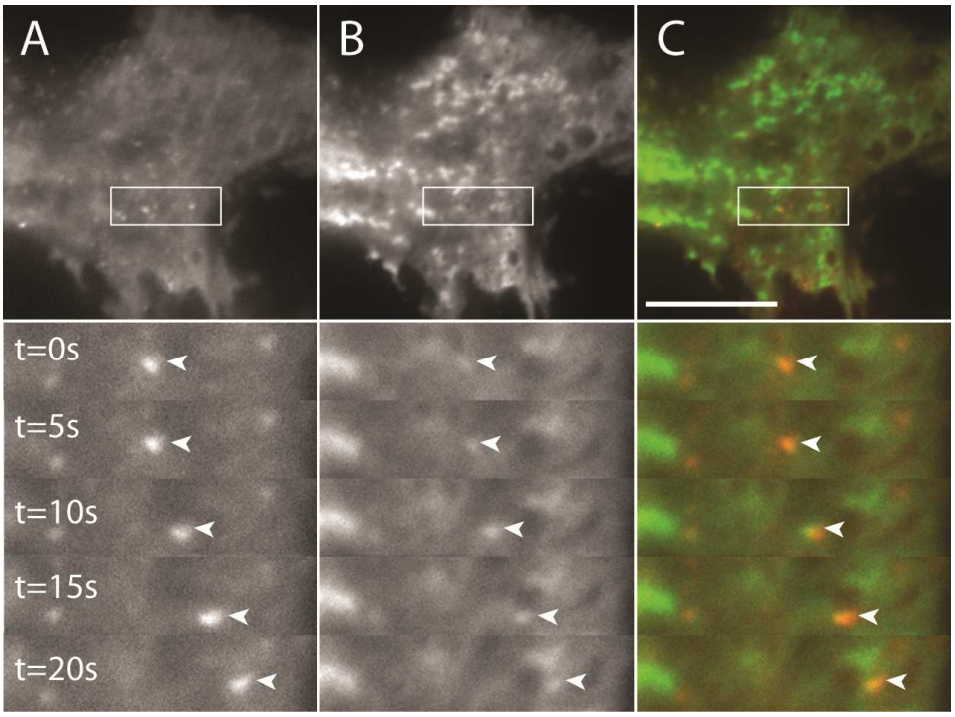

Figure 4: AAK1L and EHD3 colocalize on a highly mobile endosome subpopulation near the cell surface. MEFs were cotransfected with plasmid encoding mChr-EHD3 (A) and AAK1L-GFP (B). Live cells were imaged by TIRF microscopy. Images were sequentially captured at the indicated times (see Sup. Mov. 3). Boxed inset indicates magnified region shown below. Merged series shown in C. Arrow heads show colocalizing endosomes. Bar = 20 µm.

of siRNA treatment — a time point when the majority of cells remain adherent and adherent cells show no difference in apoptosis relative to controls (Fig. S6). In cells treated with control siRNA, β3-GFP recruitment to and concentration in focal adhesions becomes apparent within ~45 min after photobleach in HeLa cells (Fig. 5B-E). Time-lapse imaging reveals that focal adhesion formation is preceded by β3-GFP-positive tubular endosome recruitment near the site of focal adhesion formation (Sup. Mov. 5). By contrast, little recovery was observed in AAK1L-depleted cells during the same time course (Fig. 5F-J), although similar tubular endosome dynamics to control cells were observed (Sup. Mov. 6). Consistently, defects in focal adhesion recovery were also observed in EHD3-depleted cells, but not following depletion of EHD1 (Fig. 6, Sup. Mov. 7 and 8). Although β3 integrin focal adhesion recovery following photobleach agrees well with published observations (Tsuruta et al., 2002), we next tested the possibility that AAK1L or EHD3 depletion might globally impact focal adhesion formation and/or stability. This was not the case; however, since paxillin-mChr-marked focal adhesions were still observed following an extended 72 hr knockdown of either factor (Fig. S4D/G, Sup. Mov. 9). Despite the presence of focal adhesions, little colocalization between β3 integrin and paxillin was observed compared to control cells (Fig. S4C). Instead, β3 integrin was redistributed into tubular endosomes following AAK1L or EHD3 knockdown (Fig. S4E/H). Collectively, these data illustrate the important role of AAK1L and EHD3 in promoting β3 integrin delivery to the plasma membrane.

