



DIPLOMARBEIT

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The Link between Membrane Trafficking and Cell Motility

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Zusammenfassung

Um sich fortbewegen zu können, muss eine Zelle mit spezialisierten Adhäsionskomplexen fest an ihrem Substrat haften. Die größten dieser Komplexe, „Fokale Adhäsionen“, sind äußerst stabil und bilden eine molekulare Kupplung zwischen dem zellulären Zytoskelett und der extrazellulären Matrix. Zur selben Zeit zeigen diese allerdings hochgradig regulierten Molekülaustausch um zelluläre Bewegung zu ermöglichen. Darüber hinaus sind Fokale Adhäsionen auch an Signaltransduktionsprozessen beteiligt, sowohl für von der Zellumgebung ins Innere der Zelle als auch für nach außen gehende Signale. Es überrascht daher nicht, dass mehr als 150 Proteine für all diese Funktionen benötigt werden.

In dieser wissenschaftlichen Arbeit, untersuchte ich den möglichen Molekülaustausch des Fokalen Adhäsion Proteins Paxillin durch den vesikulären Transport und den Einfluss des Clathrin-Adapters EpsinR auf die Organisation der Fokalen Adhäsionen. Von einem Paxillin-Protein, dessen LIM3-Domäne durch Mutationen geschädigt ist, war bereits bekannt, dass es in Vesikeln lokalisiert ist. Ich konnte zeigen, dass dieses Protein auch in Fokalen Adhäsionen nachweisbar ist, ähnlich dem Wildtyp-Paxillin. Dies deutet an, dass die vesikuläre Lokalisation kein Artefakt ist. Darüber hinaus konnte ich mit der Hilfe von 3D-Dekonvolution Vesikel-ähnliche Strukturen beobachten, die Wildtyp-Paxillin beinhalten. Zusammen zeigen diese Erkenntnisse auf, dass vesikulärer Transport möglicherweise im Molekülaustausch von Paxillin eine Rolle spielt.

Während meiner Diplomarbeit, entdeckte ich weiters, dass EpsinR in der Nähe Fokaler Adhäsionen angereichert ist und vorübergehend mit der oben erwähnten Paxillin-Mutante interagieren kann. Die Inaktivierung von EpsinR durch RNAi führte zur Vergrößerung der Fokalen Adhäsionen und verminderter Migrationsfähigkeit von HeLa-Zellen. Allerdings zeigten Photoaktivierungs-Experimente, dass diese Defekte nicht durch eine Verminderung der Molekülaustausch-Rate erklärt werden können.

Zusätzlich habe ich zwei mit vesikulärem Transport in Verbindung stehende Proteine (CCDC51 und KIF19), die in einem siRNA Screen für Regulatoren der Fokalen Adhäsionen gefunden wurden, geklont und ihre Lokalisierung bestimmt.

Die zwei Proteine CCDC51 und KIF19 konnten im Endoplasmatischen Retikulum beziehungsweise auf Mikrotubuli lokalisiert werden.

Abstract

The migration of a cell requires its attachment to the substrate via specialised adhesion sites. The largest adhesions, “focal adhesions”, are very stable structures and form the molecular clutch between the cytoskeleton and the extracellular matrix. At the same time, they undergo highly regulated turnover to allow cell movement. Furthermore, focal adhesions are also involved in outside-in and inside-out signalling. It is not surprising that these functions require more than 150 proteins.

In the present study, I examined the possible turnover of the focal adhesion scaffolding protein paxillin through the vesicular trafficking pathway and the influence of the clathrin adaptor EpsinR on focal adhesion organisation. A mutant of paxillin containing mutations that disrupt its LIM3 domain was previously described to be localised to vesicles. I showed that this mutant also targets focal adhesions similar to wild-type paxillin, suggesting that the vesicular localisation is not an artefact. Moreover, with the help of 3D-Deconvolution I observed vesicle-like punctate structures containing wild-type paxillin. Together, these findings indicate an involvement of vesicular transport in the turnover of endogenous paxillin.

In the course of this diploma thesis, the clathrin adaptor EpsinR was found to be enriched in the vicinity of focal adhesions and to interact transiently with the paxillin mutant containing the LIM3 disruption. Moreover, EpsinR siRNA knockdown caused enlargement of focal adhesion size and reduced migratory capability of HeLa cells. However, photoactivation experiments showed that these defects could not be explained by a decrease in the turnover rate of paxillin.

In addition, I cloned and localised two vesicular transport related proteins (CCDC51 and KIF19) identified in an earlier siRNA screen for focal adhesion regulators. The two proteins CCDC51 and KIF19 localised to the endoplasmic reticulum and to microtubules respectively.

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1. Introduction

1.1 Focal Adhesions and Cell Migration

Directed cellular migration is a crucial feature in higher organisms. Starting with the development of an embryo, cellular motility is involved in physiological (embryonic development, wound healing, immune surveillance) and pathological processes (cancer and metastases). However, in order to understand these functions on the level of the organism, one has to know the molecular fundamentals of cell migration on the cellular level.

In order for cells to migrate, a complex interplay of actin filaments and microtubules is required. Actin polymerisation is the motor driving the protrusion of the cell at the front and its retraction at the rear. Beside the polymerisation, also the forces exerted by the contractile network (consisting of stable actin-filaments and the myosins) are necessary for cellular migration (Small and Resch 2005). Microtubules, however, have completely different functions. On the one hand, they are binding numerous potent regulators of the actin cytoskeleton (e.g. RhoGAPs and RhoGEFs; Broussard, Webb, and Kaverina 2008); on the other hand the regulation of cellular migration also relies on the transport of vesicles along the microtubules (Caswell and Norman 2008). Furthermore, microfilaments as well as microtubules interact with the primary sites of matrix-cytoskeleton connection, the focal adhesions (Broussard, Webb, and Kaverina 2008). Whereas actin filaments are directly bound by components of the cellular adhesions, microtubules interact mostly indirectly through regulatory factors (Kaverina, Krylyshkina, and Small 1999).

Focal adhesions provide attachment to the extracellular matrix. Although there are indications that movement without this attachment is possible (reviewed in Charras and Paluch 2008), it is most probably limited to amoeboid movement shown by the various lymphocytes and some metastasising carcinoma cells (Friedl and Wolf 2003; Lämmermann et al. 2008). Therefore, the movement utilising focal adhesions is critical for the migration of most tissue cells, especially during embryonic development and for mesenchymal cells in the adult organism.

Figure 1.1

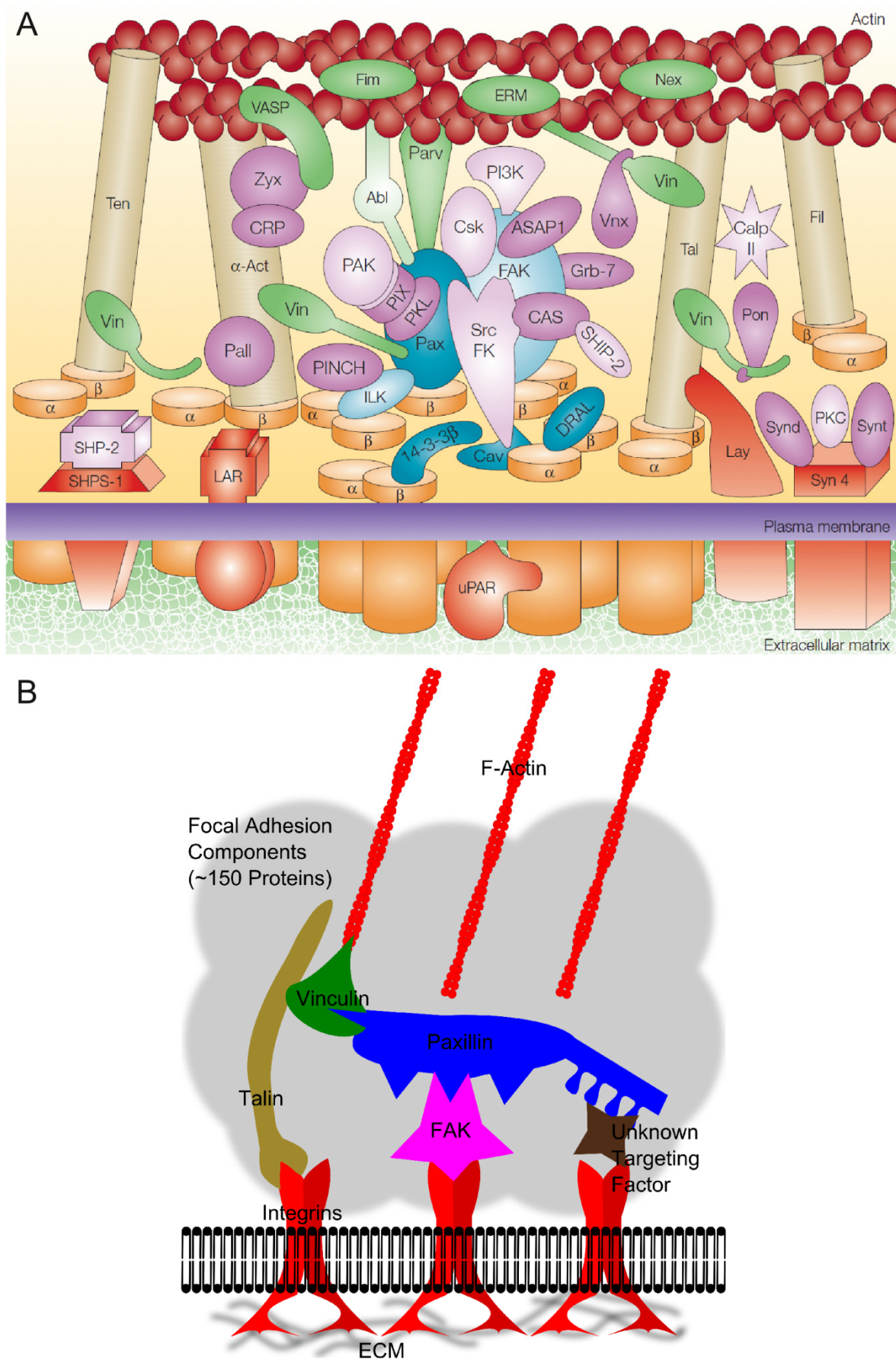


Figure 1.1: Model depicting the focal adhesion complex. **A)** Figure taken from Geiger et al. 2001. The figure shows the complexity of focal adhesions. Important molecules also shown in **B**: Integrins (α and β), Vinculin (Vin), Focal Adhesion Kinase (FAK), Talin (Tal) and Paxillin (Pax). **B)** Simplified model of the focal adhesion complex; the extracellular matrix is abbreviated with ECM. The scaffolding protein paxillin, although not shown in this figure, also can bind to integrins directly.

A structure similar to focal adhesions, the hemidesmosome, also contains the same transmembrane receptors for attachment to the extracellular matrix. Yet, their function is to provide stability for epithelial cells and not attachment during migration. Consequently, malfunctions in hemidesmosomes result in severe diseases showing epithelial instability, for example blistering diseases of the skin (Rouan et al. 2000).

Although the term focal adhesion may be used sometimes generally for all cell-matrix contacts, this is not entirely true. As reviewed by Benny Geiger (Geiger et al. 2001), focal adhesions are mature cell-matrix contacts connected with actin filaments that exert traction forces on the focal adhesion. Before maturation and connection to microfilaments, cell-substrate contacts are defined as focal contacts. When focal adhesions further mature, they align laterally along the microfilaments and form fibrillar adhesions (Broussard, Webb, and Kaverina 2008). All three types of cellular adhesions are regulated by different members of the RhoGTPase-family.

What is a focal adhesion? A focal adhesion is a structural accumulation of proteins providing direct connection between the actin cytoskeleton and the extracellular matrix (Geiger et al. 2001). Furthermore, they can transmit force from the actin flow to the extracellular matrix by a “molecular clutch”, thereby enabling the moving of the cell relative to the substrate or vice versa (Hu et al. 2007). While focal adhesions have to be very stable in order to provide sufficient attachment and to translate force, they also have to be tightly regulated during migration. This requirement results in a very strictly regulated turnover I will discuss in more detail later.

Figure 1.1 shows two typical representations of the focal adhesion complex. While in 2001 this complex was reported to contain over 50 proteins (Zamir and Geiger 2001), currently over 150 different proteins are known to participate in adhesion formation, stabilisation and dynamics (Zaidel-Bar et al. 2007). Central molecules in focal adhesions are the integrins. These transmembrane receptors provide the attachment to the outside of the cell and connect it to the cytoskeleton. For proper function, integrins form heterodimers; 18 α -subunits can pair with eight β -subunits to form 24 unique transmembrane receptors with different binding specificities (Hynes 2002).

Focal adhesions are not only passive structural elements. They are also capable of outside-in and inside-out signalling, regulating various crucial cellular functions, as shown for example for the focal adhesion kinase FAK (Ilić et al. 1995; Hagel et al. 2002). In order to achieve all these complex functions, three types of proteins have to be recruited to the focal adhesions: Structural, scaffolding and signalling molecules (BurrIDGE and Chrzanowska-Wodnicka 1996; Wozniak et al. 2004).

Structural proteins, like talin or vinculin, were known to be important for the mechanic function of focal adhesions already for a long time (BurrIDGE et al. 1988). Their basic function is the connection of the actin cytoskeleton to the integrins and consequently to the extracellular matrix. In 1990, paxillin was added to the list of focal adhesion proteins as the first scaffolding protein (Turner, Glenney, and BurrIDGE 1990). The discovery of the FAK in 1992 also introduced signalling proteins to the focal adhesion (Schaller et al. 1992). As shown in Figure 1.1A, the focal adhesion complex exhibits multiple protein-protein interactions whose regulation depends on the state of the cell.

Figure 1.1B shows a simplified view of the focal adhesion area, introducing only a few proteins important to understand the basic functions of focal adhesions. As shown, the integrin heterodimers bind the extracellular matrix and further associate with structural proteins like talin that interact with the cytoskeleton. Other structural proteins like vinculin bind indirectly to the integrins via scaffolding proteins like paxillin. This very simplified model already shows the high complexity of the focal adhesion protein interactions, although I omitted around 140 proteins additionally involved in the focal adhesion formation.

Figure 1.2

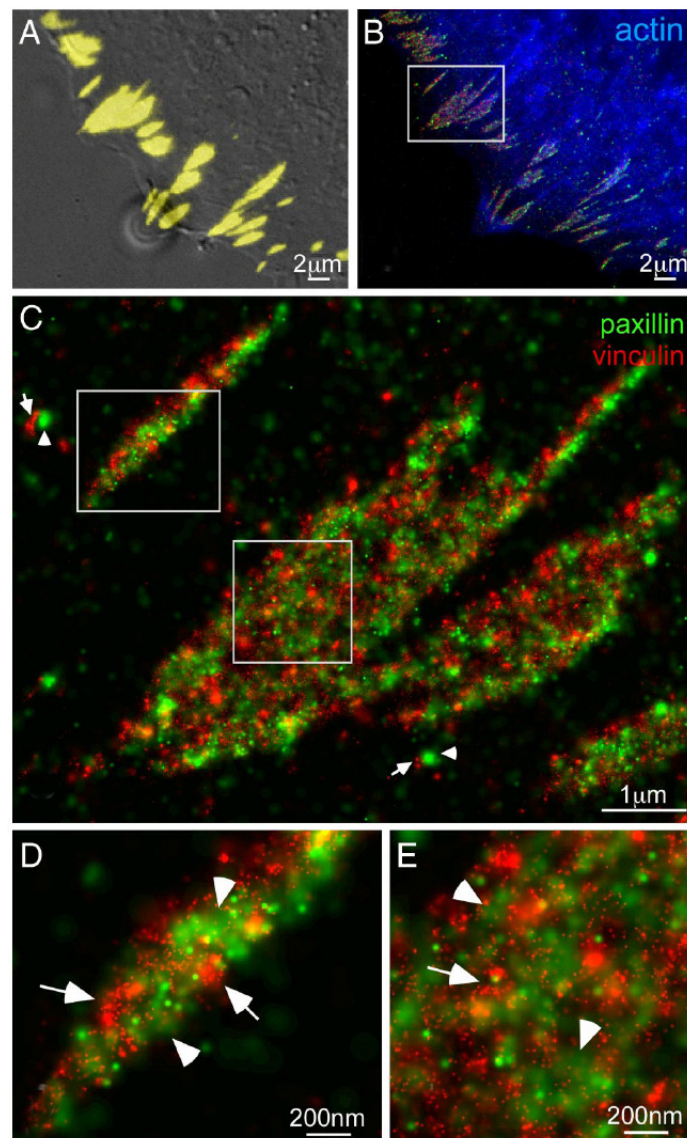


Figure 1.2: Triple-label imaging of focal adhesions with combined techniques. A) Overlaid DIC and TIRF (yellow) images of paxillin and vinculin coexpressed in an HFF-1 cell. **B)** Diffraction-limited epi-fluorescence image of mCerulean-tagged actin (blue) overlaid with PALM images of Dronpa-tagged paxillin (green) and tdEostagged vinculin (red) shows adhesion complexes at the periphery of the cell aligned with the termini of actin bundles. An expanded view **C)** of the boxed region in **B** reveals parallel arrays of interwoven paxillin and vinculin aggregates along the length of each AC, as well as possibly nascent adhesion complexes consisting of adjacent paxillin (arrowheads) and vinculin aggregates (arrows). Further magnified views **D** and **E)** of the boxed regions in **C** indicate other examples of adjacent aggregates of either paxillin (arrowheads) or vinculin (arrows) within larger adhesions. *Figure and figure legend taken from Shroff et al. 2007.*

What is the structure of a focal adhesion? Until recently it was assumed that focal adhesions are homogenous structures. However, findings by Hari Shroff and Eric Betzig propose a different model (Shroff et al. 2007; Shroff et al. 2008). Figure 1.2 shows images taken from one of their papers that analysed the localisation of various proteins localised to focal adhesions by Photoactivated Localisation Microscopy (PALM). This method relies on sequential photoactivation and photobleaching of photoactivable fluorophores with subsequent computational determination of single molecule positions. This results in superior resolution of down to 20 nm in the x- and y-direction.

As shown in Figure 1.2A, in standard microscopy setups like Total Internal Reflection Fluorescence (TIRF) microscopy, due to diffraction limitations, the ultra-structure cannot be observed. Therefore, vinculin and paxillin seem to be completely colocalised. However, application of PALM reveals a different view on focal adhesion structure (Figure 1.2B-1.2E). The main accumulations of paxillin and vinculin are localised to separate structures within the focal adhesion and overlap only partially in some areas.

The spatial separation of proteins that are even known to bind to each other directly into functional domains raises doubts about the model of a homogenous structure of focal adhesions. This for sure not only will change our knowledge of structural relationships within the adhesion area, but also may have dramatic implications for the regulation of focal adhesions. In a more structured protein complex it might be that the regulation of a few of these functional domains determines the stability of the whole focal adhesion. Although further experiments are required to comprehensively show that this focal adhesion ultra-structure represents the physiological situation, we can already start to rethink previous models of focal adhesion organisation.

1.2 The Focal Adhesion Protein Paxillin

As indicated, paxillin was among the first focal adhesion proteins discovered. Initially, it was identified as a 68 kDa protein with increased tyrosine phosphorylation in cells expressing the Src kinase of the Rous sarcoma virus (Glenney and Zokas 1989; Deakin and Turner 2008). Paxillin's role as a

scaffolding protein related to focal adhesions was established two years later by the demonstration of its binding to the vinculin tail domain (Turner, Glenney, and Burridge 1990).

As a scaffolding protein, paxillin binds to various interaction partners (Figure 1.3). Beside structural proteins like vinculin and actopaxin, also regulatory proteins such as the FAK and Src kinases, the PTP-PEST phosphatase or the RasGAP (GTPase activator protein specific for Ras) can dock to paxillin. In addition, paxillin also binds to tubulin and to the vesicular coat protein clathrin, as demonstrated by pull-down assays (Turner et al. 1999; Brown and Turner 2004).

Figure 1.3

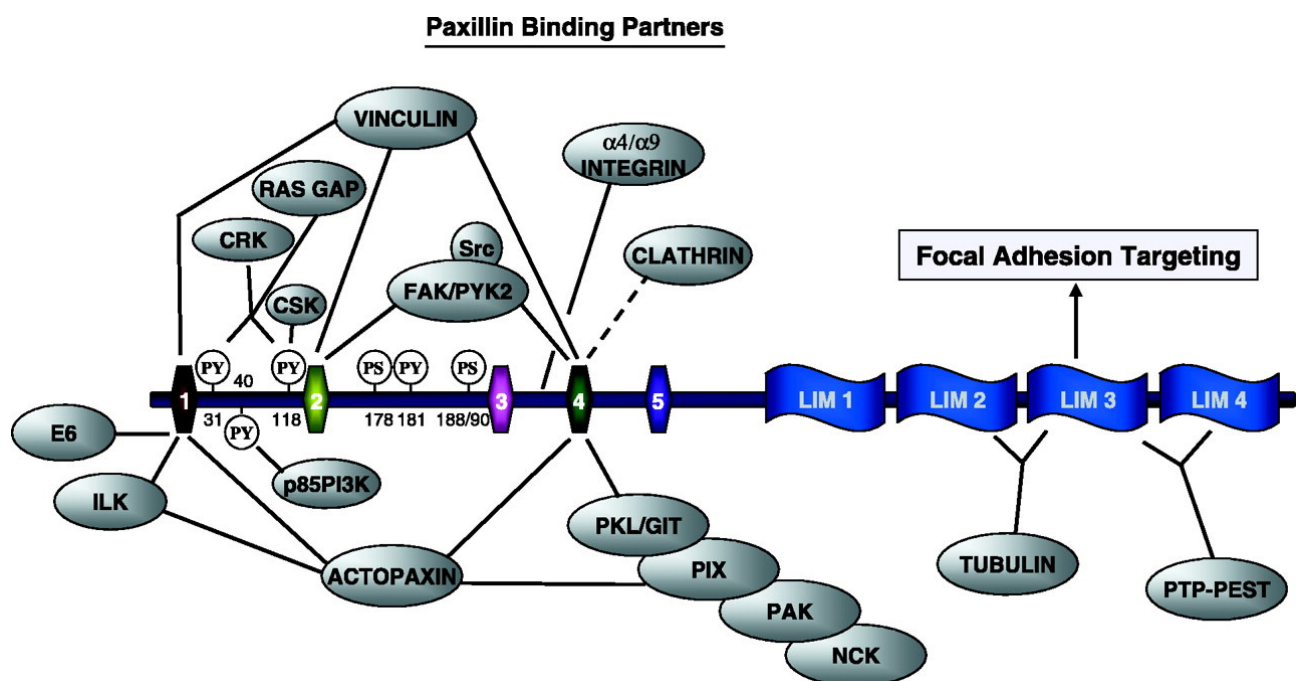


Figure 1.3: Paxillin binding partners. Figure taken from Brown and Turner 2004; various proteins bind to paxillin. The protein interactions are mediated by the LD and LIM domains and SH2 binding motifs.

Paxillin is a multi-domain protein containing five LD domains (leucine and aspartate-rich) at the N-terminus and four LIM domains (Lin11, Isl-1, Mec-3) at the C-terminus (Turner and Miller 1994; Brown, Perrotta, and Turner 1996). The LD motifs are protein-binding modules that control most of paxillin's signalling

functions. Figure 1.4 shows the role of paxillin in the signalling downstream of Rac1 (Deakin and Turner 2008). Paxillin, therefore, recruits proteins to bring them to the vicinity of other focal adhesion proteins. Furthermore, it can also bind simultaneously to proteins that interact directly with each other.

The LIM domains are protein-protein interaction domains that contain a double-zinc-finger motif (Pérez-Alvarado et al. 1994) and serve for example as binding site for the PTP-PEST phosphatase (Côté, Turner, and Tremblay 1999). Furthermore, the phosphorylation of the LIM2 and LIM3 domain was shown to influence the focal adhesion targeting of paxillin (Brown, Perrotta, and Turner 1998).

Figure 1.4

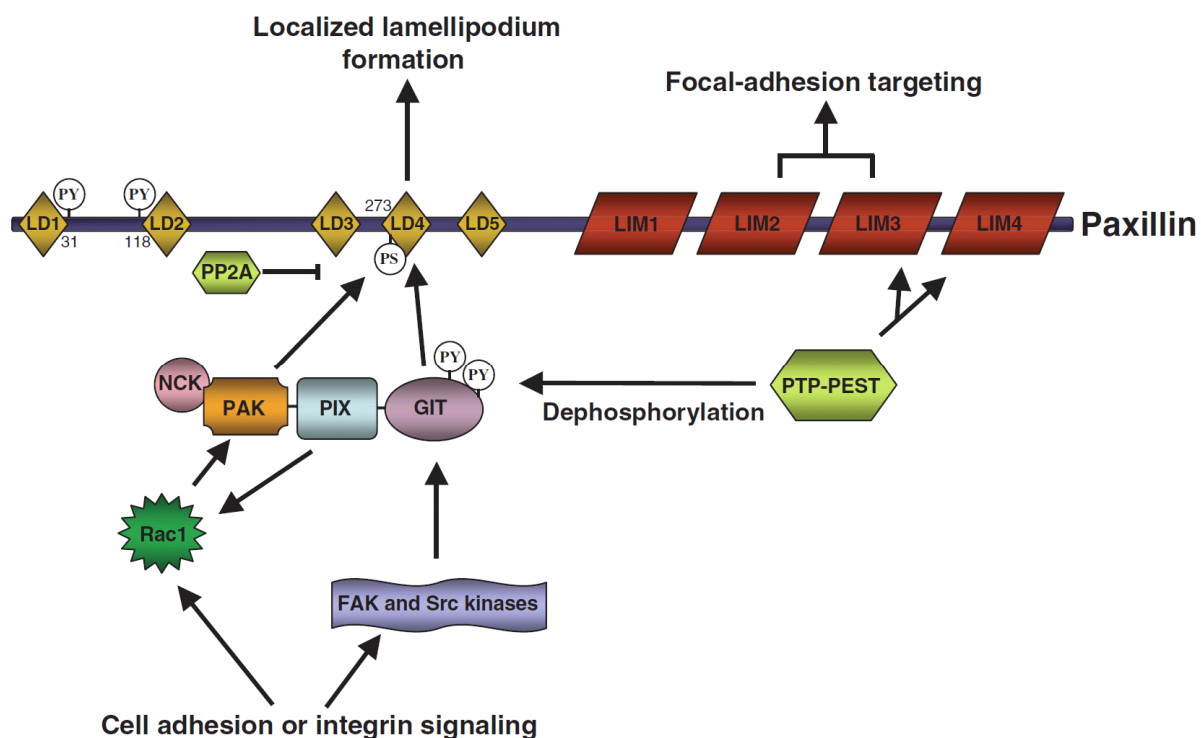


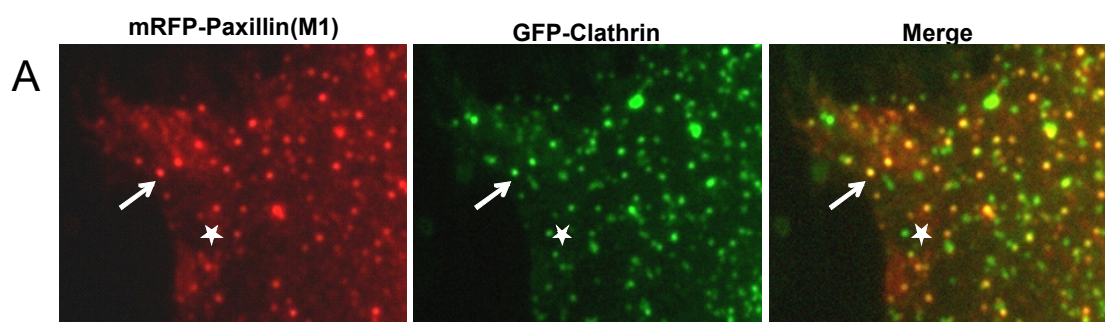
Figure 1.4: Involvement of paxillin in the signalling pathway downstream of Rac1. Figure taken from Deakin and Turner 2008. The figure shows the coordination of Rac1 signalling by the scaffolding protein paxillin. As shown, Rac1 interacts with PAK-PIX-GIT that are directly binding to paxillin. This trimeric complex is further regulated by kinases and phosphatases directly binding to paxillin.

Additionally, in the laboratory of Christopher Turner it was demonstrated that the disturbance of both zinc-fingers of the LIM3 domain abolishes the paxillin targeting of paxillin to focal adhesions (Brown, Perrotta, and Turner 1996). Over a decade later, a similar paxillin mutant was studied by Natalia Andreyeva (Andreyeva 2008). This mutant, named paxillin(M1), contained two cysteine-to-alanine conversions that destroyed the integrity of the double-zinc-finger motif in the LIM3 domain. In addition to the findings of Michael Brown, the paxillin(M1) mutant also localised to vesicular structures.

As shown in Figure 1.5A paxillin(M1) was localised to clathrin vesicles. Live-cell imaging revealed that these vesicles are highly motile and undergo multiple budding and fusion events. By the means of immunoprecipitation followed by Mass Spectrometry, binding partners of the paxillin mutant have been identified (Figure 1.5B). Beside the previously known interaction with GIT1/2, paxillin was found to interact with several components of clathrin vesicles. One of the novel paxillin binding partners was the clathrin adaptor EpsinR that was also found to interact with wild-type paxillin in immunoprecipitation experiments (Andreyeva 2008).

On the basis of the demonstrated interaction of paxillin with clathrin vesicle components, Natalia Andreyeva proposed the hypothesis that the turnover of paxillin might be regulated by vesicular transport (Andreyeva 2008). This hypothesis would be in agreement with previous data showing paxillin's binding to clathrin (Turner et al. 1999; Brown and Turner 2004) and its localisation to the Golgi apparatus (Mazaki et al. 1998).

Figure 1.5



(Figure 1.5 continued)

B Signalling complex Components of clathrin vesicles	PXN	paxillin
	GIT1	ARF-GTPase activating protein GIT1
	GIT2	ARF-GTPase activating protein GIT2
	ARHGEF7	Rho-guaninenucleotide exchange factor betaPIX
	CLTC	Clathrin heavy chain1
	CLTA	Clathrin light chainA
	CLTB	Clathrin light chainB
	CLINT1	Clathrin interacting protein EpsinR
	PIK3C2A	Phosphoinositide 3 kinase class2 alpha
	TUBB1	Tubulin beta1 chain
	HSPB1	Heat shock 27 kDa protein
	CAD	Aspartate Carbomoyltransferase

Figure 1.5: The mutant paxillin(M1) localises to clathrin vesicles. **A)** HeLa cells were co-transfected with mRFP-Paxillin(M1) (in red) and GFP-Clathrin light chain A (in green), fixed and imaged by fluorescent microscopy. Enlarged area represents examples of clathrin vesicles containing mRFP-Paxillin(M1) (marked with arrow) and without it (marked by asterisk). **B)** Identified binding partners of mRFP-Paxillin(M1) included components of the well-known GIT-PIX signaling complex as well as several components of clathrin vesicles. *Figure and figure legend adapted from Andreyeva 2008.*

1.3 Turnover of Focal Adhesions

Attachment of the cell to the extracellular matrix requires structures with molecular linkages that are stable in the short term. Focal adhesions serve this function; they normally remain stable over the course of minutes and sometimes hours. Still, the very same structures are also capable of undergoing very fast repositioning and turnover events, for example during cell migration (Figure 1.6; Broussard, Webb, and Kaverina 2008), requiring a specific regulatory machinery.

The basis for the fast switch between stable and dynamic focal adhesion states can be found on the molecular level. While the focal adhesion structure

remains stable, the majority of molecules can be turned over in the scale of minutes (Lele et al. 2006; Shroff et al. 2008).

What are the regulation mechanisms underlying the turnover of focal adhesion molecules? Before I address this question in detail for the integrins, I would like to introduce concepts for the turnover of the cytoplasmic focal adhesion component paxillin. As discussed, the exact turnover mechanisms of paxillin remain elusive. While it was reported that phosphorylation has a major impact on the paxillin localisation to focal adhesions (Brown, Perrotta, and Turner 1998), a paxillin targeting factor is still missing. For the relocation of paxillin away from the focal adhesions to the cytoplasm, mono-ubiquitylation was found to be an important signal (Didier et al. 2003). Moreover, a role of vesicular transport in paxillin turnover cannot be excluded. All aforementioned regulatory mechanisms for paxillin recycling may also be important for other focal adhesion components. Additionally, as described for zyxin, mechanical forces can influence the turnover kinetics of focal adhesion proteins (Lele et al. 2006).

