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Role of vesicular trafficking in focal adhesion turnover

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Zusammenfassung

Fokalkontakte dienen der Zelle als Verankerungen zwischen der extrazellulären Matrix mit dem Aktin Zytoskelett. Ein dynamisches Gleichgewicht von Abbau im vorderen Teil der Zelle und Aufbau von Fokalkontakten im hinteren Bereich der Zelle ist eine Grundvoraussetzung während der Zellwanderung. Trotz zahlreicher Studien, die den Regulierungsmechanismus der Fokalkontakte untersuchen, bleibt der Auf/Abbau von Fokalkontakten ungelöst.

In der vorliegenden Arbeit untersuche ich die Verbindung zwischen Vesikel Transport und Fokalkontaktumbau mithilfe eines siRNA-Microarrays. Identifizierte Gene konnten dem Vesikeltransport und Fokalkontaktumbau zugeordnet werden. Ebenso, wurden bisher noch nicht charakterisierte Gene gefunden. Weiterführende Analysen werden wertvolle Informationen über den Einfluss von Vesikeltransport auf Fokalkontaktumbau liefern.

Das Adaptor Protein Paxillin ist für die Rekrutierung von Struktur und Signalkomponenten zu Fokalkontakten nötig. Hier zeige ich, dass eine dominant negative Paxillin Mutante in einer Subpopulation von Clathrinvesikel akkumuliert und damit nahelegt, dass Paxillin im Clathrinvesikel-Weg und im Fokalkontaktumbau eine Rolle spielt. Darüberhinaus zeige ich dass, ein weiteres Protein von Clathrinvesikeln, nämlich EpsinR, in Fokalkontaktumbau involviert ist.

Abstract

Focal adhesions are sites of a cell attachment to the underlying substrate. The dynamic turnover of focal adhesions is central to the process of cell migration and includes coordinated assembly of the new adhesions at the leading edge of the cell and disassembly of old ones at the cell rear. Despite intensive studies the exact mechanism involved in focal adhesion turnover has not been resolved. During the last twenty years a role of the vesicular trafficking machinery in this process has emerged, but the identity of the involved vesicular pathway remains elusive.

In the presented study I examined a link between vesicular trafficking and focal adhesion turnover using an siRNA screening approach and investigated the role of vesicular transport in the recycling of the focal adhesion scaffold protein paxillin.

With the help of an established siRNA microarray screening platform I identified several factors affecting vesicular trafficking and focal adhesion organization simultaneously. These factors included uncharacterized molecules (for example KIF19, CCDC51, TM4SF19) and well-known vesicular components (COPI complex) that confirmed the putative link between vesicular trafficking and focal adhesions. The results of this screen provide a foundation for the further characterization of molecules putatively linking vesicular transport and focal adhesion dynamics.

The adaptor protein paxillin is responsible for recruiting structural and signaling components to focal adhesions and thereby is involved in focal adhesion turnover and cell protrusion. Previous studies indicated that paxillin localization to focal adhesions is dependent on the vesicular trafficking pathway; however its nature was still unclear. In my work I demonstrated that in the absence of a functional focal adhesion targeting domain, paxillin accumulated in a subpopulation of clathrin vesicles. It led me to the conclusion that a specific part of the clathrin pathway might play a role in paxillin recycling and therefore in focal adhesion organization. Using the siRNA knockdown approach I confirmed that a subpopulation of clathrin vesicles containing the adaptor protein EpsinR is indeed involved in focal adhesion turnover.

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Andrejeva N.S., Kovalenko O.V., Zinovkina L.A., Shanina N.A., Nadezhdina E.S. Identification of microtubule-binding site of proteinkinase LOSK/LSK. The 1st Meeting of Society of Cell Biology, St. Petersburg (Russia), 2003; was published in *Tsitologiya* 2003, Vol. 43:844-845 (in Russian).

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Table of contents

Introduction.....	13
Cell migration	14
Focal adhesion structure	16
Focal adhesion assembly	17
Focal adhesion disassembly	18
Vesicular trafficking and focal adhesion turnover	19
Integrin trafficking	22
<i>Integrin internalization</i>	23
<i>Integrin recycling</i>	24
<i>Role of the integrin trafficking in focal adhesion turnover. Future prospective.</i>	26
Overview of this study	27
PartI siRNA microarray screen as a method to identify factors involved in focal adhesion turnover	29
Introduction.....	30
Material and Methods	33
Results and Discussion	37
Conclusions and Perspectives	62
PartII Identification of paxillin vesicular recycling pathway	68
Introduction.....	69
Material and Methods	75
Results and Discussion	82
Conclusions and Perspectives	108
Summary.....	114
Bibliography	118
Supplementary materials	128
Supplementary movies.....	129
Supplementary tables.....	130

INTRODUCTION

The recognition and acknowledgment of anomalies result in crises that are a necessary precondition for the emergence of novel theories and for paradigm change.

Thomas Kuhn "The structure of scientific revolutions"

Cell migration

Cell migration has a central function in various biological processes in multicellular organisms such as embryonic development, response of the immune system to an inflammatory stimuli and wound healing during tissue repair. When cell migration becomes uncontrolled it leads to the development of different pathological changes including tumor metastasis, reduced immune defense and birth defects. Therefore to understand how cells migrate is an important issue in current research.

Cell migration requires tight coordination of actin and microtubule cytoskeleton dynamics with the formation and disassembly of the cell-extracellular matrix contacts so called adhesions (Figure 1). The steps of the cell migration include protrusion of the leading edge, formation of substrate adhesions at the front, translocation of the cell body, disassembly of the old adhesions and contraction at the rear. The structure that drives protrusion at the leading edge is called the lamellipodium. It contains a dense network of actin filaments; its coordinated polymerization at the front pushes the cell membrane forward and promotes lamellipodium extension. During cell protrusion initial contacts (called focal complexes) with the extracellular matrix (ECM) are established, some of them later develop into strong adhesions (called focal adhesions) (Ballestrem, Hinz et al. 2001; Kaverina, Krylyshkina et al. 2002; Zaidel-Bar, Ballestrem et al. 2003). Focal adhesions connect the ECM with bundles of actin filaments and serve as traction points for the forces that move the cell body forward through retraction of the cell rear (Geiger, Bershadsky et al. 2001). Retraction forces are generated by means of actomyosin contractility. Formation of the nascent focal complexes is dependent on the small GTPase Rac, whereas focal adhesion assembly requires activation of the RhoA GTPase (Nobes and Hall 1995; Rottner, Hall et al. 1999). Disassembly of focal adhesions at the rear of the cell is an essential prerequisite for translocation of the cell in the

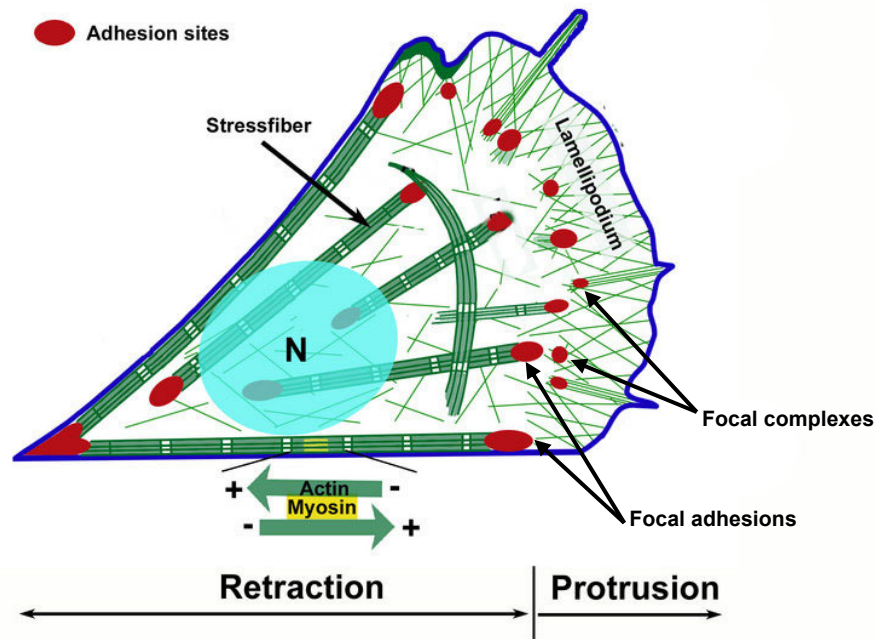


Figure 1| Schematic drawing of a migrating cell. | Protrusion of the leading edge is driven by lamellipodium containing a network of actin filaments. New focal complexes form at the leading edge of the cell; some of them later develop into strong focal adhesions connected with actin stress fibers. Retraction of the cell rear depends on the forces generated by actomyosin contractility, and accompanied by disassembly of the back adhesions. Modified from Kaverina, Krylyshkina et al.

direction of movement. Assembly of the new focal adhesions at the protruding edge of the cell and their subsequent disassembly under the cell body and at the retracting edge is referred to as focal adhesion turnover.

The role of microtubules in cell migration is less clear. An intact microtubule network is indispensable for directed migration of different cell types (Vasiliev, Gelfand et al. 1970; Xu, Wang et al. 2005). It has been proposed that microtubules deliver factors regulating focal adhesion turnover by direct targeting of focal adhesions (Kaverina, Rottner et al. 1998; Kaverina, Krylyshkina et al. 1999). Additionally microtubules were suggested to be involved in the maintenance of the level of RhoA activity (Ren, Kiosses et al. 1999).

The regulation of actin and microtubule networks during cell migration have been discussed in detail in several reviews (Kaverina, Krylyshkina et al. 2002; Palazzo and Gundersen 2002; Small, Geiger et al. 2002; Wehrle-Haller and Imhof 2003). In my introduction I will concentrate on the mechanism of focal adhesion turnover.

Focal adhesion structure

Focal adhesions were first identified by interference reflection microscopy as specialized relatively immobile foci providing attachment of the cell to the substrate (Abercrombie and Dunn 1975; Izzard and Lochner 1980). The cell to substrate distance at focal adhesions appeared to be 10-15 nm. Since then it became well established that focal adhesions represent molecular complexes which link extracellular matrix via transmembrane receptors to the actin cytoskeleton (Zamir and Geiger 2001). More than 50 focal adhesion proteins were identified and their number still continues to grow. The components of focal adhesions can be divided into three groups according to their location: extracellular, transmembrane and cytoplasmic (Table 1).