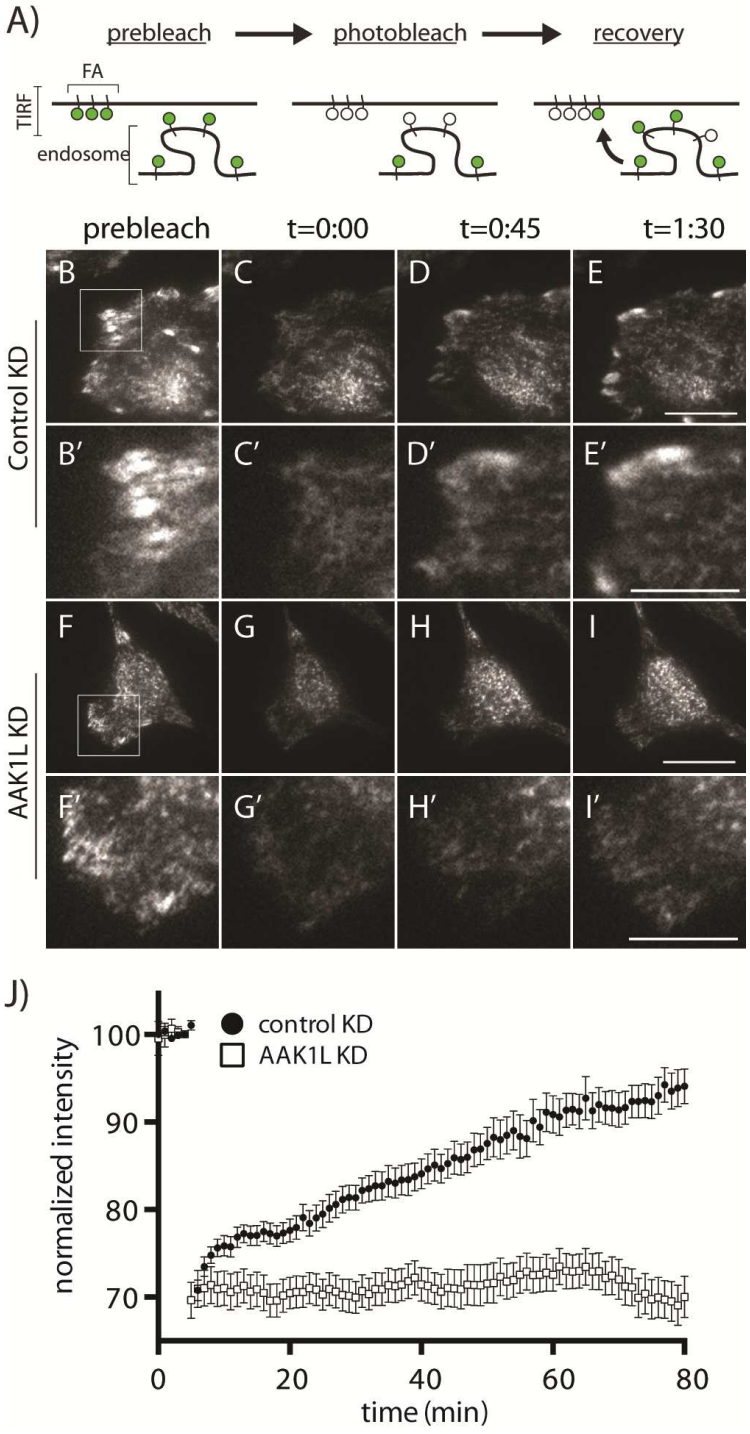

Figure 5: AAK1L depletion delays β3 integrin delivery to focal adhesions

Figure 5: AAK1L depletion delays β3 integrin delivery to focal adhesions. HeLa cells were transfected with control- or AAK1L-specific siRNAs. After 36 hr cells were transfected with plasmid encoding β3-GFP and live cells were imaged by TIRF microscopy. A) Diagram of the experimental design indicating the cell surface photobleach and recovery time-course. Time-course of β3-GFP focal adhesion formation in control (B-E) or AAK1L (F-I) siRNA treated cells (see Sup. Mov. 5 and 6). Boxed regions are shown in higher magnification in the prime-lettered boxes. Images are representative of at least 10 different cells from 3 independent experiments. Bar = 10 µm. J) Time-course quantification (see Materials and Methods) of β3-GFP fluorescence recovery in focal adhesions for each indicated condition. Error bars indicate ±SD.

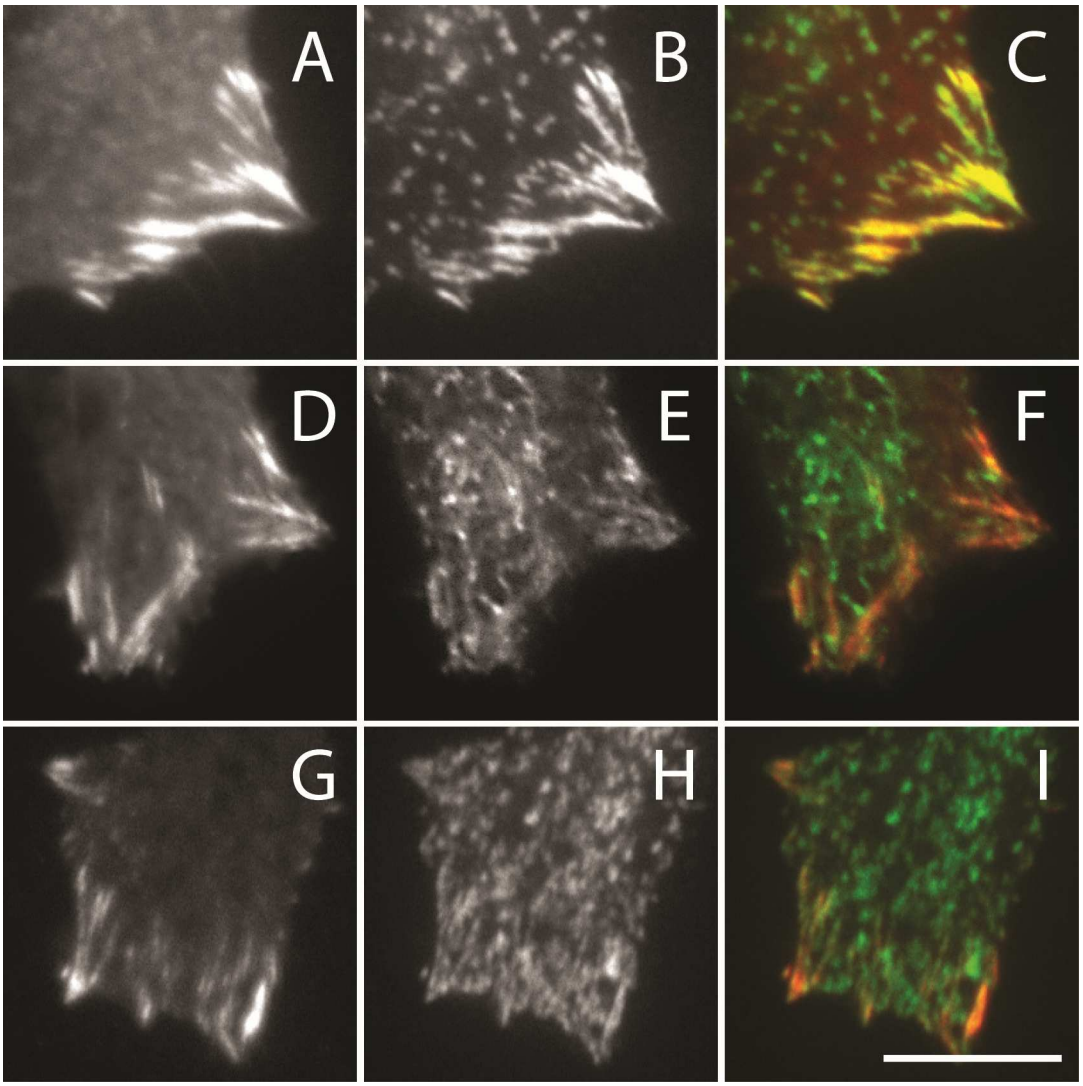

Figure S4: β3 integrin is redistributed from focal adhesions to tubular endosomes following AAK1L or EHD3 depletion. HeLa cells were treated with control siRNAs (A-C) or those targeting AAK1L (D-F) or EHD3 (G-H) for in presence of Z-VAD-FMK. After 60 hr incubation, cells were transfected with plasmid encoding paxillin-mChr (A, D, G) and β3 integrin-GFP (B, E, H). Live cells were then visualized at 72 hr by TIRF microscopy. Bar = 20 µm.