Figure 1.6

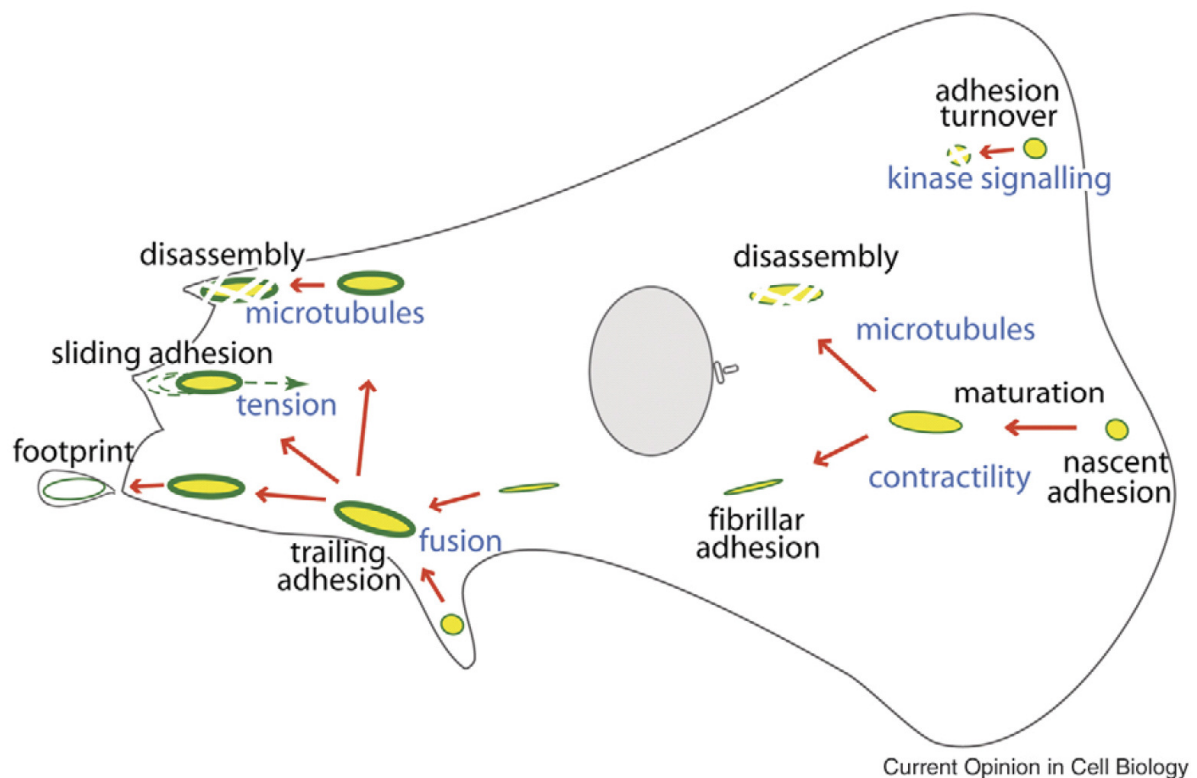


Figure 1.6: Model of asymmetric adhesion dynamics. Nascent adhesions formed at the front either undergo turnover, which is predominantly controlled by kinase signaling, or mature in response to contractile forces. Mature adhesions can disassemble in a microtubule-dependent manner or be transformed into fibrillar adhesions. Trailing adhesions arise as a result of fusion of additional nascent adhesions and remaining fibrillar adhesions. Once formed, trailing adhesions slide because of tension from attached stress fibers and either eventually disassemble or detach in the form of membrane ‘footprints’. *Figure and figure legend taken from Broussard, Webb and Kaverina 2008.*

As a matter of fact, the turnover of the transmembrane protein integrin has different requirements than the one of cytoplasmic proteins. Foremost, vesicular transport is required for the delivery, removal and recycling of integrins.

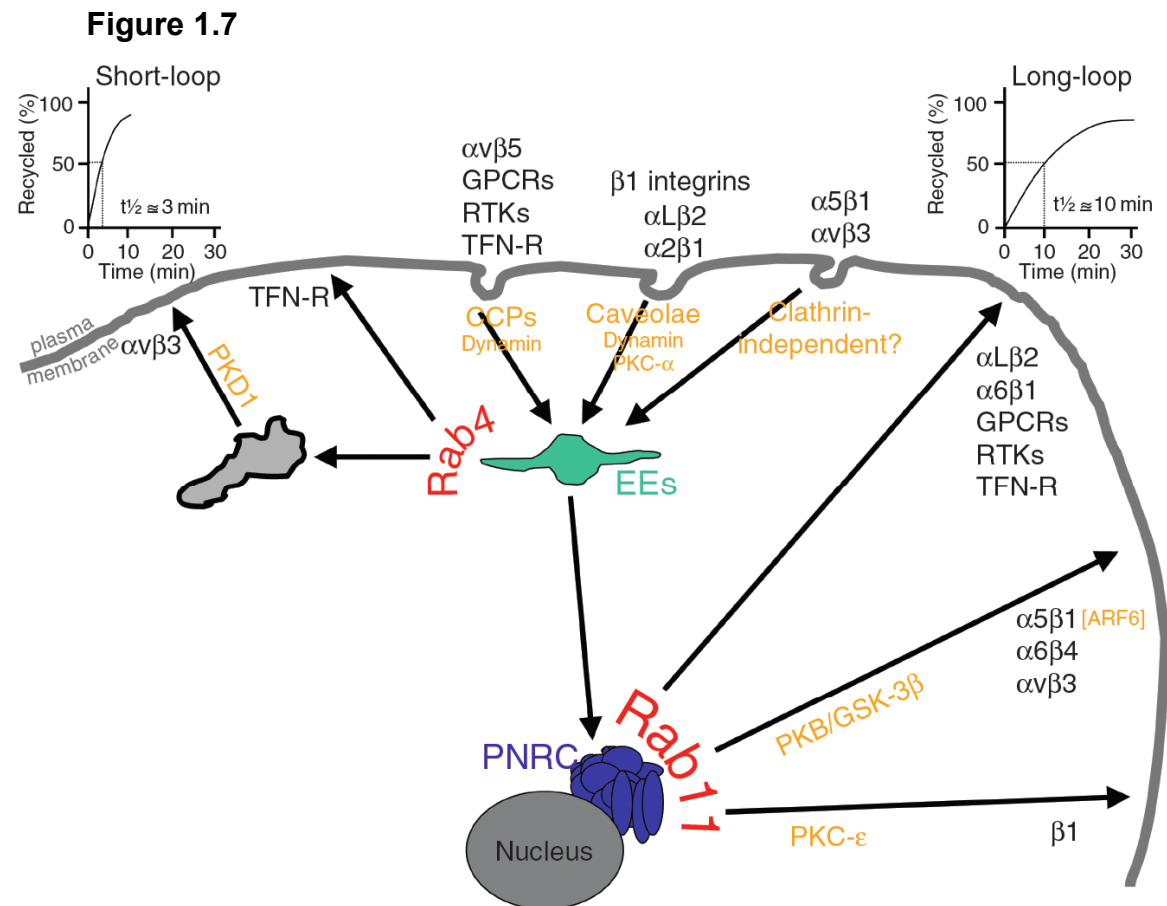


Figure 1.7: Schematic Summary of the Rab GTPase and kinase control of integrin trafficking. Figure taken from Caswell and Norman 2006. The figure illustrates

(Figure 1.7 continued) the various vesicular pathways by which integrins can be recycled. CCPs: clathrin coated pits. EEs: early endosomes. PNRC: perinuclear recycling centre.

The recycling of integrins is a phenomenon that is known already for quite a long time. It is a very efficient and fast process, however, also very selective (Bretscher 1992; Bretscher and Lutter 1988; Caswell and Norman 2006). Upon internalisation, dependent on clathrin, caveolae and additional clathrin-independent mechanisms, the integrins can be recycled through the so-called short- and long-loop (Figure 1.7). The choice of the recycling route depends on the nature of the integrin heterodimers. After the transport of the receptors to early endosomes, Rab4 and Rab11 GTPases are responsible for the further recycling by the short- or long-loop, respectively.

However, in 2008 Jim Norman already had to change parts of the model represented on Figure 1.7 (Lecture on the ELSO meeting in Nice 2008). While the basic assumptions still remained the same, the integrin heterodimers can switch the recycling route according to environmental conditions. Furthermore, the two recycling pathways can also vary significantly in the time needed for one cycle. This demonstrates the complex and interwoven vesicular transport system used for the recycling of matrix receptors of focal adhesions.

1.4 The Vesicular Coat Protein Clathrin and Its Adaptor EpsinR

Vesicular trafficking is involved in numerous cellular processes. It is required for the delivery of proteins and peptides to organelles or the extracellular environment and also for the recycling of bulk membrane. In higher organisms, critical functions like synaptic transmission or the functions of immune cells rely on exo- and endocytosis. It is, therefore, not surprising that researchers working in the field of vesicular transport face an overwhelming level of complexity and an enormous number of proteins involved in this processes.

Figure 1.8

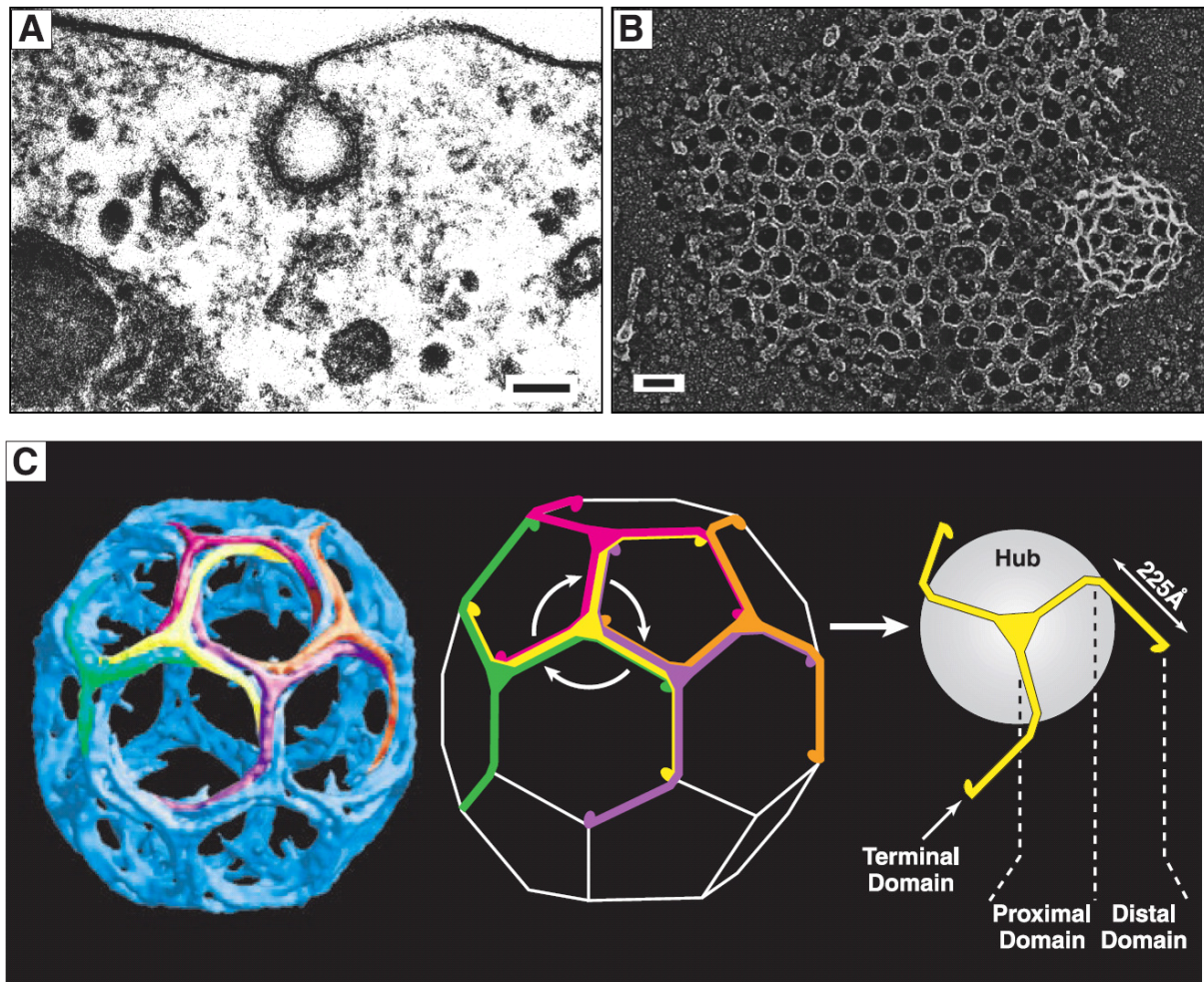


Figure 1.8: Clathrin structure. Taken from Marsh and McMahon 1999. **A)** TEM (Transmission Electron Microscopy) image of a clathrin-coated vesicle. **B)** SEM (Scanning Electron Microscopy) image of a clathrin rich region with a budding vesicle. **C)** Model of the clathrin cage.

Nevertheless, all the vesicular trafficking pathways are challenged by the same mechanistic problems. In order to form a vesicle, the membrane has to be curved, the budding has to be induced. (McMahon and Gallop 2005). Many different proteins are known that can induce this curvature inside the membrane and the majority of vesicles are additionally coated by proteins facilitating this process. The main proponent of this family of coating proteins is clathrin. The smallest subunit of a clathrin coat is a triskelion formed by a heterohexamer, consisting of three clathrin heavy chain (CHC) and three accessory clathrin light chain (CLC) proteins (Kirchhausen 2000). In electron microscopy, the clathrin

lattice can be seen localised around budding vesicles and also at the membrane, pre-assembled in hotspots of vesicular budding (Figure 1.8A and 1.8B; Marsh and McMahon 1999). In a reconstruction the clathrin lattices form a rounded cage resembling a polyhedron (Figure 1.8C).

Figure 1.9

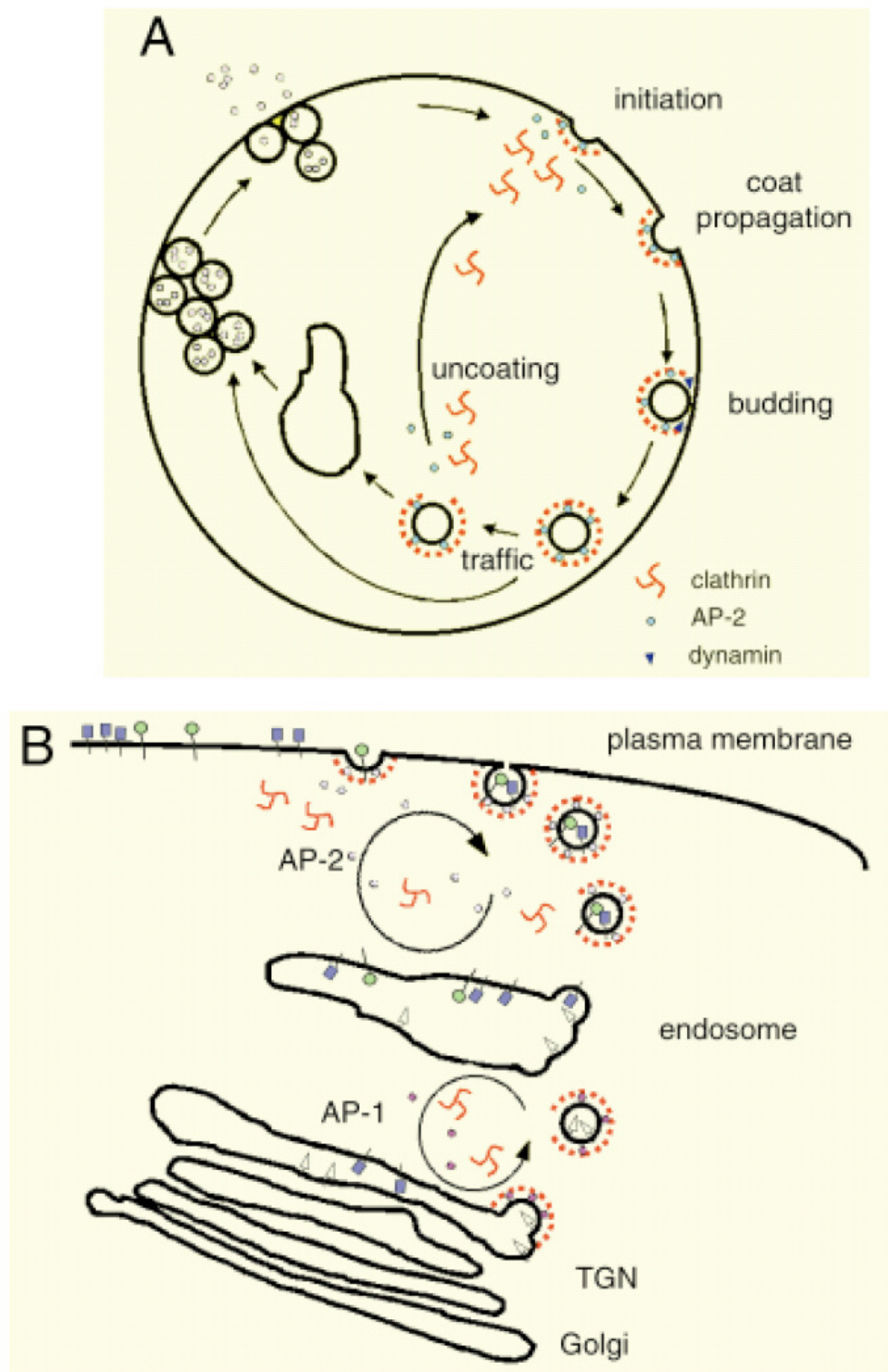


Figure 1.9: Clathrin function in exo- and endocytic pathways. Taken from Kirchhausen 2000. **A)** General model showing the function of clathrin. **B)** Model showing the involvement of clathrin and two of its adaptors (AP1 and AP2) in exo- and endocytosis.

Clathrin is found on many cellular membranes. While it is absent from the endoplasmic reticulum and the cis-Golgi apparatus, it can be found on the trans-Golgi apparatus, all types of endosomes and the plasma membrane. (Figure 1.9B; Kirchhausen 2000). Therefore, the clathrin functions are multi-faceted and often interdependent. Mechanistically, clathrin participates in the budding process (McMahon and Gallop 2005) and then immediately dissociates from a new vesicle (Figure 1.9A).

However, it can also stay on some types of vesicles for longer periods of time. Thus, clathrin positive punctate structures can be seen after an immunostaining for endogenous clathrin or the expression of a fluorophore tagged clathrin construct.

The recruitment of clathrin to the membrane is dependent on several clathrin adaptors. They can be classified into four polymeric adaptor protein complexes (AP1, AP2, AP3 and AP4; Hirst and Robinson 1998; Boehm and Bonifacio 2002) and monomeric adaptors. In contrast to clathrin, these adaptors are only localised to particular subsets of the clathrin buds (Figure 1.9B; showing the proteins AP1 and AP2).

Figure 1.10

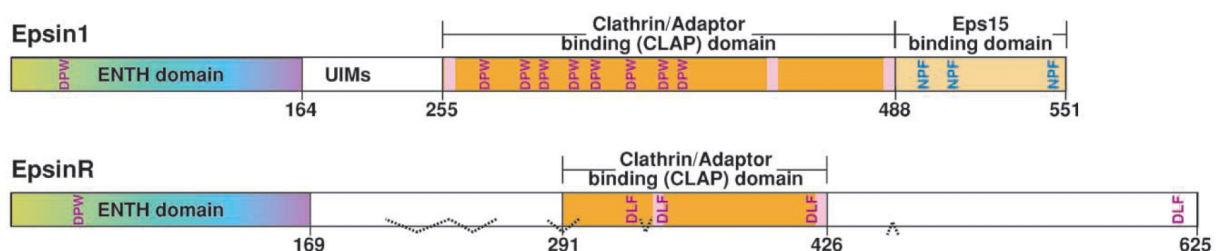


Figure 1.10: Domain structure of EpsinR compared to epsin1. Taken from Mills et al. 2003. The figure shows the difference in domain architecture between EpsinR and epsin1. Note that EpsinR is missing the UIMs (ubiquitin interaction motifs) and the Eps15 binding domain.

A central group of the monomeric adaptors are the epsins. They were originally identified as binding partners of Eps15 (Epidermal growth factor receptor pathway substrate 15). However, now they are described as a family of proteins containing an ENTH (epsin-NH₂-terminal homology; responsible for phosphatidylinositol phosphate binding) and a clathrin/adaptor binding (CLAP) domain (Mills et al. 2003). In 2002, an epsin related protein, lacking the Eps15 binding motifs, was identified and termed EpsinR (Ford et al. 2002).

EpsinR contains an ENTH domain that differs in its lipid binding specificity from other epsins (Ford et al. 2002). The presented CLAP domain is also distinct and it is missing the ubiquitin interaction motif (important for interaction with ubiquitin and the mono-ubiquitylation of the epsins themselves; Figure 1.10). EpsinR is evolutionary conserved and has homologues in *Drosophila* and *C. elegans* (Mills et al. 2003).

Figure 1.11

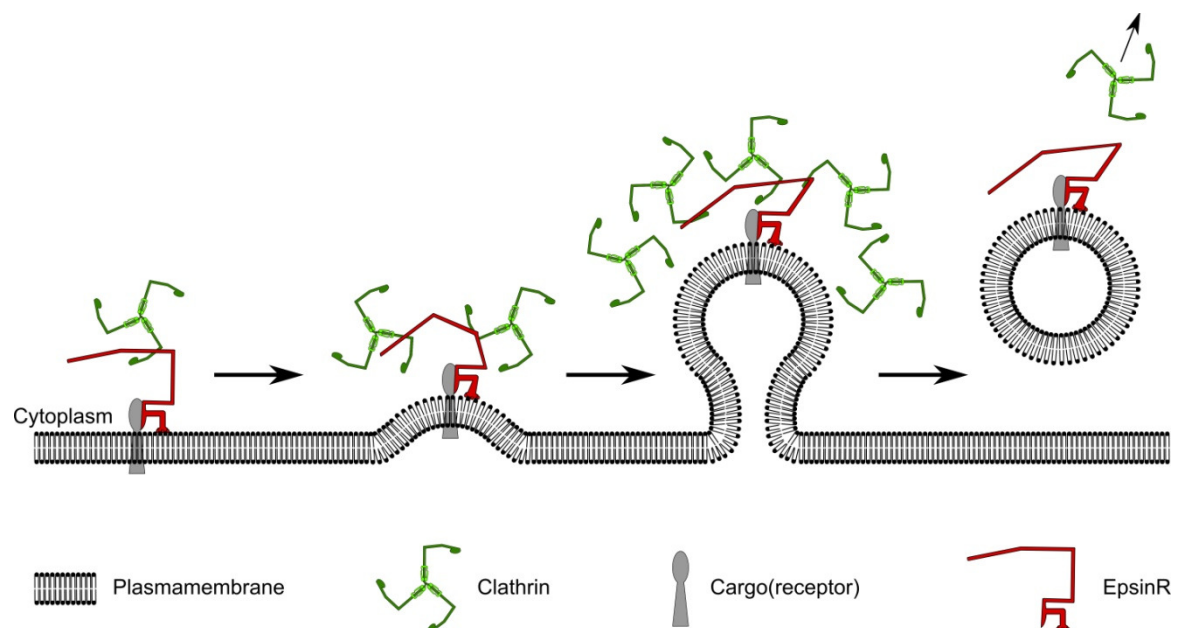


Figure 1.11: The activity of EpsinR during the budding of clathrin-coated vesicles. The figure shows the recruitment of clathrin by EpsinR that is binding to the plasma membrane by its ENTH domain while interacting with its cargo.

EpsinR was shown to bind and interact with the adaptor complex AP1, but not with AP2 and AP3 and to be preferentially localised to the trans-Golgi apparatus (Kalthoff et al. 2002; Mills et al. 2003; Hirst et al. 2003). However, the siRNA knockdown of EpsinR does not influence a classical function of AP1, the sorting of lysosomal enzymes (Hirst et al. 2003). In recent characterisations of this protein, two papers were analysing the function of EpsinR in more detail, yielding further insights into the function of this protein. First, EpsinR can act as an adaptor for the SNARE protein Vit1b (Hirst et al. 2004). In the same paper, it was also shown that AP1 and EpsinR depend partially on each other for the integration into clathrin coated vesicles. Second, the role of EpsinR in retrograde transport was demonstrated (Saint-Pol et al. 2004). Whereas the loss of AP1 function did not influence the assayed trafficking from early and recycling endosomes to the trans-Golgi network, EpsinR knockdown inhibited this process.

In conclusion it can be said that EpsinR is a clathrin adaptor possessing redundant and independent functions with respect to the role of AP1. Furthermore, it is localised not only to the trans-Golgi area, but also to other endogenous membranes like recycling endosomes. A simplified model of EpsinR function in vesicular budding is shown in Figure 1.11.

1.5 Advanced Imaging Methods

One of the most important techniques in modern cell biology is the visualisation of proteins with the help of fluorescent molecules (reviewed in Lippincott-Schwartz and Patterson 2003). Information about the localisation and dynamics of a protein inside a cell contributes in a major way towards clarifying its function. Today, imaging possibilities go far beyond simply looking at the cell in a conventional wide-field microscope.

1.5.1 Total Internal Reflection Fluorescence Microscopy

One factor limiting image quality in wide-field imaging is out-of-focus fluorescence of proteins residing outside the imaging plane. Principally there are two possible ways to solve this problem on the level of the microscope equipment. First, the fluorescent light coming from the planes beyond or below the imaging plane can be excluded from the detector by a pinhole. Second, the planes beyond or below are not excited and the fluorescence is coming only from the imaging plane. Confocal microscopy is based on the first approach.

Figure 1.12

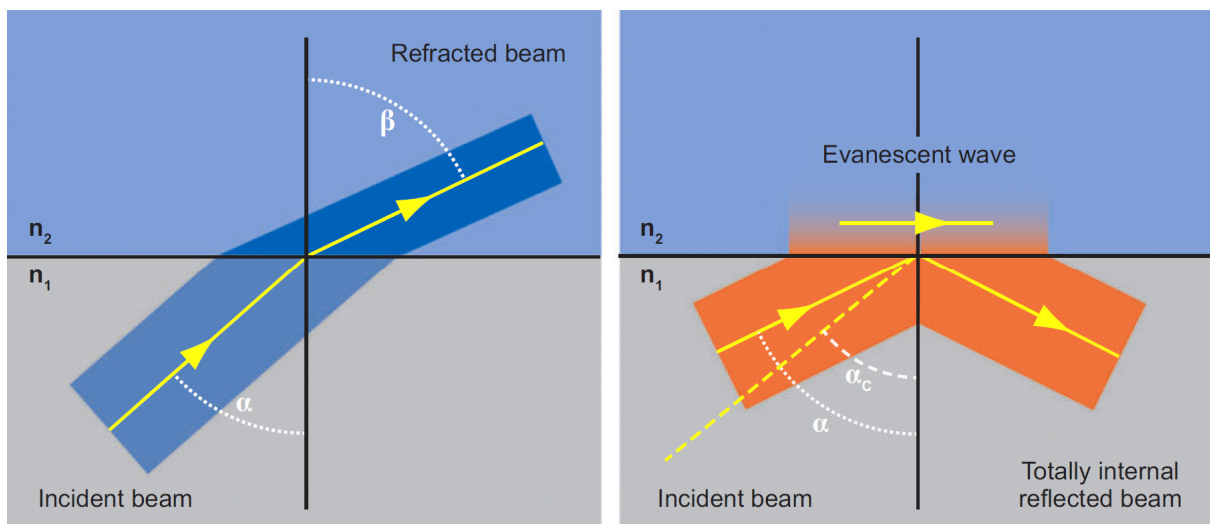


Figure 1.12: From refraction to total internal reflection (TIR). Figure taken from Groves et al. 2008. If the incident angle α of an incoming beam is below the critical angle α_c , the beam will be refracted. The outgoing beam will be propagated with the new angle β (which depends on the two indices of refraction n_1 and n_2). If the incident angle is beyond the critical angle, the beam will not be able to pass the interface between the two media and will be totally reflected. However, an evanescent wave will be generated, penetrating into the medium not entered by the incoming beam.

One principal method using the second approach is Total Internal Reflection Fluorescence (TIRF) microscopy. This type of microscopy exists now for over two decades and was already excellently reviewed by Axelrod (Axelrod, Burghardt, and Thompson 1984; Axelrod 1989; Axelrod 2001). The basic principle of TIRF

microscopy is based on a phenomenon which is even taught in school: the total internal reflection of an incident beam at the interface of two media with different indices of refraction (Figure 1.12). In the presence of total internal reflection, an evanescent wave will penetrate into the second medium. This evanescent wave of light has the same wavelength as the incident beam; however, its intensity decays exponentially. Therefore, in a standard experimental setup only 200 to 300 nm will be illuminated. If the incident beam would be capable of exciting a fluorophore, then the evanescent wave does it as well. Therefore, this method can be used to visualise only the basal side of a specimen.

Due to the dependency on total internal reflection, only fluorophores adjacent to the glass-water interface can be visualised. This is both an advantage, but also a limitation of TIRF microscopy. For cell biology this means that only the basal membrane and a small layer of the adjacent cytoplasm of a cell can be visualised. Therefore, this technique is most relevant in the fields of cell biology where substrate interactions are of interest. The vesicular machinery and vesicle dynamics present at the basal cellular membrane were studied in great detail with this method (Groves, Parthasarathy, and Forstner 2008). Also processes within the plasma membrane such as receptors dynamics or lipid rafts were described using TIRF microscopy (Schütz et al. 2000; Sako et al. 2003). As already described, the components of the focal adhesion complex are located in the basal membrane and in close proximity to it. Therefore, this technique has been established as the principal method to visualise cell-matrix contacts of all sorts.

1.5.2 3D-Deconvolution

In addition to the microscopic methods, also computational possibilities exist to correct for out-of-focus fluorescence. One of these computational methods is Deconvolution in three dimensions (3D-Deconvolution). Out-of-focus fluorescence is not erratic but follows an optical law. If a point is seen outside its focus plane, in x- and y-dimension a so-called Airy disc will be visible (Figure 1.13; (McNally et al. 1999). If a 3D reconstruction of such an imaging series representing a point at several optical planes is viewed in x and z, a double cone

is visible. This spreading of a point into an Airy disc in out of focus planes is called a point spread function (PSF). Due to the optical aberrations in a microscopy setup, an actual PSF will have a symmetry that differs from a theoretical PSF (Figure 1.13B and 1.13D).

The measured PSF is true for every single point in an image acquired in the particular microscopy system. Therefore, with complex mathematical algorithms it is possible to process for every plane in a z-series an image without out-of-focus light. For this, the information of the adjacent planes and the PSF are used to remove the Airy discs of every individual point in out-of-focus planes.

Figure 1.13

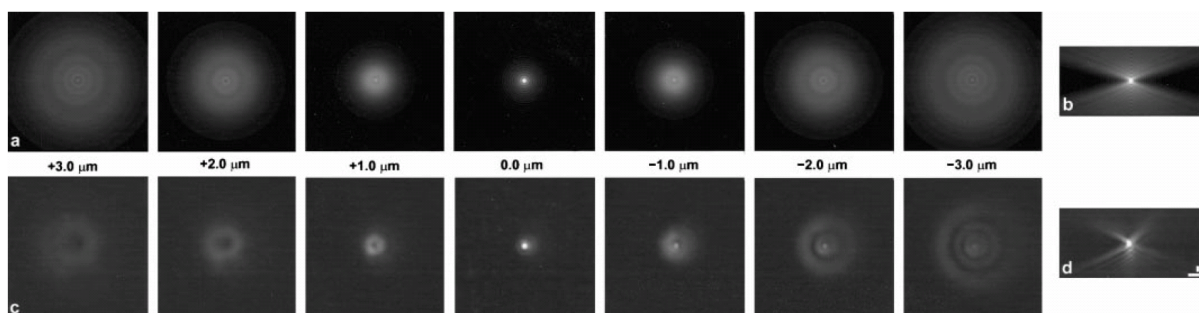


Figure 1.13: Sequential focal planes for predicted and measured point spread functions (PSFs). Figure taken from McNally et al. 1999. **A)** Sequential planes through a theoretical PSF. **B)** XZ-view of **A**. **C)** Sequential planes through a measured PSF. **D)** XZ-view of **C**.