The major transmembrane receptors of focal adhesions are integrins. Integrins are heterodimeric receptors generated by selective pairing between α and β subunits. There are 24 distinct integrin receptors that possess different binding affinity to various ECM ligands (Hynes 1992). The type of integrins found within a given focal adhesions depends on the ECM to which cells are attached. The most studied adhesions to fibronectin contain $\alpha 5 \beta 1$ -integrin as a major receptor. In addition to integrins there are a number of other transmembrane proteins involved in the organization of focal adhesions such as tetraspanins, proteoglycans (syndecan 4), growth factor receptors and matrix metalloproteases (Miranti and Brugge 2002).

Integrins do not bind actin directly and do not have any enzymatic activity; instead they recruit a number of proteins that form a focal adhesion plaque providing connection to the actin cytoskeleton and performing signaling functions. Focal adhesion plaque proteins can be subdivided into several categories: structural proteins, adaptor or scaffolding proteins and enzymes (Lo 2006; Allison L. Berrier 2007). Structural proteins, including talin, tensin, filamin and others, usually interact with integrins' cytoplasmic tails or with actin directly and serve as the focal adhesion backbone. Adaptors (for example paxillin, PINCH, zyxin) link integrin-associated proteins with actin or signaling molecules. Finally, enzymes including tyrosine kinases (FAK, Src), phosphatases (PTP-1B) and modulators of small GTPases (GITs, ASAP1) transform and

Focal adhesion proteins		
Location	Focal adhesion proteins	
Extracellular	Collagen, fibronectin, heparan sulfate, laminin, proteoglycan, vitronectin	
Transmembrane	Integrins 18 α and 8 β (24 combinations in humans), LAR-PTP receptor, layilin, syndecan-4	
Cytoplasmic	Structural	Actin, α -actinin, EAST, ezrin, filamin, fimbrin, kindling, lasp-1, LIM nebullette, MENA, meosin, nexilin, paladin, parvin, profilin, ponsin, radixin, talin, tensin, tenuin, VASP, vinculin, vinexin
	Enzymatic	Protein tyrosine kinase: Abl, Csk, FAK, Pyk2, Src Protein serine/threonine kinase: ILK, PAK, PKC Protein phosphatase: SHP-2, PTP-1B, ILKAP Modulators of small GTPase: ASAP1, DLC-1, Graf, PKL, PSGAP, RC-GAP72 Others: calpain II, PI3-K, PLC γ
	Adapters	p130Cas, caveolin-1, Crk, CRP, cten, DOCK180, DRAL, FRNK, Grb 7, Hic-5, LIP.1, LPP, Mig-2, migfilin, paxillin, PINCH, syndesmos, syntenin, tes, Trip 6, zyxin

Table 1 | Known focal adhesion components. Adapted from Lo, 2006.

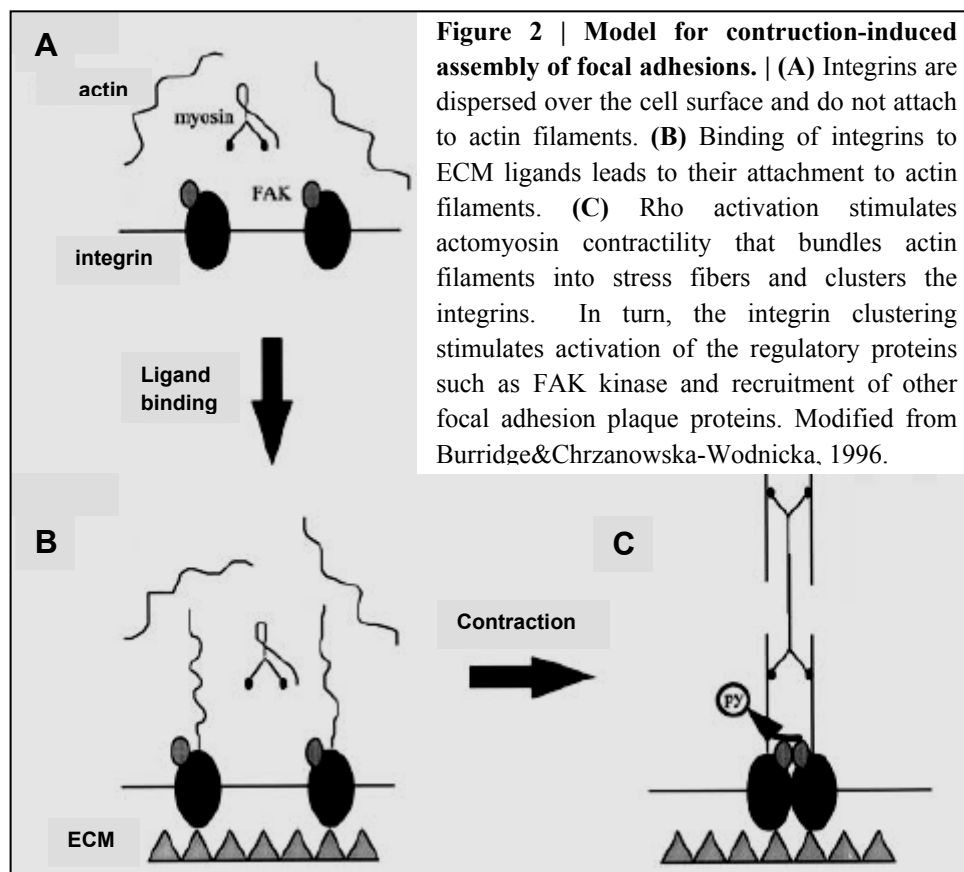
transport signals from integrins to the actin cytoskeleton and other cellular compartments, and regulate focal adhesion turnover. Among focal adhesion components there are also modulators of actin polymerization (VASP, profilin), indicating a role of focal adhesions in the control of actin polymerization.

Despite extensive studies the mechanism of focal adhesion targeting remains poorly understood even for some well-known components. For example the identity of the focal adhesion docking molecules for the important scaffolding proteins paxillin and zyxin still has not been recognized.

Focal adhesion assembly

The cornerstone of the assembly of focal adhesions is the clustering of integrins that is mediated from outside of the cell by binding to the ECM ligands, and from the inside by intracellular tension generated by actin filaments (Burrige and Chrzanowska-Wodnicka 1996). Figure 2 demonstrates the model of contraction-induced focal adhesion formation. First integrins bind to ECM, become activated and attach to the actin filaments. This process is accompanied by RhoA activation that in turn leads to the activation of myosin II and the stimulation of actomyosin

contractility. The resulting tension bundles actin filaments into stress fibers and clusters the attached integrins. Clustering of the integrins induces further recruitment of early signaling and scaffolding molecules such as FAK, paxillin, talin and subsequently later ones such as zyxin and tensin (Miyamoto, Teramoto et al. 1995; Zaidel-Bar, Ballestrem et al. 2003). Whether proteins are recruited to focal adhesions individually or in preformed complexes remains to be established.



Focal adhesion disassembly

In contrast to focal adhesion assembly there is no accepted model of focal adhesion disassembly. In my laboratory it has been suggested several years ago that focal adhesion disassembly requires delivery of so called “relaxing” factors that is dependent on microtubules (Kaverina, Krylyshkina et al. 1999; Kaverina, Krylyshkina et al. 2002). Those “relaxing” factors were expected to inhibit RhoA and actomyosin contractility locally in the area of focal adhesions thereby

inducing their disassembly. Since activation of RhoA promotes assembly of focal adhesions, it was logical to believe that a reversal of the RhoA activation would lead to their disassembly. However it was recently demonstrated that focal adhesion disassembly can occur in cells with constitutively active RhoA (Ezratty, Partridge et al. 2005). Moreover in mitotic cells the loss of focal adhesions is also accompanied by high levels of RhoA activity (Maddox and Burridge 2003).

Ezratty and co-workers proposed a new model where focal adhesion disassembly was mediated by endocytosis (Ezratty, Partridge et al. 2005). They found that microtubule-induced focal adhesion disassembly was dependent on the large GTPase dynamin, a key player of the cellular endocytic machinery. The idea of focal adhesion disassembly by endocytosis is consistent with the studies demonstrating endocytic recycling of integrins. However it is difficult to imagine how endocytic vesicles would form in the presence of numerous focal adhesion plaque proteins.

Vesicular trafficking and focal adhesion turnover

Vesicular trafficking is a fundamental process that controls the architecture of different intracellular compartments and the communication between the cell and its environment. A simplified model of vesicular trafficking includes two major transport routes: endocytosis and exocytosis (Figure 3). Endocytosis represents the formation and inward movement of plasma membrane vesicles. It can be subdivided into clathrin-dependent and clathrin independent endocytotic pathways. Clathrin-dependent endocytosis involves the assembly of vesicles coated with clathrin (Hirst and Robinson 1998; Kirchhausen 2000; Mousavi, MalerÃ, et al. 2004). Clathrin-independent endocytosis uses an alternative protein coat or does not require a protein coat for the formation of endocytic vesicles. For example caveolae-dependent endocytosis occurs through plasma membrane invaginations that are enriched in cholesterol and sphingolipids (Candice M. Thomas 2008).