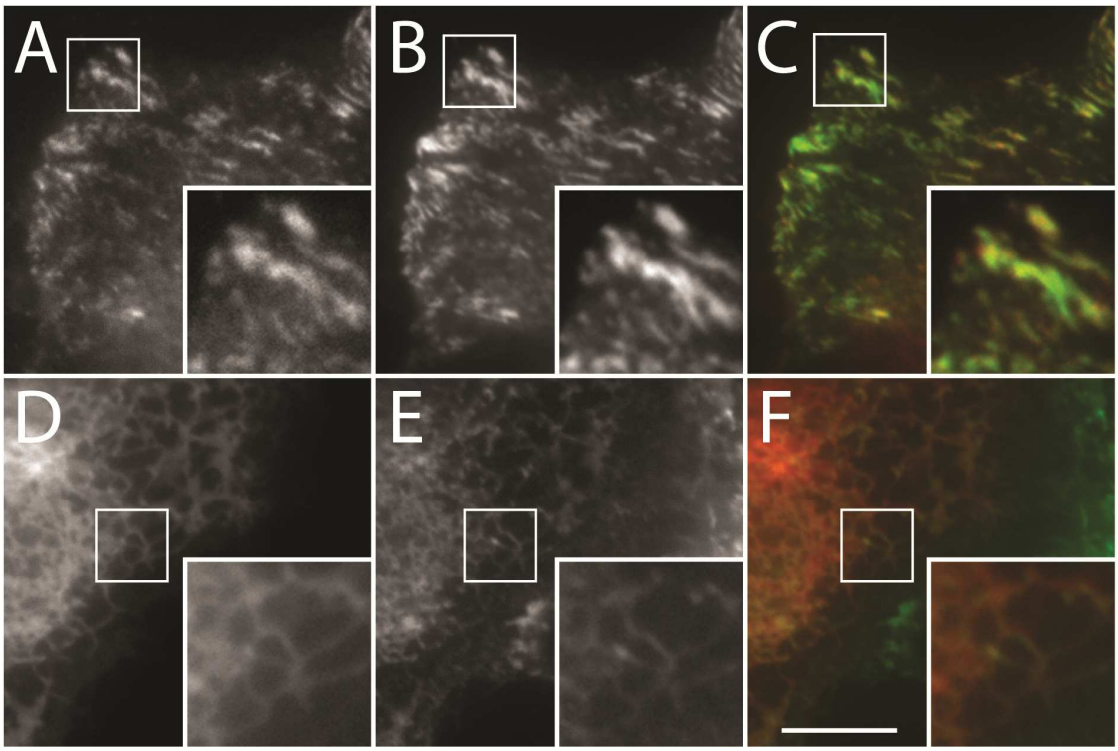

Figure S5: Colocalization analysis of recombinant αv integrin-mChr and β3 integrin-GFP. HeLa cells were cotransfected with plasmid encoding αv integrin-mChr (A, D) and β3 integrin-GFP (B, E). Live cells were visualized by TIRF microscopy, where colocalization was observed in focal adhesions (A-C) and tubular endosomes after focal adhesion photobleach (D-F). Insets represent boxed regions at higher magnification. Bar = 20 µm.

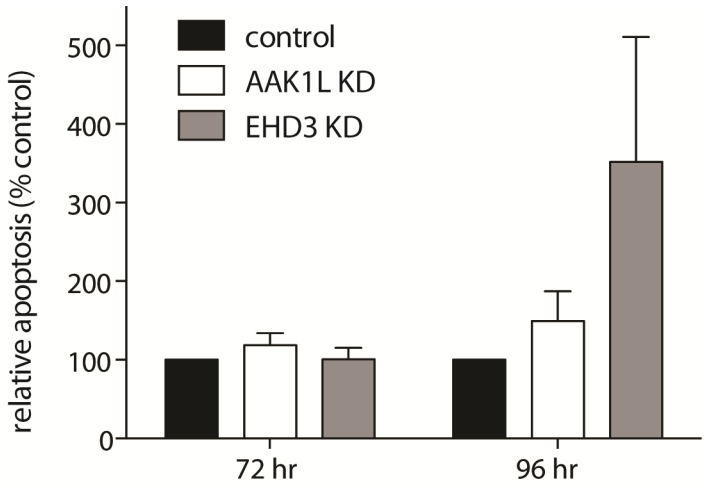

Figure S6: Apoptosis time-course for adherent cells. HeLa cells were treated with control siRNA or those targeting AAK1L or EHD3 for the indicated times. Cells that remained adherent to dishes were then evaluated for apoptosis using the Annexin V-APC apoptosis assay (see Materials and Methods).

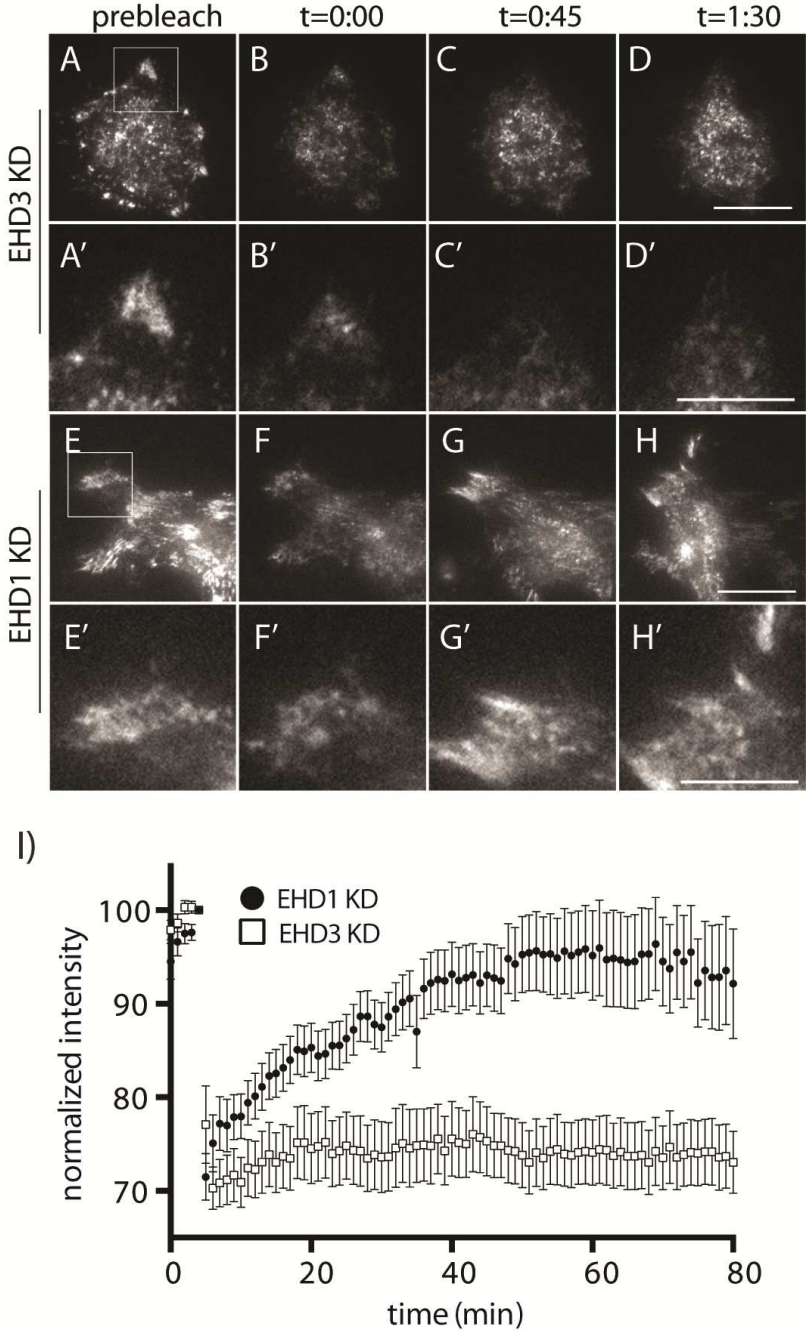

Figure 6: The rate of β3 integrin delivery to focal adhesions is delayed in EHD3-, but not EHD1-depleted cells.

Figure 6: The rate of β3 integrin delivery to focal adhesions is delayed in EHD3-, but not EHD1-depleted cells. HeLa cells transfected with siRNA targeting EHD3 (A-D) or EHD1 (E-H). After 36 hr, cells were transfected with plasmid encoding β3-GFP and live cells were imaged by TIRF at the indicated times (see Sup. Mov. 7 and 8). Boxed regions are shown in higher magnification in the prime-lettered boxes. Images are representative of at least 10 different cells from 3 independent experiments. Bar = 10 µm. I) Time-course quantification of β3-GFP fluorescence recovery in focal adhesions for each indicated condition. Error bars indicate ±SD.