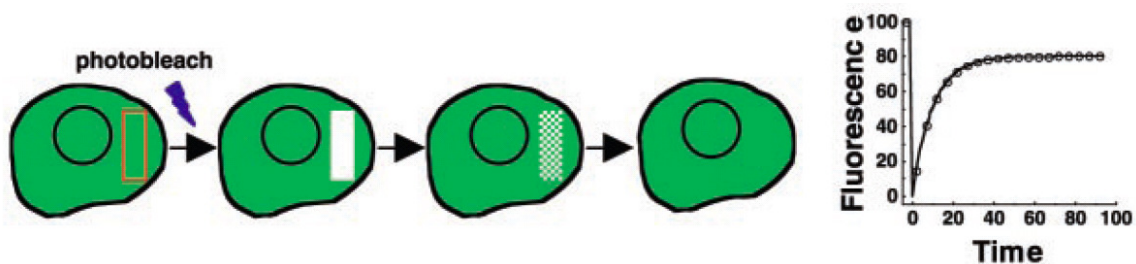
This method allows visualising structures normally masked with out-of-focus fluorescence. However, one has to be very careful, when using this technique. In order to interpret Deconvolution images correctly, the user has to be experienced and able to distinguish between artefacts of the processing steps and real objects. Still, if handled professionally, 3D-Deconvolution allows the detection of structures masked in an unprocessed fluorescent image.

1.5.3 Fluorescence Recovery After Photobleaching and Photoactivation

The discovery of the green fluorescent protein (GFP) and its introduction as a tool for live-cell microscopy had an enormous impact on cell biology. Not surprising, Martin Chalfie, Osamu Shimomura and Roger Y. Tsien were awarded the Nobel Prize in Chemistry for the discovery and development of GFP as a universal laboratory tool in 2008. Since its initial use, the experimental setups and the fluorophores have become more sophisticated. One group of methods using these advancements is applied for the measurement of protein dynamics in live-cell microscopy.

Figure 1.14

A Fluorescence Recovery After Photobleaching (FRAP)



B Photoactivation

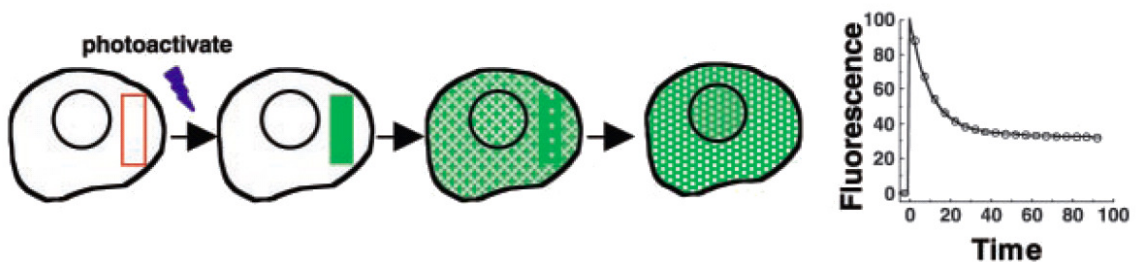


Figure 1.14: The basic principle of FRAP and photoactivation. Figure adapted from Lippincott-Schwartz and Patterson 2003. **A)** Fluorescence Recovery After Photobleaching (FRAP) in a cell and the corresponding recovery curve. **B)** photoactivation (PA) in a cell and the corresponding curve of fluorescence loss.

Various techniques are currently in use to elucidate the kinetics of protein binding and diffusion, two of which are Fluorescence Recovery After

Photobleaching (FRAP) and photoactivation (PA) (reviewed in Lippincott-Schwartz and Patterson 2003). Both techniques rely on the same principle. A localized subset of proteins is modified using laser light and becomes distinct from the rest of the population. Then, the exchange of modified proteins by native ones is measured and analysed (Figure 1.14). Yet, the two methods require different systems in order to perform the measurements.

Figure 1.15

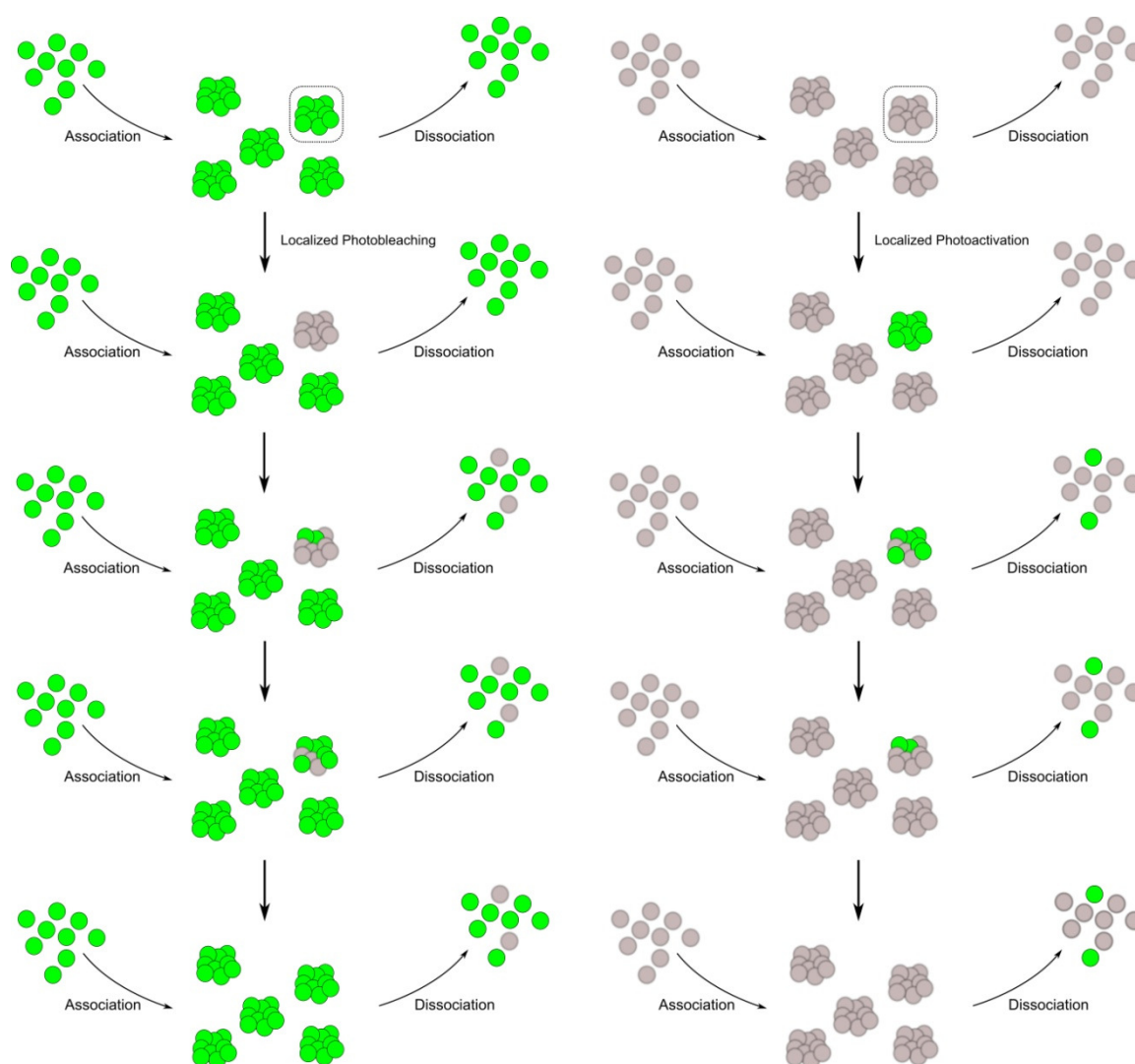


Figure 1.15: Comparison of the molecular basis of FRAP and PA. Figure demonstrates the difference in molecular dynamics for FRAP (left) and PA (right). Green-coloured circles indicate the fluorescent molecules; grey-coloured circles the photobleached/inactivated molecules.

FRAP uses photobleaching to inactivate the fluorophore attached to the protein of interest (Figure 1.15). Over the course of time, the bleached molecules will be replaced by unbleached ones. In order to perform photobleaching, only an imaging system with the capability of directing a laser beam onto the specimen, through an appropriated field aperture or by a point-scanner is required.

Photoactivation is based on the use of photoactivable proteins, tagged to the protein of interest. These proteins are only visible upon photoactivation with laser light (Figure 1.15). Over the course of time, the activated molecules are replaced by inactivated ones. For photoactivation as well as for FRAP a point-scanner laser is used. However, the wavelength of the laser utilised for photoactivation, in contrast to photobleaching, differs from the wavelength required for imaging (photoactivation uses mostly light with a shorter wavelength).

Although both techniques seem to rely on the same mechanistic principle, there are important differences. First, FRAP measures the association, PA the dissociation rates of proteins. These rates may be similar in a balanced system, but differ in an imbalanced situation. Second, FRAP uses an observed structure for the analysis. In contrast most photoactivable proteins are not visible prior to activation. Therefore, for the identification of appropriate structures for analysis, an additional marker has to be transfected. This may be a disadvantage, because a different protein may not colocalise well enough for analysis. Furthermore, if the same protein is used to avoid this, the additional expression may influence the results obtained. However, photo-switchable proteins (fluorophores that change their emitted wavelength upon photoactivation) have the potential to overcome these limitations.

Nevertheless, PA also has its advantages. One has to keep in mind that, due to special environmental conditions (e.g. pH, oxidation states, binding proteins), the photobleaching may also be reversible (Lippincott-Schwartz and Patterson 2003). Additionally, recent findings of Kathrin Heinze (ELMI meeting 2008) indicate a possible influence of FRAP on the binding and unbinding kinetics of fluorophore-tagged proteins due to the high laser power usually used for photobleaching.

There are several other parameters, beside this unbinding effect of FRAP, that may influence the quantitative results and make the measurements difficult

(Lippincott-Schwartz and Patterson 2003). For example also minor variations in the viscosity of the cytoplasm may lead to false assumptions relevant for the analysis of kinetic curves. Therefore, the quantitative calculation of kinetic parameters based on kinetic curves from FRAP/PA has to be done very carefully. Nevertheless, especially for comparison of protein kinetics in different experimental conditions FRAP and PA are useful techniques, already successfully applied in numerous publications.

2. Material and Methods

Cell culture. HeLa cells (gift of Yuko Kiyosune; KaN Research Institute, Kyoto) were maintained in standard cell culture conditions (37°C/5%CO₂) in low glucose DMEM (Gibco #31885-023) supplemented with 10% Fetal Calf Serum (PAA Laboratories) and penicillin/streptomycin (Gibco #15140-122).

DNA transfection and DNA constructs. HeLa cells were transfected with Superfect (Quiagen) according to the manufacturer's protocol. Human EGFP-Paxillin was provided by Ravi Salgia (Salgia et al. 1995). EGFP-Clathrin Light Chain A was kindly provided by Klemens Rottner (HZI, Braunschweig Ger). EpsinR-EGFP and the D34G/R67L dominant negative mutant constructs were provided by Harvey T. McMahon (Mills et al. 2003; MRC Cambridge UK). Paxillin(M1) and mRFP-tagged paxillin wild-type constructs were cloned by Natalia Andreyeva (Andreyeva 2008). The mCherry-tagged vinculin construct was cloned by Maria Nemethova in my laboratory.

Cloning of novel DNA constructs. PAGFP-Paxillin. Coding sequence of human paxillin was amplified from EGFP-Paxillin using primers incorporating NheI and XhoI restriction sites (Forward: 5'-GGA AGT GAA CCG TCA GAT CCG CTA G-3'; Reverse: 5'-ATC TCG AGG GAC TTG TAC AGC TC-3') and ligated into a PAGFP-Actin containing vector (kindly provided by Klemens Rottner) replacing the actin coding sequence. **CCDC51 and KIF19 constructs.** Coding sequences of human CCDC51 and KIF19 were amplified from full-length ORF clones (ImaGenes and Geneservice respectively) using primers incorporating BglII and Sall (pEGFP C1) and BglII and EcoRI (pEGFP N1) for CCDC51 and XhoI and EcoRI (both, pEGFP C1 and N1) for KIF19 (CCDC51/C1: Forward: 5'-TAA GAT CTA TGA TGG GGC GCA GC-3'; Reverse: 5'-AAG TCG ACG CTG GCT TTG AAT AGC-3'; CCDC51/N1: Forward: 5'-TAA GAT CTA ACG ATT GGT CGG GCC A-3'; Reverse: 5'-CCG AAT TCT GCT GGC TTT GAA TAG C-3'; KIF19/C1: Forward: 5'-ATC TCG AGT TAT GAA GGA CAG CGG-3'; Reverse: 5'-TAG AAT TCG TAA CCT GCT CAC CCT G-3'; KIF19/N1: Forward: 5'-AAC TCG

AGA TGA AGG ACA GCG GG-3'; Reverse: 5'-TTG AAT TCT AAC CTG CTC ACC CTG TC-3') and ligated into pEGFP C1 and N1 (Clonetech) as indicated.

DH5 α transformation was done with competent bacteria stored at -20°C and prepared by Hannah Neumeier in my laboratory. The bacteria were thawed on ice and then 2 μ l of DNA were added to 50 μ l of bacteria. After 30 minutes on ice the cells were exposed to a heat-shock for 40 seconds at 42°C and then incubated on ice for 2 minutes. The cells recovered in 350 μ l of pre-warmed LB Medium (prepared in the IMBA media kitchen) at 37°C on a shaker (350 rpm) for one hour. The bacteria were then plated on appropriate selection plates and incubated over night on 37°C. PCR fragment purification and gel extraction were done according to the manufacturer's protocol (Quiagen). Mini, Midi and MaxiPrep (Quiagen) were done according to the manufacturer's instructions.

siRNA transfection. HeLa cells were seeded in 12-well plates (1.0×10^5 cells per well) 24 hours before transfection. The siRNA oligonucleotides targeting human clathrin heavy chain (UAAUCCAAUUCGAAGACCAAU, Motley et al. 2003) and human EpsinR (AAUACAGAU AUGGUCCAGAAA, Hirst et al. 2004) were produced by Ambion and stored at -20°C as 50 μ M working stocks. Mixtures of 6 μ l Oligofectamine (Invitrogen) with 24 μ l OPTIMEM (Gibco) and 3 μ l siRNA with 100 μ l OPTIMEM were prepared separately and incubated at room temperature for 7 minutes. Solutions were then combined by gentle pipetting and left for 20 minutes at room temperature. The normal medium was aspirated off the HeLa cells and replaced by 800 μ l of standard medium without antibiotics. The transfection mix was distributed drop-wise to the whole surface of the well. On the next day cells were split on coverslips or to live-cell imaging chambers (LabTek chambers, Nunc) coated with fibronectin (10-50 μ g/ml, Roche). siRNA knockdown of EpsinR was examined after 72 hours. For Clathrin Heavy Chain siRNA knockdown, a second transfection was performed after two days to increase the knockdown efficiency. Experiments were carried out two days after the second siRNA transfection.

Nocodazole treatment. Nocodazole (stored at -20°C in DMSO) was diluted to the final concentration of 100/300 nM for TMA and 8 μ M for PA in standard medium.

Transferrin uptake assay. HeLa cells were starved for 45 minutes in low glucose DMEM (Gibco) containing 0.2% BSA. Then, they were incubated with the same medium containing 25 µg/ml transferrin labelled with Alexa Fluor 568 for 15 minutes, washed, fixed and stained according to the immunostaining protocol. For the live-cell imaging, HeLa cells prepared in the starvation medium were incubated for one minute with transferrin. Thereafter, they were rinsed and imaged again in the starvation medium.

HeLa cell lysis. HeLa cells were rinsed once with 1xPBS (prepared in the IMBA media kitchen) and then lysed with 100-200 µl of lysis buffer (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 75 mM NaCl, 0.05 mM DTT, 1% Triton X-100, 10% glycerol, 1 tablet Complete Protease Inhibitor (Roche) in 25 ml). The cells were then scraped off the plastic vigorously. The lysate was then incubated 25 minutes on ice and afterwards centrifuged for 15 minutes (16000xg/4°C). The supernatant was then taken for subsequent SDS-PAGE and Western blotting or stored at -20°C for later use.

SDS-PAGE and Western Blotting. Equal amounts of proteins (as determined by the Bradford protein assay) were diluted with 4x Loading Dye (200 mM Tris-HCl pH 6.8, 400 mM β-Mercaptoethanol, 4% SDS, 40% glycerol, 0.01% Bromphenol Blue) and loaded on a poly-acrylamid gel (for four gels: **10% resolving gel**: 8 ml Aqua dest., 6.6 ml 30% Rothiphorese gel 30 (Roth), 5ml 1.5M Tris-HCl pH 8.8, 200 µl 10% SDS, 10 µl TEMED, 100 µl 10% APS; **4% stacking gel**: 3 ml Aqua dest., 660 µl of Rothiphorese gel 30 (Roth), 1.3 ml 0.5M Tris-HCl pH 6.8, 50 ml 10% SDS, 5 µl TEMED, 25 µl 10% APS). The gel was run at 20 mA/40 mA for stacking/resolving gel in Running buffer (250 mM Tris-HCl, 1.92 M Glycin, 1% SDS for 10x stock). The gel was then equilibrated in Transfer buffer (48 mM Tris-base, 39 mM Glycin pH 2.6, 0.037% SDS, 20% methanol). The nitro-cellulose membrane (Millipore) was equilibrated in methanol. Then a semi-dry blot was assembled upside down (3x wet Whatman paper – gel – membrane – 3x wet Whatman paper). The blotting was run for one hour at a voltage kept below 10 V and an appropriate electric current (surface of the blot in square centimeter multiplied by 1.0-1.3). After transfer, the membrane was blocked in TBS-T (TBS: 0.2 M Tris-Cl, 1.37 M NaCl, pH to 7.6 with HCl for 10x

stock, TBS-T: 0.1% Tween 20 in 1xTBS) containing 5%w/v non-fat-dry milk powder. Subsequently, the membrane was incubated with the primary antibody for one hour, washed three times for seven minutes with TBS-T and incubated for one more hour with the secondary antibody (HRP-conjugated; Jackson immunoresearch laboratories). The blot was washed three times for seven minutes with TBS-T and then incubated with Amersham ECL Western Blotting Detection Reagent (GE Healthcare) for one minute and developed with a photosensitive film (GE Healthcare). If a membrane was reused and stained with a different antibody, it was incubated with Re-blot Plus Strong Solution (Chemicon) diluted 1:10 in ddH₂O and washed twice for five minutes in blocking solution.

Immunostainings, antibodies and reagents. For immunostaining, cells were split on coverslips coated with fibronectin (10 µg/ml, Roche). Cells were fixed in 4% PFA (para-Formaldehyde) for 15 minutes or for 5 minutes in 4%PFA and 3% Triton X-100 followed by 20 minutes in 4% PFA (for paxillin staining alone or with actin; subsequent permeabilisation was then omitted). For the staining, cells were permeabilised with 0.05% Triton X-100 in 1xPBS for 5 minutes. After washing for three times five minutes with 1xPBS the cell were blocked for 15 minutes (with 5% Donor Horse Serum, 1% BSA in 1xPBS, pH 7.3), rinsed once with 1xPBS and then incubated with Image-iT FX signal enhancer (Molecular Probes Invitrogen) for 30 minutes. After rinsing with 1xPBS, the coverslips were incubated for one hour with primary antibody, washed three times ten minutes with 1xPBS and incubated for one hour with the secondary antibody. Cell nuclei were stained if necessary with 1 µg/ml Hoechst 33342 dye (Sigma-Aldrich) for ten minutes. Upon Immunostaining or transfection, coverslips were mounted on glass-slides using the Prolong Gold reagent (Molecular Probes) and dried at room temperature over night.

The following antibodies were used: monoclonal paxillin (BD Transduction Laboratories #P13520) and clathrin heavy chain (X-22; kindly provided by Harvey T. McMahon); polyclonal paxillin (Santa Cruz Biotechnology (H-114): sc-5574) and EpsinR (ProteinTech Group, Chicago, #10470-1-AP; Ra48 kindly provided by Harvey T. McMahon). The secondary antibodies Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 568 and Alexa Fluor 598(SFX) were purchased from Molecular

Probes, Invitrogen. Actin was labeled with Alexa Fluor 568-Phalloidin (Molecular Probes, Invitrogen).

Figure 2.1

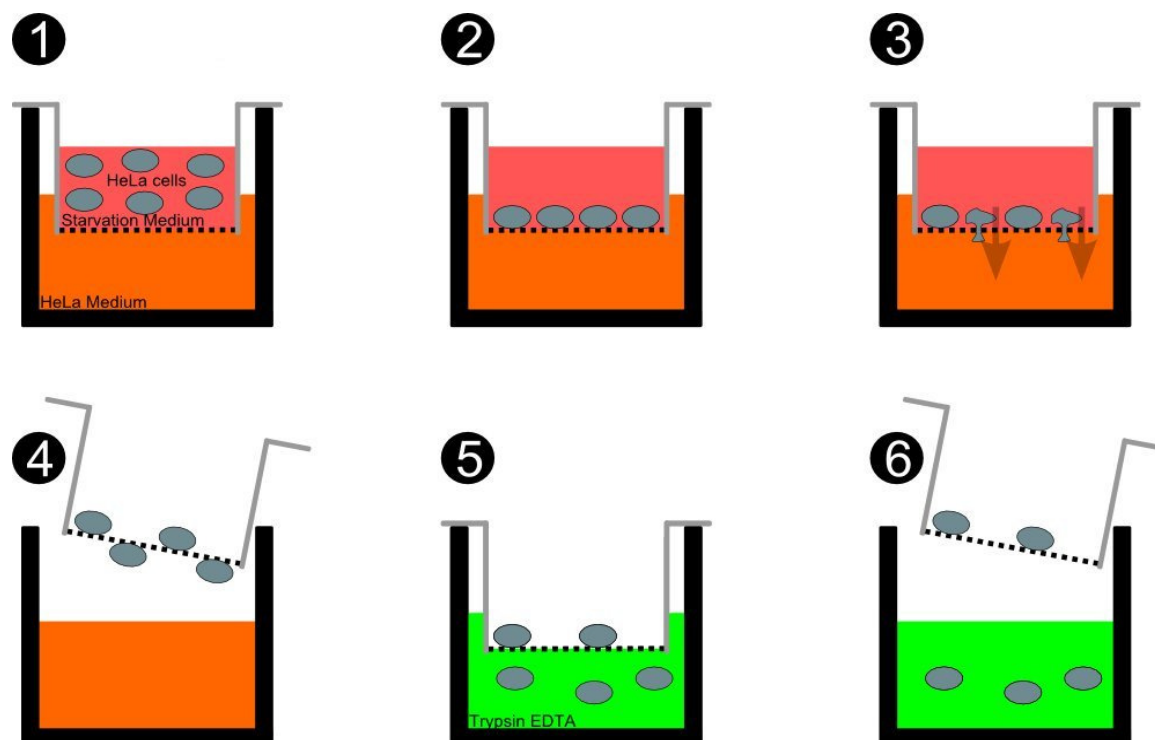


Figure 2.1: Work-flow of a Transwell Migration Assay. 1) Starved HeLa cells are added in starvation medium to the insert and put into a well containing standard HeLa Medium with 10% FCS. 2) The cells attach to the porous membrane. 3) The cells migrate through the membrane over night. Note that only part of the cells is able to migrate in this time. 4) The insert is removed from the well. 5) The insert is put into a new well containing Trypsin EDTA; the cells attaching to the lower side of the membrane will be mobilised from the porous membrane. 6) The insert is removed and the cells that have migrated and remained in the Trypsin EDTA are counted.

Transwell Migration Assay. The Transwell Migration Assay was performed in transwell 12-well plates (8 μm pore size, Greiner). Cells were starved in low glucose DMEM (Gibco) containing 0.2% BSA overnight. Afterward $3\text{--}4 \times 10^5$ cells were plated in the starvation medium to the inserts. The well itself was filled with standard medium containing 10% FCS, and cells were allowed to migrate overnight. Inserts were transferred into fresh 12-well plates filled with Trypsin-EDTA and incubated for 10 minutes at 37°C . Cells were detached from the

membrane by gentle shaking and tipping of the plate, the inserts were removed and cells left in the wells were counted with a hemocytometer. Figure 2.1 shows a scheme for the experimental work-flow.

Spreading assay. HeLa cells (after siRNA treatment) were trypsinized and seeded in standard medium on coverslips coated with Fibronectin (50 µg/ml, Roche). After 15, 30 and 60 minutes coverslips for each condition were fixed and stained with the antibodies of interest.

Microscopy. For the analysis of immunostainings an upright Axioplan2 (Zeiss) equipped with a Mercury lamp/LED lamps was used; wide-field live-cell imaging for subsequent 3D-Deconvolution was done on an AxioObserver microscope (Zeiss) with LED lamps. Both systems used a CoolSnapHQ camera (Photometrics) for detection.

For TIRF microscopy two different setups have been used. Both systems had a Cascade 512B camera (Photometrics) with an upstream Dual-View (Visitron). The first system was an Axiovert 200 upgraded with a Visitron TIRF system. The second system was a self-made TIRF system on a Nikon stand developed by Kurt Anderson (Beatson Institute of Cancer Research, Glasgow UK). Images were acquired with the MetaMorph software (Molecular Devices, MDS Analytical Technology).

Photoactivation (PA). Photoactivation experiments were performed using a LSM510 Duo confocal microscope on an AxioObserver (Zeiss) stand. Photoactivation was done in point-scanner mode, while the imaging was done with the 5-Live system and AxioVision software (Zeiss).

For photoactivation, HeLa cells were transfected with PAGFP-Paxillin and mCherry-Vinculin constructs. Imaging was started with one frame per second. After the third frame an area containing an adhesion and adjacent cytoplasm was activated with three to five pulses of a 405 laser (25% laser power). Then, imaging was resumed and finished after 180 frames (three minutes) in total. The obtained videos were analysed with MetaMorph (Molecular Devices, MDS Analytical Technology) and the average intensity values of the photoactivated paxillin in the area of an adhesion for the imaged time was exported to MS Excel

(Microsoft) for analysis. For every adhesion area, an equally activated cytoplasmic area adjacent to the analysed adhesion was subtracted to exclude measurement of cytoplasmic photoactivated paxillin.

3D-Deconvolution. For 3D-Deconvolution, three to seven z-planes were acquired. The distance between the z-planes was dependent on the experimental conditions (objective, wavelength used, indices of refraction) and calculated by the calculator for proper optical sampling, located on the homepage of SVI (<http://www.svi.nl/support/wiki/NyquistCalculator>). For the Deconvolution the program Huygens Essential (SVI) was used. The PSFs used were acquired at the corresponding system before the experiments (by Pawel Pasierbek, Biooptics, IMP/IMBA, Vienna).

Image analysis and post-processing. For standard image analysis and post-processing MetaMorph (Molecular Devices, MDS Analytical Technology), ImageJ and The Gimp softwares were used. Only non-destructive image processing was applied that did only enhance the contrast of structures. Maximum Projection Images were made in ImageJ. For this, time-lapse videos were combined into one single frame; for every pixel the maximum value displayed in the time-lapse video was plotted. Therefore, Maximum Projection visualises the behaviour of structures over time in one image. In some figures the time-lapse videos were split into three equal parts (for example 60 frames were split into three parts containing the frames 1-20, 21-40 and 41-60). For each part the Maximum Projection Image was made and coloured differently. Subsequently, the three pictures were merged into one RGB picture that showed stable structures in white. Moving structures, however, could be seen as coloured footprints.

Adhesion measurements. HeLa cells were fixed and stained with paxillin antibody to visualise focal adhesions and Hoechst stain to visualise nuclei as described. The images were acquired using the automated acquisition system present in the wide-field microscope described before for live-cell imaging. Auto-focus and proper acquisition were ensured by a MetaMorph (Molecular Devices, MDS Analytical Technology) journal. The images were then analysed by an

algorithm developed by Karin Aumayr (Biooptics, IMP/IMBA, Vienna) for Definiens (Definiens). This algorithm was trained to recognise the cell adhesions and the nucleus, subsequently classifying them as objects of various sizes. This information was then transferred to MS-Excel (Microsoft) for further analysis of the data.

Figures and illustrations. Figures were made with the help of the Scribus software. The illustrations were drawn in Inkscape. The post-processing of pictures was done in The Gimp.

3. Results

3.1. Paxillin Is Connected to Vesicular Structures

As discussed, Natalia Andreyeva worked with a similar construct used in the experiments done in the lab of Christopher Turner (Brown, Perrotta, and Turner 1996). As previously described by these workers, the paxillin mutant paxillin(M1) (containing two point mutations corrupting both zinc-fingers of the LIM3 domain) showed reduced localisation to focal adhesions. However, it additionally accumulated in punctate structures that were discovered to be clathrin vesicles. Due to this unexpected out-come experiments were performed to further characterise the vesicular structures found (Andreyeva 2008). Natalia Andreyeva's findings resulted in the hypothesis that the vesicles containing paxillin(M1) are physiological vesicles, albeit with increased size but whose existence reflects the connection of vesicular transport also with wild-type paxillin.

One part of my diploma project was to further analyse the paxillin(M1) mutant and its turnover relative to wild-type paxillin. My aim was to investigate paxillin's direct connection to vesicular transport. For this purpose, I decided to start by observing the behaviour of both constructs with various microscopic methods for time-lapse imaging.

Figure 3.1A displays a representative time-lapse imaging series of a cell transfected with GFP-tagged wild-type paxillin. As expected, the fluorophore-fusion protein localised to the focal adhesions; also a cytoplasmic background can be seen, especially in the assumed area of the Golgi apparatus/perinuclear recycling compartment (GA/PNRC). The largest cell adhesions, focal adhesions (Schlessinger and Geiger 1983; Burridge 1986), are long-lasting structures that remain stable over minutes (this is especially true for normally non-migrating cells like HeLa cells). Therefore, in the time frame used in Figure 3.1A (123.9 seconds), neither assembly, nor disassembly can be observed directly.

In contrast to the localisation of the wild-type paxillin, the paxillin(M1) mutant was targeted to punctate, mobile structures (Figure 3.1B), some of which could be labelled for markers of clathrin-coated vesicles (Andreyeva 2008). In Figure

3.1D the movement of individual vesicles can be seen as a coloured footprint of the individual structures. Vesicles that did not move remain white in this maximum projection image and tend to be larger than the mobile vesicles. One explanation for these enlarged vesicular complexes is the trapping of paxillin(M1) due to overexpression. Alternatively, mutations in the LIM3 domain could stabilise the vesicles (Andreyeva 2008).