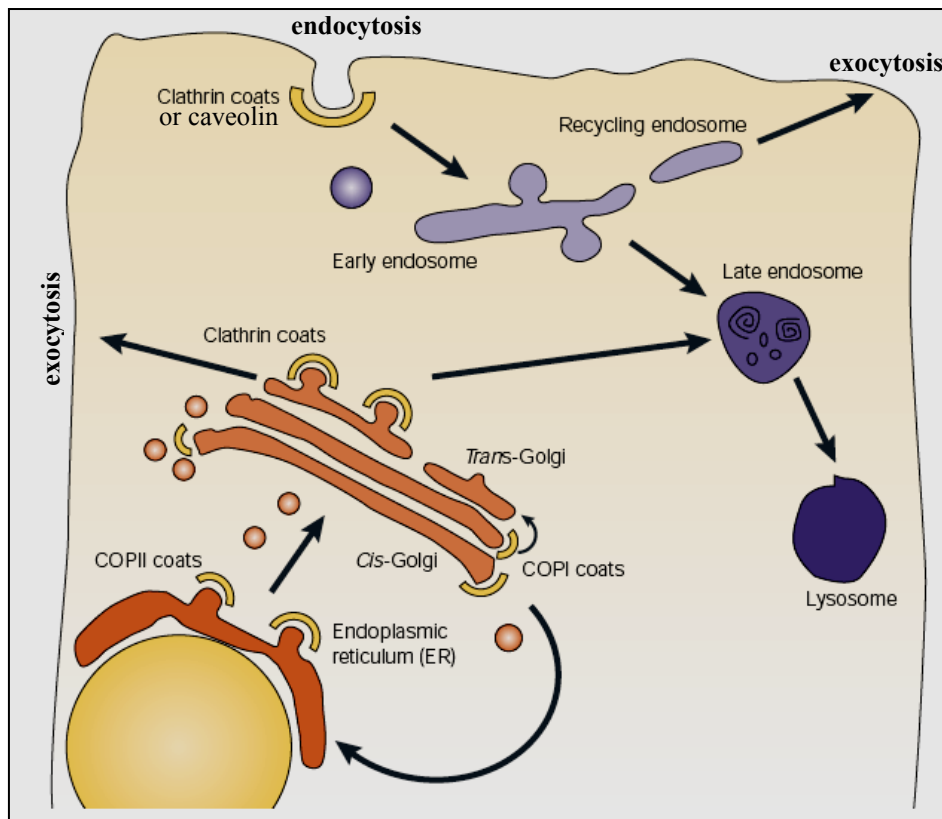


Figure 3| Simplified model of vesicular trafficking in eukaryotic cells. | In the endocytic pathway molecules are internalized at the plasma membrane in a clathrin-dependent or – independent manner and delivered to the early endosomes. Then they are recycled back to the membrane and undergo exocytosis or they are transported to late endosomes and lysosomes. Exocytosis is also involved in the delivery of newly synthesized molecules from the endoplasmic reticulum (ER) to the plasma membrane. Those molecules are transported first from the ER to the Golgi in COPII-coated vesicles, then from the *cis*-Golgi to the *trans*-Golgi cisterns involving COPI coat proteins, and finally to the plasma membrane likely in a clathrin-dependent manner. Adapted from Kirchhausen, 2000.

Exocytosis includes delivery of the newly synthesized or recycled proteins to the plasma membrane. The vesicles of the early exocytic pathway are formed with participation of coat protein complexes I and II (COPI and COPII) (McMahon and Mills 2004; Béthune, Wieland et al. 2006; Kirchhausen 2007). It has been recently shown that clathrin vesicles also take part in protein recycling (Deborde, Perret et al. 2008).

Specific coat proteins together with their adaptor proteins recruit different cargos and determine the intracellular destination of the endocytic or exocytic vesicles. The assembly of coats and vesicle formation, budding, transport, tethering and fusion are under control of the Arf and Rab GTPases and their effectors (Zerial and McBride 2001; D'Souza-Schorey and Chavrier 2006).

Vesicular fusion events are in addition dependent on soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which establish a specific connection between the transported vesicle and targeted membrane (Duman and Forte 2003).

It was long ago proposed that polarized vesicular trafficking toward the leading edge and subsequent exocytosis is an essential condition for directional cell movement (Figure 4) (Kupfer A, Kronebusch PJ et al. 1987; Bretscher 1996). Exocytosis at the protruding edge could provide a new membrane mass for the cell extension and deliver new cell-surface receptors together with adhesion and cytoskeletal components. There are a number of studies demonstrating polarized exocytosis of vesicles toward the leading edge including direct observation of recycling receptors in moving fibroblasts (Bretscher 1983; Hopkins, Gibson et al. 1994; Schmoranzler, Kreitzer et al. 2003).

In conformity with exocytosis polarized toward the cell front, endocytosis was proposed to occur mainly at the cell rear, contributing to the cycling of bulk membrane or adhesion molecules (Bretscher 1996). It was indeed demonstrated that clathrin endocytic vesicles were distributed towards the trailing edge of moving T-lymphocytes (Samaniego, Sanchez-Martin et al. 2007). Surprisingly in migrating fibroblasts clathrin-mediated endocytosis on the contrary was concentrated towards the leading edge (Rappoport and Simon 2003). It is possible that endocytosis plays a different role during distinct types of cell migration (rapid amoeboid lymphocyte movement and slow fibroblast movement).

Despite numerous studies the exact mechanism whereby vesicular

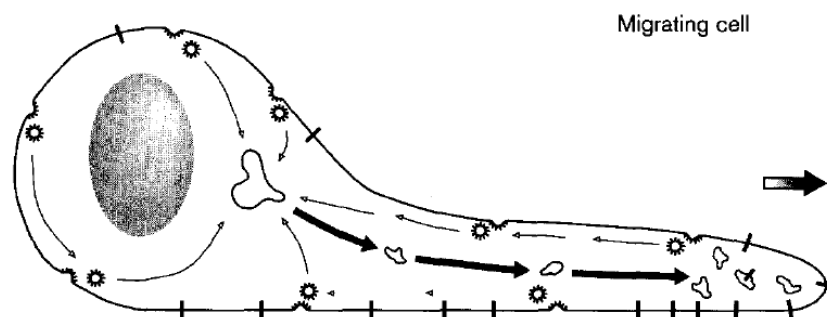


Figure 4| Schematic drawing of the vesicular trafficking route in a migrating cell. | In a migrating cell exocytosis is polarized toward the cell's leading edge. Adopted from Bretcher&Aguado-Velasco, 1996.

trafficking contributes to cell migration remains obscure. However the importance of vesicular trafficking for cell polarization and migration is widely accepted and highlighted by various experiments. For example Bershadsky and Futerman used brefeldinA treatment to disrupt the Golgi apparatus and inhibit vesicle transport and observed a reduction in lamellipodium activity and loss of cell polarization (Bershadsky and Futerman 1994). More recently overexpression of the dominant-mutants of Rab11 that blocked endocytic recycling was found to produce impaired cell polarity and disorganized motility (Prigozhina and Waterman-Storer 2006).

One way by which vesicular trafficking could influence cell migration is through the regulation of focal adhesion turnover. As mentioned before, a block in cellular endocytosis inhibited focal adhesion disassembly. And from the other side, vesicular recycling could deliver focal adhesion components to the leading edge of cells. A role of vesicular endocytosis and recycling was intensively studied and well-accepted for the membrane components of focal adhesions, integrins. It is less clear whether the delivery of other focal adhesion molecules including regulatory and scaffolding proteins is also dependent on a vesicular trafficking pathway. There are however some indications that the focal adhesion scaffolding protein paxillin might be transported through a vesicular route. Paxillin recycling will be discussed in detail in the separate introduction for the second part of this work.

Integrin trafficking

On the basis of biochemical assays it was almost 20 years ago shown that certain integrin receptors are internalized from the plasma membrane to the endosomal compartments and then transported back to the cell surface (Bretscher 1992). The initial idea was that integrin receptors are internalized at the retracting end of the cell and transported toward the leading edge where they undergo exocytosis and serve for the establishment of new adhesions. Since then the molecular mechanism of the integrin recycling and its role in cell migration has been studied in detail. However, the identification of the molecular machinery responsible for integrin trafficking still remains incomplete.

Studies of integrin recycling are hampered by several factors. First, there is not one common trafficking route for the recycling of all integrin heterodimers. Different integrin heterodimers can apparently use different internalization and recycling pathways. Moreover it has been demonstrated that in the absence of one trafficking pathway integrin receptors can be switched to another trafficking route (J. Norman, ELSO2008 congress). The diversity of the integrin recycling pathways makes it difficult to reconcile the data available into one general model.

Second, the data on integrin recycling came primarily from biochemical experiments. The most common assay addressing integrin recycling involves monitoring the dynamics of integrins labeled at the cell surface with specific antibodies. However the use of antibodies recognizing different conformational states of the same integrin receptor (“active” or “inactive” conformations) has led to the accumulation of contradictory results regarding pathways involved in receptor trafficking (Liu, He et al. 2007; Nishimura and Kaibuchi 2007). In addition there exist no convincing observations of integrins recycling through vesicular pathway in live cells that could be explained in part by low maturation efficiency of the overexpressed integrins fused to fluorescent proteins.

Recent research on the integrin trafficking machinery is comprehensively summarized in several reviews (Caswell and Norman 2006; Pellinen and Ivaska 2006; Caswell and Norman 2008). Below I will specifically discuss evidence for a role of integrin internalization and integrin recycling in focal adhesion turnover.

Integrin internalization

It has been for some time considered that integrins appear to be internalized in a clathrin-independent manner by a protein kinase C alpha (PKC α)-dependent caveolae pathway (for review see (Caswell and Norman 2006)). However new evidences indicate that β 1-integrins internalization could be regulated by the endocytic clathrin-adaptor AP2 (Liu, He et al. 2007; Nishimura and Kaibuchi 2007). For example a recent study demonstrated that the cargo-specific adaptor Numb found in AP2-clathrin vesicles controls endocytosis of β 1-integrins by direct binding (Nishimura and Kaibuchi 2007). Moreover the same studies showed that suppression of the clathrin trafficking pathway by means of siRNA led to a reduction of the endocytosis rate of β 1- and β 3-

integrins. The latest hypothesis suggests that the same integrins could be recruited and internalized in both clathrin- and caveolae-vesicular structures depending on the circumstances and their location in the cell (Caswell and Norman 2008).