We next tested the hypothesis that AAK1L and EHD3 function in a rapid recycling pathway to deliver β3 integrin to the cell surface using a cell surface biotinylation-based assay (Roberts et al., 2001). Consistent with our live-cell imaging results, we observed a marked reduction in β3 integrin rapid recycling rate following depletion of either AAK1L or EHD3 (Fig. S7A). Based on these results, we conclude that AAK1L and EHD3 are critical for rapid recycling of β3 integrin. Given the reduced rapid recycling rate, we anticipated that β3 integrin cell surface expression would be altered following AAK1L or EHD3 depletion. However, when β3 integrin cell surface levels were analyzed by flow cytometry, we failed to detect a statistically significant difference between control and AAK1L- or EHD3-depleted cells (Fig. 7D). This latter observation is consistent with published studies where perturbations in integrin recycling or endosomal transport failed to correlate with alterations in integrin cell surface levels (Caswell et al., 2007; White et al., 2007; Fang et al., 2010).

β3 integrin recycling requires AAK1L kinase activity

Published observations illustrate the importance of kinases in regulating integrin recycling and delivery to nascent focal adhesions (Woods et al., 2004). Thus, to extend our analysis, we asked if AAK1L kinase activity was essential for maintaining αvβ3 integrin-mediated cell adhesion in HeLa cells. To do so, cells were depleted of AAK1L using siRNA targeting the 5' UTR to disrupt cell adhesion. Cells were then infected with

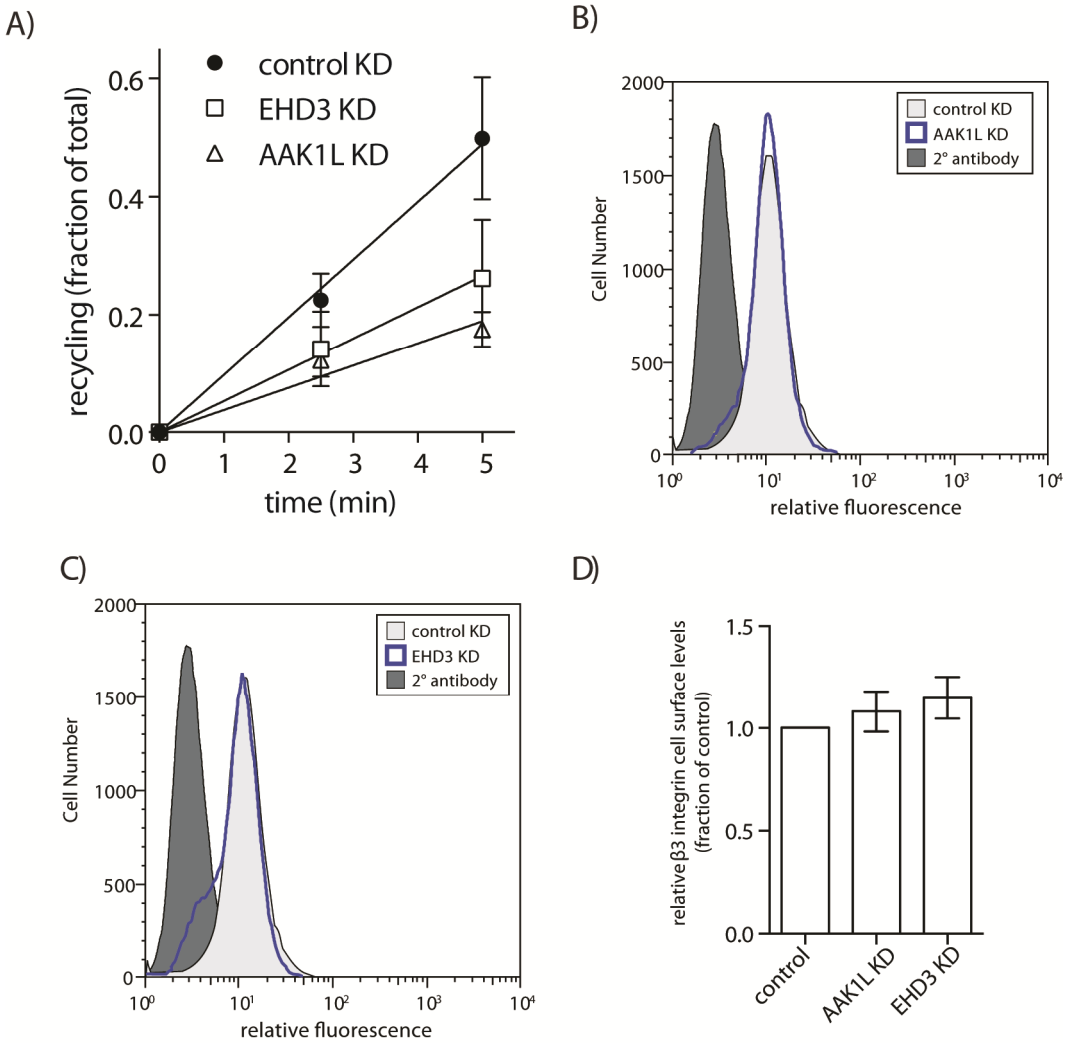

Figure S7: AAK1L and EHD3 promote β3 integrin rapid recycling.

Figure S7: AAK1L and EHD3 promote β3 integrin rapid recycling. A) The rapid recycling assay was performed in serum starved HeLa cells that were treated with control siRNA or those targeting AAK1L or EHD3 as indicated for 72 hr. Surface proteins were then biotinylated with 0.2 mg/mL NHS-SS-Biotin at 4°C for 30 min. Biotinylated cell surface proteins were allowed to internalize for 15 min at 22°C and rapid recycling was evaluated for the indicated times in the presence of serum containing media. MesNa treatment at 4°C was used to remove remaining cell surface biotin. Recycling kinetics were plotted from 3 independent experiments. B-D) β3 integrin cell surface levels were evaluated in AAK1L- (B) or EHD3- (C) depleted HeLa cells using flow cytometry counting 50,000 cells and compared to cells treated with control siRNA (control KD) or cells treated with secondary antibody alone, example flow charts are shown (B/C). For clarity, the siRNA and 2° antibody controls for AAK1L and EHD3 depletion were duplicated and shown in each chart. Data from 5 independent experiments were plotted (D). β3 integrin antibody (#555752*)* used for flow cytometry was obtained from BD Biosciences. Error bars indicate ± SEM. Additional information is available in the Materials and Methods.