Figure 3.1

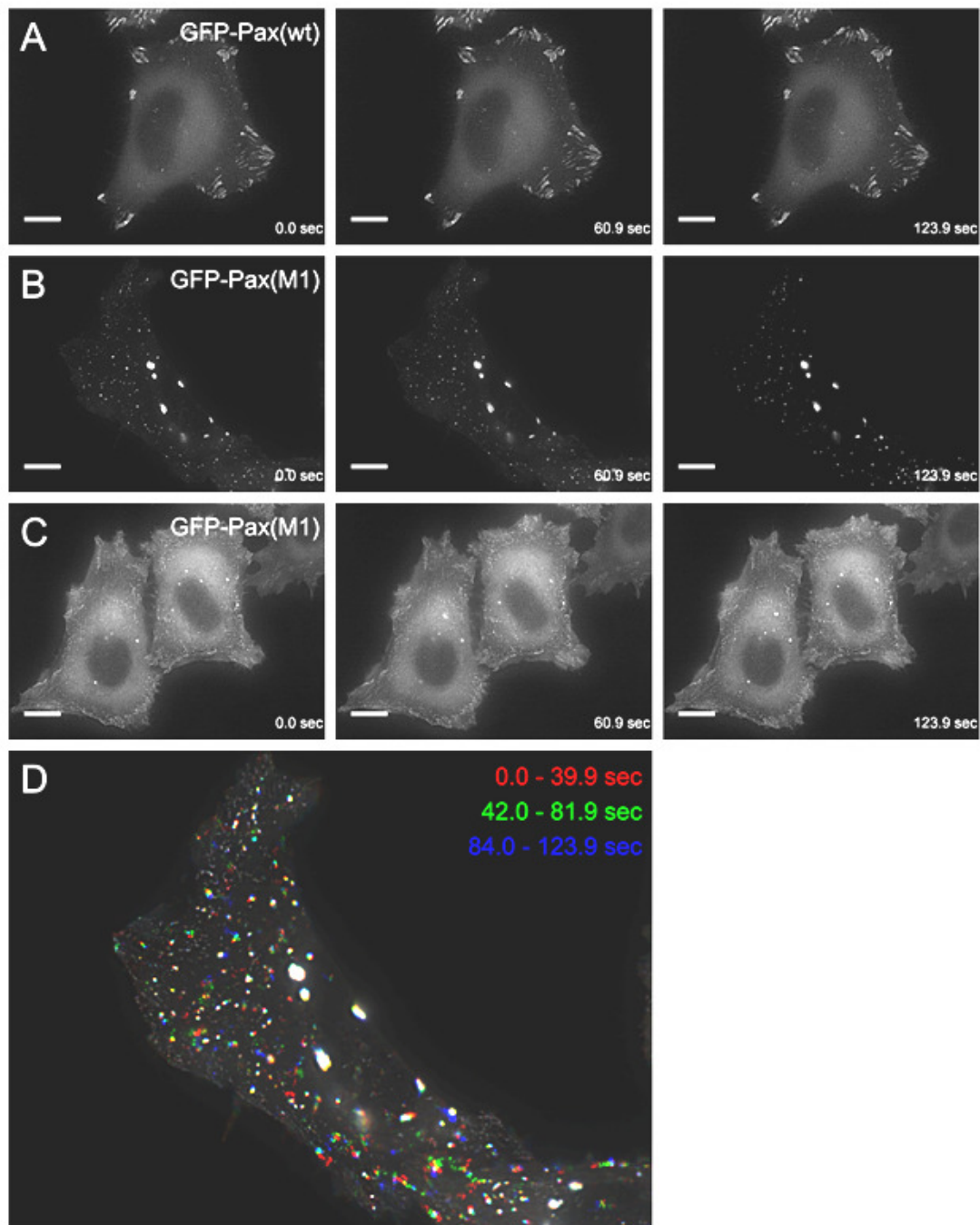


Figure 3.1: Distribution of GFP-tagged wild-type paxillin and mutant paxillin(M1). Time-lapse images of HeLa cells transfected with GFP-tagged wild-type paxillin (GFP-Pax(wt)), **A**) and mutant paxillin(M1) (GFP-Pax(M1), **B** and **C**) acquired in a wide-field setup followed by 3D-Deconvolution. Time points of the individual frames are indicated at the lower right; the scale bar in all the images equals 10 μ m. **A**) Wild-type paxillin is targeted to the focal adhesions and shows residual cytoplasmic staining accumulated around the nucleus. **B**) Mutant paxillin(M1) is targeted to vesicular structures. **C**) A subpopulation of cells transfected with mutant paxillin (M1) with weaker localisation to vesicular structures exhibits residual targeting to the focal adhesion area. **D**) Maximum projection of 60 time-lapse images of the cell shown in **B**. The movement of the vesicular structures over time can be seen as a coloured footprint (0.0 to 39.9 seconds in red, 42.0 to 81.9 seconds in green and 84.0 to 123.9 seconds in blue). Stable structures are depicted as white due to the overlay of the same area in all three colours.

Beside the vesicular localisation, the most striking difference between wild-type and mutant paxillin is the disappearance of the latter from the focal adhesions. In the first study of paxillin containing LIM3 domain mutations, it was reported that the mutant paxillin containing the same mutations as paxillin(M1) is completely lost from cellular adhesions (Brown, Perrotta, and Turner 1996). In my experiments, however, a subpopulation of cells had considerable amounts of fluorescent protein targeted to focal adhesions (Figure 3.1C). Due to weaker vesicular structures, the focal adhesions can be observed, although they are still very faint compared to the cytoplasmic background. As already reported, the vesicles were previously overlooked as a result of the experimental conditions and the use of only fixed cells (Andreyeva 2008). In addition the earlier studies utilised chicken paxillin, transfected into mammalian cells and a chicken specific antibody for detection (Brown, Perrotta, and Turner 1996). This likely resulted in inferior resolution compared to the current experimental setups. Still, I could show that the focal adhesion targeting can only be observed in cells that contain a small number of (comparable) faint vesicular structures.

Since this weak focal adhesion targeting could be seen in all cells, I considered that the adhesion localisation might be real, but simply masked by the vesicles as well as by the imaging noise in the normal wide-field microscopy setup used. Fortunately, the majority of vesicles are located in higher optical

planes than the focal adhesions. Therefore, I applied a different microscopy method exclusively visualising the cell-substratum interface: total internal fluorescence (TIRF) microscopy.

Figure 3.2

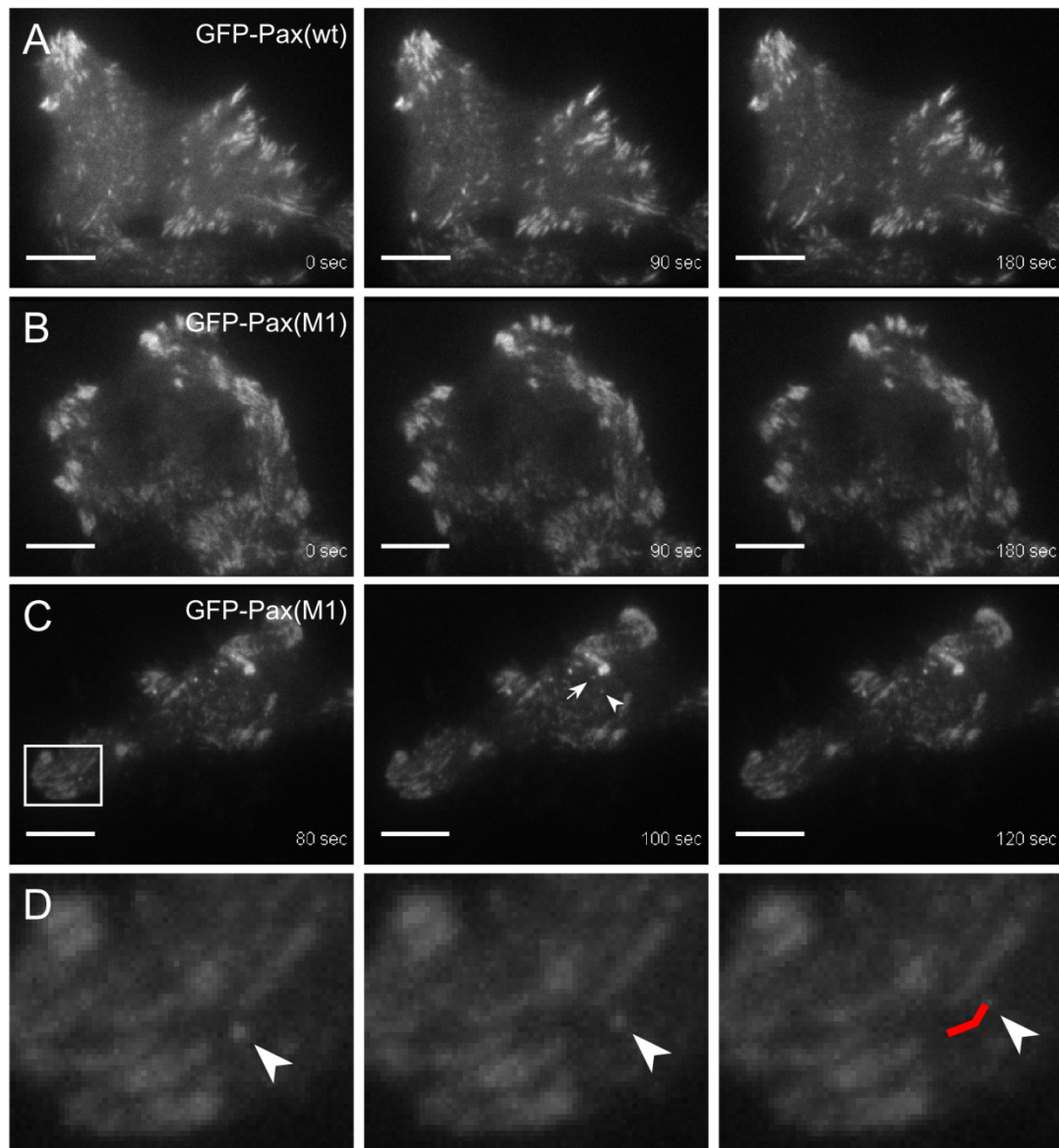


Figure 3.2: The mutant paxillin(M1) also localises to focal adhesions. Time-lapse images of HeLa cells transfected with GFP-Pax(wt) (**A**) and the mutant GFP-Pax(M1) (**B**, **C** and **D**) acquired with total internal reflection (TIRF) microscopy. Time points of the individual frames are indicated at the lower right; the scale bar in all the images equals 10 μ m. **C**) The arrow in the second frame (100 seconds) indicates a vesicle appearing in this frame and disappearing before the third frame (120 seconds). The arrowhead indicates a vesicle present in the first (80 seconds) and second frame

(Figure 3.2 continued) that disappears before the third frame. **D)** Blow up of the boxed region in the first frame in **C**; the arrowhead indicates a vesicle moving during the three indicated frames; the red line in the third frame indicates the distance the vesicle moved during 40 seconds.

As shown in Figure 3.2A, wild-type paxillin localised to focal adhesions, as previously seen in Figure 3.1A. In contrast, the paxillin(M1) mutant showed a changed distribution (Figure 3.1B), when observed in TIRF microscopy. Although the signal was weaker than for the wild-type isoform, the mutant protein was clearly localised to focal adhesions. Furthermore, although most mobile vesicles (and all of the bright stationary structures) remained above the imaging plane, some moving vesicles (Figures 3.2C and 3.2D) could still be detected. From observations I made in several experiments, it was apparent that these vesicles also interacted with focal adhesions.

Since Brown and Turner did not have access to TIRF microscopy they overlooked the adhesion localisation of paxillin(M1). The double point mutation in the LIM3 domain causes paxillin to be highly concentrated in the vesicular compartment, but the mutant still binds to focal adhesions and may, therefore, retain its normal function. This further supports the hypothesis that the vesicles, although highly enriched for the mutant, could reflect the physiological situation: soluble focal adhesion components like paxillin are incorporated in vesicular structures.

To assure the proper localisation of paxillin in the adhesion complex, the equilibrium of paxillin between adhesions and vesicles has to be controlled (Figure 3.3). The presented model assumes wild-type paxillin to be mainly targeted to the adhesion area, but with a minor fraction in the vesicular compartment. The mutations introduced in the paxillin(M1) protein shift this balance in favour of the vesicular compartment.

Figure 3.3

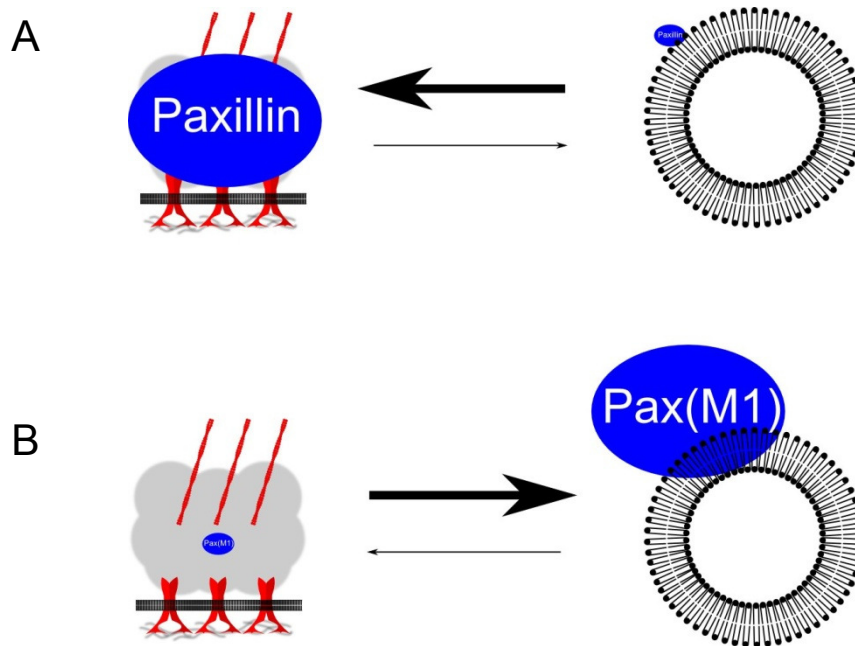


Figure 3.3: The Paxillin Equilibrium. Localisation of wild-type paxillin and the paxillin(M1) mutant to focal adhesions and vesicular structures (hypothetical model). **A)** Endogenous wild-type Paxillin is predominantly concentrated at the focal adhesions and only a minor fraction of the protein interacts with vesicles. **B)** In contrast, the mutant is highly enriched in the vesicular compartment and only residually localized to focal adhesions.

Although this shift can be caused by less efficient binding of mutant paxillin to the adhesion area, the stability by which paxillin(M1) binds to the vesicles leaves this possibility less likely. A mere inefficiency in binding would only increase the concentration of cytoplasmic proteins. Therefore, in a situation of overexpression of for example fluorophore-tagged paxillin, the vesicles should also appear. However this cannot be observed. Most probably, the double zinc-finger mutation influences the efficiency of vesicle interactions directly. Unfortunately, these findings do not clarify the mechanism of vesicular enrichment of the mutant; yet, they help to understand the relationship between paxillin and the vesicular structures. Furthermore, this model implies the

existence of wild-type paxillin in vesicular structures; theoretically, mostly localised to small, optically and temporally not easily resolvable vesicles.

Figure 3.4

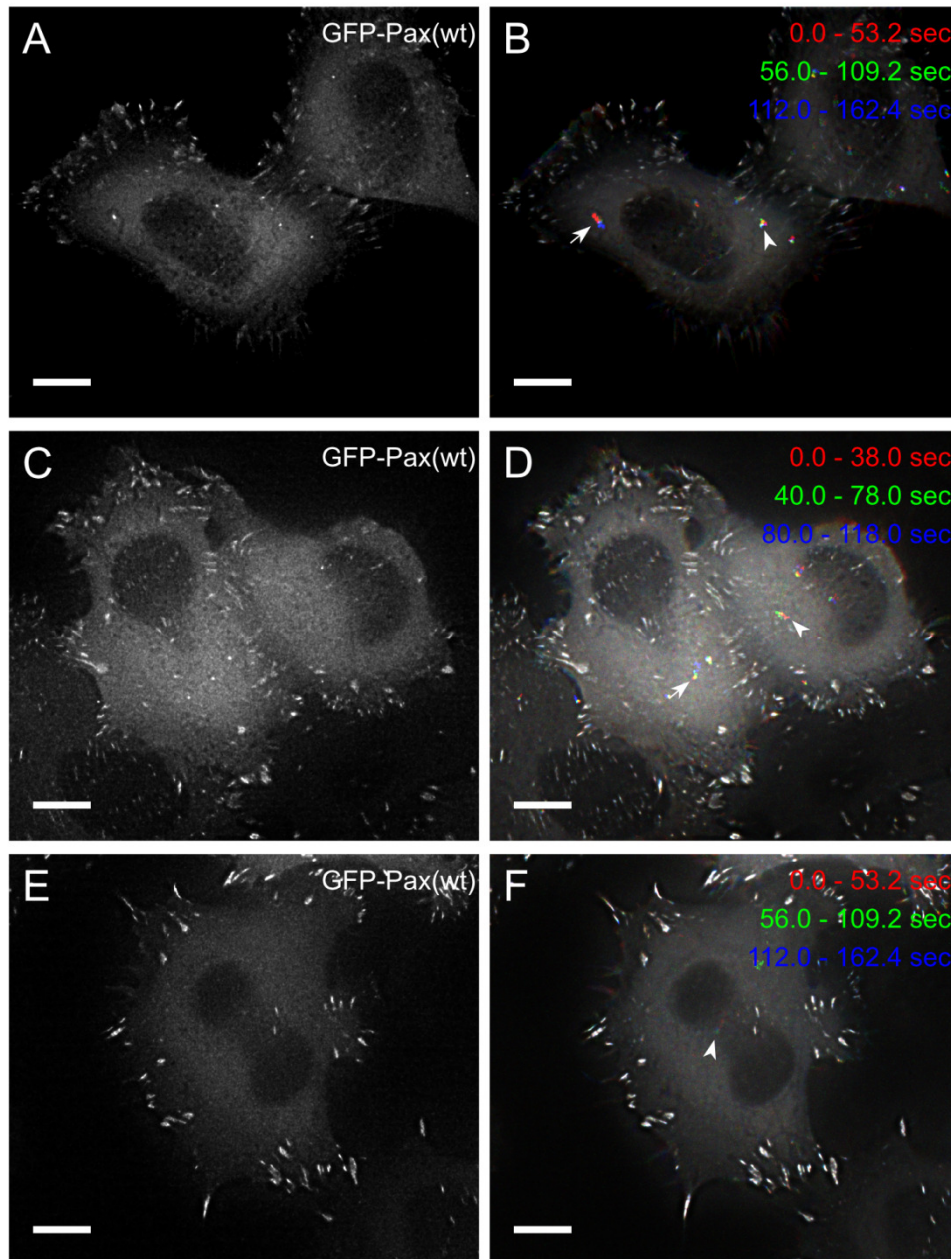


Figure 3.4: Wild-type paxillin localises to mobile vesicular structures. First frames (**A**, **C** and **E**) and maximum projections of 60 frames (split into three parts that are coloured differently as indicated at the upper right; **B**, **D** and **F**) of the corresponding time-lapse images of HeLa cells transfected with GFP-Pax(wt) acquired with a wide-field setup after 3D-Deconvolution; scale bar in all the images equals 10 μ m. Note that for **B**, **D** and **F** moving vesicles result in coloured footprints, stable structures like adhesions

(Figure 3.4 continued) result in white colour (compare to Figure 3.1D). The arrows and arrowheads indicate various vesicles that are moving differently. Various variations of movement patterns can be observed; most vesicles change their velocity and some also their direction in the indicated time frame. This further strengthens the argument that these punctate structures are physiological vesicles.

In an attempt to resolve wild-type paxillin vesicles, I used 3D-Deconvolution that allows visualisation of small structures even in the presence of noise. In order to optimise the resolution of the vesicles, optical slices through the entire cell were included in the imaging process. And, indeed, wild-type paxillin punctate structures were observed. Figure 3.4 shows three representative cells and their differentially coloured maximum projections for the corresponding time lapse images. In Figures 3.4 B, 3.4D and 3.4F arrows and arrowheads mark sample vesicular structures (seen as coloured footprints, as in Figure 3.1D).

The punctate structures exhibit variations in movement, size and speed; excluding unspecific protein aggregates that would be expected to be less motile. The 3D-Deconvolution was controlled with the corresponding fluorophore without a fusion protein, under which conditions no vesicle-like structures were detected (data not shown)

In order to further clarify the nature of these vesicles, colocalisation experiments with vesicular markers were required. Since the mutant paxillin(M1) vesicles were identified to be clathrin vesicles, the most reasonable protein to check for interaction was clathrin itself. Moreover, Natalia Andreyeva previously showed that the mutant and wild-type isoforms are targeted to the same vesicles if co-expressed in HeLa cells; these were also shown to be positive for clathrin (Andreyeva 2008). Therefore, I applied the 3D-Deconvolution approach to determine whether wild-type paxillin vesicles are colocalised with clathrin.

However, as shown in Figures 3.5A, B and C, stable colocalisation of RFP-Pax(wt) and GFP-Clathrin was not detectable. Yet, paxillin overlapping with the clathrin signal for several seconds could be seen. This indicates possible transient interactions between these vesicles; it further implies that the focal

adhesion protein, indeed, might enter the clathrin pathway. A reason for only transient interactions could be that the observable vesicles are paxillin reservoirs that only interact with clathrin shortly during budding of small and fast vesicles. If paxillin is localised only to such vesicles positive for clathrin, they would only be detectable by imaging at high temporal resolution. The paxillin(M1) mutant containing vesicles could be locked in a state that stabilises the interaction with paxillin, allowing their detection.

Although the interaction data of paxillin and clathrin vesicles in the cell periphery did not show any long-term colocalisation, it cannot be excluded that they will exhibit stable interactions near the GA/PNRC. As already discussed, clathrin does not bind stably to all types of vesicles. This could be important, if vesicular transport is not only involved in regulation and recycling, but also in the delivery of the focal adhesion component.

As noted in previous studies (Mazaki et al. 1998; Turner et al. 1999; Andreyeva 2008), it is known that wild-type paxillin not only precipitates clathrin in immunoprecipitation experiments, but also localises to the GA/PNRC. This data already suggested a connection of paxillin to a vesicular compartment. Hitherto, paxillin wild-type vesicles have not been described. The reason for this is mainly the impossibility of resolving these structures in most microscopy setups. Furthermore, although in immunostainings these limitations could have been overcome, granular cytoplasmic paxillin signal after antibody-staining is often disregarded as an artefact.

As already indicated in the introduction, Natalia Andreyeva described the interactions of paxillin with various vesicle-related proteins (Andreyeva 2008). A very promising candidate was the clathrin adaptor EpsinR. Significantly EpsinR localises only to a subset of clathrin vesicles and is involved in only a part of the clathrin trafficking pathway. I, thus, investigated the influence of EpsinR on focal adhesion organisation to assess whether EpsinR-associated vesicles play a role in paxillin turnover

Figure 3.5

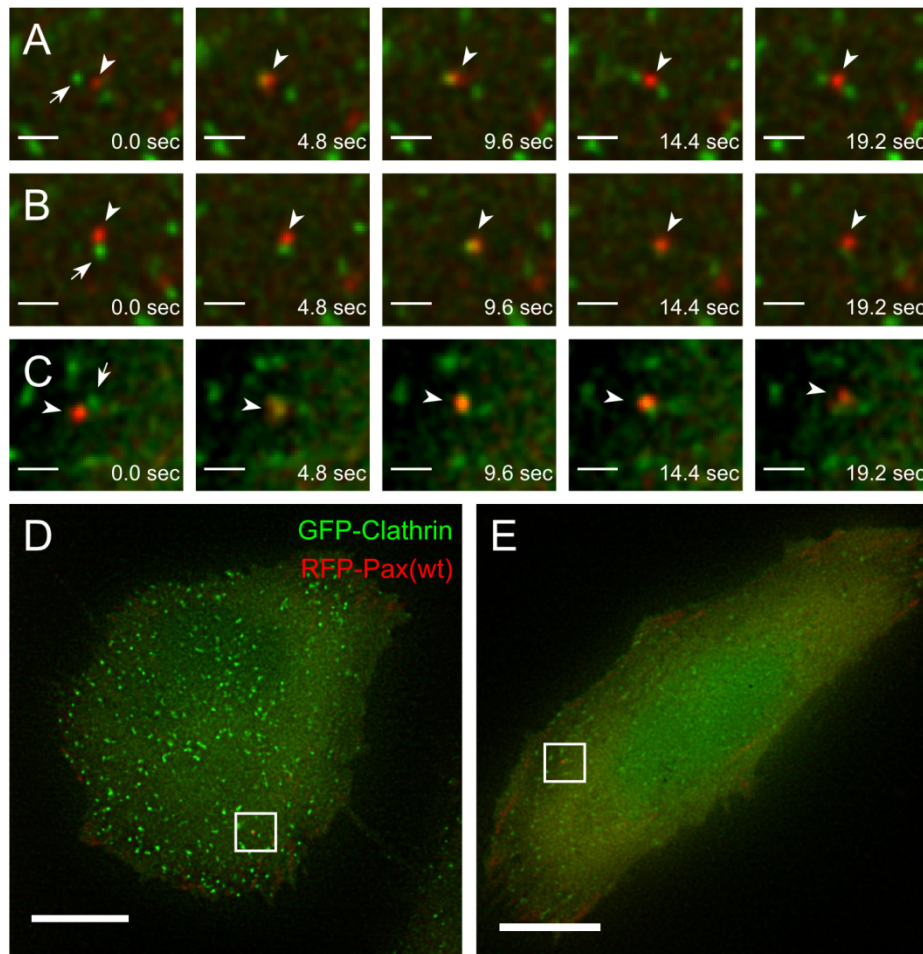


Figure 3.5: Wild-type paxillin vesicles do not colocalise, but interact with clathrin vesicles. **A, B** and **C**) Time lapse images of HeLa cells transfected with GFP-Clathrin and RFP-tagged wild-type paxillin (RFP-Pax(wt)) acquired in a wide-field setup after 3D-Deconvolution. The arrowheads indicate the position of the wild-type paxillin vesicle in each frame. These vesicles show no permanent colocalisation with clathrin vesicles (indicated by an arrow in the first frames of each time-lapse imaging series). However, they share positions with them over seconds, suggesting a temporary interaction. Relative time-points are indicated at the lower right; scale bar in all images equals 1 μm . **D** and **E**) Corresponding first frames for the time-lapse images in **A**, **B** and **C**. Boxed region in **D** corresponds to figures **A** and **B** (at different time points); Boxed region in **E** to figure **C**. Scale bar in both images equals 10 μm .

3.2. EpsinR and Focal Adhesions

As discussed in the introduction, EpsinR is mainly localised to endogenous membranes (Golgi apparatus, endosomes). Therefore, its main functions are trans-Golgi, inter-vesicle and retrograde transport. I wanted to assess its possible function in focal adhesion turnover first by localisation studies of an EpsinR-GFP construct expressed in HeLa cells.

In the context of viral invasion, increased endocytic activity upon activation of FAK was already described (Krishnan et al. 2006). An increased number of early endosomes and various recycling vesicles might then be localised close to focal adhesions. Thus, a first step to connect EpsinR and focal adhesion turnover was to check for targeting of EpsinR to these early and recycling endosomes.

The binding of transferrin to the transferrin receptor and its subsequent internalisation is an established method to visualise endocytosis, vesicular transport and recycling processes. Accordingly, I used Alexa-568-tagged transferrin to mark endocytic vesicles. This I used to analyse whether the accumulation of endosomes around the cell adhesions is also present in our model system.

When HeLa cells were shortly incubated with the labelled transferrin, endocytic vesicles could be detected immediately after washing (Figure 3.6A; focal adhesions are visualised by GFP-tagged paxillin). However, it is important to note that the observed vesicles do not represent the individual vesicles of transferrin directly after endocytosis. Due to their small size and low number of labelled molecules, it is not possible to resolve them. The observed signal represents the first accumulation of several endocytic vesicles in early endosomes (personal communication with Harvey T. McMahon; unpublished data).

To display the vesicles around the focal adhesions over time, representative time-lapse images were combined to maximum projection images (Figures 3.6B, 3.6D and 3.6F; for details on maximum projections, see Materials and Methods). Indeed, transferrin positive vesicles accumulated in the vicinity of focal adhesions (Figure 3.6B; the arrow and the arrowhead indicate such accumulations on the picture). Theoretically, in areas without focal adhesions existing vesicles could go

out of the imaging plane (see Introduction, TIRF microscopy) due to invagination of the membrane. However, the absence of signal in some areas around adhesions contradicts this argument and suggests specificity of the observed signal. In addition, a similar pattern can also be seen for cells that are fixed after transferrin uptake and imaged in a wide-field setup (data not shown).

Figure 3.6

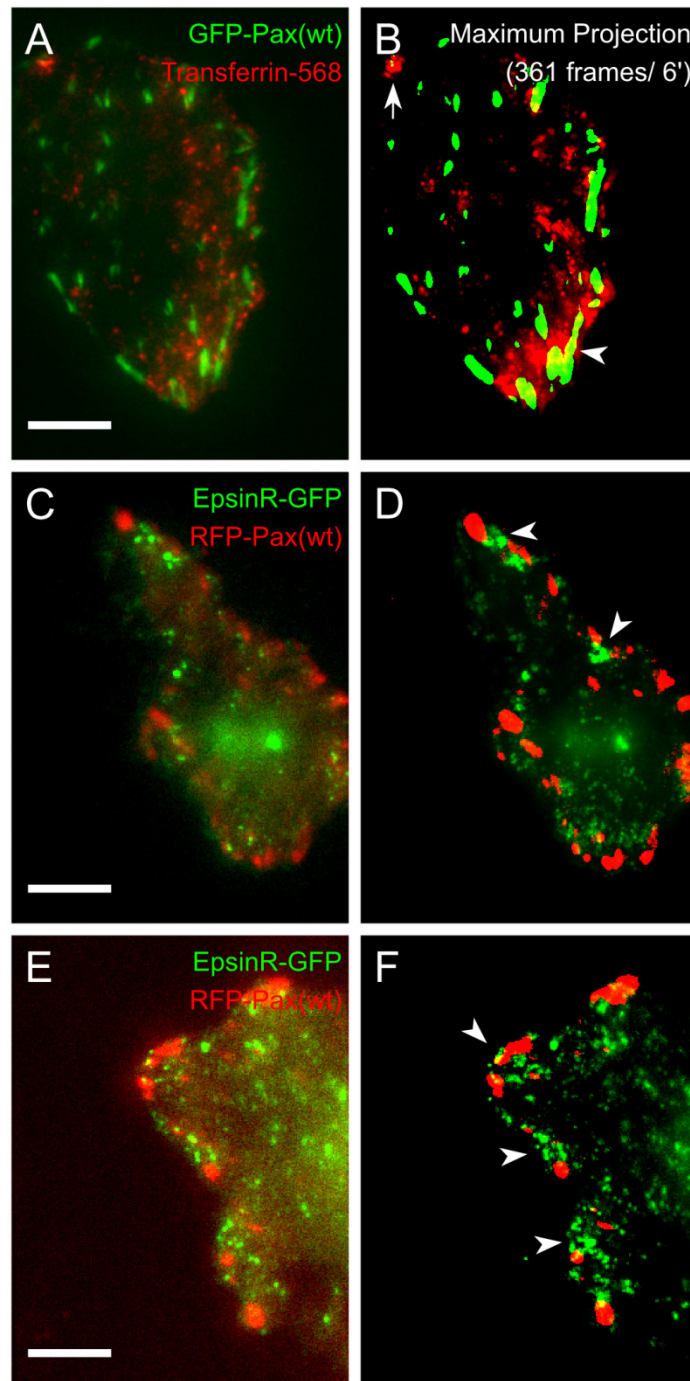


Figure 3.6: Transferrin and EpsinR activity is concentrated around the focal adhesion area. First frames (**A, C and E**) and maximum projections (**B, D and F**) of the corresponding time-lapse images (361 frames for six minutes) acquired with total internal reflection (TIRF) microscopy; scale bar in all the images equals 10 μm . In the maximum projections the contrast was highly increased for the adhesions to mark adhesion area. **A)** A HeLa cell transfected with GFP-Pax(wt) was incubated for 15 seconds with transferrin coupled to Alexa-568 (25 $\mu\text{g/ml}$; Transferrin-568) and then imaged after washing. **B)** The arrow indicates a small dot-like adhesion with strong vesicular activity around it; the arrowhead marks an area with big adhesion area and strong transferrin accumulation. **C and E)** HeLa cell transfected with EpsinR-GFP and RFP-Pax(wt) **D and F)** Arrowheads indicate areas of EpsinR accumulation around focal adhesion areas.