Despite numerous studies on integrin endocytosis there were just a few straight forward experiments showing a connection between the rate of clathrin- or caveolin-dependent integrin internalization and focal adhesion turnover. For example a block in integrin endocytosis led to the enlargement of focal adhesions accompanied by their stabilization and reduced disassembly rate (Ezratty, Partridge et al. 2005). Ezratty and co-workers demonstrated that siRNA knockdown of dynamin, a GTPase responsible for both clathrin-dependent and – independent endocytosis, caused an increase of β 1-integrin containing focal adhesions and inhibited their microtubule-induced disassembly. Previously it has been shown that a dominant-negative mutant of dynamin blocks β 1-integrin internalization (Ng, Shima D et al. 1999). Taken together these data lead to the conclusion that dynamin-dependent integrin internalization is an essential step in the focal adhesion disassembly process.

Additionally, enlargement of focal adhesions was observed upon inhibition of Rab21 function (Pellinen, Arjonen et al. 2006). Rab21 is a small GTPase that was found to be a positive regulator of β 1-integrin endocytosis. Therefore an increase in focal adhesion size is a general consequence of suppression of integrin internalization.

Integrin recycling

Integrin heterodimers internalize into early endosomes from which they are transported directly back to the plasma membrane in a Rab4-dependent manner (short loop) or they first proceed to the perinuclear recycling compartment (PNRC) and only then return to the membrane through a Rab11-dependent pathway (long loop). The short and the long loop pathways were shown to transport different integrin heterodimers and to be dependent on the different regulatory signals. Recently it has been demonstrated that downregulation of α 5 β 1 trafficking through the long loop led to the increase in α v β 3 integrin recycling dependent on the short loop (J.Norman, ELSO2008 congress). That means that these two pathways are interdependent and likely can

substitute for each other. Therefore experiments where one of the two pathways is suppressed with the aim to reveal its role in integrin recycling and focal adhesion turnover should be interpreted with caution.

The link between integrin recycling to plasma membrane and focal adhesion turnover was investigated directly only in a limited number of studies. Most of the studies examined the influence of blocking integrin recycling on cell migration or cell spreading and did not address changes in focal adhesion turnover. Logically blocking integrin transport to the plasma membrane should decrease the size of focal adhesions. Surprisingly, the interference with integrin recycling routes on the contrary produced enlargement of focal adhesions.

Woods and colleagues showed that a block in Rab4-mediated recycling of $\alpha v\beta 3$ integrins by suppression of protein kinase D 1 (PKD1) led to reduced incorporation of integrins in newly forming focal adhesions at the leading edge and the appearance of prominent adhesions uniformly distributed at the ventral cell surface (Woods, White et al. 2004). Similarly a block of Rab11-dependent recycling upon expression of a Rab11 dominant-negative mutant (Rab11-GDP) was found to induce the formation of densely packed focal adhesions not only in protruding lamellipodium but uniformly at the cell perimeter (Prigozhina and Waterman-Storer 2006). Though Prigozhina and colleges did not investigate the effect of Rab11-GDP overexpression on integrin recycling in the cells examined, the inhibition of $\beta 1$ -integrin trafficking upon expression of Rab11-GDP mutant was previously shown by another research group (Powelka, Sun et al. 2004).

Recently, an enlargement of focal adhesions was correlated with the inhibition of $\beta 1$ -integrin recycling and was also observed upon siRNA knockdown of EHD1, a protein which regulates return of the endocytosed cargo to the plasma membrane from the PNRC (Jovic, Naslavsky et al. 2007). The increase in focal adhesion size was shown to be a consequence of impaired focal adhesion disassembly. Accordingly, it has been proposed that interference with EHD1-dependent trafficking affects recycling of not only integrin receptors but also some focal adhesion disassembly factors (Jovic, Naslavsky et al. 2007).

Another group of endocytotic proteins that was found to be involved in integrin trafficking is the SNARE (soluble NSF attachment protein receptors,

where NSF stands for N-ethylmaleimide-sensitive factor) family of membrane proteins. The main function of SNAREs is membrane fusion. It has been demonstrated that inhibition of SNARE function impaired recycling of $\alpha 5 \beta 1$ -integrins and led to an increased number of large focal adhesions (Gonon, Skalski et al. 2005; Skalski and Coppelino 2005). Coppelino and co-workers hypothesized that SNARE-mediated transport is involved in the delivery of factors that regulate focal adhesion size downstream of RhoA activation.

Therefore enlargement of focal adhesions is a general effect observed upon interference with integrin recycling pathways at different levels. There are several theoretical explanations of how decreased transport of integrins to the cell surface results in large focal adhesions, however the exact reason is not clear.

Role of the integrin trafficking in focal adhesion turnover.

Future prospective.

In summary, integrin internalization is required for the disassembly of focal adhesions. However further experiments to distinguish the function of clathrin-dependent and clathrin-independent endocytic routes in focal adhesion disassembly would be necessary. The role of the integrin recycling in focal adhesion turnover has to be addressed more carefully. It seems clear that recycling of integrins is essential for the formation of new focal adhesions at the polarized edge of cells. To answer the question about the requirement of integrin recycling for focal adhesion assembly in general, it would be helpful to inspect the situation when both, short and long, integrin trafficking loops are inhibited. Since it is likely that vesicular recycling is also involved in the transport of focal adhesion regulators such as disassembly factors, a new experimental approach to inhibit not the entire pathway but specifically its interaction with given integrins should be established. Thus it is important to promote identification of the novel proteins which are involved exclusively in the trafficking of integrins as for example the cargo-adaptor Numb.

In addition development of imaging techniques that would allow observation of integrin internalization and recycling in live cells is a next critical step in revealing the role of integrin trafficking in focal adhesion turnover. The latest advances in this field include for example the generation of

photoactivatable GFP- $\alpha 5$ integrin that makes it possible to analyze integrin dynamics in real-time (Caswell, Spence et al. 2007).

Overview of this study

According to the foregoing introduction focal adhesion turnover is an essential part of cell migration. Resolving the pathway involved in the regulation of focal adhesion assembly and disassembly will provide a deeper understanding of the mechanism of cell movement. Over the last twenty years numerous data highlighting a role of vesicular trafficking machinery in focal adhesion turnover have accumulated. It has been widely accepted that the rate of intracellular integrin recycling depends on vesicular transport and influences focal adhesion dynamics and cell migration. The likelihood exists that focal adhesion scaffold and regulatory proteins also undergo vesicular recycling. However the nature of the vesicular trafficking pathway involved in focal adhesion turnover has not been specified and the exact players of this pathway remain elusive. In the presented study I examined a link between vesicular trafficking and focal adhesion turnover using two different approaches.

In the first part of my work I established a siRNA microarray screen for identification of the factors involved in focal adhesion turnover. This method allows fast analysis of the entire groups and families of proteins regarding their influence on focal adhesions. I took an advantage of microarray RNAi replicas and assayed for proteins affecting vesicular trafficking pathway and focal adhesion organization simultaneously. Despite a number of limitations that emerged during the siRNA screen, I identified several uncharacterized as well as known vesicular trafficking regulators as factors involved in focal adhesion organization. This work was done in collaboration with Rainer Pepperkok and Jez Simpson at the EMBL, Heidelberg, Germany.

In the second part of my study I attempted to answer the question whether vesicular trafficking is involved in the recycling of the focal adhesion scaffold protein paxillin. Paxillin recycling to focal adhesions has earlier been

proposed to be dependent on the vesicular trafficking pathway. However the identity of the putative vesicular structures responsible for the paxillin transport was not determined. I demonstrated that in the absence of the functional focal adhesion targeting domain, paxillin accumulated in a subpopulation of clathrin vesicles. This and other findings (described in Part 2) led me to the conclusion that a specific part of the clathrin pathway plays a role in paxillin turnover and therefore in focal adhesion organization.

PART I

SIRNA MICROARRAY SCREEN AS A METHOD TO IDENTIFY FACTORS INVOLVED IN FOCAL ADHESION TURNOVER

Introduction

RNA interference (RNAi) is a cellular response to double-stranded RNA (dsRNA) leading to sequence-specific inhibition of gene expression. This phenomenon is based on the degradation of mRNA which has a region complementary to the dsRNA nucleotide sequence. The RNAi silencing was first described and applied for gene silencing in invertebrates: *Caenorabditis elegans* and *Drosophila melanogaster* cells. With the discovery of small interfering RNAs containing only 20-23 nucleotides (siRNAs) it became possible to overcome the mammalian interferon response to dsRNAs and achieve efficient RNAi gene knockdown in mammalian cells (Caplen, Parrish et al. 2001; Elbashir, Lendeckel et al. 2001). Currently the siRNA technique is one of the most common and convenient methods to study gene functions by analyzing knockdown phenotypes.

At the beginning RNAi was used for down-regulation of individual genes (for review, see (McManus and Sharp 2002)), however soon large-scale RNAi screening techniques were introduced since RNAi made possible silencing of almost any gene with a known sequence (for review, see (Friedman and Perrimon 2004)). Development of an automated approach for siRNA delivery and for automated phenotype analysis was a necessary step toward establishment of efficient high-throughput RNAi screens. Initially for mammalian culture cells delivery of siRNAs was achieved by conventional lipid-based transfection in 96- or 384-well plates using liquid-handling robotics (Pelkmans and Zerial 2005). Nowadays transfection in multiwell format is nearly completely replaced by more cost and time efficient microarray “reverse transfection”.

“Reverse transfection” was first exploited by Ziauddin and Sabatini for cDNA transfection (Ziauddin and Sabatini 2001), and afterward modified by several laboratories for siRNA delivery (Kumar, Conklin et al. 2003; Erfle, Simpson et al. 2004; Neumann, Held et al. 2006). In the “reverse-transfection” protocol mammalian cells are cultured on a glass slide that has been previously printed with different synthesized siRNA oligonucleotides. Cells that overlay each printed spot take up the siRNA from the glass surface, which then causes sequence-specific inhibition of gene expression. Thus each siRNA spot defines

an area of cells with suppression of an individual gene. Microarray-based siRNA platforms make possible performance and subsequent analysis of hundreds or even thousands of gene knockdowns just on one microscopy slide that provides increased data acquisition speed by microscopy and reduced cost owing to the miniaturized amounts of reagents needed. Moreover siRNA transfection occurs in homogeneous cell population in a highly parallel manner that improves the accuracy of the screening data. In addition several microarray platforms may be simultaneously produced and stored, and subsequently used as directly comparable replicas in the same screen or for the different screens.