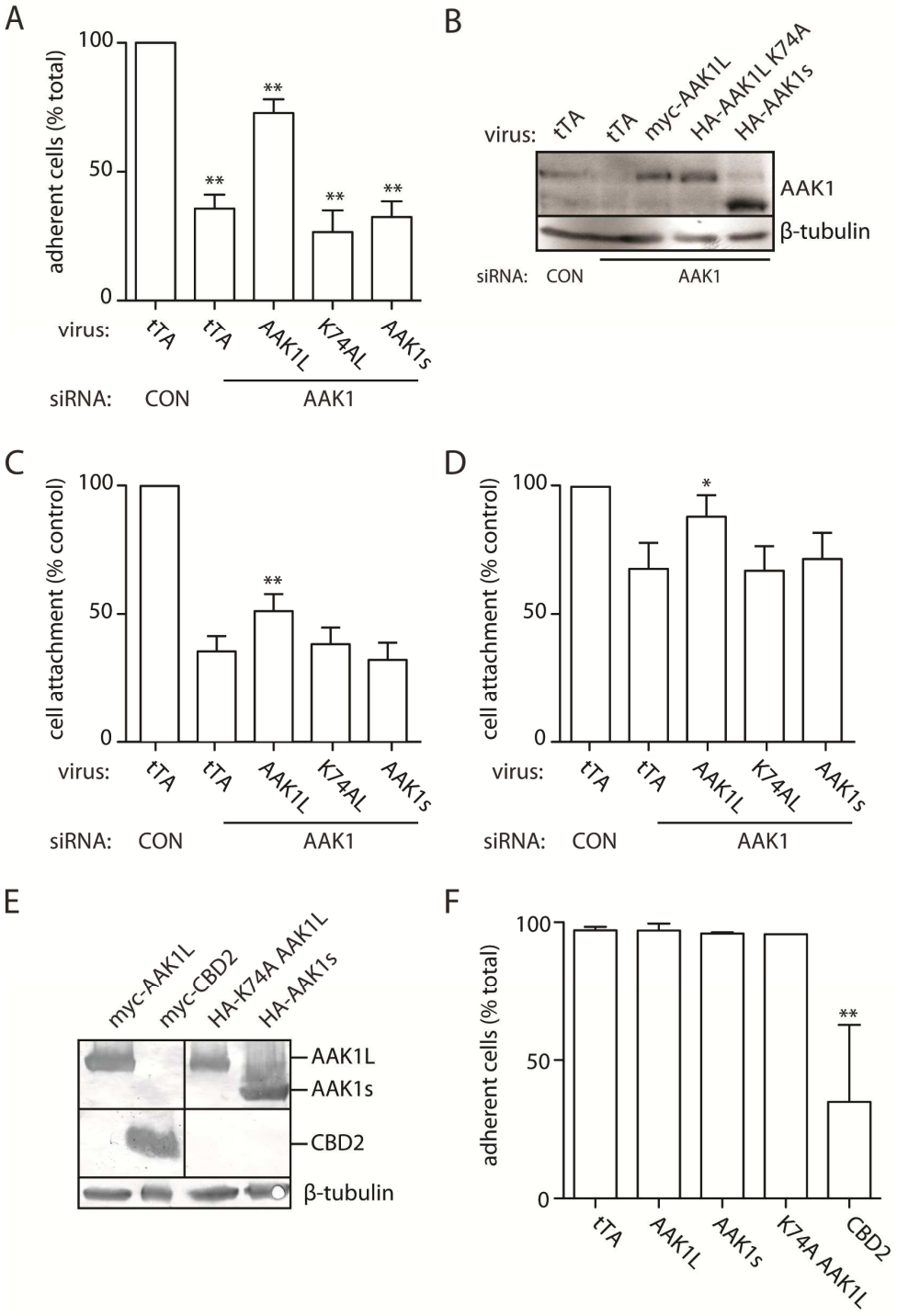

Figure 7: Cell adhesion requires AAK1L kinase activity

Figure 7: Cell adhesion requires AAK1L kinase activity. A) tTA HeLa cells were transfected with control or AAK1L specific siRNAs. After 36 hr, cells were infected with adenovirus encoding tTA (control), AAK1L, K74A AAK1L (kinase dead), or AAK1s in the presence of 15 ng/mL tetracycline to limit protein expression. Cells were evaluated for adhesion defects following an additional 24 hr incubation. B) Immunoblot analysis of protein expression levels for the indicated forms of AAK1 in (A). C/D) tTA HeLa cells were treated as indicated in (A), removed from dishes using PBS/EDTA and replated on vitronectin- (C) or fibronectin-covered (D) dishes to evaluate cell attachment. E) tTA HeLa cells were infected with adenovirus encoding the indicated AAK1 form in the absence of tetracycline, evaluated by immunoblot for protein expression levels, and assayed for defects in cell adhesion (F). Error bars represent ± SD of three independent experiments. * and ** indicates a significance of $p<0.05$ and $p<0.005$ for a paired *t* test.

adenovirus encoding various siRNA-resistant forms of AAK1L, the expression of which was under control of a tetracycline-regulatable promoter to limit protein expression to near endogenous levels. We reasoned that if kinase activity was essential to αvβ3 integrin transport, kinase dead AAK1L would be unable to rescue the adhesion defect on culture dishes following AAK1L depletion. Indeed, kinase dead AAK1L (K74A AAK1L), like infection controls, failed to rescue cell adhesion defects, while a significant rescue was observed following WT AAK1L expression (Fig. 7A). Our previous findings indicate that the carboxy-terminal clathrin binding domain (CBD2) is critical for AAK1L function (Henderson & Conner, 2007). Consistent with this, cell adhesion defects were not rescued following adenovirus-mediated expression of the short AAK1 isoform (AAK1s), which lacks CBD2. To extend our analysis, we next tested the ability of siRNA-resistant AAK1L to restore cell attachment to vitronectin or fibronectin in AAK1L-depleted cells. By comparison to the rescue in cell adhesion to tissue culture dishes (Fig. 7A), the degree of rescue for vitronectin and fibronectin attachment was reduced, although statistically significant relative to control cells or those expressing kinase dead AAK1L or AAK1s (Fig. 7C/D). We interpret the reduced rescue in vitronectin and fibronectin attachment to reveal suboptimal kinase expression conditions where siRNA-resistant AAK1L expression was sufficient to maintain cell adhesion to culture dishes, but not optimal for the robust reestablishment of new extracellular matrix attachments following cell replating.

The inability of AAK1s to rescue cell adhesion defects following depletion of

endogenous AAK1L suggested that protein interactions mediated through CBD2 are important for AAK1L function in integrin recycling. We next tested the hypothesis that CBD2 might compete for critical factors mediating β3 integrin transport in a dominant-negative fashion following overexpression, thereby disrupting cell adhesion to culture dishes. Indeed, CBD2 overexpression led to cell adhesion defects, while no defects were observed following overexpression of WT AAK1L, K74A AAK1L, or AAK1s (Fig. 7E). To confirm that CBD2 disrupts β3 integrin recycling, we tested β3-GFP focal adhesion formation in CBD2-overexpressing cells after photobleach by TIRF in live cells.