From the accumulation of transferrin around focal adhesions, I would predict EpsinR localisation, if it was involved in focal adhesion turnover, also in their vicinity. Figures 3.6C and 3.6E show two representative cells used for this analysis. The maximum projections of cells co-transfected with GFP-tagged EpsinR and RFP-tagged paxillin are shown in Figures 3.6D and 3.6F. Although the accumulation of EpsinR is not as clearly concentrated around focal adhesions as for transferrin, still the strongest signal can be seen there (as indicated by the arrowheads). Moreover, beside the cell adhesion vicinity, the EpsinR is mainly located in the centre of the cells. This was also confirmed by correlative wide-field imaging (data not shown); this observed region corresponds to the GA/PNRC. By TIRF microscopy only a small part of the GA/PNRC is close enough to the substrate to fall within the evanescent wave, which leads to a selective enhancement of the EpsinR signal in the vicinity of the adhesions.

The localisation of EpsinR to the vicinity of focal adhesions supports its postulated involvement in paxillin turnover. Due to the previously shown binding in immunoprecipitation (Andreyeva 2008), I investigated a possible interaction of EpsinR and the mutant paxillin(M1). However, immunostainings of transfected cells did not confirm this. As shown in Figure 3.7C, the paxillin mutant and endogenous EpsinR did not show colocalisation in fixed cells. Furthermore, whereas clathrin and paxillin(M1) (Figure 3.7I) or clathrin and EpsinR (Figure

3.7H) colocalised, EpsinR and paxillin(M1) are targeted to different subsets of clathrin vesicles in the same cell (Figure 3.7G).

Figure 3.7

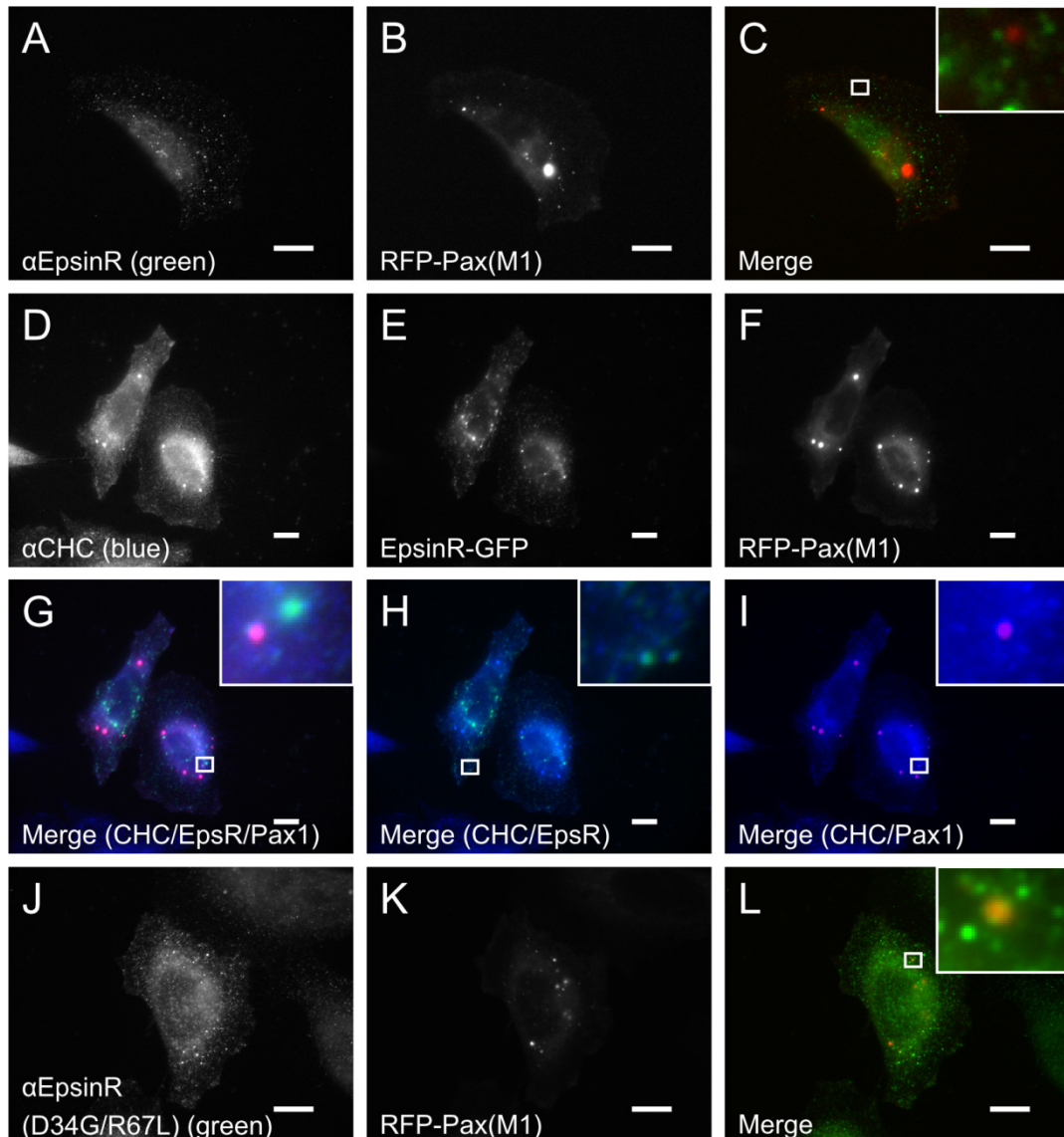


Figure 3.7: The dominant negative mutant EpsinR D34G/R67L does colocalise with the mutant paxillin(M1), but wild-type EpsinR does not. Images showing various immunostainings of transfected HeLa cells were acquired in a wide-field setup. Scale bar in all images equals 10 μ m; boxed regions are presented as a blow up in the upper right corner of each picture. **A, B and C)** Immunostaining for EpsinR of a cell transfected with the mutant RFP-Pax(M1). Shown are the separate channels (**A** and **B**) and a merge of the two channels (**C**). Note that EpsinR and paxillin(M1) do not colocalise, as seen in the box. **D – I)** Immunostaining for clathrin heavy chain of a cell

(Figure 3.7 continued) transfected with EpsinR-GFP and the mutant RFP-Pax(M1). Shown are the separate channels (**D**, **E** and **F**) and a merge of all three channels (**G**) or two of the channels (**H** and **I**). Note that, although EpsinR and paxillin(M1) are not interacting themselves, they are both colocalised with clathrin. **J**, **K** and **L**) Immunostaining for EpsinR of a cell transfected with the mutant RFP-Pax(M1) and the dominant negative mutant EpsinR D34G/R67L. Shown are the separate channels (**J** and **K**) and a merge of the two channels (**L**). Note that EpsinR and paxillin(M1) do colocalise in the presence of the mutant EpsinR construct, as seen in the box.

However, if the paxillin mutant is co-transfected with the dominant negative EpsinR D34G/R67L construct, colocalisation could be observed (Figure 3.7L). In this mutant, described by researchers in the laboratory of Harvey T. McMahon (Mills et al. 2003), two point mutations were introduced in its ENTH domain. This weakens its binding to lipids, imparting the recruitment of the protein to the membrane. Consequently, EpsinR is relocated to irregularly sized vesicular structures, accompanied by various interaction partners; therefore, acting as a dominant negative mutant. As shown, the dominant mutant EpsinR construct colocalised partially with the mutant paxillin.

What could be the reason for this? Considering the immunoprecipitation data, an interaction (at least indirectly) of EpsinR and wild-type paxillin/mutant paxillin(M1) was already shown. Since the dominant negative mutant is not recruited appropriately to the membranes, also the regulation of binding to its interaction partners may be inefficient. Therefore, a normally short-lived interaction between EpsinR and paxillin(M1) can last longer in this situation. Consequently, a different approach to resolve their interactions would be live-cell imaging. By observing vesicles over a longer period of time, the probability to record an interaction event should increase.

Figure 3.8

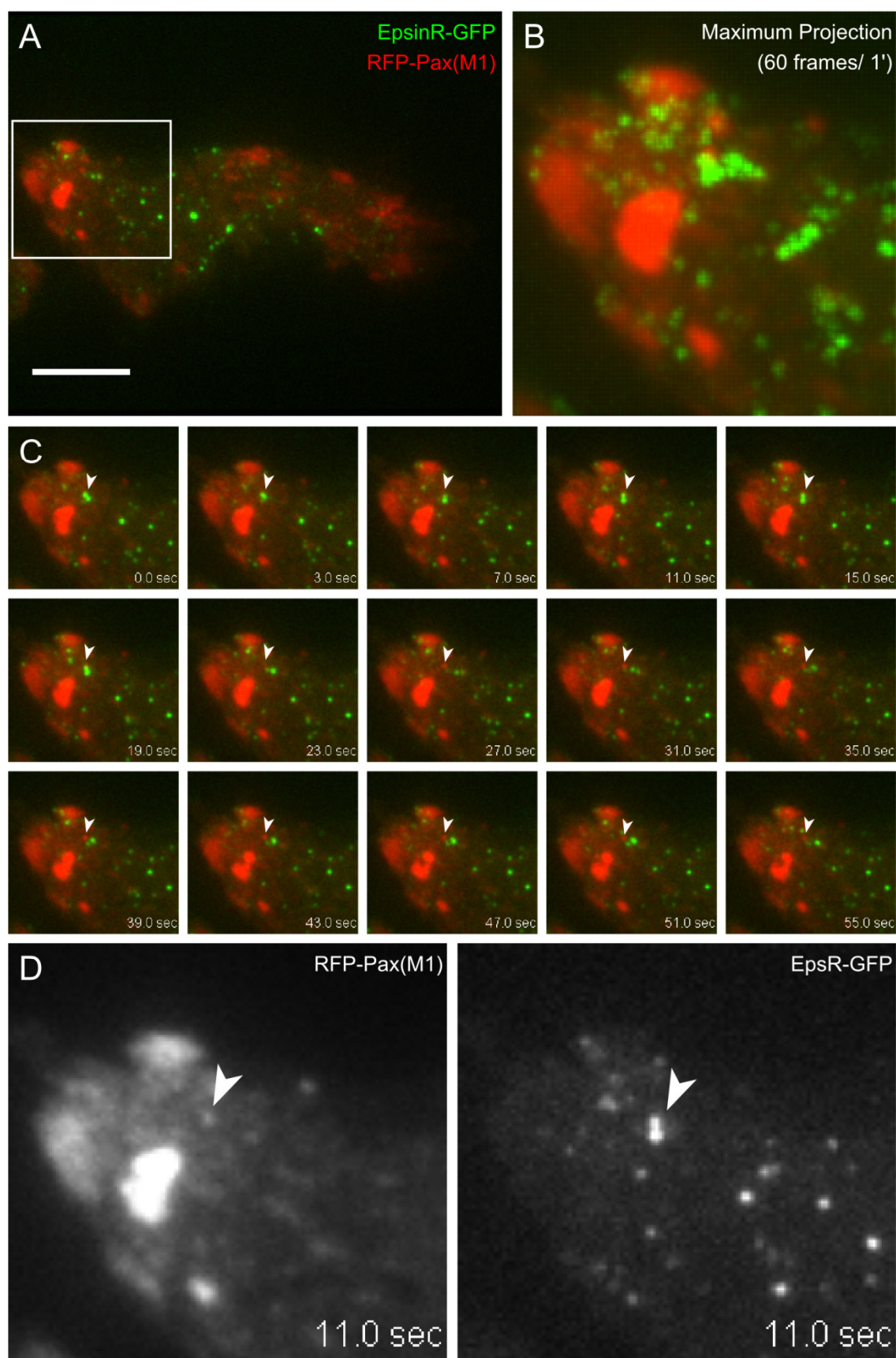


Figure 3.8: EpsinR and the mutant paxillin(M1) do not colocalise, but interact transiently. Time lapse images of a HeLa cell transfected with EpsinR-GFP and mutant RFP-Pax(M1) acquired with total internal reflection fluorescence (TIRF) microscopy. Relative time-points are indicated at the lower right (**C** and **D**); scale bar in **A** equals 10 μ m. **A**) First frame of the time-lapse images, boxed region is seen in **B**, **C** and **D**. **B**) Maximum projection of the corresponding 60 frames for 1 minute. **C**) Selected images of the time lapse; the arrowhead indicates a mutant paxillin(M1) vesicle (and the area it will form in the first two frames). Note that for most of the frames EpsinR and paxillin(M1) do not colocalise, but are resting adjacent to each other. **D**) Blow up with separated channels for the fourth frame of **C**; the arrowhead indicates again the position of the mutant paxillin(M1) vesicle. Note that at 11.00sec during the sequence a paxillin positive vesicle can be seen and colocalises with the EpsinR positive vesicle (arrowhead). This indicates a possible action of EpsinR in the budding of paxillin vesicles, which is in accordance with the known functions of EpsinR.

For this purpose, we co-transfected HeLa cells with RFP-tagged paxillin(M1) and GFP-tagged EpsinR. Subsequently the cells were analysed by TIRF microscopy. The corresponding vesicular structures were observed for one minute (Figure 3.8). The maximum projection of the time-lapse images (Figure 3.8B) shows increased accumulation of EpsinR near the adhesions. Moreover, in this area a paxillin(M1) vesicle could be observed (the arrowheads in Figure 3.8C indicate its position).

Figure 3.8D shows a blow up of the fourth frame with separated channels. As indicated by the arrowhead, the two proteins overlapped in this frame, further indicating the suggested transient interaction between paxillin(M1) and EpsinR. Even more, the mutant paxillin signal is not present at the beginning, but starts to form during the first 10 seconds of the imaging period. This indicates a possible function of EpsinR in the budding of paxillin off the focal adhesion area.

An inherent advantage, but also limitation of TIRF microscopy is that structures beyond a thin optical slice at the basal side of the cell are not seen. Therefore, a vesicle residing in a higher layer of the cell moving into the imaged section would show a gradual intensity increase during its appearance; this, easily, could be mistaken for budding. However, the vesicle marked in Figure 3.8

is very stable over the imaging period. Therefore, the possibility of a downward moving vesicle is less likely in this case.

3.3. Loss of EpsinR and Clathrin Function and Its Impact on Focal Adhesions

My experiments so far localised EpsinR near the focal adhesions and indicated its transient interaction with paxillin(M1) vesicles. However, the physiological implications of these findings, in respect to focal adhesions, were still unknown. To further delve into this problem, I decided to analyse the loss-of-function phenotype for EpsinR and clathrin. The latter was used as an internal control, since EpsinR only defines a subset of clathrin vesicles. Therefore, the loss of clathrin function should resemble the phenotypes of loss of EpsinR function.

In order to induce loss of function phenotypes, I performed siRNA knockdowns with oligos specifically designed against EpsinR and clathrin heavy chain (CHC). The efficiency of these knockdowns was tested by immunostaining for the respective antibodies and transferrin uptake (Figures 3.9A-L).

Upon the knockdown of CHC the reduction in the protein levels can be seen clearly in the antibody staining. Furthermore the efficiency of the knockdown is confirmed by reduced uptake of transferrin for the knockdown cells (Figure 3.9D). This was expected, since the latter is highly dependent on the clathrin machinery. EpsinR reduction in protein level cannot easily be seen with the immunostaining (Figure 3.9J), probably because of the sensitivity of the antibody, rendering it difficult to distinguish between variations in the protein level. Additionally, also EpsinR localises strongly to the GA/PNRC; this signal is also existent for the knockdown cells and differences cannot be seen easily in this area. However, still cells with reduced protein level can be observed. Furthermore, cells showing this reduction also exhibited a localisation defect of transferrin (Figure 3.9K; indicated by the arrowheads); it accumulated at the periphery of the cells, but not in the perinuclear area as in the control cells. The most probably reason is the disruption of the EpsinR function in retrograde transport; therefore, although taken up, transferrin cannot be redistributed to the centre of the cell.

Figure 3.9

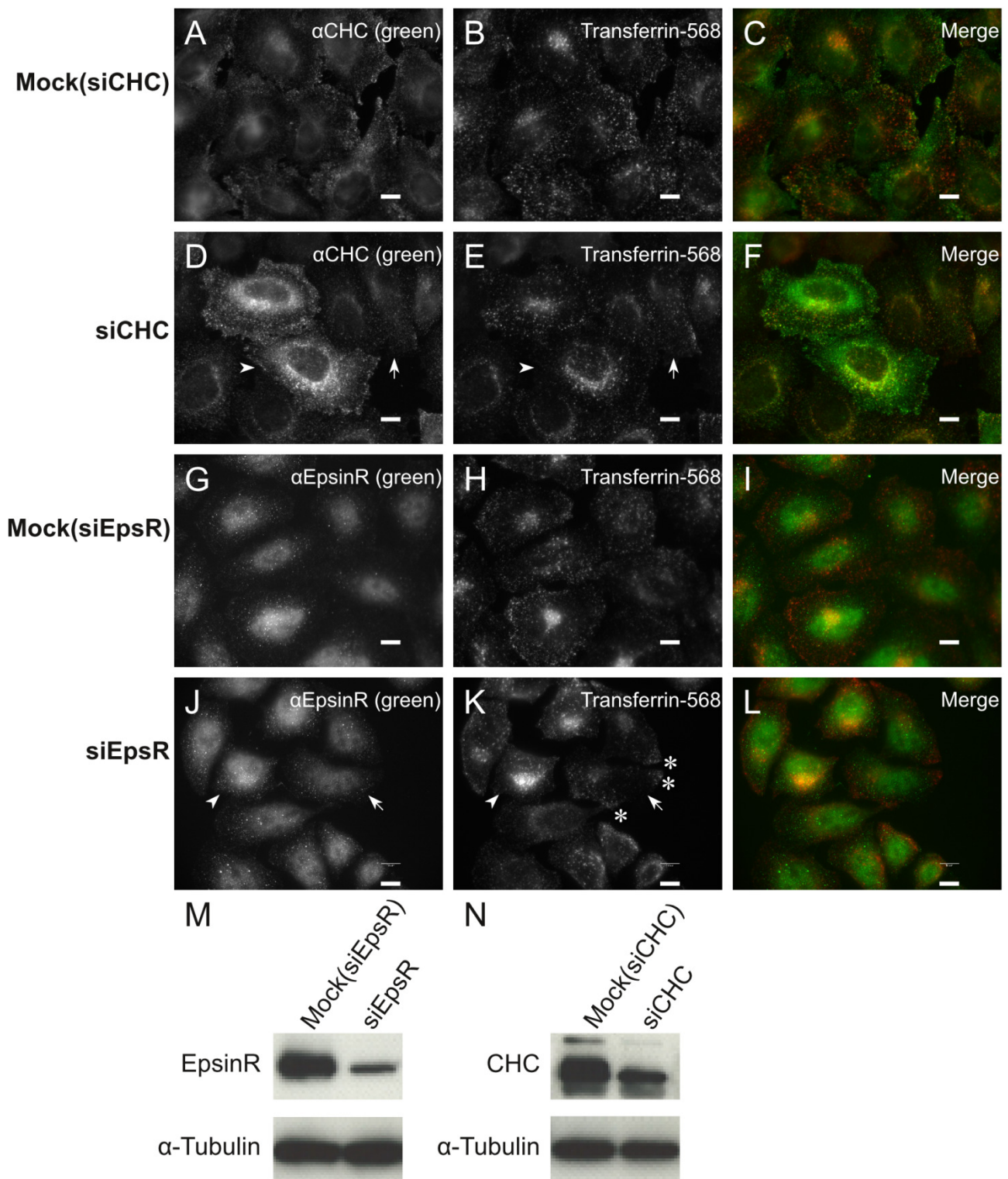


Figure 3.9: siRNA knockdown of EpsinR and CHC results in defective Transferrin uptake. A – L) Images showing various immunostainings of HeLa cells incubated with Transferrin-568 (25 μg/ml) for 15 minutes before fixation were acquired in a wide-field setup. Scale bar in all images equals 10 μm. The cells were treated as

(Figure 3.9 continued) indicated on the left side. Mock(siCHC): mock transfection, left for two days and an additional two days after a second mock transfection. siCHC: siRNA knockdown for two days and an additional two days after a second siRNA transfection. Mock(siEpsR): mock transfection, left for three days. siEpsR: siRNA knockdown for three days. For further information on siRNA knockdown protocols see also “2. Materials and Methods”. **A – F**) Immunostaining for clathrin after Mock(siCHC) (**A**, **B** and **C**) or siCHC (**D**, **E** and **F**) and Transferrin-568 incubation. Shown are the separate channels (**A** and **B**; **D** and **E**) and a merge of the two channels (**C**; **F**). The arrow and the arrowhead in **D** and **E**, indicate a cell with knockdown and wild-type levels of CHC, respectively. Note that the knockdown cells show a reduced uptake of transferrin (**E**) compared to wild-type. **G – L**) Immunostaining for EpsinR after Mock(siEpsR) (**G**, **H** and **I**) or siEpsR (**J**, **K** and **L**) and Transferrin-568 incubation. Shown are the separate channels (**G** and **H**; **J** and **K**) and a merge of the two channels (**I**; **L**). The arrow and the arrowhead in **J** and **K**, indicate a cell with knockdown and wild-type levels of EpsinR, respectively. Note that the knockdown cells show a disturbed distribution of transferrin (**K**) compared to wild-type (direct comparison can only be made between cells in the same image) towards the cell periphery (marked with an asterisk in **H**), presumably due to defective delivery to the perinuclear recycling centre. **M** and **N**) Western Blots showing the reduction in protein levels for EpsinR (**M**) and CHC (**N**) after the respective siRNA knockdowns and compared to the respective mock control. Additionally, for both figures a tubulin staining is shown as a loading control. Note that the protein bands on the Western Blots show a decrease in total amount after the respective knockdown compared to the relevant mock control.

In addition to the immunostainings, efficiency of the knockdowns was also analysed with Western Blots. As shown in Figures 3.9M and 3.9N, the protein level of the proteins knocked down was reduced significantly compared to the mock control. The α -tubulin loading control indicated that this reduction is, indeed, due to a specific decrease of the respective protein.

The foregoing data indicates that I was able to specifically induce EpsinR and clathrin loss-of-function phenotypes using RNAi. To study the role of EpsinR and clathrin in focal adhesion turnover, as a first step, paxillin distribution after knock-down was analysed by immunostaining (Figure 3.10). Upon knockdown of clathrin (Figure 3.10D), focal adhesions, stained with anti-paxillin antibody,

increased in size and shifted to the cell periphery. This was accompanied by loss of small adhesions from the centre.

Upon knockdown of EpsinR the adhesions also changed their appearance. Figure 3.10K indicates cells with wild-type (arrowhead) and reduced levels (arrow) of EpsinR. The corresponding staining for paxillin shows an increase in adhesion size, even stronger than the phenotype for CHC knockdown cells. Moreover, also the lateralisation of adhesions and loss of small adhesions in the centre of the cells was observed. However, the two last phenotypes were milder compared to the knockdown of CHC; this once more suggests the higher specificity of EpsinR compared to clathrin.

Figure 3.10

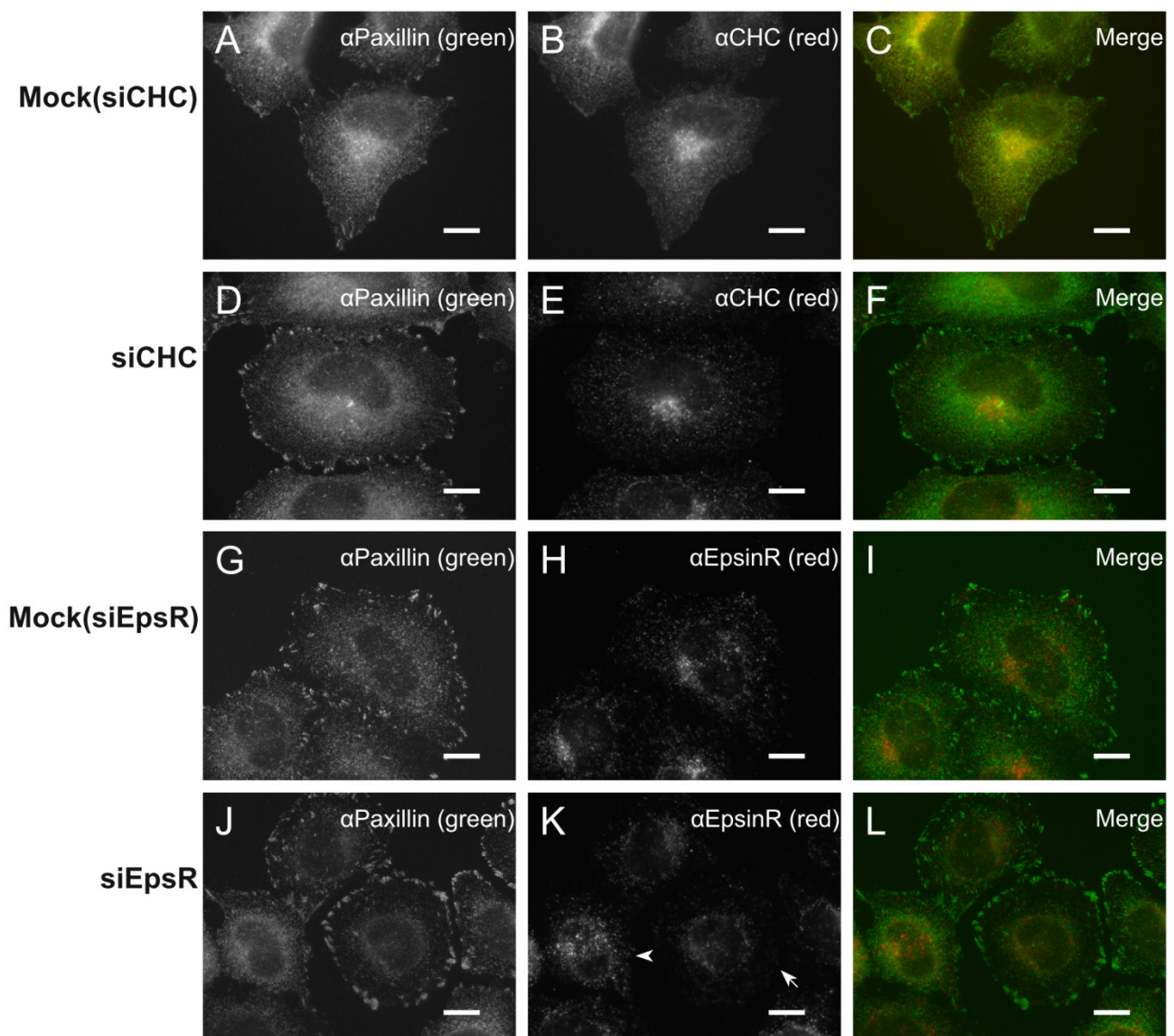


Figure 3.10: siRNA knockdown of EpsinR and CHC increases the focal adhesion size. Images showing various immunostainings of HeLa cells were acquired in a wide-field setup. Scale bar in all images equals 10 μ m. The cells were treated as indicated on the left side (for explanations see Figure 3.9). **A – F)** Immunostaining for paxillin (**A – F**) and clathrin after Mock(siCHC) (**A, B** and **C**) or siCHC (**D, E** and **F**). Shown are the separate channels (**A** and **B**; **D** and **E**) and a merge of the two channels (**C**; **F**). Note that the knockdown cells show increased size and lateralisation of the focal adhesions stained by paxillin. **G – L)** Immunostaining for paxillin (**G – L**) and EpsinR after Mock(siEpsR) (**G, H** and **I**) or siEpsR (**J, K** and **L**). Shown are the separate channels (**G** and **H**; **J** and **K**) and a merge of the two channels (**I**; **L**). The arrow and the arrowhead in **K**, indicate a cell with knockdown and wild-type levels of EpsinR respectively. Note that the knockdown cells show increased size and lateralisation of the focal adhesions stained by paxillin.

Knockdown cells stained for actin (with fluorophore-tagged Phalloidin) showed the difference between the EpsinR and CHC knockdown phenotypes more clearly (Figure 3.11). Knockdown of CHC had more drastic effects on the organisation of the actin cytoskeleton (Figure 3.11B). The microfilaments were bundled in strong stress fibres parallel to the edges of the (frequently rounded) cells and disappeared from the centre of the cell. Although actin fibres of the EpsinR knockdown cells exhibited strong, lateral stress fibres, this phenotype was milder compared to CHC knockdown.

Whereas the presented phenotypes could be reproduced several times convincingly, the magnitude of the adhesion area increase was variable. The size of adhesions is dependent on type and concentration of the matrix the cells are growing on. Furthermore, the number of replatings also changes this parameter. Even in different cells of the same culture it can vary quite drastically. Although I did the microscopic analysis of the phenotypes very carefully with these considerations in mind, I could not exclude a bias, because of the selection of cells analysed.

Figure 3.11

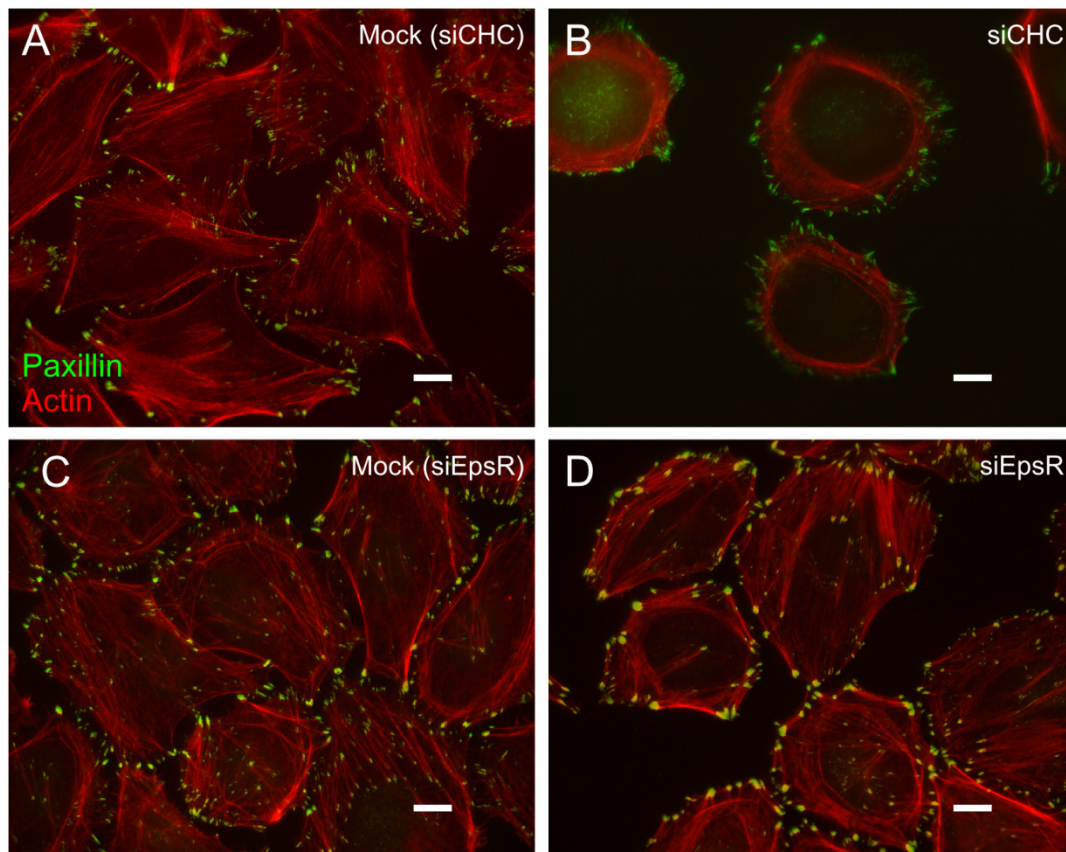


Figure 3.11: siRNA knockdown of EpsinR and CHC induces increase of focal adhesion size. Images showing immunostainings of HeLa cells for paxillin and staining with Phalloidin for actin were acquired in a wide-field setup. Scale bar in all images equals 10 μm . The cells were treated as indicated on the upper right (for explanations see Figure 3.9). **A)** Mock (siCHC) treated cells. **B)** siCHC treated cells show increase and lateralisation of focal adhesions; additionally, also the actin filaments show strong lateralisation and bundling along the cell edges. **C)** Mock (siEpsR) treated cells. **D)** siEpsR treated cells show increased focal adhesion size and milder lateralisation of focal adhesion; moreover, also the actin filaments are not strongly lateralised. However, the extend of focal adhesion and actin lateralisation depends on the strength of the knockdown and may vary between knockdown batches; the increase in focal adhesion size, though, is constant.