Despite the appealing advantages of microarray siRNA platforms for high-throughput analysis of gene functions the number of published screens using this technique is limited. The main reasons for that include first of all a requirement of quite expensive and sophisticated robotic equipment, as well as costly libraries of synthesized siRNA oligonucleotides. Therefore only well-funded institutions and laboratories with ambitious goals of performing wide-genome screens have the possibility and motivation to establish siRNA microarray production. For example two wide-genome screens were recently initiated: Mitocheck screen for genes involved in mitosis sponsored by the European Commission and a screen for protein trafficking regulators in the Rainer Pepperkok laboratory in EMBL, Heidelberg. However during past three years only their assays for identification of desired genes have been published whereas the analysis of collected data remains uncompleted (Neumann, Held et al. 2006; Simpson, Cetin et al. 2007). This is explained by the issue that development of a robust trustable assay for siRNA screening together with automated data analysis programs is a challenging and time consuming process. The choice of the right assay may predetermine the outcome of the screen and its significance. Handling of the collected data and its accurate analysis with subsequent validation experiments also requires considerable time and effort. Despite improvements in techniques siRNA microarrays are still too expensive for application on a genome-wide scale.

Alternative strategies have in consequence been employed involving smaller siRNA screens for analysis of a limited but still sizeable group of genes

using easily scored assays. Such screens could be performed by smaller laboratories in collaboration with the main centers or institutes where large-scale siRNA screening technology is well established. Furthermore the amount of synthesized oligonucleotides in siRNA libraries sold by the companies such as Ambion is sufficient for printing multiple microarray platforms than necessary for a single screen. Sharing of microarray resources among laboratories therefore leads to a more effective approach for discovering new gene functions via siRNA platforms.

In my work I took advantage of the willing collaboration of Rainer Pepperkok's group in EMBL, Heidelberg and used their established siRNA microarray facility and to look for genes involved in focal adhesion regulation in human cell culture. My primary aim was to systematically analyze distinct groups and families of proteins regarding their influence on focal adhesions rather than perform a wide-genome siRNA screen. The microarray siRNA screening technique had not been previously exploited for analysis of focal adhesion structures. In the first part of my thesis work I have demonstrated the capability of this technique to identify novel factors playing a role in focal adhesion regulation.

Materials and methods

1. Cell culture and treatment

HeLa cells (gift of Yuko Kiyosue, KaN Research Institute, Kyoto) were maintained in DMEM (Gibco #31885-023) supplemented with 10% Fetal Calf Serum (PAA Laboratories) and penicillin/streptomycin (Gibco #15140-122). HeLa cell line stably expressing EGFP-paxillin was maintained in complete HeLa medium supplemented with 800 ug/ml G418 (generated by N. Andreyeva).

To assay an effect of Brefeldin A (Sigma) on focal adhesions it was diluted in the complete medium to a final concentration 10 ug/ml and cells were incubated overnight.

2. Preparation of siRNA microarrays

Chemically synthesized 21nt RNA duplexes were obtained from Ambion Europe, Ltd. Preparation and spotting of siRNA mixtures onto single-well chambered LabTek glass coverslips (NalgeneNunc, Rochester, NY, USA) were performed as previously described (Erffle, Simpson et al. 2004; Neumann, Held et al. 2006). Each LabTek coverslip contained 348 spots in a 12x32 format with a spot diameter of 400 um and a spot-to-spot distance of 1125 um. Spotted arrays were stored in plastic boxes containing silica gel at room temperature.

3. Immunostaining of cell arrays

HeLa cells (1.0×10^5 cells) were plated to the dried microarrays in a total volume of 1.5 ml complete medium. siRNA reverse transfection did not require any further manipulation; cells were transfected with siRNA oligos simply by growing on the siRNA spots. After 50 hours incubation (37°C, 5% CO₂) cells were fixed in a mixture of 3% Triton X-100 and 3% PFA for 3 minutes followed by fixation in 3%PFA for 35 minutes. Subsequently arrays were washed with 1xPBS for a few minutes and stained for 1 hour at room temperature with Paxillin monoclonal antibody (BD Biosciences). After 15 minutes wash (3 times 5 minutes with 1xPBS) arrays were incubated for 1 hour with Alexa-488 anti-mouse antibody (Molecular Probes) mixed with Alexa-568 Phalloidin (Molecular Probes). Cell nuclei were stained with 1 ug/ml Hoechst 33342 dye (Sigma-Aldrich). After washing (3 times 10 minutes with 1xPBS), arrays were stored in 1xPBS at 4°C.

4. Image acquisition

Cell arrays were imaged with a 25x air objective on a scan^R fluorescence microscope (Olympus Biosystems) equipped as described (Simpson, Cetin et al. 2007). Acquisition started from the first siRNA spot labeled with indelible ink and continued automatically based on spot-to-spot distance. All images were acquired with the help of scan^R software using gradual autofocus mode.

5. Validation by “conventional” siRNA transfection

HeLa cells were seeded in 12-well plates (1.0×10^5 cells per well) 24 hours before transfection. siRNAs oligonucleotides (Supplementary Table 1) including Silencer Negative Control #2 siRNA (catalog # 4613) were produced by Ambion and stored at -20°C as 50 uM working stocks. Mixtures of 6 ul Oligofectamine (Invitrogen) with 24 ul OPTIMEM (Gibco) and 3 ul siRNA with 100 ul OPTIMEM were prepared separately and incubated at room temperature for 7 minutes. Solutions were combined by gentle pipetting up and down and left for 20 minutes at room temperature. Culture medium was changed to the medium without antibiotics. Afterward transfection mix was carefully added to the center of each well. On the next day cells were split on coverslips coated with fibronectin (10 ug/ul, Roche). Fixation and immunostaining procedures were performed in the same way as for cell microarrays. Coverslips were mounted on the slides with Prolong Gold (Molecular Probes) and dried at room temperature at least over night. siRNA knockdown times varied from 50 to 55 hours.

6. Validation by RT-qPCR

HeLa cells were subjected to siRNA transfection in 12-well format, split the next day into 6-well dishes and incubated for 50-55 hours. Total RNA was extracted and purified using High Pure RNA isolation Kit (Roche). cDNA was produced by reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen) and Random primers (Invitrogen) according to the manufacturer's protocol. SybrGreen method was chosen for real-time PCR with gene-specific primers including primers for housekeeping gene GAPDH (Supplementary table 7). Real-time PCR reactions were carried out on iCycler (BioRad) by repeating the following amplification cycle 40 times: denaturing at 95°C for 15 s followed by annealing at 56°C for 30 s and extension at 72°C for 30 s. Degree of

siRNA knockdown was calculated by comparing the amplification level of the gene of interest, normalized through the level of the housekeeper gene, between samples transfected with a specific siRNA and negative control samples.

7. Nocodazole wash-out assay

HeLa cells (1.0×10^5 cells) were plated to the dried microarrays in triplicate. After 50 hours incubation one microarray platform was fixed. The cells on the other microarrays were starved for 1 hour in DMEM (supplied with 0.2% BSA) and treated with 8 μ M nocodazole in DMEM with 0.2%BSA for 4 hours. Afterward one replica of the two was fixed. The last microarray was rinsed with DMEM several times, left for two hours in DMEM with 0.2%BSA and finally also fixed. Fixation and following paxillin and actin staining was performed according to the standard protocol described above. All microarray incubation steps during assay were performed at 37°C with 5% CO₂.

8. Monitoring of focal adhesion dynamics in siRNA-treated cells

Hela cells with stable expression of EGFP-paxillin were split on fibronectin-coated (10 μ g/ml of fibronectin (Roche) in 1xPBS) coverslips and transfected with negative control (Silencer Negative Control #2 siRNA, Ambion) or beta-COP siRNAs (GGAUCUUCAACAUCUAAU; Simpson, Cetin et al., 2007). After 55 hours incubation time live-cell videos were acquired on a Zeiss Axiovert 200M inverted microscope equipped with a rear-illuminated, cooled CCD camera (Micromax or Cascade, Roper), halogen lamp, and filter wheel and shutters controlled with Metamorph software. Images were collected in green fluorescent channel with 30 s between frames using 100x1.4 NA Plan-Apochroma objective. During imaging cells were maintained in an open heating chamber (Warner instruments) at 37°C in the complete medium supplemented with 15 mM HEPES.

9. Cell spreading assay

HeLa cells were transfected with negative control or beta-COP siRNAs and after 55 hours incubation time replated onto fibronectin-coated (50 ug/ml fibronectin, Roche) coverslips. Following two hours incubation cells were fixed and stained with paxillin and phalloidin as described eralier.

Results and Discussion

siRNA microarray as a method to identify focal adhesion regulators

To test if it is possible to use siRNA microarray method for detection of factors involved in focal adhesion turnover, I created microarray platforms containing siRNAs to down-regulate expression of genes known or expected to play a role in cell-substrate adhesion and cell migration. Based on a literature search I selected 125 genes coding diverse focal adhesion scaffold and regulatory proteins (Supplementary Table 2). I also wanted to include in the preliminary screen complete protein families and systematically characterize all their members regarding influence on focal adhesions. Two protein groups were selected: Rho family GTPases including their regulators and molecular motor families together with microtubule tip proteins.

The three most extensively studied RhoGTPases - RhoA, CDC42 and Rac1 - have long been accepted as key players in the coordination of actin, microtubule and focal adhesion reorganization during cell migration (for review see (Ridley 2001)). Recent research indicates that some of the other members of this family as well as their regulators also affect cell cytoskeleton architecture (for review see (Wennerberg and Der 2004)). Regulators of RhoGTPases include three subgroups which control GTP hydrolysis reaction: RhoGAPs (GTPase Activating Proteins catalyzing GTP hydrolysis), RhoGEFs (GTPase Exchange Factors promoting exchange of GDP for GTP) and RhoGDIs (Guanine nucleotide Dissociation Inhibitors preventing GDP release in the cytosol) (Supplementary Table 3).