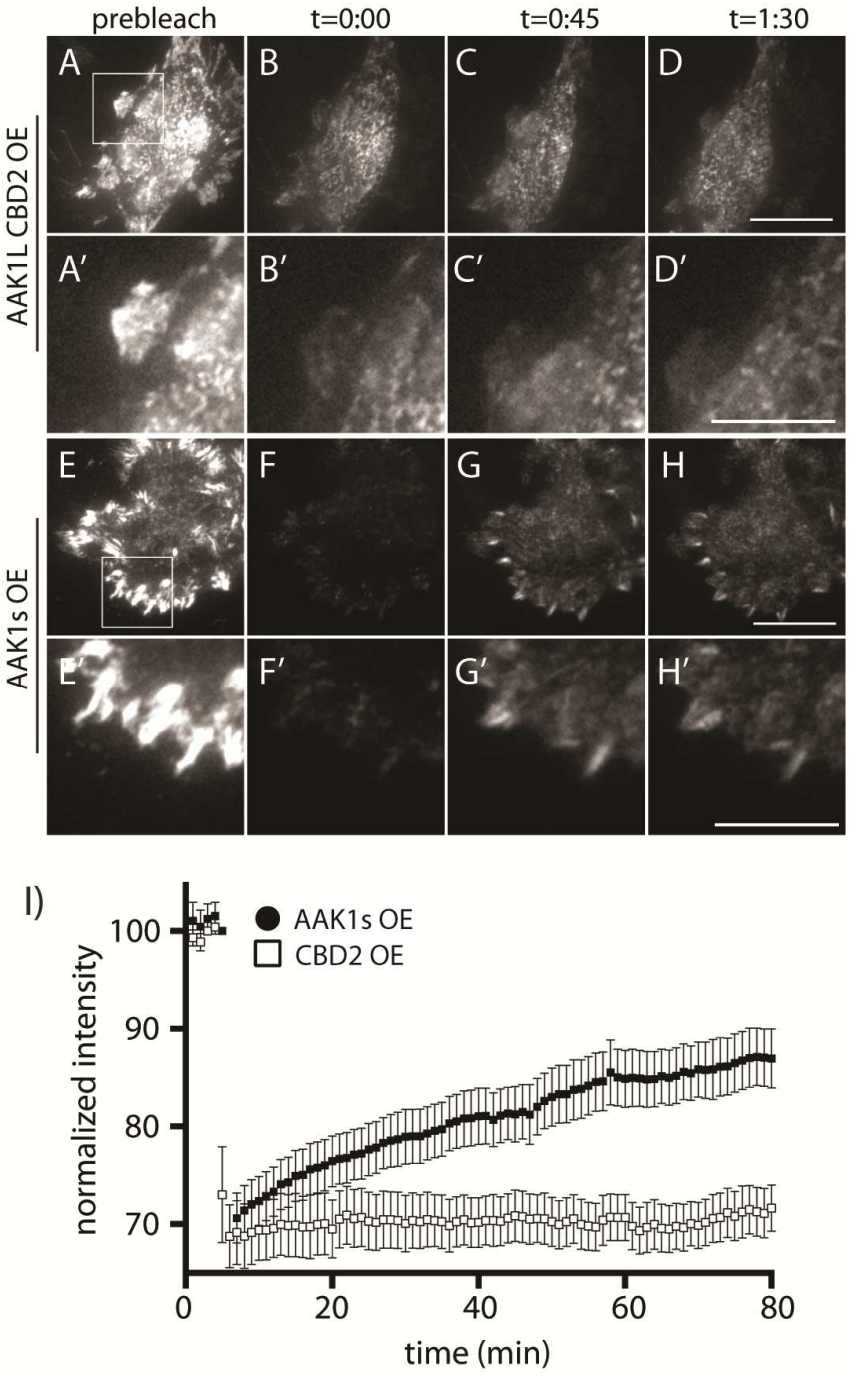

Figure 8: AAK1L CBD2 overexpression (OE) delays β3 integrin delivery to focal adhesions.

Figure 8: AAK1L CBD2 overexpression (OE) delays β3 integrin delivery to focal adhesions. β3-GFP-expressing tTA HeLa cells were infected with adenovirus encoding AAK1L CBD2 (A-D, Sup. Mov. 10) or AAK1s (E-H, Sup. Mov. 11). Live cells were then evaluated for focal adhesion formation following photobleach, as described in Fig. 5. Boxed regions are shown in higher magnification in the prime-lettered boxes. Images are representative of at least 10 different cells from 3 independent experiments. Bar = 10 µm. I) Time-course quantification of β3-GFP fluorescence recovery in focal adhesions for each indicated condition. Error bars indicate ±SD.

Consistent with adhesion data, focal adhesion formation was impaired in cells overexpressing CBD2. By contrast, focal adhesions readily formed within 45 min in cells overexpressing AAK1s (Fig. 8, Sup. Mov. 10 and 11). Based on these findings, we conclude that AAK1L kinase activity and the carboxy-terminal region are essential for promoting β3 integrin recycling to maintain cell adhesion.

Section E: Discussion

Phosphorylation regulates integrin recycling

A growing number of studies position phosphorylation as a key regulatory mechanism in governing integrin recycling from the EE/SE (Roberts et al., 2004; White et al., 2007; Woods et al., 2004). Here we provide evidence demonstrating a previously unknown role for AAK1L in rapid/short-loop β3 integrin recycling. Loss-of-function analyses reveal that AAK1L is critical for maintaining appropriate β3 integrin cell surface distribution sufficient to support cell adhesion to vitronectin and fibronectin. Additionally, rescue studies, combined with the dominant-negative impact of CBD2 overexpression on β3 integrin recycling and cell adhesion, indicate that kinase activity and CBD2 are essential in mediating AAK1L-dependent integrin recycling events. This latter observation is intriguing given that CBD2 supports interaction with clathrin, the binding to which stimulates AAK1L kinase activity toward several of its known targets, AP-1, AP-2, and Numb1 (Conner et al., 2003; Henderson & Conner, 2007; Sorensen & Conner, 2008). *In vitro* reconstitution studies indicate that clathrin and AP-1 promote formation of

endosome-derived vesicles via a rab4-mediated pathway (Pagano et al., 2004). Given the well-described role for rab4 in β3 integrin rapid recycling (Roberts et al., 2001), it is tempting to speculate that AAK1L-dependent phosphorylation is critical for AP-1-mediated packaging of β3 integrin into transport vesicles for recycling. However, given adhesion defects on culture dishes were not observed in HeLa cells following AP-1 depletion (data not shown), we do not favor this model.

Alternatively, AP-2 has also been implicated in post-endocytic sorting events for β1 integrin and the major histocompatibility complex class I through an Arf6-mediated pathway (Lau and Chou, 2008). However, AP-2 also performs a predominant role in promoting β1 integrin endocytosis (Teckchandani et al., 2009). Similarly, Numb1 functions at multiple receptor transport steps. Loss-of-function studies indicate that Numb1 is critical for β1 integrin internalization (Teckchandani et al., 2009) and endosomal sorting decisions, where it directs Notch to the degradative pathway (McGill et al., 2009). Therefore, resolving the potential connection between AAK1L and AP-2 or Numb1 in β3 integrin recycling has proved challenging, especially given that cell adhesion on culture dishes was unperturbed following AP-2 or Numb1 depletion (Teckchandani et al., 2009). It is also possible; however, that AAK1L targets other factors that coordinate β3 integrin recycling.