Consequently, the most reliable way to confirm changes in focal adhesion size was to perform statistical analysis. For this purpose, the selection of images had first to be automated. This could be easily achieved by using an automated

microscope, acquiring hundreds of pictures in one run. Second, the analysis of a large number of adhesions required an automated image analysis, measuring and listing focal adhesion size. For this purpose, I used a program algorithm developed by Karin Aumayr (Head of the Biooptics Department of the IMP/IMBA Vienna) that analysed raw pictures of HeLa cells stained for paxillin (Figure 3.12A) and the nuclei (Hoechst-stain; picture not shown). It recognised reliably the area of single adhesions and nuclei for each picture (Figures 3.12B and 3.12C) and exported this information for further analysis. Figure 3.12C shows an example picture after analysis with the program; the recognised objects (except for the nucleus) were listed in different size categories (according to the pixel size).

Figure 3.12

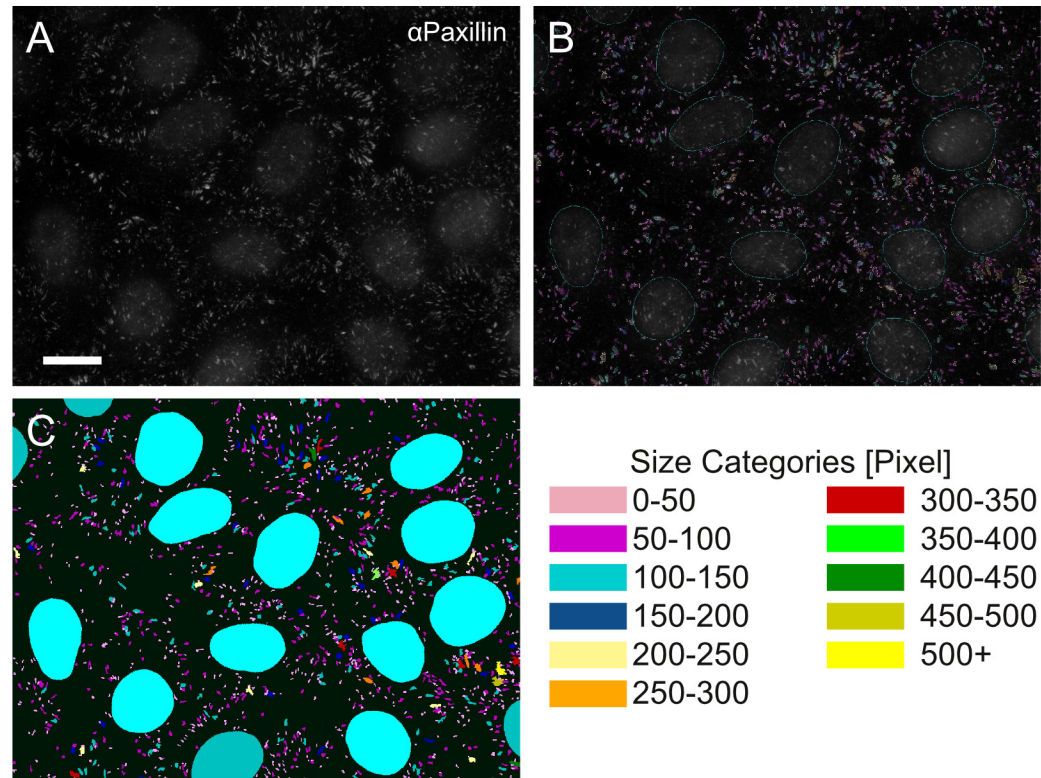


Figure 3.12: Mock(siEpsR) treated HeLa cells and the measured adhesion areas. Images showing an immunostaining for paxillin of HeLa cells before (A) and after analysis with Karin Aumayr’s program algorithm (B and C) were acquired in an automated wide-field setup. Scale bar in A equals 10 μm . B) In addition to the image depicted in A, the outline of the adhesions recognised by the program are shown. The colours correspond to the table shown in C. Additionally, the outline of the nuclei is

(**Figure 3.12 continued**) drawn (based on a Hoechst-staining, not shown). **C**) The greyscale image still present in **B** is removed and the outlines are filled with the appropriate colours. The list on the right shows the pixel-size categories and the corresponding colours.

Figure 3.13

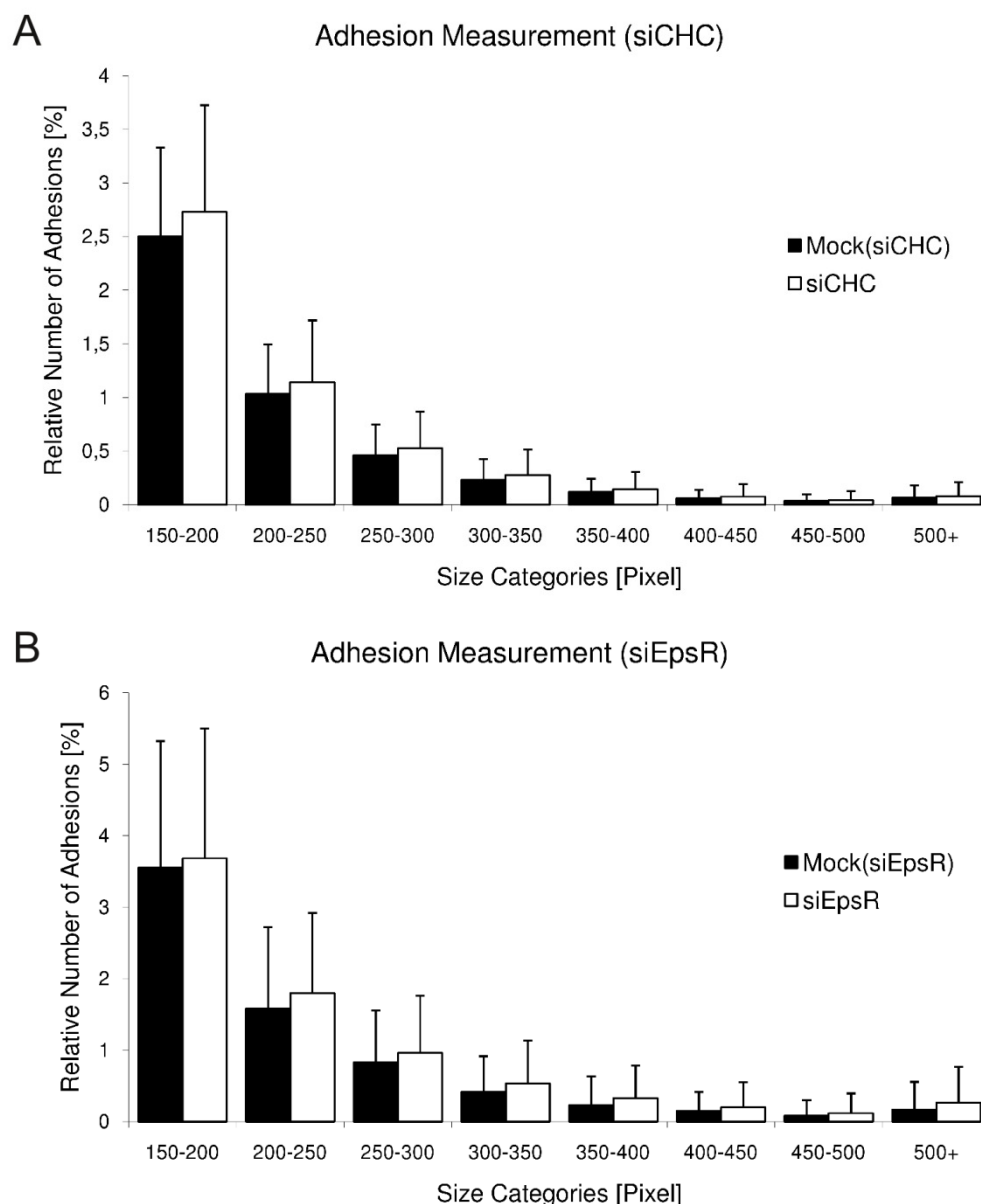


Figure 3.13: siRNA Knockdown of EpsinR and CHC increases the number of larger sized adhesions. Histograms showing the distribution of adhesion to size categories defined by pixel size after siRNA knockdown (for explanations of the different conditions see Figure 3.9); the first three categories were left out for better representation of the data. The x-axis represents the relative number of adhesions [%] in

(Figure 3.13 continued) a category; the y- axis shows the size category defined by pixel size in the original images. The raw pictures were analysed by a program routine in Definiens (Definiens), developed by Karin Aumayr. **A)** Closed bars represent data from Mock(siCHC) treated cells; open bars represent data from siCHC treated cells. n>7800 cells for both conditions **B)** Closed bars represent data from Mock(siEpsR) treated cells; open bars represent data from siEpsR treated cells. n>1900 cells for both conditions.

Figure 3.13 shows the final data of an automated analysis of focal adhesion size. After knockdown of CHC, the number of adhesions in the bigger size categories was increased (Figure 3.13A), the number of smaller adhesions reduced (data not shown). This corresponds to my previous findings from immunostainings. For EpsinR knockdown (Figure 3.13B) due to a different density of cells in the experiment used for this analysis, the values were a bit shifted compared to Figure 3.13A. Yet, also EpsinR knockdown cells showed the same tendency of increased adhesion size in all categories as CHC knockdown cells.

Therefore, according to the automated adhesion measurement, it can be concluded that Knockdown of EpsinR and CHC leads to an enlargement of the focal adhesions. Although this already connected EpsinR vesicles to the regulation of cell adhesions, neither the impact on cellular functions, nor the underlying mechanisms of the knockdown phenotype were obvious. Therefore, I decided to examine cells after siRNA knockdown of EpsinR for differences in cellular migration, turnover kinetics of individual molecules and whole adhesions and cell spreading.

For these experiments, I used Nocodazole treated cells as a control. Due to the visible phenotype, the increase of focal adhesions, Nocodazole treatment seemed to be an obvious choice. Upon treatment of Nocodazole, cells increase the size of their focal adhesions and arrange them preferentially at the edges (Figure 3.14). One reason for this is increased RhoGTPase activity (Bershadsky et al. 1996; Enomoto 1996). However, the exact mechanisms are not known. Previous findings also showed a direct interaction of microtubules and focal adhesions (Kaverina, Krylyshkina, and Small 1999). Therefore, the mechanism

underlying this phenomenon also may be due to the interaction of microtubule associated proteins with the focal adhesions. Furthermore, considering the observations made in the course of this work, there well may be an additional contribution resulting from the abolishment of vesicular transport by microtubule disruption.

To assay cellular migration, I decided to use a Transwell Migration Assay (TMA), also known in the literature as Boyden Chamber assay. In this experimental setup cells are forced to migrate through a porous membrane. In contrast to wound-healing assays that can measure colloidal sheet migration of cells in 2D, the TMA assays the migration of individual cells in 3D (therefore, sometimes in the literature these are also called 2D and 3D migration assay, respectively).

Figure 3.14

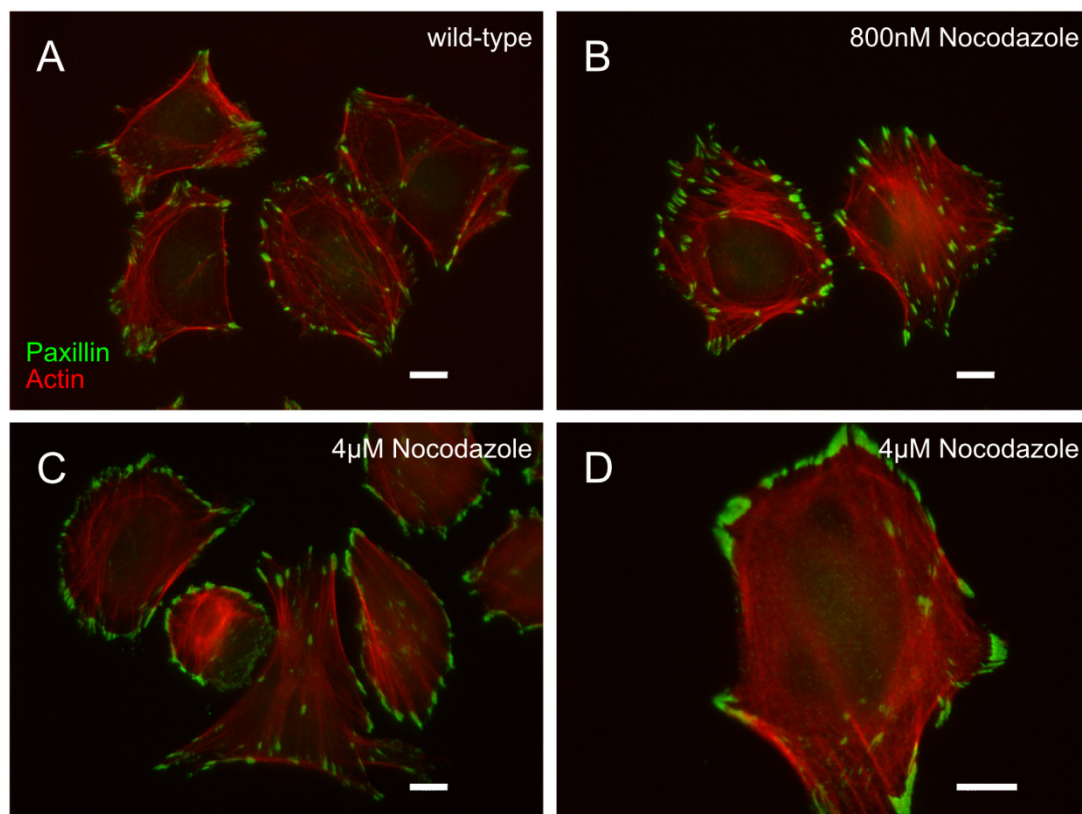


Figure 3.14: Prolonged Nocodazole treatment results in increase in focal adhesion size. Images showing immunostainings of HeLa cells for paxillin and staining with Phalloidin for actin were acquired in a wide-field setup. Scale bar in all images equals 10 μm . **A)** Immunostaining of wild-type cells. **B)** Immunostaining of cells treated with 800 nM Nocodazole over night. **C)** Immunostaining of cells treated with 4 μM Nocodazole over night. **D)** Immunostaining of cells treated with 4 μM Nocodazole over night in a higher magnification (100x objective; **A**, **B** and **C** were acquired with a 63x objective). Note for **B**, **C** and **D** the size-increased and the lateralisation of focal adhesions. Both effects are stronger for the higher concentration of Nocodazole.

Figure 3.15

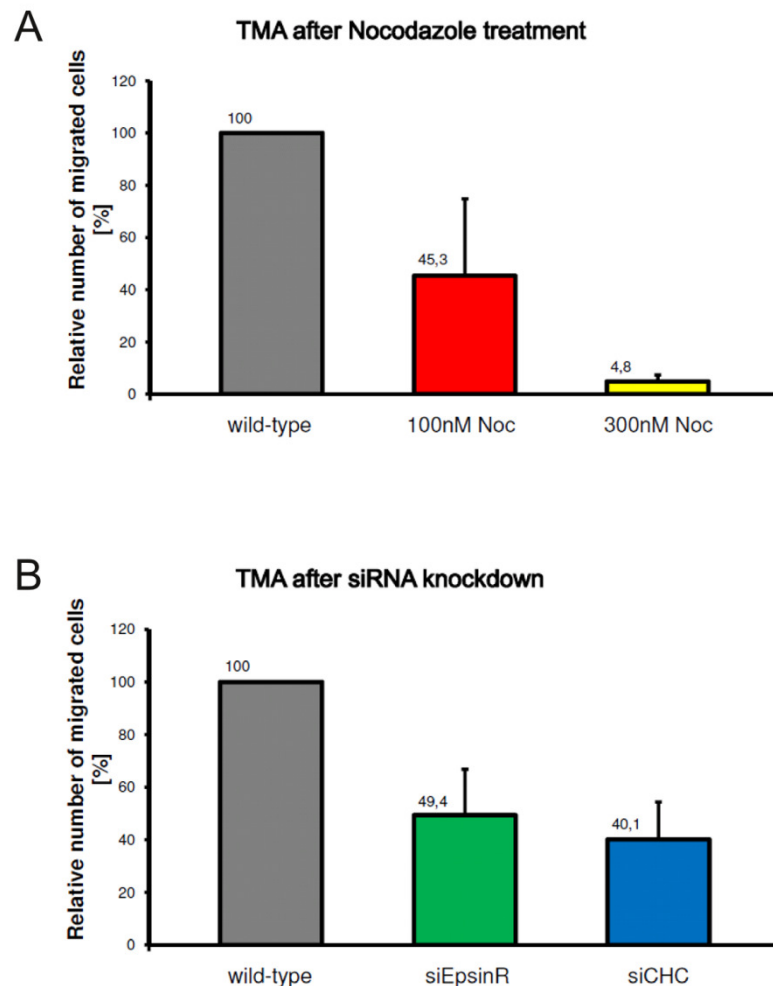


Figure 3.15: siRNA knockdown of EpsinR and CHC decreases the motility of HeLa cells in a Transwell Migration Assay (TMA). Histograms showing the relative number of migrated cells in various conditions; wild-type is normalised to 100%. All the

(**Figure 3.15 continued**) shown bars are the product of at least two independent experiments done in triplicates or quadruplicates. **A)** As a control for the migration assay, 100 and 300 nM Nocodazole were applied during the migration period. This resulted in around 50% reduction of migrated cells for the lower and around 95% reduction for the higher Nocodazole concentration compared to the wild-type. **B)** After siRNA knockdown (for explanations of the different conditions see Figure 3.9) of EpsinR and clathrin, the number of migrated cells was reduced by around 50% and 60%, respectively.

To test for the sensitivity of the TMA, I treated cells with 100 and 300 nM Nocodazole during the migration period and compared them to untreated ones. The concentrations were lower by a factor 80 and 30, respectively, compared to the normally used concentration for Nocodazole treatment (around 8 μ M). This should ensure that the cells are still capable of (impaired) migration and not completely immobile (Liao, Nagasaki, and Gundersen 1995). As seen in Figure 3.15A, this was achieved. Compared to the control, treatment with 100 nM Nocodazole reduced the number of migrated cells by around 55%; 300 nM Nocodazole reduced the number of migrated cells by around 95%. Therefore, the method should be sensitive enough to detect changes in the migration capability of cells treated with siRNAs against EpsinR and CHC.

As shown in Figure 3.15B, the knockdown of EpsinR resulted in around 50% less migrated cells. This reduction is comparable to treatment with 100 nM Nocodazole. Correspondingly, the knockdown of CHC also decreased the migration rate by around 60%. Due to the insignificant difference between the two knockdowns, a general conclusion can be drawn: whereas the knockdown of CHC disturbs the entire clathrin vesicular system, its impact on cell motility is almost the same as for the EpsinR knockdown. Therefore, one of the more critical clathrin pathways interacting with cell migration is EpsinR-dependent.

Although the TMA revealed one functional consequence of EpsinR loss, the mechanism underlying this phenomenon remained unclear. To uncover the role of EpsinR in focal adhesion turnover, I decided to examine turnover dynamics of paxillin. If EpsinR was involved in paxillin recycling, its knockdown would alter the kinetics of this process. Two main methods to address this question are FRAP

and PA (see also the introduction). Since PA reflects the dissociation of the protein of interest not only in a steady-state situation, I decided to use the latter for my experiments.

Figure 3.16

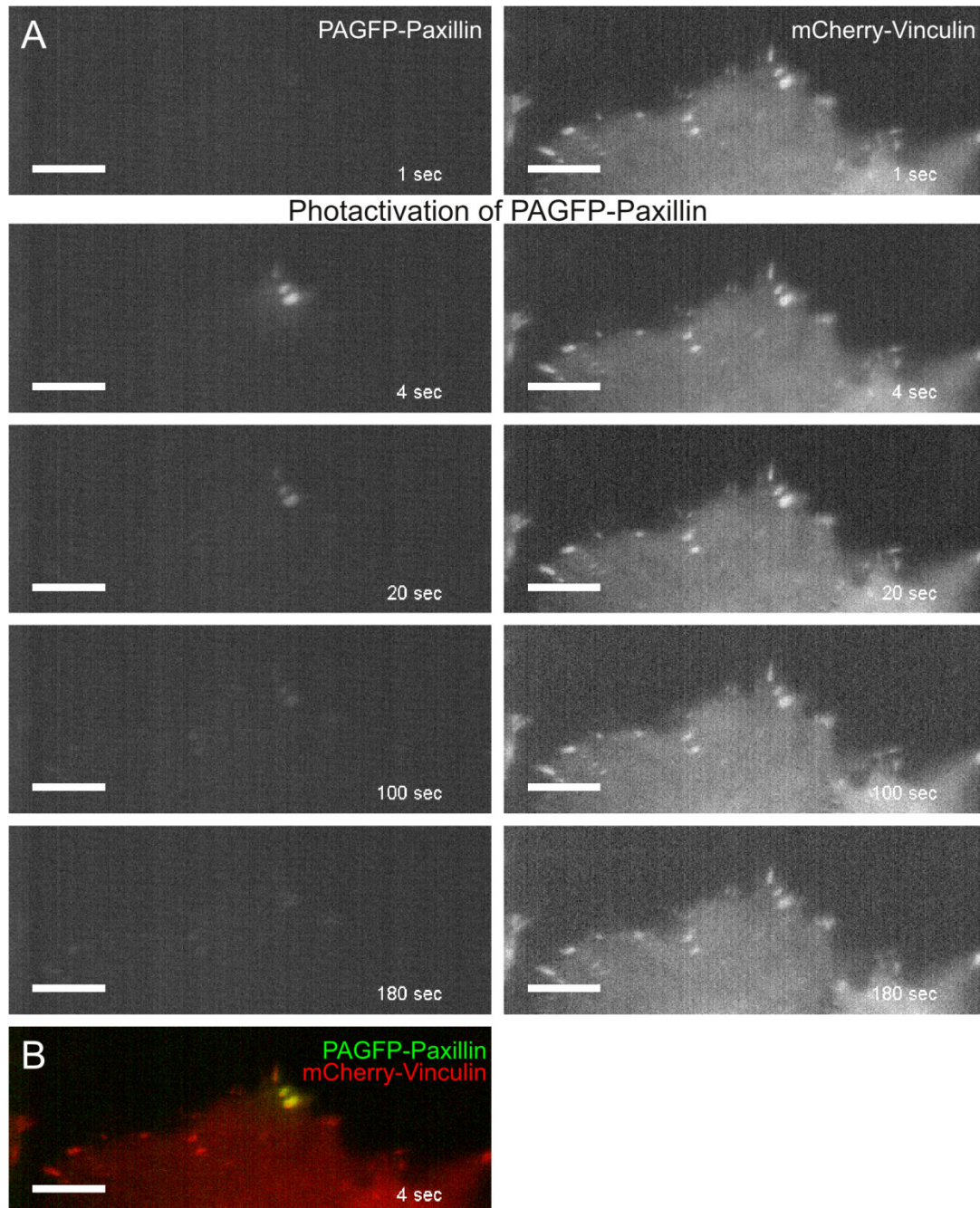


Figure 3.16: A typical photoactivation experiment. Time-lapse images of a HeLa cell double-transfected with mCherry-Vinculin and photoactivable GFP paxillin (PAGFP-Paxillin) were acquired using a Laser Scanning Microscope (LSM) in line-scanner mode. Time points of the individual frames are indicated at the lower right; the scale bar in all the images equals 10 μm . **A)** The channel of PAGFP-Pax is shown on the left; the channel of mCherry-Vinculin on the right. Note, while the mCherry-Vinculin signal remains stable over time, the PAGFP-Paxillin signal is only visible after the activation by a 405 nm laser pulse in the area of two adhesions and disappears again over time. **B)** Merge of both channels shown in A at the indicated time point.

Figure 3.17

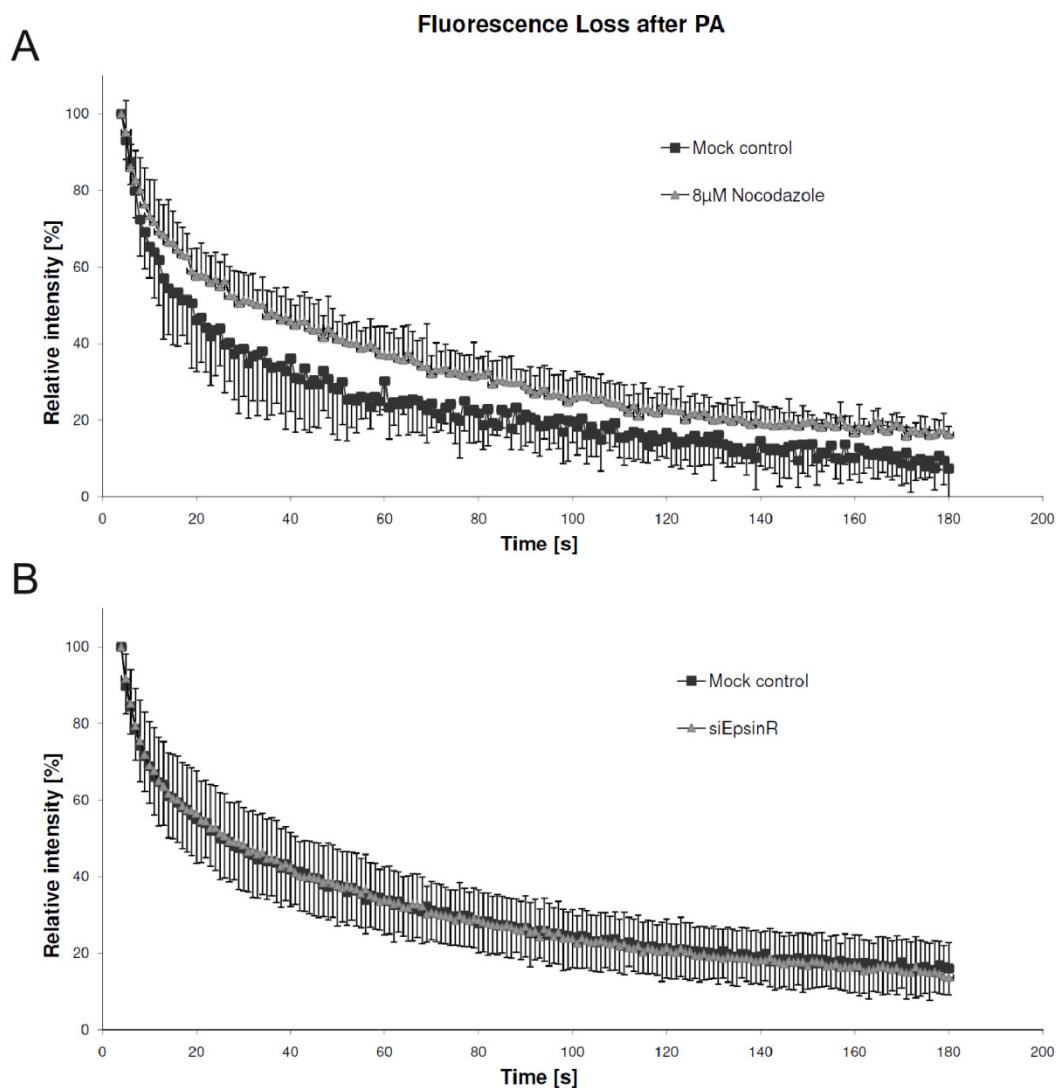


Figure 3.17: Unlike Nocodazole treatment, siRNA knockdown of EpsinR does not influence dissociation dynamics of paxillin. Diagram plotting relative intensity of the PAGFP-Paxillin signal after photoactivation (see Figure 3.16; 100% at t=4 seconds). The curves represent loss of photoactivated paxillin from the adhesions, yielding thereby the dissociation kinetics of paxillin in the wild-type situation, after Nocodazole treatment (8 μ M) and upon siRNA knockdown of EpsinR. Error bars are shown in negative direction for the mock control, in positive direction for Nocodazole treatment and siRNA knockdown. **A)** Loss of photoactivated paxillin for mock control (n=6 adhesions) and Nocodazole treated (n=6 adhesions) cells. Note the decreased dissociation speed of paxillin after Nocodazole treatment. **B)** Loss of photoactivated paxillin for mock control (n=33 adhesions in two independent experiments) and siEpsR treated cells (n=34 adhesions in two experiments). Note that the two curves, unlike in **A**, are identical and show no difference in dissociation kinetics.

Unfortunately, I did not succeed in establishing a stable cell line expressing the photoactivable construct. Therefore, transient transfection before the experiment was necessary. However, clathrin deficient cells are not capable of taking up DNA precipitates for transfection. Therefore, we had to limit our photoactivation studies to EpsinR deficient cells.

Figure 3.16 represents a typical photoactivation experiment. While the mCherry-tagged vinculin (used for identification of suitable adhesions), remained stable for the imaging time of three minutes, photoactivable GFP-tagged paxillin (PAGFP-Paxillin) was not visible before photoactivation (between the frames for three and four seconds).

After photoactivation the activated paxillin signal disappeared, due to dissociation from the activated focal adhesion and subsequent dilution in the pool of inactivated PAGFP-Paxillin and the endogenous protein. Both fluorophore-tagged proteins colocalised well to the focal adhesions (Figure 3.16B) which are normally stable over the time period of three minutes. Anyway, mCherry Vinculin could be used to monitor the focal adhesion positioning during the experiment. As a read-out, the dissociation curves for the control cells were compared to Nocodazole treated and EpsinR knockdown cells. It was expected that Nocodazole treated cells would show impaired, therefore, slower turnover compared to control cells and could be used as a control for the method.

Indeed, Nocodazole treatment did change the turnover kinetics of paxillin in steady-state adhesions (Figure 3.17A). Compared to wild-type, drug-treated cells exhibited a dissociation which differed in two aspects: first, the half-time of dissociation was clearly increased, indicating a general slowdown of the kinetics. This leads to the assumption that loss of microtubules has an immediate effect on the fast turnover of paxillin. Second, the immobile fraction (immobile in the time-scale of three minutes) was also increased. This indicates that the fraction of molecules exchanged by slow turnover is increased. Although these two phenotypes are most probably directly related, they have different impacts on the kinetics of turnover.

For EpsinR knockdown (cells were identified by their enlarged focal adhesion), in contrast, as shown in Figure 3.17B, none of these changes could be detected. The dissociation curves from control and knockdown adhesions perfectly fitted, indicating no change in the paxillin turnover dynamics. Therefore, a malfunction in dissociation of paxillin molecules in the fast turnover due to EpsinR knockdown cannot be the causing agent for the focal adhesion size increase.

Beside the molecular turnover of single molecules, the turnover of entire adhesions may also yield further insights into the function of EpsinR. Although this would not clarify the molecular mechanisms underlying the observed phenotypes, it could help to clarify the cellular mechanisms resulting in the reduced migration capability after siRNA knockdown.

For this purpose, I imaged HeLa cells transfected with GFP-tagged paxillin for one hour. As shown in Figure 3.18A, the HeLa cells were extending lamellipodia and formed novel adhesions after this extension. The boxed region indicates a large adhesion, sliding upon tension and splitting into two smaller adhesions without disintegration. Additionally, note the concentration of the cytoplasmic signal around the nucleus, presumably at the GA/PNRC.