In my laboratory it was previously shown that inhibition of the motor protein kinesin-1 caused dramatic enlargement of focal adhesions (Krylyshkina, Kaverina et al. 2002). It was therefore suggested that motor-dependent delivery of regulatory proteins along actin or microtubule tracks to adhesion sites may influence adhesion turnover. That is why I chose to perform siRNA knockdown of kinesin and myosin motor families and microtubule tip proteins with the aim to investigate their possible involvement in focal adhesion rearrangement (Supplementary Table 4).

In total I selected 386 genes for the primary siRNA microarray experiment.

At least two different siRNAs for each gene were designed and produced by Ambion Europe, Ltd and spotted randomly on to four LabTeck slides together with negative control scrambled siRNA and positive control siRNA (Supplementary Table 5). HeLa cells were plated on top of the slides where they took up the arrayed siRNA oligonucleotides upon reverse transfection. After 50 hours cells were fixed and stained with Hoechst 33342, an antibody against focal adhesion protein paxillin and phalloidin to visualize nuclei, focal adhesions and actin cytoskeleton in blue, green and red channels respectively. A 50 hour incubation period was chosen as a compromise between time sufficient for

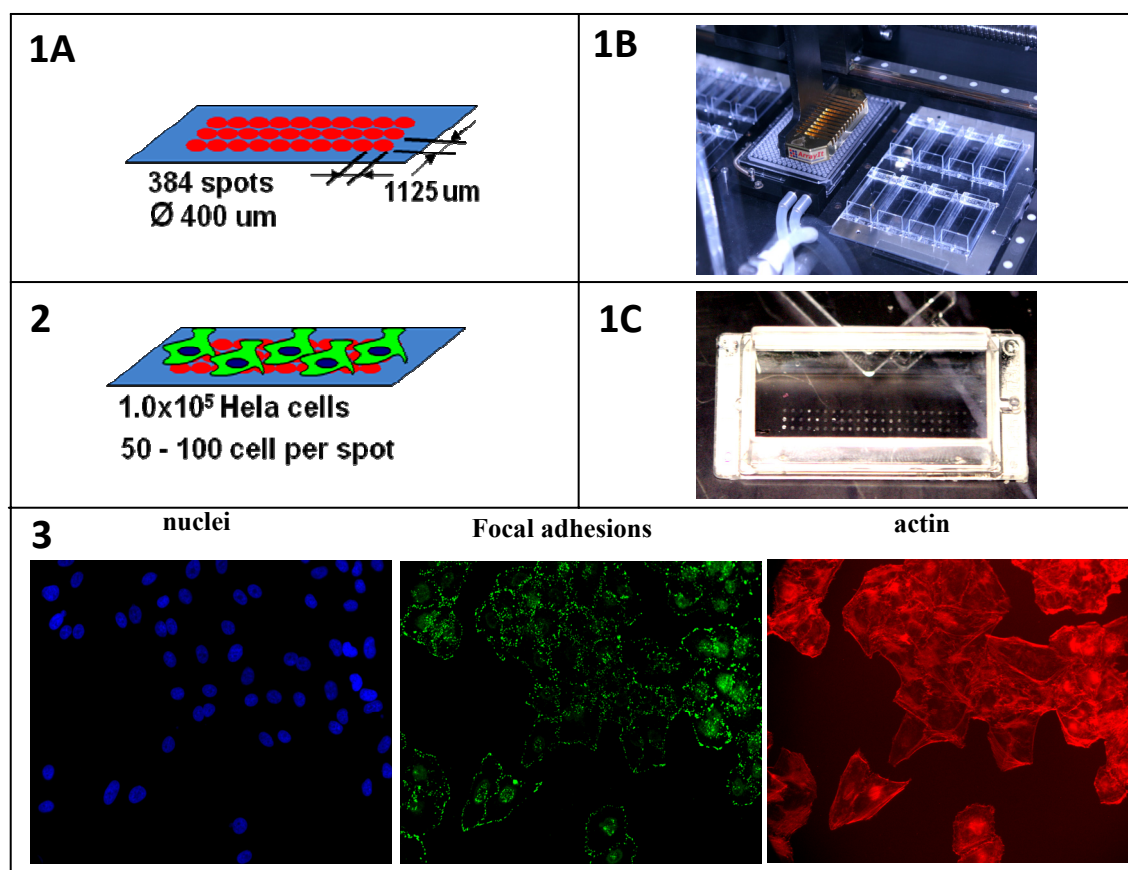


Figure 1 | Main steps in a siRNA microarray screening. | 1) Transfection mixtures containing siRNA oligonucleotides together with transfection reagent are automatically printed on LabTek chamber slide (1A. schematic drawing of spotted LabTek slide, 1B. photograph of the contact printer, 1C. photograph of spotted LabTek chamber); 2) HeLa cells are seeded on to dried microarrays (schematic drawing); 3) After 50 hours incubation time cells are fixed and stained to visualize structures of interest (nuclei, focal adhesions and actin). Three images from blue, green and red fluorescent channels are acquired with the help of automated microscopy from each siRNA spot.

protein depletion upon siRNA knockdown and the growth of cell to an optimal cell density for subsequent phenotypic analysis. Images from three fluorescent channels were acquired from the central area of each microarray spot with the help of automated microscopy. With the 25x objective it was possible to image 50-100 cells per spot and achieve a resolution high enough to analyze structures as small as focal adhesions (Figure 1).

As a positive control we used siRNA oligonucleotide targeting INCENP, inner centromer protein involved in cytokinesis. This siRNA was previously shown to give a robust and clearly recognized phenotype that was characterized by appearance of multinucleated cells (Neumann, Held et al. 2006). In each experiment we observed that at least 50% of all cells located on the INCENP siRNA spots contained two or more nuclei (Figure 2). This provided an internal control to show that printed siRNA platforms were functional and had a high transfection efficiency rate. Negative control scrambled siRNA that did not recognize any mammalian mRNAs showed no phenotypic defects.

Automated quantitative image analysis is considered to be the most unbiased approach for the evaluation of siRNA knockdown phenotypes and scoring of hits. Therefore I tried to develop image analysis program that would be able to recognize focal adhesions and classify phenotypes according to their number, size and distribution. Unfortunately the programs I tested such as MatLab and image-analysis software Cellenger (Definiens) were very sensitive to focus alterations and even with slightly out-of-focus images could not correctly

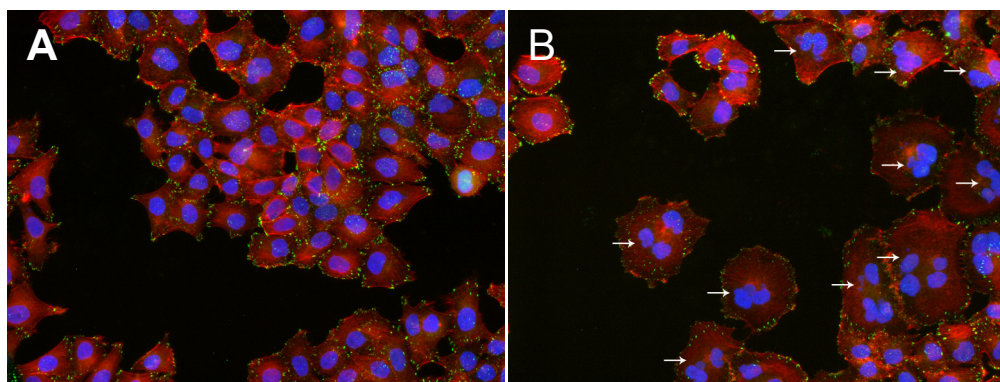


Figure 2 | Knockdown of INCENP results in appearance of multinucleated cells. | Images of cells acquired from microarray spots with printed INCENP siRNA were manually analyzed. Upon INCENP knockdown at least 50% of cells contained two or more nuclei which were visualized by Hoechst 33342 staining (marked by arrows) (B). Control cells transfected with scrambled siRNA contained only one nucleus (A).

detect focal adhesion structures. This turned out to be a considerable problem since the automated acquisition program could not always accurately focus on focal adhesions. At first I attempted in collaboration with mathematicians to develop more sophisticated recognition algorithms but finally decided to carry out visual phenotypic analysis by eye. Visual scoring of hits turned out to be a reproducible approach to identify novel focal adhesion regulators in a high-throughput microscopy assay.

I performed blind visual inspection of acquired images and assigned phenotypes diverging from the negative control to two categories based on the size of focal adhesions: 1) with enlarged focal adhesions and 2) with smaller focal adhesions. I chose the size of adhesions as the main feature for classification because first of all it could be relatively easily estimated by eye. Secondly, I assumed that defects in focal adhesion turnover were reflected in their dimensions: deregulation of focal adhesion assembly would lead to the decrease in focal adhesion size, whereas defects in the process of disassembly would cause enlargement of adhesion structures. To make analysis more reliable I performed and analyzed three replicate experiments. A gene was assigned to one of the categories if it was similarly scored in at least two replicate arrays and if the identical defect was seen in at least 50% of the acquired cells.