AAK1L belongs to the Ark1/Prk1 family of serine/threonine kinases, which, in yeast, targets actin regulatory proteins implicated in endocytosis (Cope et al., 1999; Engqvist-

Goldstein & Drubin, 2003; Henry et al., 2003; Sekiya-Kawasaki et al., 2003; Watson et al., 2001). A role for AAK1L in regulating actin dynamics in mammalian systems is currently unknown. However, our live-cell TIRF imaging reveals the presence of AAK1L on endosomes directly subjacent to the plasma membrane, which align along actin filaments. This suggests a functional connection between rapid recycling endosomes and the actin cytoskeleton. Indeed, rapid recycling of transferrin and β2-adrenergic receptors is dependent on the actin cytoskeleton (Millman et al., 2008; Yan et al., 2005). Linking constitutive recycling endosomes to the actin cytoskeleton is thought to be accomplished by the CART complex (Yan et al., 2005). Actin-dependent recycling is also regulated by supervillin, which links peripheral endosomes to the actin cytoskeleton and promotes rapid recycling of both β1 and β3 integrins (Fang et al., 2010). Given the requirement of AAK1L activity in transferrin receptor (Henderson & Conner, 2007) and β3 integrin recycling, its localization to actin-aligned endosomes, and its homology to actin-regulating kinases, we speculate that AAK1L may function to regulate endosome linkages to the actin cytoskeleton to facilitate endosome recycling. Our future efforts will be directed at resolving this possibility.

EHD3-dependent sorting decisions from the EE/SE

EHD protein family members have distinct roles in endocytic transport of numerous receptor types (Grant & Caplan, 2008). Here we provide evidence demonstrating a previously unappreciated role for EHD3 in directing β3 integrin recycling from the EE/SE to the plasma membrane. Consistent with a general recycling role, EHD3-

deficient cells have reduced plasma membrane delivery of the Na^+/Ca^+ exchanger in cardiomyocytes (Gudmundsson et al., 2010), although the intracellular transport pathway was not resolved in this study. Our observations, combined with published reports, suggest that EHD3 directs myriad integral membrane proteins from the EE/SE to their proper destination. For example, EHD3 depletion disrupts Shiga toxin delivery from the EE/SE to the Golgi (Naslavsky et al., 2009) and impairs transferrin receptor targeting to the ERC (Naslavsky et al., 2006). While the EHD3 mechanism of action is currently unknown, the EHD ATP-binding G domain is critical for phospholipid binding and membrane recruitment (Blume et al., 2007; Daumke et al., 2007; Naslavsky et al., 2007). Following recruitment to membranes, the EH domain might serve as a nucleation site to recruit auxiliary factors that promote cargo sorting within the EE/SE. Indeed, the EHD3 binding partner, rabenosyn-5, is essential for Shiga toxin targeting to the Golgi following internalization (Naslavsky et al., 2009). Similarly, EHD3 acts in concert with Rab11-FIP2 to direct transferrin receptor to the ERC (Naslavsky et al., 2006).

What auxiliary factor(s) might be critical for EHD3-mediated β3 integrin recycling from the EE/SE? Based on our findings, AAK1L is an obvious candidate given its colocalization with EHD3 on dynamic endosomes directly subjacent to the plasma membrane and the observation that EHD3 or AAK1L loss-of-function leads to β3 integrin recycling defects. However, our current results do not allow us to conclude a mechanistic relationship between these two factors. Since the majority of AAK1L-positive endosomes lack EHD3, it is possible that AAK1L and EHD3 act within different

early endosome subcompartments to regulate β3 integrin transport. It is also possible that EHD3 function is directly regulated by AAK1L kinase activity. Alternatively, given that EHD3 localizes to microtubule-dependent endosome tubules (Galperin et al., 2002) and AAK1L endosomes align with actin filaments, we speculate that EHD3 and AAK1L may promote recycling endosome transfer from the microtubule to actin cytoskeletal network for plasma membrane delivery. Determining the potential mechanistic relationship between AAK1L and EHD3 in promoting β3 integrin recycling will necessitate additional investigation.

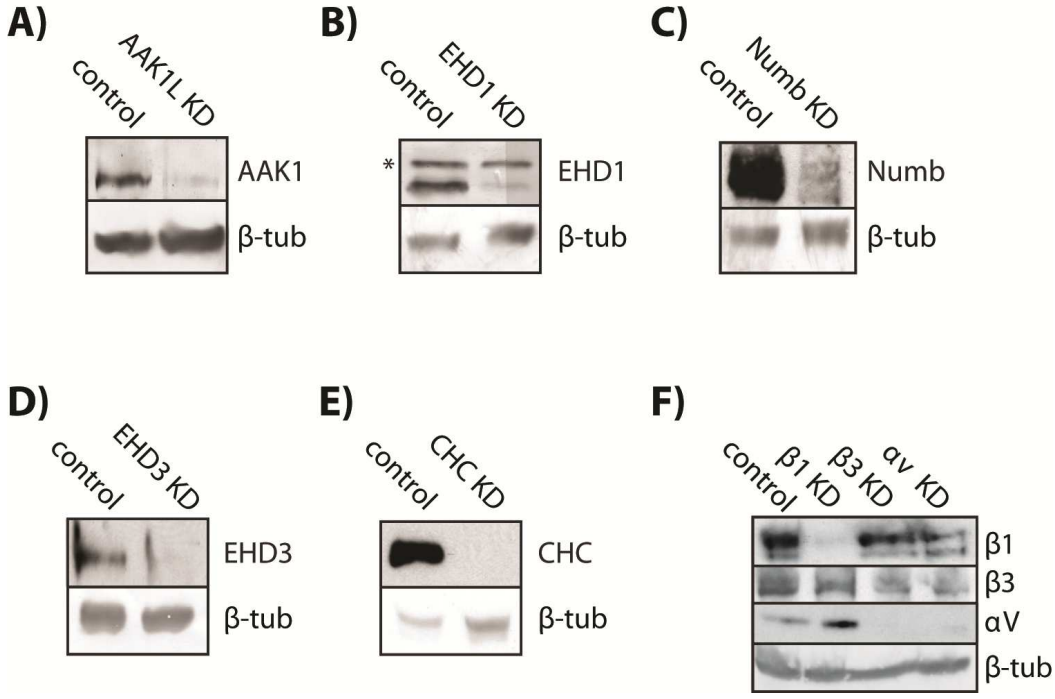

Figure S8: Immunoblot analysis of tTA HeLa cell lysates following treatment with control or siRNAs targeting the indicated factor. Blots were probed for beta tubulin as a protein loading control. Asterisk in B indicates a non-specific cross-reacting band.

Chapter IV: Conclusions and future directions

Integrin transit through the EE/SE promotes cell function and survival

After endocytosis, integrin sorting decisions are made at the early/sorting endosome (EE/SE), which directs integrins to the lysosome for degradation or returns integrins to the plasma membrane via the short- or long-loop recycling pathways. While originally postulated that receptors only simply sorted by geometry in the endosome, increasing evidence recognizes endosomal sorting as a highly regulated process (Maxfield & McGraw, 2004; Bridgewater et al., 2012). I provide evidence that AAK1L and EHD3 are important EE/SE factors that regulate integrin rapid recycling to the plasma membrane.