Figure 3.18

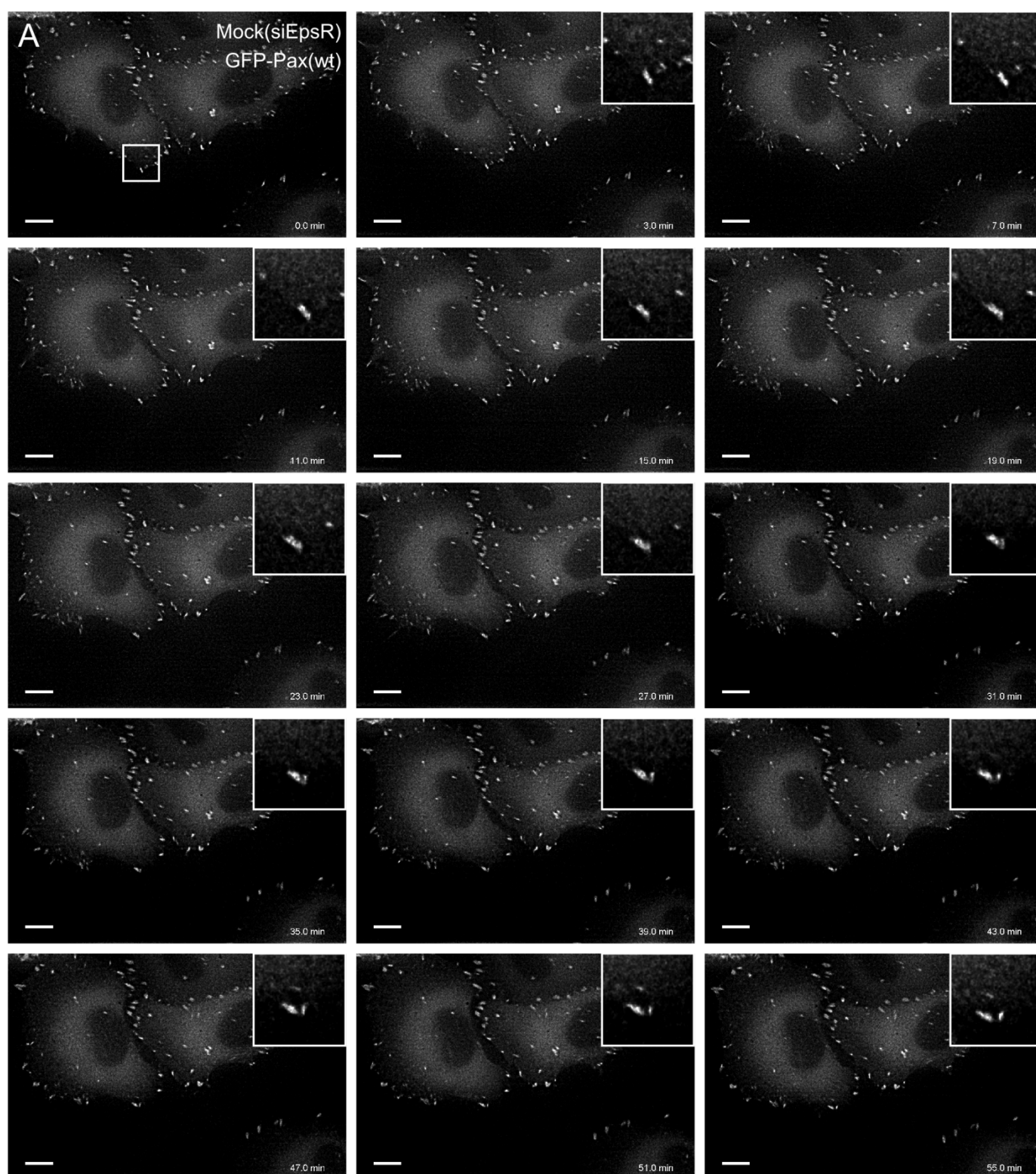
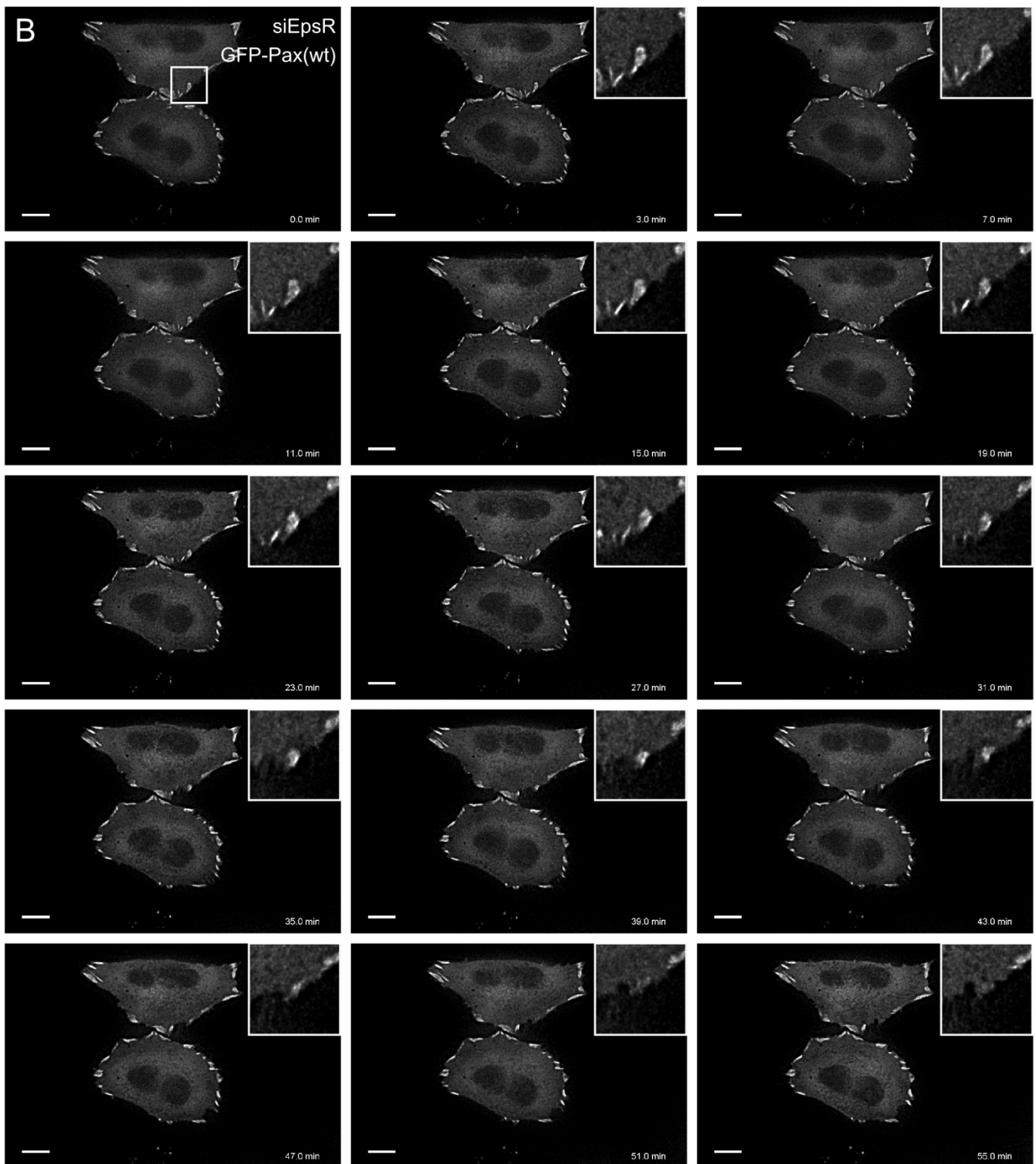


Figure 3.18: Long-term turnover of adhesions is slowed down by loss of EpsinR. Time-lapse images of a HeLa cell transfected with GFP-Pax(wt) after siRNA knockdown (for explanations of the different conditions see Figure 3.9) acquired in a wide-field setup followed by 3D-Deconvolution. Time points of the individual frames are

Figure 3.18 continued



(Figure 3.18 continued) indicated at the lower right; the scale bar in all the images equals 10 μm ; boxed regions are presented as a blow up in the upper right corner of each picture. **A)** Time lapse showing cells after Mock(siEpsR). Cells are retracting and reforming adhesions in the time frame of 55 minutes. The large adhesion in the boxed

(Figure 3.18 continued) region is sliding and splitting into two separate adhesions upon supposed application of tension. **B)** Time lapse showing cells after EpsinR knockdown. Cells are retracting some adhesions; however, no new adhesions are formed in the time frame of 55 minutes. The large adhesion in the boxed region is starting to slide, comparable to the shown adhesion in **A**; however, it then starts to disassemble and disappears completely after 51 minutes.

In contrast, cells after siRNA knockdown of EpsinR (cells were again identified by their enlarged focal adhesions) did not form new adhesions in the analysed time period (Figure 3.18B); this could be the reason for the decreased motility seen in the TMA. Additionally, for the example given in the boxed region, the generally oversized adhesions tended to dissolve upon stress and did not slide and split as seen for the wild-type. Furthermore, note the uniform staining of cytoplasm by paxillin compared to the control cells. This indicates a reduced localisation of paxillin to the GA/PNRC upon loss of EpsinR function. However, this could also be caused by the possible disruption of this organelle by the knockdown of EpsinR (compare to the overexpression phenotype; Mills et al. 2003). Either way, this once more points out the connection of wild-type paxillin to the vesicular compartment. In addition, the intensity ratio between focal adhesion and cytoplasm for the paxillin seems to be different between wild-type and knockdown (Figure 3.18). This could be observed in several cells, therefore, indicating that, upon knockdown of EpsinR, the resulting increase in adhesion size area eventually leads to a reduction of the cytoplasmic paxillin pool.

However, as described before, this kind of qualitative read-out is not statistically objective. Therefore, an automated acquisition of cells combined with a computational analysis would be desirable. Possible read-outs would include: cell area changes over time or positional changes of the cell body, particle tracking of adhesions to distinguish between their disintegration, sliding and splitting, and measurement of intensity ratios between cell adhesions and the cytoplasm. As an alternative, also the treatment of drugs could be helpful. Upon application of a well-described drug, stable experimental condition can be generated.

If EpsinR is involved in the formation of cell-matrix contacts, a possible functional read-out for its loss could be the spreading of HeLa cells. As shown in Figure 3.19, mock control cells or cells treated with siRNA against EpsinR and CHC were seeded on fibronectin-coated cover slips and fixed 15, 30 and 60 minutes thereafter. To visualise spreading, the cells were stained for paxillin; in addition, the respective antibodies were used to identify cells with efficient knockdown of EpsinR and CHC.

However, as seen in Figures 3.19M and 3.19N, even though the cells with reduced protein levels can be identified easily, no obvious difference between mock control and siRNA knockdown cells, in regard to their spreading efficiency, can be seen. Therefore, it can be assumed that neither clathrin nor EpsinR play a functional role during the spreading of cells. This, most probably, suggests the involvement of other vesicular transport machineries in this process, since for the delivery and recycling of integrins vesicular transport is absolutely required.

Although I showed the importance of EpsinR for focal adhesion size and cellular migration, still the mechanistic foundations for this remain elusive. One critical aspect for this is the regulation of paxillin. It is known that its function is dependent on the phosphorylation state. However, as discussed in the introduction, neither the targeting factor to focal adhesions nor any specific modifications are known that are relevant for paxillin localisation. The only signal so far known to regulate relocation of paxillin from the adhesion structures is mono-ubiquitinylation by the RING-finger protein 5 (RNF5; Didier et al. 2003). Therefore, an interesting aspect would be to reveal which signals are related to EpsinR dependent paxillin dynamics.

As shown in Figure 3.20, upon knockdown of either EpsinR or clathrin, in addition to the usual paxillin signal (arrow) on Western Blot a higher band is visible (arrowhead). This band might indicate a modification of paxillin taking place in the absence of the EpsinR pathway. Unfortunately, I did not succeed so far in the identification of this unknown modification. However, I suggest that the most reasonable choice for this modification would be mono-ubiquitinylation, since this is, indeed, a signal for paxillin to relocate from focal adhesions to the cytoplasm.

Figure 3.19

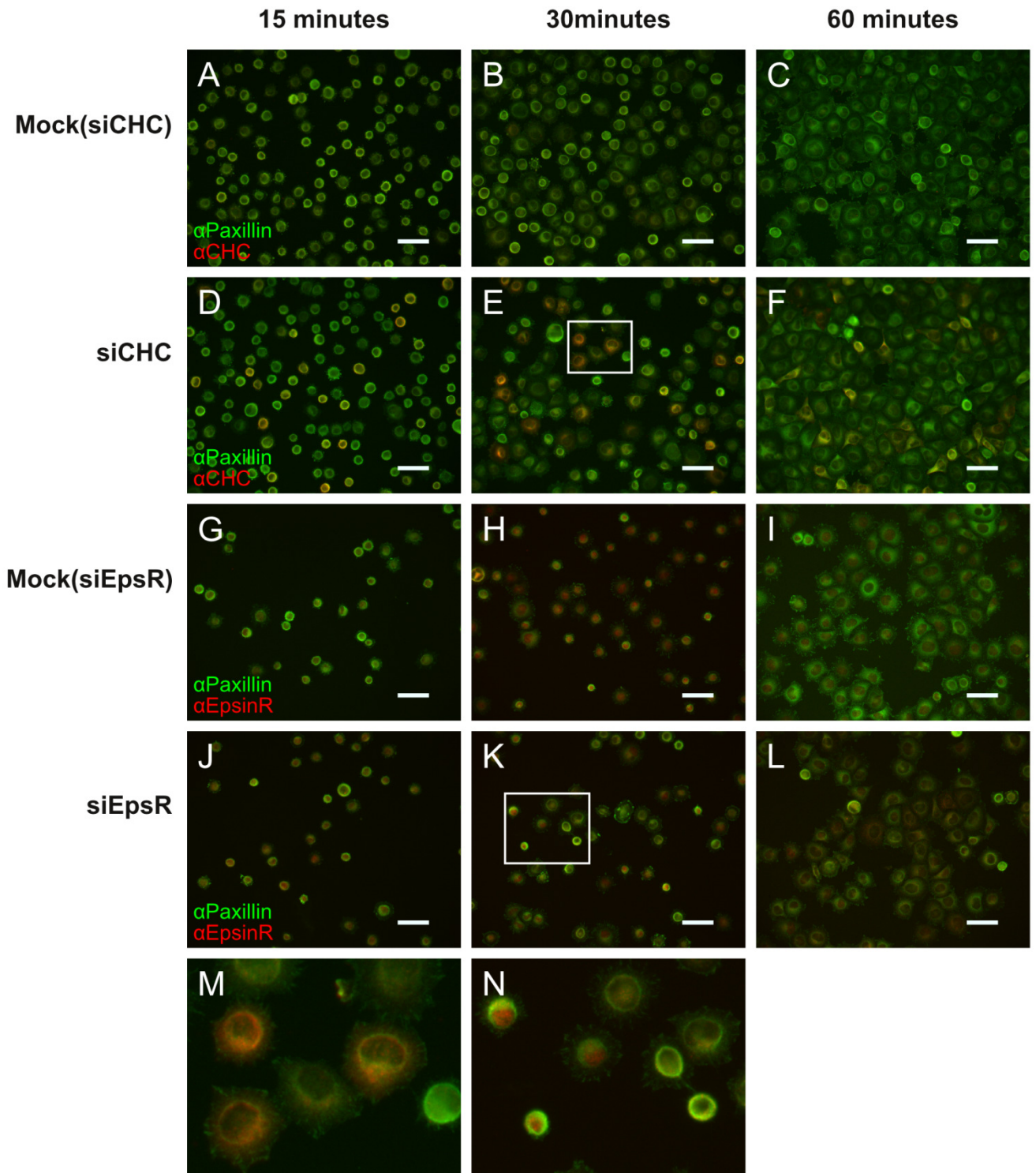


Figure 3.19: siRNA knockdown of EpsinR and CHC does not influence spreading dynamics of HeLa cells. Images showing immunostainings of HeLa cells for paxillin (**A – N**), Clathrin (**A – F** and **M**) and EpsinR (**G – L** and **N**) were acquired in a wide-field setup. Scale bar in all images equals 50 μm . The cells were treated as indicated on the left side (For explanations see Figure 3.9). **A – L**) Cells were seeded on fibronectin and then fixed at the time points indicated on the top of the figure.

(**Figure 3.19 continued**) **M**) Blow up of the boxed region in **E**. **N**) Blow up of the boxed region in **K**. Note that for siCHC and siEpsR differences in the staining intensity for clathrin and EpsinR, respectively, indicate whether the cell is expressing wild-type or reduced levels of the corresponding protein. As it can be seen from the figure, there is no obvious difference in spreading behaviour of HeLa cells upon knockdown of clathrin or EpsinR.

Figure 3.20

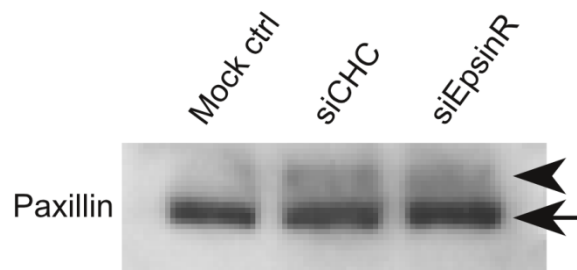


Figure 3.20: siRNA knockdown of EpsinR and CHC induces an unknown modification of paxillin, seen in Western Blot. Western Blot shows the appearance of an additional paxillin band after the respective siRNA knockdown indicated on the top (for explanations see Figure 3.9). The arrow indicates the normal height paxillin is running; the arrowhead indicates the appearance of the additional higher band upon both siRNA knockdowns.

3.4. Screening Hits

In 2006, a novel siRNA screen was published by scientists from the laboratory of Rainer Pepperkok (Simpson et al. 2007). This screen was intended to identify novel proteins involved in vesicular transport, based on the secretion profiles of a viral protein. Natalia Andreyeva in my laboratory performed a follow-up screen based on these results, looking for knockdown phenotypes that additionally caused defects in focal adhesion assembly or disassembly (Andreyeva 2008).

Figure 3.21

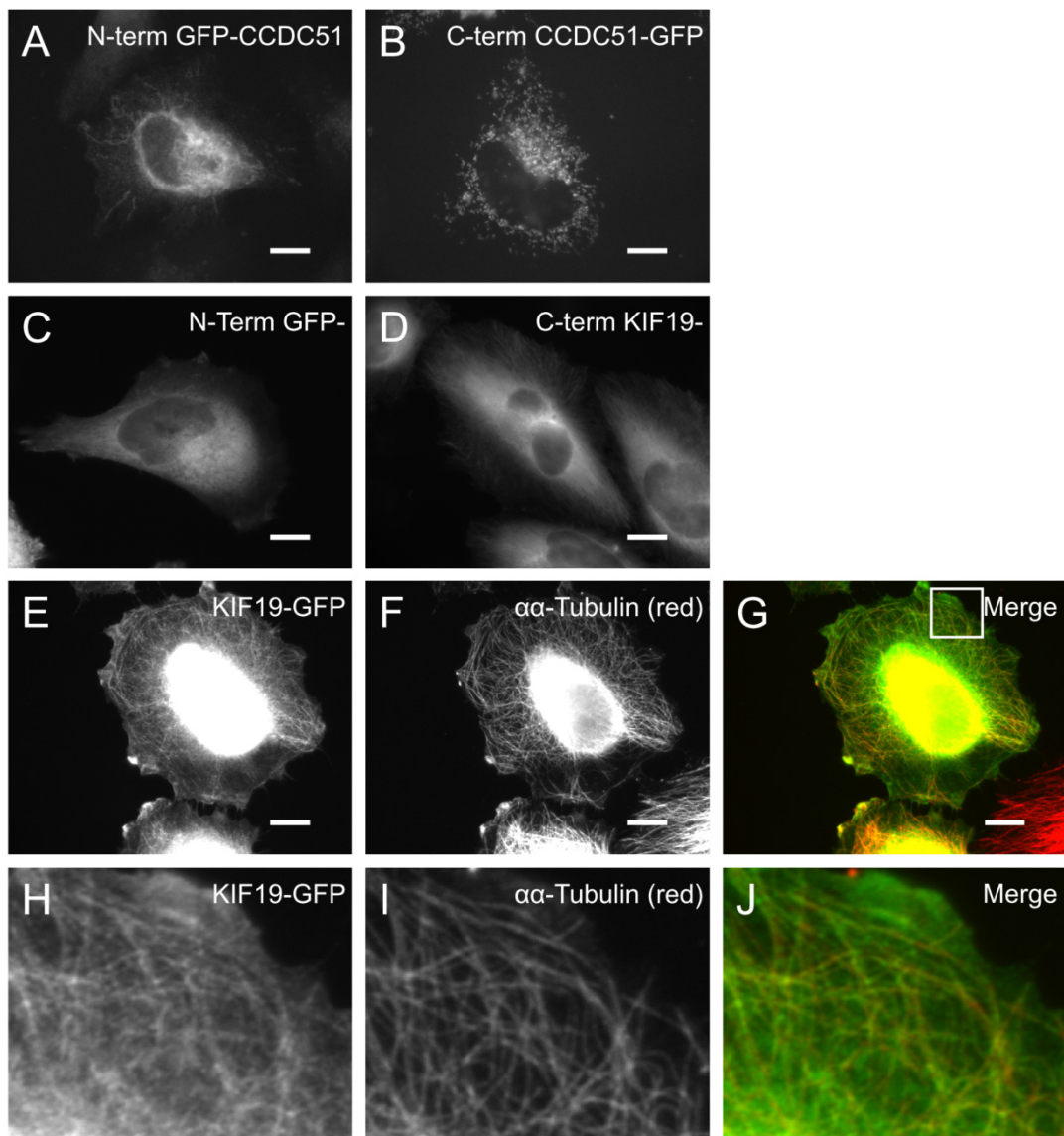


Figure 3.21: CCDC51 and KIF19 localisation in fixed cells. Images showing fixed HeLa cells after transfection with various constructs were acquired in a wide-field setup. Scale bar in all images equals 10 μ m. **A – D)** Images of fixed cells after transfection of GFP-tagged CCDC51 (**A** and **B**) and KIF19 (**C** and **D**); further the images compare the constructs with N-terminal (**A** and **C**) to the constructs with C-terminal GFP tag (**B** and **D**). Note the different localisation of the various constructs. **E, F** and **G**) Immunostaining for α -tubulin after transfection with GFP-tagged KIF-19; shown are the separate channels (**E** and **F**) and a merge of the two channels (**G**). **H, I** and **J**) Blow up of the boxed region in **G** separated into the individual channels (**H** and **I**).

Several proteins were identified by this screen with an impact on vesicular transport as well as integrity of cellular adhesions (for a complete list see Andreyeva 2008). The most promising novel hits were analysed by Maria Novatchkova (Bioinformatics IMP/IMBA Vienna) and two of them were chosen for further analysis: CCDC51 and KIF19.

CCDC51 (coiled-coil domain containing 51) is a highly conserved 45 kDa protein. Due to its coiled-coil domain predictions are very difficult. However, according to bioinformatic analysis it might have a flavin monooxygenase domain as well as a t-SNARE superfamily sequence (www.ensembl.org). Yet, other predictions also assign a mitochondrial targeting signal to it. Nevertheless, a coiled-coil domain protein with a putative t-SNARE domain would be very likely involved in vesicular transport.

Kif19, kinesin family member 19, is a member of the microtubule motor family of kinesins. The main isoform (62 kDa) is highly conserved and the classical motif for kinesin family members can be found. Indeed, a microtubule motor has a high probability to be involved in vesicular trafficking and focal adhesion organisation.

Both proteins were cloned into the vectors pEGFP-C1 and pEGFP-N1 to analyse their localisation. As shown in Figure 3.21A, the fusion protein GFP-CCDC51, with the GFP fused to the N-terminus of the protein, localised efficiently to tubular structures all over the cell. These structures represent most probably the endoplasmic reticulum. The construct tagged with a C-terminal GFP, in contrast, did not localise to tubular structures, but to irregularly sized dots, highly concentrated around the GAPNRC area (Figure 3.21B). This could indicate a defective protein clogged in the intracellular membranous compartment that may even disrupt the normal function of this compartment. The different localisation of the two constructs results from the position of the fluorophore tag. Depending on the site of fusion, it may influence the conformation and functionality of CCDC51 (fluorophore tags can block interaction sites or even prevent proper folding). Although I propose the tubular localisation to be representing the native distribution of the protein, still antibody staining of the endogenous protein would be necessary to conclusively prove this.

Figure 3.22

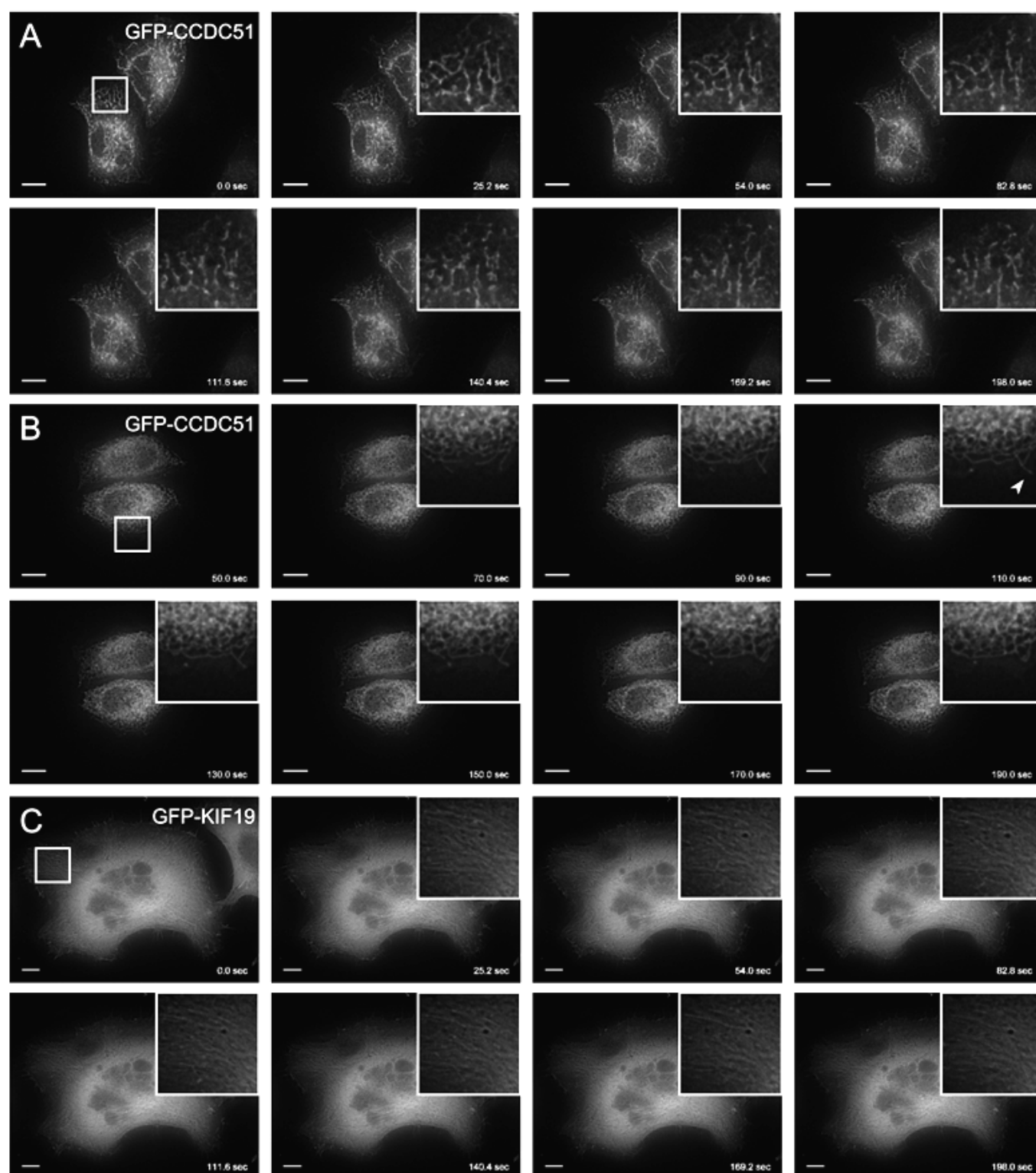


Figure 3.22: CCDC51 and KIF19 dynamics in live-cell imaging. Time-lapse images of HeLa cells transfected with N-terminally GFP-tagged CCDC51 (**A** and **B**) and KIF19 (**C**) acquired in a wide-field setup followed by 3D-Deconvolution. Time points of the individual frames are indicated at the lower right; the scale bar in all the images equals 10 μm ; boxed regions are presented as a blow up in the upper right corner of

(Figure 3.22 continued) each picture after the first frame. Note that KIF19, unlike CCDC51, remains stable over the imaging time of nearly 200 seconds. As already proposed, also the live-cell imaging further indicates the postulated localisation to the endoplasmic reticulum for CCDC51 and to microtubules for KIF19.

For KIF19, the fusion protein GFP-KIF19, with the GFP fused to the N-terminus, localised mainly to the cytoplasm (Figure 3.21C). If the fluorophore was fused to the C-terminus, however, the fraction of proteins with cytoplasmic localisation decreased. Instead, the protein localised to filamentous structures (Figure 3.21D). These filaments had the appearance of microtubules, in accordance to the predicted kinesin function. This was confirmed by staining for α -tubulin after transfection with the KIF19 construct (Figures 3.21E, 3.21F and 3.21G). As also seen in the blow up (Figures 3.21H, 3.21I and 3.21J), KIF19, indeed, localised to microtubules labelled by the tubulin staining.

To further analyse the localisation and function of the two candidate proteins, I decided to observe their dynamics in live cells using time-lapse imaging. Whereas CCDC51 tubules (Figure 3.22A and 3.22B) are highly dynamic, KIF19 labelled microtubules remain stable for the indicated time. For CCDC51, this is in accordance to the predicted localisation, since the endoplasmic reticulum is highly motile. Although microtubules normally show dynamic instability and are, therefore very mobile, this mobility can only be seen with the help of a tip-label. Therefore, the stable appearance of the microtubules marked by KIF19 is not unexpected.

Furthermore, for CCDC51, single tubules can be observed protruding to the cell periphery, shortly interacting with the surroundings (Figure 3.22B; arrowhead). Recent findings already postulated an interaction between the endoplasmic reticulum and cell-matrix adhesions that facilitates the activity of a focal adhesion phosphatase (Hernández et al. 2006). This could be a general mechanism for the control of cell-matrix contacts and CCDC51 may be one of the proteins important for the endoplasmic reticulum targeting.

For KIF19, the relation between function and localisation is more obvious. As a predicted microtubule motor, its association with microtubules is not surprising. Since, as already discussed for previous experiments and in the

introduction, the connection of vesicular transport and focal adhesion regulation is multifaceted and complex, various transport molecules are required for delivery of the regulatory vesicles. The identified motor protein KIF19 might be one of these transporters.

4. Discussion and Further Prospects

4.1. Vesicular Transport and Its Impact on Paxillin Turnover

Delivering a transmembrane molecule to the plasma membrane of a cell requires the vesicular transport system. Therefore, the targeting of the main adhesion receptors, integrins, to the membrane is dependent on vesicles. As already discussed in the introduction, the delivery, removal and recycling are important regulatory mechanisms for focal adhesion turnover.

Although integrins provide the attachment of the cell to the extracellular matrix, they are insufficient to form a functional focal adhesions. Beside them, a cohort of structural, scaffolding and signalling molecules are required. These proteins have to be regulated as efficiently as integrins to assure adequate turnover of cellular adhesions. In contrast to the transmembrane receptors, they are normally soluble, cytoplasmic proteins. Thus, their regulation can be controlled in various ways that are independent of vesicular trafficking. For example, since several proteins associated with focal adhesions are kinases (FAK, Src, ILK), phosphorylation is critical for the localisation of various focal adhesion components, including paxillin (Brown, Perrotta, and Turner 1998; Zamir and Geiger 2001).

Yet, the question remains whether protein modifications are sufficient for the regulation of focal adhesion turnover. Not only adhesions at distinct sites of the cell have to be regulated differently; moreover, proteins in adjacent adhesions or even in the same one have to be controlled independently. Utilisation of the existent vesicular trafficking system for the regulation of the soluble adhesion components would be an additional, logical strategy.

The binding of focal adhesion components is determined by an internal hierarchy. Therefore, not all the molecules have to be regulated very specifically. One of the central molecules, scaffolding various proteins inside the adhesion, is paxillin. Using confocal reflection microscopy it has been demonstrated that paxillin is recruited to adhesion areas before integrins (Jim Norman; personal communication). Consequently, the efficient regulation of paxillin by vesicular transport can be expected. Indeed, as reported earlier, paxillin not only

colocalises with the Golgi apparatus (Mazaki et al. 1998), but also co-precipitates with clathrin components (Turner et al. 1999; Andreyeva 2008). However, up to this moment, localisation of wild-type paxillin to distinct vesicular structures has not been reported.

In the course of this diploma work, I confirmed the previously described localisation of a paxillin mutant containing two amino acid substitutions, disrupting both zinc-fingers in the LIM3 domain. This construct localises only residually to focal adhesions and mainly is targeted to vesicular structures, positive for clathrin. This fact further strengthens the hypothesis that paxillin localisation might be regulated by vesicular transport. Moreover, with the help of 3D-Deconvolution I was able to visualise wild-type paxillin containing vesicles. Although they do not colocalise with clathrin, still they were found to interact transiently with clathrin vesicles.