Knockdown of 10% of the screened genes (38 out of 386) showed visible changes in focal adhesion size. Two thirds of the scored siRNAs (22 out of 38) lead to the reduction in focal adhesion size rather than enlargement (Table 1) and corresponded to putative positive regulators. In addition to the adhesion alterations I could see a broad spectrum of distinct defects in cellular morphology and actin cytoskeleton organization. To simplify classification of the phenotypes I concentrated only on the two opposite defects on actin distribution: appearance of strong stress fibers and loss of organized actin

Group	#	Gene	Focal Adhesions	Actin	siRNAs	Published phenotypes	Validated phenotypes	RT-PCR
RhoGTPases	1	ARHN	Bigger		1 out of 2			
	2	ARHGAP5	Bigger	SF	1 out of 4			
	3	DLC1	Bigger		1 out of 6			
	4	ABR	Bigger	SF	1 out of 4			
	5	AKAP13	Bigger	SF	1 out of 6			
	6	TIAM1	Bigger		1 out of 2			
	7	GEFT	Bigger	SF	1 out of 4			
Focal Adhesion	8	ANXA2	Bigger	SF	1 out of 6			
	9	TPM1	Bigger	SF	1 out of 8			
	10	FLNC	Bigger	SF	1 out of 2			
	11	TM4SF6	Bigger		1 out of 2			
	12	PAK2	Bigger	SF	1 out of 2			
	13	PAK4	Bigger	SF	1 out of 12			
	14	TLN1	Bigger	SF	1 out of 2			
	15	PXN	Bigger		1 out of 2			
RhoGTPases	16	CDC42	Smaller	Disorganized	2 out of 4			
	17	Rac1	Smaller	Disorganized	2 out of 6			
	18	RhoB	Smaller	Disorganized	1 out of 2			
	19	ARHGAP4	Smaller	Disorganized	1 out of 2			
	20	SRGAP1	Smaller	Disorganized	1 out of 2			
Motors	21	KIF11	Smaller		1 out of 2			
	22	KIF1A	Smaller	Disorganized	1 out of 2			
	24	MYH9	Smaller	Disorganized	2 out of 2			
Focal Adhesion	25	MACF1	Smaller	Disorganized	1 out of 4			
	26	MLPH	Smaller	Disorganized	1 out of 2			
	27	AP1B1	Smaller		1 out of 2			
	28	ITG5B	Smaller		1 out of 2			
	29	ILK	Smaller		1 out of 6			
	30	FYN	Smaller		1 out of 6			
	31	FBLP-1	Smaller		1 out of 6			
	32	PTK2	Smaller		1 out of 4			
	33	NF2	Smaller	Disorganized	1 out of 8			
	34	PIK3R1	Smaller		1 out of 5			
	35	PRKCA	Smaller		1 out of 2			
	36	MYLIP	Smaller	Disorganized	1 out of 2			
	37	NCK2	Smaller	Disorganized	1 out of 6			
	38	MAPK1	Smaller	Disorganized	1 out of 4			

Table 1 | List of the scored genes with annotation of the siRNA knockdown phenotypes. | The “Focal adhesion” column shows the effect of gene knockdown on focal adhesion size. The “Actin” column shows the effect of gene knockdown on the organization of the actin cytoskeleton (SF – formation of prominent stress fibers, Disorganized – loss of organized actin filaments). “siRNAs” column indicates how many different siRNA oligonucleotides for each gene were spotted onto microarrays and how many of them gave similar defective phenotypes (for example 2 siRNA out of 4 spotted produced similar phenotype). The last three columns indicate how many gene knockdown phenotypes were previously published, were validated by conventional siRNA transfection or by RT-PCR.

filaments (in Table 1 marked as Disorganized actin). A clear correlation was observed between focal adhesion enlargement and formation of actin stress fibers, while smaller focal adhesions were in half of the cases accompanied by the loss of organized actin. These observations were consistent with the demonstration that the level of actin-myosin contractility affects focal adhesion organization (Burridge and Chrzanowska-Wodnicka 1996).

To test whether the microarray siRNA phenotypes were reproducible I randomly selected several genes from the hit list and repeated knockdown with exactly the same oligonucleotides but using “conventional” oligofectamine transfection. 13 out of 15 chosen oligonucleotides caused focal adhesions alterations identical to the ones seen on the microarray chips. Figure 3 represents comparison of the original microarray adhesion phenotypes that were scored with 25x magnification and reproduced phenotypes that were acquired with 63x magnification. The difference between enlarged and diminished focal adhesions in comparison with the control phenotype became even more obvious when images were acquired with 63x magnification upon individual siRNA transfections. The aforementioned correlation between focal adhesion size and actin organization was also reproduced (Figure 4). Two oligonucleotides targeting PTK2 and MLPH genes caused the defects in cell morphology and focal adhesion pattern but focal adhesion size remained similar to control phenotype.

For 6 siRNAs I measured the mRNA knockdown efficiency in transfected cells by quantitative reverse-transcriptase PCR (qRT-PCR). I found that 4 siRNAs (Rac1, ITGB5, MACF1, MYH9) suppressed the mRNA level of the targeted gene by 70-80% (Figure 5). For Cdc42 the residual mRNA level was less than 50% and for RhoB - around 80%. Since the protein and mRNA levels should mirror each other I assumed that the observed siRNA knockdown phenotypes resulted from the loss of corresponding protein function. However the appearance of visible loss-of-function phenotype does not always correlate with mRNA level and depends on the properties of particular protein. In the case of CDC42 and RhoB for example even a small reduction in mRNA level was sufficient to induce defects in focal adhesion organization.

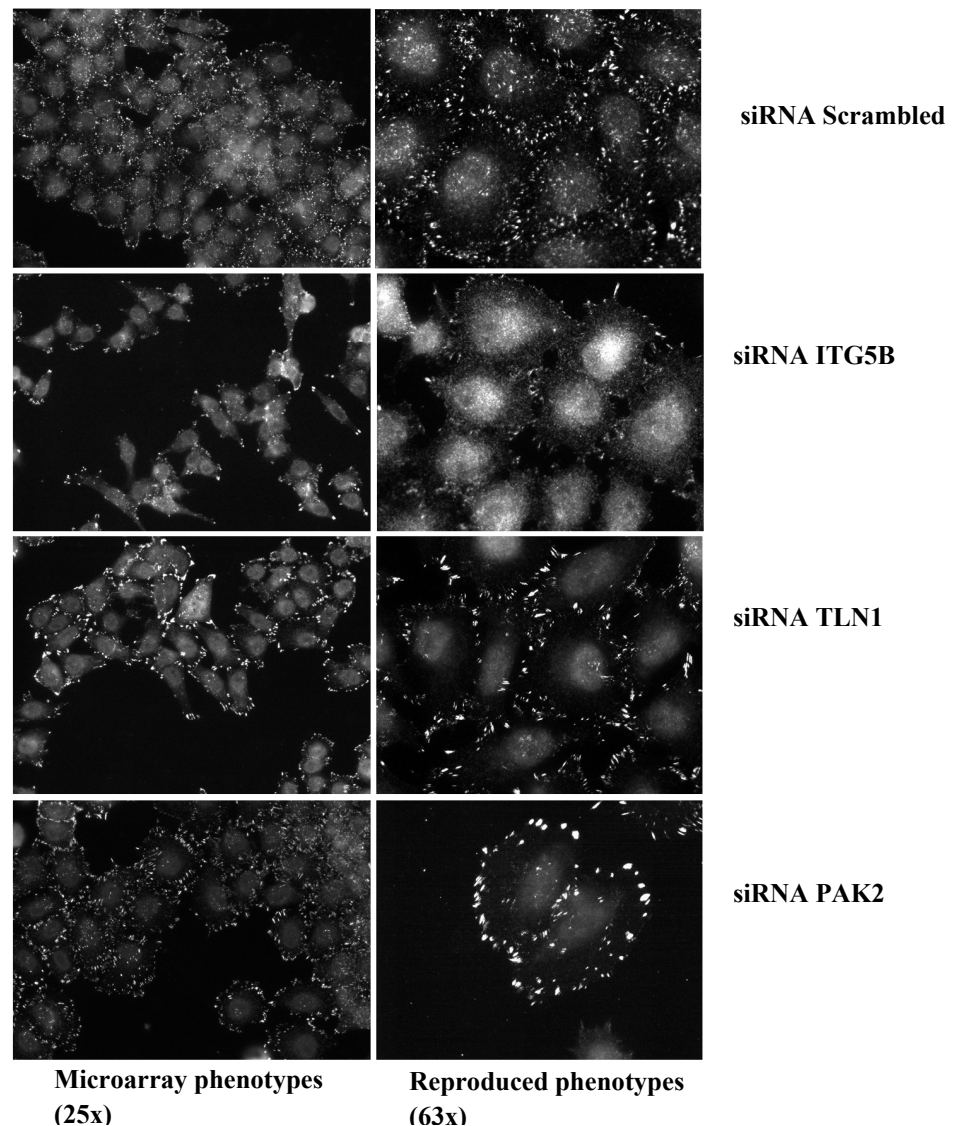


Figure 3 | Defective focal adhesion phenotypes were reproduced by “conventional” siRNA transfection. | Left column shows selective images that were acquired during microarray screen at 25x magnification. Right column represents images of the cells exposed to “conventional” transfection with identical siRNA oligonucleotides and acquired at 63x magnification. For example knockdown of beta5-integrin (ITGB5) by “conventional” siRNA transfection confirmed reduction in focal adhesion size detected in the microarray screen. Knockdown of talin1 (TLN1) and PAK2 kinase lead to the reproduced enlargement of adhesions. Focal adhesions were visualized by immunostaining with anti-paxillin antibody.