Targeting focal adhesions with a TIRF photobleach/recovery approach, we selectively imaged right at and right below the ventral plasma membrane and observed that bleached focal adhesions in AAK1L- and EHD3-depleted mammalian cells recovered much more slowly than focal adhesions in control-siRNA-depleted cells or in cells depleted of EHD1, a long-loop recycling factor (Chapter 3). This demonstrates the important role that AAK1L and EHD3, early endosomal factors, have in promoting β3 integrin return to the plasma membrane. Similarly for PDGF-stimulated short-loop recycling, depletion of protein kinase D1 (PKD1) reduced the amount of β3 integrin recruited to focal adhesions at the leading edge (Woods et al., 2004). These data indicate that disruptions in integrin sorting at the early endosome have repercussions for β3

integrin delivery to the functional unit of adherence, focal adhesions.

It is unclear whether or not integrins are delivered directly to focal adhesions. In the control experiments of both previous reports (Chapter 3; Woods et al., 2004), recovery of bleached focal adhesions occurred around 45 minutes, which is similar to the photobleach recovery timing of integrins previously reported (Tsuruta et al., 2002). Because transit through the short-loop recycling pathway takes approximately 15 minutes, it suggests that integrins are not delivered and incorporated directly into the focal adhesion. We observe tubular endosome structures near the ventral plasma membrane that extend directly underneath focal adhesions (Chapter 3) and would anticipate focal adhesion recovery times to be similar to recycling times if the integrin returned directly to the adhesion. One hypothesis is that integrins are delivered to the plasma membrane near the focal adhesion and a regulated process exists to incorporate the integrins into the adhesion. Indeed, nanostructure analysis of focal adhesions indicates that β3 integrin freely and rapidly diffuses in the plasma membrane near the focal adhesion, but within the focal adhesion, β3 integrin is stationary and immobilized. The evidence suggests that integrin incorporation into a focal adhesion is a process that involves increased integrin binding to ECM, activation and integrin interaction with cytoplasmic proteins that mediate actin cytoskeleton linkages. However, these processes can contribute to the dynamic remodeling of an adhesion that has been observed on a timescale of seconds to a few minutes (Rossier et al., 2012). These data suggest that integrins are not incorporated directly into the focal adhesion and dissecting the

relationship between focal adhesions and trafficking will be the focus of future studies.

Depletion of either AAK1L or EHD3 led to loss of HeLa cell adherence to plastic dishes and on both vitronectin and fibronectin ligands (Chapter 3). Prolonged siRNA-mediated treatments for EHD3 and AAK1L results in increased apoptosis (Chapter 3) which was anticipated given that integrin-ECM adhesion is required for survival in many cell types (Meredith & Schwartz, 1997). Depletion of a long-loop recycling factor (EHD1) or ESCRT lysosomal sorting factors (Tsg101) in HeLa cells (Chapters 2 & 3) did not lead to loss of cell adhesion or reductions in survival in the time frame observed. This indicates that receptor passage through the early endosome is imperative for the promotion of cell survival.

When investigating which integrins were mediating cell adhesion in HeLa cells, depletion of β3 integrin, not the more predominant β1 integrin, resulted in cell adhesion defects to plastic dishes (Chapters 2 & 3). Depletion of β3 integrin's primary heterodimer partner, αv, also led to loss of cell adhesion in HeLa cells (Chapter 3), which has been reported in previous findings (Ballana et al., 2009). Different cell types express different combinations of integrin heterodimers, allowing the cell to have a nuanced, specialized response to its extracellular environment (Hynes, 2002). However, this has made it difficult to ascertain whether integrin trafficking defects and their disruption of cell function translates broadly across different cell lines or even in comparisons of trafficking studies done in cancerous versus non-cancerous cell lines. However, it is clear

in all cell types, that changes in integrin trafficking affects motility and that communication between growth factor receptors and integrin receptors has an important role in regulating integrin trafficking (Ivaska & Heino, 2011). Depletion of β3 or αv integrin leads to loss of adhesion in HeLa cells, a cervical cancer cell line (Chapters 2 & 3; Ballana et al., 2009); however, mouse embryonic fibroblast (MEF) knockout cell lines for β3 and αv integrin have been created (Bader et al., 1998; Hodivala-Dilke et al., 1999; Schmidt et al., 2013), which supports the hypothesis that certain integrin heterodimers mediate cell survival signaling in a cell-type-specific manner. Future work will focus on determining which signaling pathways that promote cell survival are perturbed in cells with rapid recycling defects.

Sorting of activated versus inactivated receptors

Using a siRNA-mediated loss-of-function approach, I find that maintenance of active-conformation β1 integrin levels at the plasma membrane requires AAK1L (Chapter 2). These observations are supported by additional findings that depletion of EE/SE-associated sorting factors results in reductions in levels of activated β1 integrin at the cell surface (Tiwari et al., 2011; Steinberg et al., 2012). This indicates that sorting at the EE/SE is an important transport step for activated integrins. However, it could be possible that these factors have a role at the EE/SE that is resensitizing or repriming integrins to be in the activated, or high-affinity ligand binding, state. It has been demonstrated that expression of a dominant-negative Rab4, an early endosomal localized GTPase, prevents the recycling and resensitization of the β_2-adrenergic receptor (β_2AR)

from the early endosome (Seachrist et al., 2000). The mechanism of resensitization of integrins for ligand has been proposed for growth factor-promoted short-loop recycling of β3 integrin and long-loop recycling of β1 integrin in fibroblasts to explain their contributions and influence on cell migration (White et al., 2007). However, recent studies provide evidence that integrins in an activated state can undergo endocytosis and recycling, and are transported through Rab4-, 5- and 11-positive compartments, which is contrary to what happens to β_2AR, which is desensitized through internalization (Seachrist et al., 2000; Odley et al., 2004; Arjonen et al., 2012; Steinberg et al., 2012).

Depletion of EE/SE factors leads to increased integrin transport to the lysosome (Chapter 2; Tiwari et al., 2011; Steinberg et al., 2012). Likewise, when transit to the lysosome is disrupted, via siRNA-mediated depletion of lysosomal-sorting factors, activated integrins accumulate in an EE/SE-positive compartment and their degradation is decreased (Chapter 2; Lobert et al., 2010). This suggests that receptor recycling is positively promoted by EE/SE-associated factors, like AAK1L, and that depletion of these factors leads to increased transit time at the EE/SE. Integrins are retained at the EE/SE and and mis-sort to other transport pathways. Increased degradation is observed, indicating that transport to the lysosome is increased. However, while reduction of integrin amount is significant, it is not zero, indicating that integrins can be sorted to other pathways to eventually return to the plasma membrane. Integrins (in both conformations) can transit both short- and long-loop recycling pathways (Roberts et al., 2001; Arjonen et al., 2012). Depletion of AAK1L leads to activated integrin

accumulation in a perinuclear compartment and leads to accumulation of the transferrin receptor in the PNRC (Chapter 2; Henderson & Conner, 2007). The identification of the integrin-positive perinuclear compartment in AAK1L-depleted cells is unknown (Chapter 2) and future work will determine whether this compartment is positive for PNRC- or LE/lysosomal-positive markers or a combination of both. It is possible that AAK1L-depletion leads to increased integrin transit to the PNRC and from there, integrin is sorted to the degradative pathway; however, depletion of factors that are imperative for integrin exit from the PNRC do not lead to reductions in integrin levels (Li et al., 2005; Jović et al., 2007), which indicates that receptor fate is determined at the EE/SE.

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