What is the consequence of these findings? The obtained data indicate the physiological connection of paxillin and vesicles and imply that a cytoplasmic focal adhesion proteins may be transported through the same pathway as integrins. This adds a further layer of complexity to the turnover of these proteins.

One of the major problems for the studying of paxillin containing vesicles is their small size and high motility. In order to resolve such vesicular structures properly, a high temporal resolution is required. Furthermore, to visualise them, 3D-Deconvolution would be the method of choice. However, due to a basic requirement of 3D-Deconvolution (acquisition of several z-planes for one time point), this method cannot provide the high temporal resolution that would be required. Therefore, the paxillin(M1) mutant is a convenient tool that might help to discover the nature of paxillin containing vesicles. However, the LIM3 domain mutations also might stabilise interactions with the vesicular compartment. Thus, some observed interactions may not be as stable and relevant for endogenous paxillin as for the paxillin(M1) mutant.

Another pending question in this context concerns the paxillin(M1). In earlier studies it was assumed that the paxillin LIM3 domain binds to a focal adhesion targeting factor. However, this factor has not been identified for over a decade

and so far the exact mechanism for paxillin targeting to focal adhesions is not clear.

Does such a factor really exist? Paxillin exhibits interactions with various proteins in the focal adhesion complex. Thus, it could be possible that one of these proteins is the critical recruitment factor. However, this is a hen and egg problem. Despite the extensive research done on this problem, the exact hierarchy of focal adhesion formation is still not known. It may even be possible that, indeed, paxillin does not have a unique recruitment factor, but the interaction with its numerous binding partners is critical for its targeting to focal adhesions.

Anyhow, the function of the paxillin LIM3 domain remains elusive. As shown by my own and previous experiments by Natalia Andreyeva, it seems very unlikely that its sole function is the targeting to focal adhesions. This previous hypothesis could not explain the appearance of paxillin containing vesicular structures. Furthermore, even in the presence of the LIM3 domain mutations (or the absence of the whole domain) paxillin could be detected at focal adhesions.

In previous studies, the localisation of single paxillin domains (Efimov et al. 2008) was analysed. It was shown, that the individual LIM3 or LIM2 domains were targeted inefficiently to focal adhesions. The combination of the two domains, however, has been described to be very efficient in its targeting to cell adhesions. Although this observation postulates that the LIM3 domain is involved in the localisation of paxillin to the cellular adhesions, I would like to propose another possibility. It has been discovered by Natalia Andreyeva that different LIM3 domain mutations have different phenotypes in regard to the paxillin targeting to adhesions and vesicles. All of these mutants showed a decreased localisation of paxillin to focal adhesions and an accumulation in vesicles. Furthermore, the two phenotypes showed an inverse correlation, meaning, the more paxillin was targeted to vesicles, the less it was localised to the focal adhesions and vice versa. Already the disruption of either the first or the second zinc-finger alone exhibited different phenotypes; the double zinc-finger disruption, as in paxillin(M1), showed the highest targeting rate of paxillin mutant to the vesicular structures (Andreyeva 2008).

However, interestingly, the complete deletion of the LIM3 domain has less effect on paxillin targeting to focal adhesions than the double mutation. This could indicate that the focal adhesion targeting deficiency is an indirect effect of

alterations in this domain. Since the zinc-fingers are structural elements, repeated several times in paxillin, the disturbance of the secondary structure could influence adjacent domains' functions (e.g. the LIM2 domain that may also be important for focal adhesion targeting). Whereas the deletion of the whole LIM3 domain has negative effects on the functions of adjacent domains, still the phenotype is milder than the mere disruption of the structural integrity of the two zinc-fingers.

Although this could explain the described discrepancies, still the function of the LIM3 domain itself remains elusive. A hint for its function could come from the stabilisation of clathrin on paxillin(M1) vesicles. Whereas the clathrin coat quickly dissociates from the majority of budding vesicles, it tends to stay on early endosomal structures for prolonged periods of time (Harvey T. McMahon, personal communication). Thus, it can be hypothesized that the enlarged vesicular structures, seen upon expression of paxillin(M1) are early endosomes. For the integration into the recycling pathways, however, paxillin should be targeted to recycling endosomes. Therefore, one possible reason for the vesicular accumulation of paxillin with a mutated LIM3 domain is a defect in the transfer from early to recycling endosomes.

Yet, still a lot of questions remain open. First, the nature of vesicles containing wild-type and mutant paxillin should be further characterised. For this, the straight-forward strategy would be to perform colocalisation experiments with vesicular markers. Although these experiments had already been started, still several of these proteins remain to be tested. However, for the vesicles containing wild-type paxillin this is not an easy task to perform. Since they are difficult to observe, special techniques to describe them are required. Although the paxillin(M1) can help to identify components of these vesicles, it is not sufficient; still all these interactions also have to be shown for vesicles containing wild-type paxillin. Therefore, conditions to induce vesicles containing wild-type paxillin have to be found.

For example, one particular condition was described in the lab of Harvey T. McMahon (unpublished results, personal communication). The overexpression of a novel protein containing a GTPase activating domain (GRAF1 was shown to induce paxillin's redistribution to vesicular structures. However, also in more

physiological conditions this has been eventually observed. Preliminary studies in tumour cells cultivated on top of epithelium cells also revealed these vesicles (Natalia Andreyeva, personal communication). Therefore, it is likely that in three-dimensional cell migration the reduced size and number of adhesions also result in an increased localisation of paxillin to the vesicular compartment. This would further stress the importance of using three dimensional cell culture models, since motility in 3D-substrates is closer to the physiological state than two dimensional cell migration.

Additionally, the interaction of paxillin(M1) with integrins in the vesicular compartment is of great interest. Preliminary integrin internalisation assays performed in our laboratory indicate a partial colocalisation of these two proteins in vesicular structures.

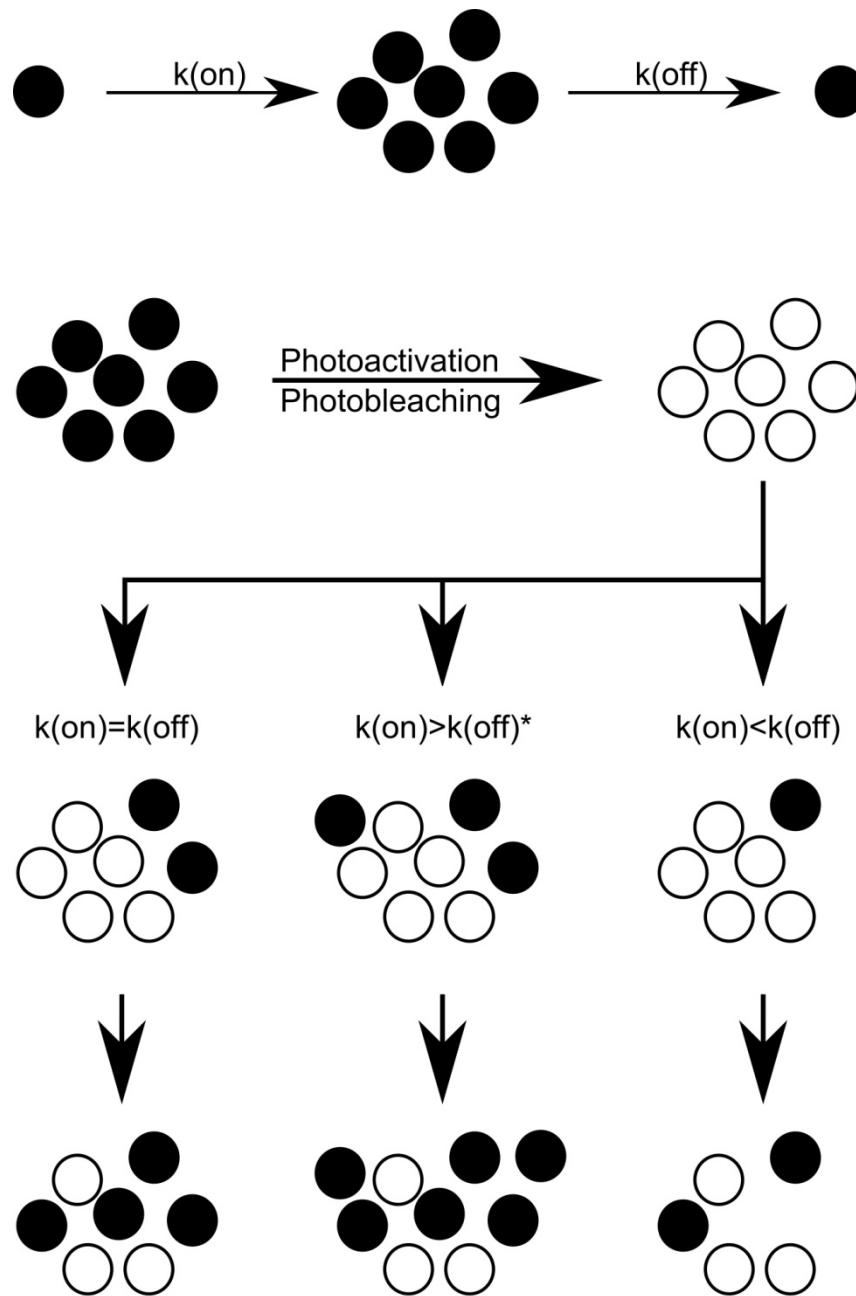
Therefore, in future, the facilitated visualisation of vesicles containing wild-type paxillin, three dimensional migration and integrin recycling assays would help to define the role of vesicular transport in paxillin turnover and unveil the complex mechanisms of its regulation.

4.2. The Role of EpsinR in Focal Adhesion turnover

In parallel to studies of the localisation of focal adhesion components to vesicles, I focused on a role of distinct vesicular proteins in cell migration and adhesion. As shown in my experiments, I tried to unveil the function of EpsinR in focal adhesion turnover. Whereas the functional consequences of its depletion upon siRNA knockdown were striking (increased focal adhesion size, reduced migration) the mechanisms causing these phenotypes remained elusive.

In the course of my work, I postulated that the most probable reason for the enlarged adhesions upon EpsinR knockdown could be an imbalance in the turnover of focal adhesion components, most probably paxillin. It was shown that any disturbance of the integrin turnover results in an increase of focal adhesion size (Gonon et al. 2005; Skalski and Coppolino 2005; Jović et al. 2007). However, knockdown of EpsinR did not influence the fast turnover of paxillin. Yet, this does not mean that the assumptions regarding paxillin turnover were entirely incorrect.

Figure 4.1



* If the number of binding sites is limited in the system, $k(\text{on}) > k(\text{off})$ will be the same as $k(\text{on}) = k(\text{off})$.

Figure 4.1: The difference of FRAP and PA in an imbalanced system. The figure represents a structure composed of several identical proteins. New proteins bind to this structure with the kinetic constant $k(\text{on})$ and dissociate with the constant $k(\text{off})$. Closed circles and open circles represent proteins before and after photoactivation/photobleaching respectively. Photoactivated/photobleached proteins are replaced by native ones over the course of time. If $k(\text{on}) = k(\text{off})$ the number of incoming and outgoing molecules is the same, therefore no difference in FRAP and PA will be seen. However,

(Figure 4.1 continued) if $k(\text{on}) > k(\text{off})$ or $k(\text{on}) < k(\text{off})$, the difference between binding and unbinding molecules results in different measured values in FRAP and PA. Note that in the special case of limited binding sites $k(\text{on}) > k(\text{off})$ will be the same as $k(\text{on}) = k(\text{off})$.

First, a possible reason for the absence of changes in paxillin dynamics upon EpsinR knockdown could be caused by the utilised technique. As shown in Figure 4.1, the basic principle of FRAP and PA to measure protein dynamics is similar. Therefore, in a steady-state situation, when the number of binding and unbinding proteins is the same, the kinetic curves measured by PA and FRAP are identical. Yet, in an imbalanced situation, due to the fact that only either incoming or leaving proteins are visualised, PA results might be different from FRAP results. Therefore, it would be interesting to perform FRAP experiments with the same experimental settings as in my PA experiments.

Second, in the analysed time frame, the turnover of a substantial fraction of paxillin in a respective focal adhesion could be measured. Still, a small stable fraction remained in the time frame of three minutes. Yet, this stable fraction will be also replaced, if analysed for a longer period of time. Thus, long-term turnover of paxillin at the focal adhesions was not possible to examine using a PA/FRAP approach.

Third, as discussed in the introduction, the main role of EpsinR was previously reported as a trafficking protein between internal membrane compartments. Although my preliminary experiments correlating TIRF and wide-field microscopy, showed EpsinR vesicles appearing and disappearing at the plasma membrane, the function of EpsinR at the plasma membrane has not yet been proven. Thus, the role of EpsinR in focal adhesion turnover could be a more indirect one that does not influence the immediate turnover of paxillin but the trafficking between membrane compartments.

Fourth, since all the adhesions analysed in my experiments were stable for the three minutes used in my photoactivation experiments, it cannot be excluded that EpsinR (and maybe vesicular transport in general) does not play a major role in the maintenance of focal adhesion organisation. Therefore, its involvement could be only necessary upon stress such as the assembly and disassembly of focal adhesions in cell migration.

Figure 4.2

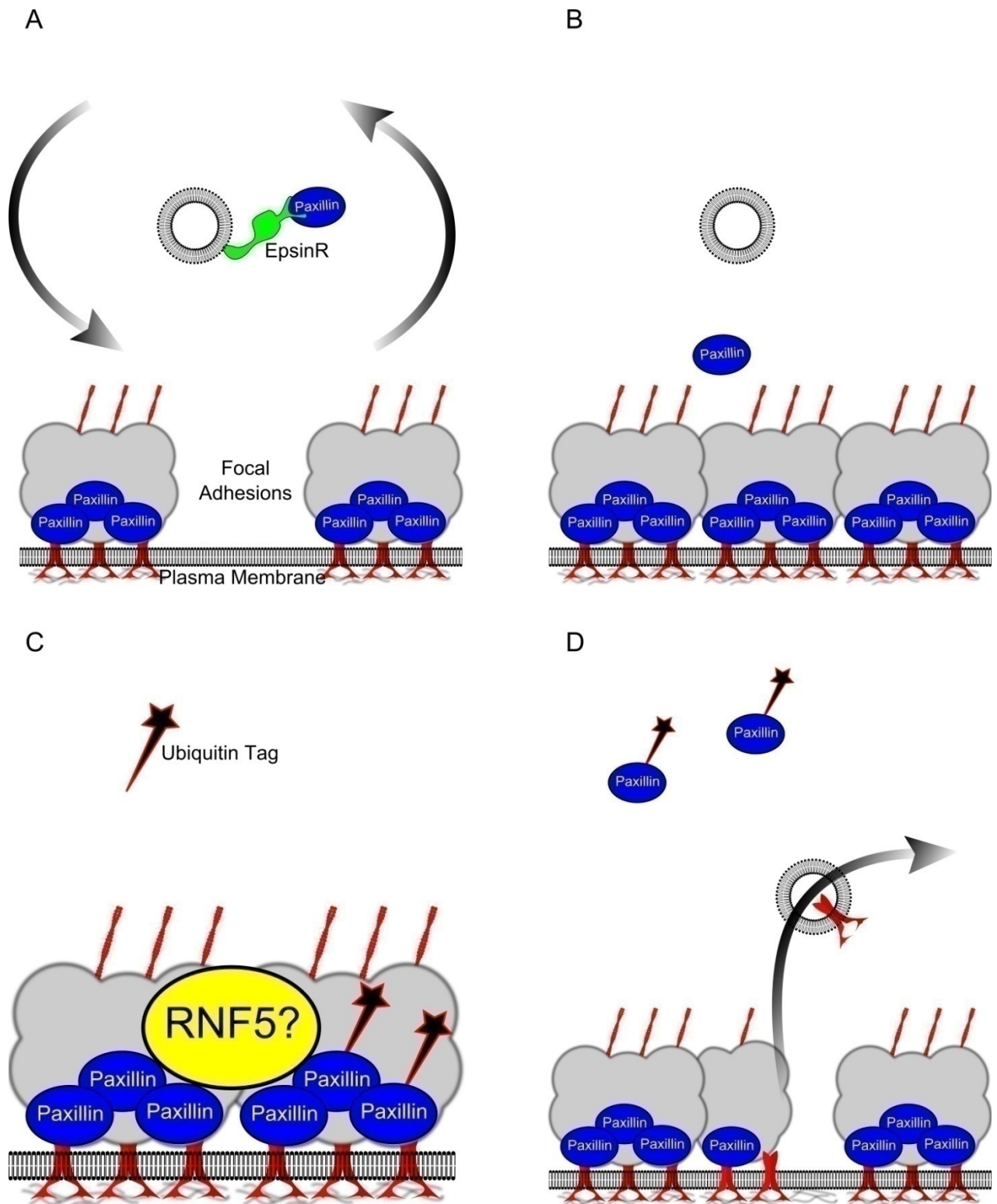


Figure 4.2: The possible role of ubiquitylation in paxillin regulation. A) In a steady-state situation EpsinR helps to regulate the paxillin turnover. **B)** Upon loss of EpsinR function, paxillin accumulates in focal adhesions and increases their size. **C)** In

(Figure 4.2 continued) order to counter paxillin accumulation, alternative regulatory mechanisms are switched on, one of which might include the mono-ubiquitinylation of paxillin. Most probably RNF5 is the ligase used for this (Didier et al. 2003). **D)** Although this mechanism is capable of removing a portion of the excessive paxillin molecules from the focal adhesions, it is not as efficient as the normal regulation process; the focal adhesion size still increases.

Fifth, another possibility would be that EpsinR is not involved in the turnover of paxillin but another focal adhesion molecule. The most likely candidate for this would be integrins. Therefore, experiments measuring the turnover of transmembrane receptors would help to clarify this issue. However the binding of EpsinR to paxillin in immunoprecipitation experiments renders this alternative less likely (Andreyeva 2008).

A very interesting aspect, only briefly described in my experiments was the appearance of an additional paxillin band on the Western Blot upon EpsinR or CHC knockdown. As already discussed, a possible reason for this would be the mono-ubiquitinylation of paxillin. Figure 4.2 shows a hypothetical model for the role of ubiquitinylation in paxillin turnover. As shown in the paper of Didier et al. in 2003, the RNF5 protein is capable of inducing paxillin ubiquitinylation that leads to the redistribution of paxillin from the focal adhesions. Therefore, if the knockdown of EpsinR and CHC, indeed, disturbs paxillin turnover, it has to be controlled by alternative mechanisms like, for example, up regulation of paxillin ubiquitinylation.

What is the exact role of EpsinR in focal adhesion turnover? An alternative hypothesis is the “catcher model”. As shown in Figure 4.3 one of the main functions of EpsinR in this model is to bind paxillin diffused in the cytoplasm. This would decrease the concentration of free paxillin in the cytoplasm available for turnover at the focal adhesions. Upon the knockdown of EpsinR the concentration of paxillin is elevated, leading to the enlargement of focal adhesions.

Figure 4.3

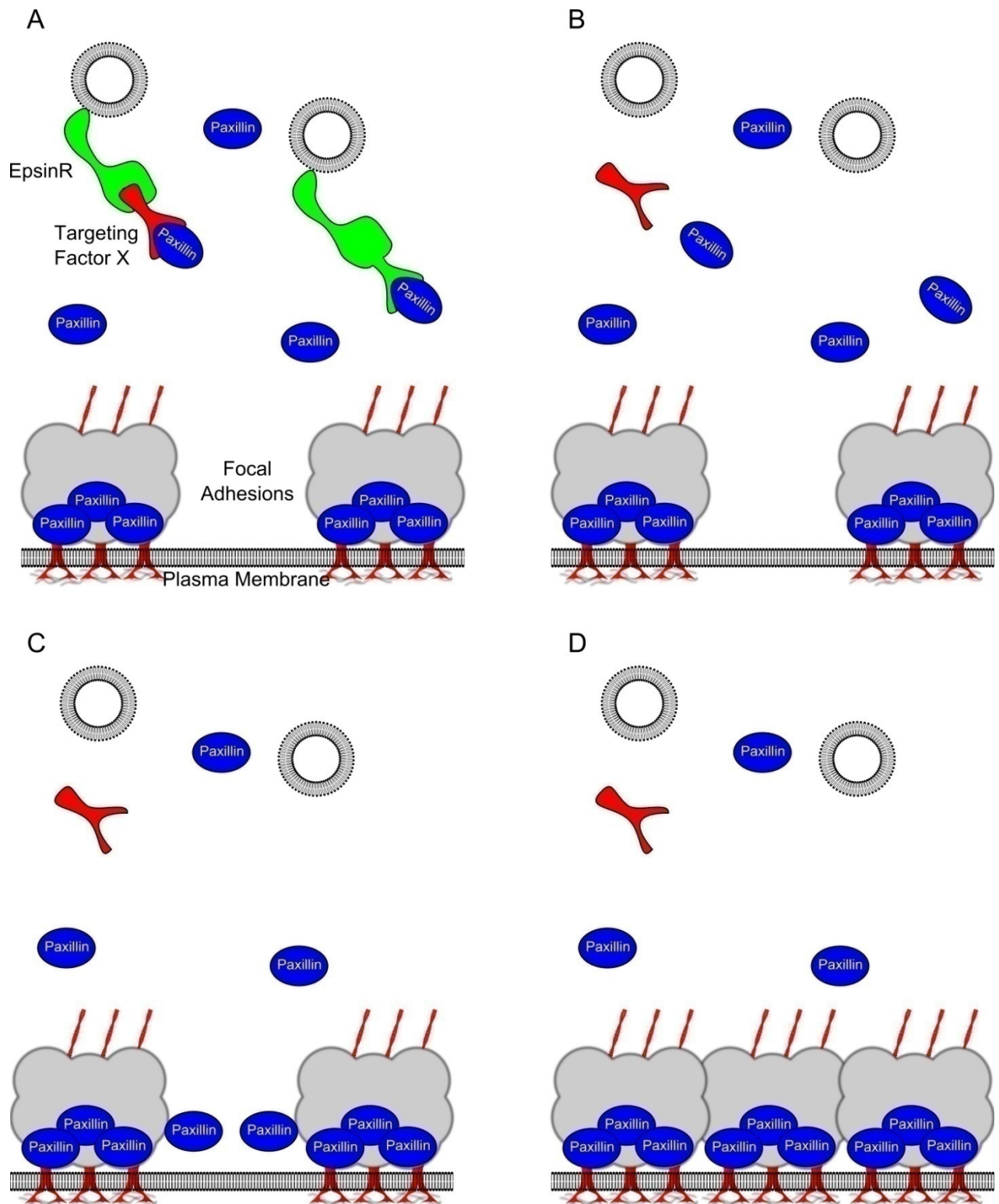


Figure 4.3: Catcher Model. A) This model proposes that one of the main functions of EpsinR is the binding to paxillin (directly or indirectly through a targeting factor X). Thereby the concentration of free paxillin molecules, available for focal adhesion binding,

(Figure 4.3 continued) is limited. **B)** Upon knockdown of EpsinR the concentration of free cytoplasmic paxillin would increase. **C)** An increased number of paxillin molecules would then be targeted to the focal adhesions. **D)** This recruits other focal adhesion components and induces the general increase of focal adhesion size.

Is there any proof for such an involvement of EpsinR in paxillin turnover? One line of evidence is coming from observations made with the help of live-cell microscopy. In my long-term turnover experiments, the ratio of focal adhesion intensity and the cytoplasmic background (probably containing paxillin bound to small vesicles) is similar for most control cells. However, EpsinR knockdown cells show a decreased background, indicating loss of the cytoplasmic fraction of paxillin.

Another line of evidence is coming from recent observations made by Benny Geiger and colleagues (unpublished results, currently reviewed). On the basis of the data obtained in FRAP experiments (using high-end microscopy setups that allow precise beam localisation and beam size regulation), they formulated a hypothesis of a diffusion gradient of focal adhesion components around focal adhesions. In this model they postulated that such a diffusion gradient exists not only around adhesions in x- and y-direction, but also above focal adhesions. This gradient could be formed by vesicular structures concentrated around cell adhesions, if they were able to bind to the soluble focal adhesion components.

If we try to integrate the information about vesicular transport and focal adhesion turnover existing so far, we obtain a similar model as for integrins. In the model shown in Figure 4.4, I try to summarise the various findings made in the course of this work.

Figure 4.4

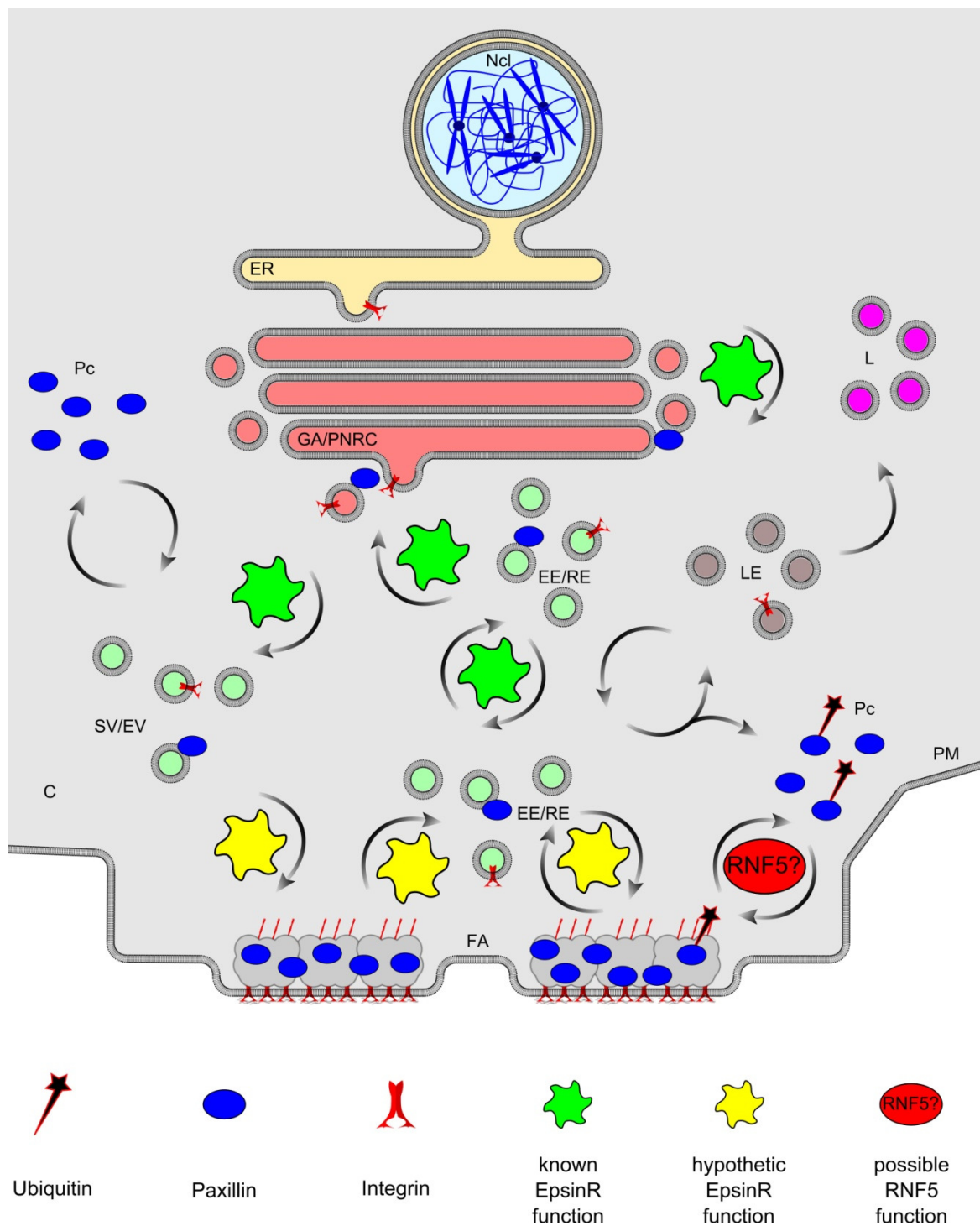


Figure 4.4: Integrated model for paxillin turnover including the role of EpsinR. Figure shows the possible interaction of the various vesicular compartments with focal adhesion components. The green propeller indicates EpsinR functions that are already known; the yellow one, hypothetic interactions including the plasma membrane. Ncl: Nucleus; ER: Endoplasmic Reticulum; GA/PNRC: Golgi Apparatus/Perinuclear

(Figure 4.4 continued) Recycling Centre; Pc: cytoplasmic fraction of Paxillin; SV/EV: Secretory and Exocytic Vesicles; FA: Focal Adhesion; EE/RE: Early Endosomes and Recycling Endosomes; LE: Late Endosomes; L: Lysosomes. Note that the vesicular compartment's size is exaggerated, compared to the other organelles, especially the nucleus.

As shown, it is assumed in this model that paxillin is present freely in the cytoplasm and, in addition, localised to the perinuclear membrane compartments (including the Golgi apparatus). Upon budding from these organelles, paxillin accompanies transmembrane proteins like integrins to secretory/exocytic vesicles. These are targeted to the plasma membrane, or, more specific for integrin and paxillin, to the focal adhesions. From there integrins and possibly paxillin are endocytosed back to the early endosomes and transported further to recycling endosomes. Depending on the situation, these proteins then are recycled back to the focal adhesions or via the long recycling loop transferred to the PNRC. Alternatively, the early endosomes mature to late endosomes and further to lysosomes, where proteins are degraded. At this point, most probably membrane associated cytoplasmic proteins like paxillin dissociate. In addition to the vesicular pathways, also the direct turnover of paxillin by mono-ubiquitylation and additional mechanisms (e.g. phosphorylation) regulates the protein's binding to focal adhesions directly from the cytoplasm.

What are the future experiments that should be done to further understand EpsinR functions? First, the EpsinR dominant negative mutant can be used to reproduce the results obtained from siRNA knockdown of EpsinR. Since the paxillin(M1) mutant already was shown to colocalise with this construct, follow-up experiments using the wild-type paxillin would be helpful to clarify the interactions between EpsinR and paxillin in the vesicular compartment.

Second, as already discussed before, FRAP experiments are required to show the role of EpsinR in the short-term turnover of paxillin. Third, the integrin internalisation assay will be useful to clarify whether EpsinR is, indeed, involved in integrin transport. Beside immunostainings also live-cell imaging of the integrins could be performed to address this question. Fourth, the ubiquitylation

of paxillin could be a quite interesting and important mode of its regulation. Thus, immunoprecipitation experiments to pull down modified paxillin should be made to further address the question of the chemical modification inducing the additional paxillin band after knockdown of EpsinR and clathrin.

4.3. The Screening Hits CCDC51 and KIF19

I cloned and characterised two proteins (CCDC51 and KIF19) that were picked up in the siRNA screen searching for proteins whose knockdown affects vesicular trafficking as well as focal adhesion organisation (Andreyeva 2008). The localisation of these proteins possibly reflects their function in the turnover of focal adhesions and, simultaneously, in vesicular transport. Yet, before further characterisation experiments are performed, first, the siRNA knockdown phenotypes have to be reproduced in a standard experimental setup. In addition, the phenotypes also have to be reproduced with siRNAs targeting different sites of the mRNA coding the same proteins. To reveal CCDC61 and KIF19 functions, cell migration, cell spreading, long-term focal adhesion turnover and maybe turnover kinetics of focal adhesion components have to be analysed upon siRNA knockdown.

Further colocalisation experiments using live-cell imaging and immunostaining can be performed. Especially the proper localisation of CCDC51 to the endoplasmic reticulum has to be confirmed. Similar to the experiments performed by Hernández and colleagues (Hernández et al. 2006), the interaction of endoplasmic reticulum containing CCDC51 with focal adhesions could give further insights into its function. For KIF19, the transported cargos have to be found. Therefore, live-cell imaging of vesicular markers to assay vesicle dynamics upon knockdown of KIF19 could yield further insights. A parallel approach to determine the function of the two candidate proteins would be the analysis of their interaction partners using immunoprecipitation followed by Mass Spectrometry.

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