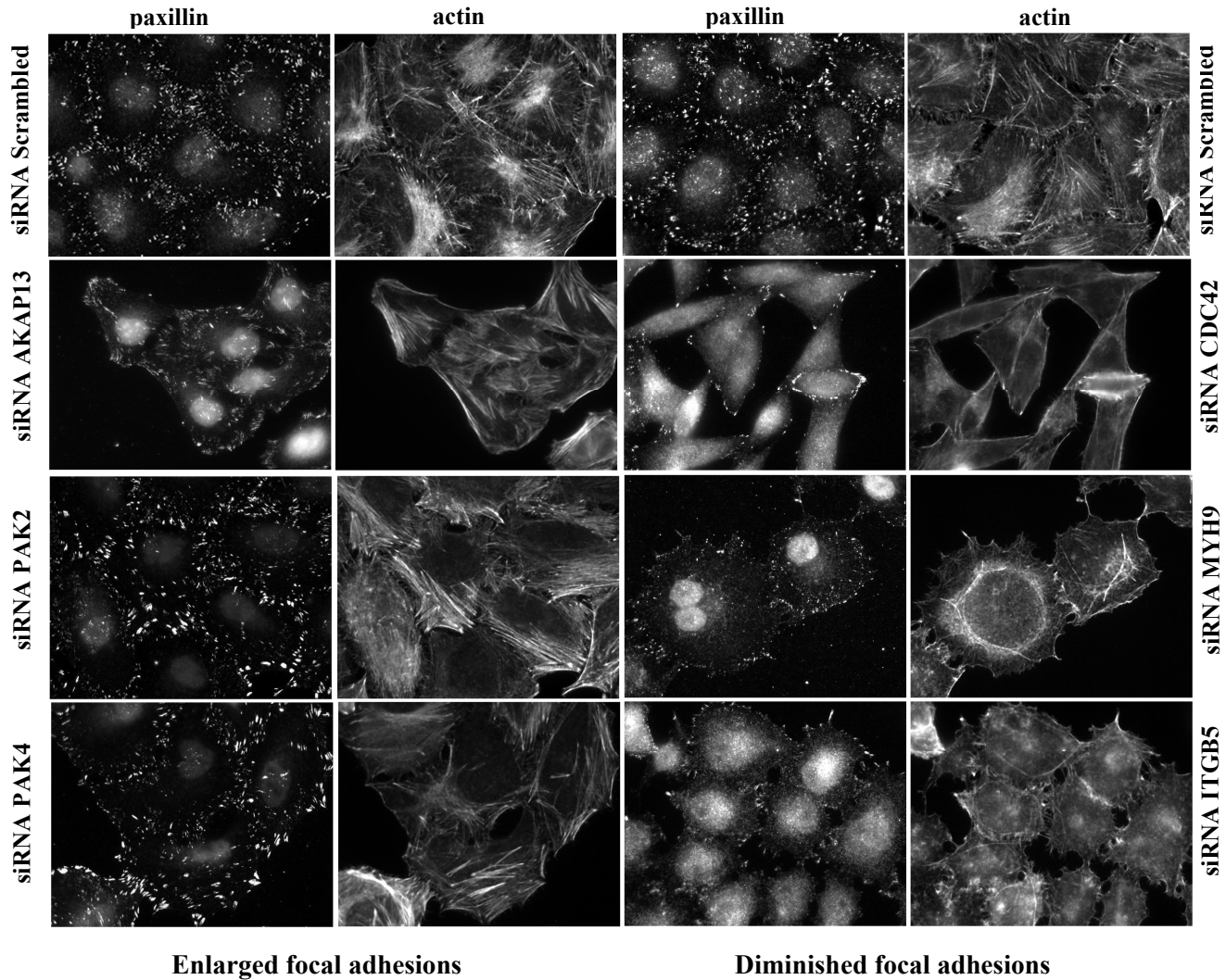


Figure 4 | Reproduced siRNA phenotypes confirmed interconnection between focal adhesion size and actin cytoskeleton organization. | Two left columns show examples of enlarged focal adhesions accompanied by formation of strong actin stress fibers upon siRNA knockdown of AKAP13, PAK2 and PAK4 genes. Two right columns demonstrate reduced focal adhesions correlated with disarrangement of actin filaments upon suppression of CDC42, MYH9 and ITGB5 genes. Focal adhesions were visualized by anti-paxillin antibody staining, actin was stained with phalloidin. siRNA knockdown was performed using “conventional” siRNA transfection. Images were acquired at 63x magnification.

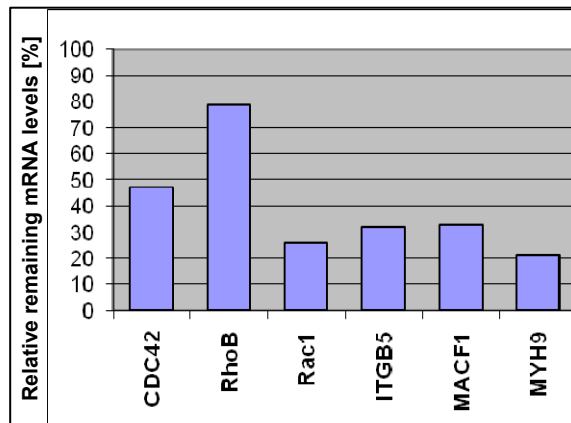


Figure 5 | RT-PCR analysis confirmed siRNA knock-down efficiency. | siRNA knockdown was performed by “conventional” transfection. Remaining mRNA expression levels were determined 50 hours after transfection and are shown as an average of the three independent experiments.

Among the identified 38 genes were 5 for which the effect of siRNA knockdown on focal adhesion size was previously studied by independent research groups. For example down-regulation of the main RhoGTPase Rac1 or class II nonmuscle myosin heavy chain MYH9 was shown to prevent formation of focal adhesions and lead to a significant reduction in focal adhesion size (Yip, El-Sibai et al. 2007; Li, Friedmann et al. 2008), and depletion of p21-associated kinase PAK2 caused RhoA activation and an increase in focal adhesion size (Coniglio, Zavarella et al. 2008). As the microarray phenotypes resembled the published ones I concluded that the established microarray siRNA platforms could be suitable to detect genes directly or indirectly affecting focal adhesion size. While I was searching for the published data regarding influence of gene knockdowns on focal adhesion organization particular on focal adhesion size I noticed that very often researches limited their studies of siRNA gene knockdowns to general characterization of cell migration or cell spreading. But the changes in focal adhesions and actin cytoskeleton, main cellular components involved in these processes, remained unexplored. siRNA depletion of integrin-linked kinase (ILK) was shown to impair spreading of HeLa cells on fibronectin as well as migration in a Transwell motility assay (Fukuda, Chen et al. 2003). One of the possible reasons for the described ILK-knockdown defects might be deregulation of focal adhesion turnover. And indeed the microarray screen detected a decrease in focal adhesion size upon ILK siRNA transfection. Therefore focal adhesion microarray data provides a basis to identify genes potentially involved in cell adhesion and migration.

While I was able to identify proteins affecting focal adhesion size, I expected to score more than 10% genes since the majority of chosen siRNAs targeted genes that were shown or predicted to play a role in cell migration, spreading or cytoskeleton architecture. A representative example of the gene which was not scored in the primary screen but was expected to be scored is dynamin-2 (DNM2), a GTPase involved in endocytosis. In the laboratory of G. Gundersen it was demonstrated that siRNA depletion of dynamin-2 lead to a twofold enlargement of focal adhesions due to its apparent function in focal adhesion disassembly (Ezratty, Partridge et al. 2005). However when I performed dynamin-2 siRNA knockdown in HeLa cells by conventional transfection with previously published siRNA oligonucleotide, a significant increase in focal adhesion size was observed (Figure 6), confirming the Ezratty et al. data.

One of the possible reasons why fewer genes than expected were scored might be the manual annotation of the hits. During visual image inspection only the most obvious phenotypes were considered, whereas less visible changes in focal adhesion size were ignored. In addition it was complicated to evaluate phenotypes of the siRNA spots with higher cell density where cells were closely packed; that could also be a source of mistakes during visual scoring.

Another potential reason for low hit number could be a low efficiency of siRNA knockdowns due to suboptimal design of the siRNA oligonucleotides. I counted the number of different siRNA sequences targeting the same gene which

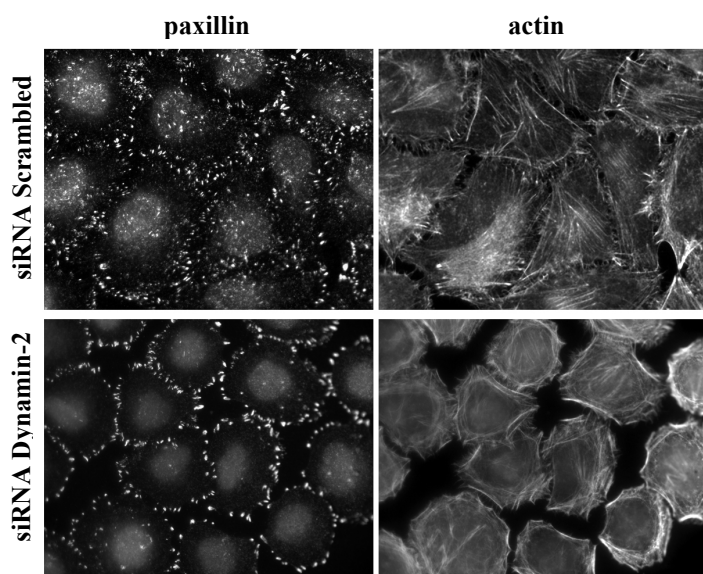


Figure 6 | siRNA knockdown of dynamin 2 produces enlargement of focal adhesions. | Knockdown of dynamin 2 was performed by “conventional” siRNA transfection using siRNA oligonucleotide published by G. Gunderson laboratory (Ezratty et al., 2005). Focal adhesions were visualized by anti-paxillin antibody staining, actin was stained with phalloidin. Images were acquired at 63x magnification.

produced similar focal adhesion phenotypes (Table 1). For only 3 out of the 38 scored genes were there two distinct siRNAs which caused identical knockdown defects. For the rest of the genes only one oligonucleotide gave a clear phenotype, despite the use of more than 4 siRNAs for 50% of the targets. Discounting mistakes of visual scoring I could conclude that only 30% of the designed oligonucleotides efficiently produced a loss of function phenotype.

The siRNA knockdown efficiency and appearance of loss-of-function phenotype is also dependent on the protein's half-life. Depletion of stable, long-lived proteins could require prolonged incubation time upon siRNA transfection. In the established microarray screen cell density was the limiting factor and restricted the knockdown period to around 50 hours. Thereby some of the genes coding stable proteins that affect adhesion turnover could have escaped detection.

In conclusion I demonstrated that the siRNA microarray technique could be applied for detection of proteins involved in focal adhesion organization. However the method allows only a superficial rather than detailed systematic analysis, at least with the experimental design employed here. Nevertheless it may be useful for fast identification of novel genes affecting selected processes.

Monitoring of focal adhesion assembly and disassembly processes on siRNA microarrays

In my assay for focal adhesion regulators I assumed that the size of adhesions would reflect the defects either in their assembly or disassembly processes. With the aim to more easily distinguish between focal adhesion assembly and disassembly regulators I investigated ways to amplify these two events separately in cells exposed to siRNA action.

It was already shown some time ago discovered that disruption of microtubules in fibroblasts or epithelial cells by nocodazole typically causes amplification in focal adhesion size and formation of strong stress fibers owing to increased RhoGTPase activity (Bershadsky, Chausovsky et al. 1996; Enomoto 1996). Gunderson and colleagues recently demonstrated that upon microtubule re-polymerization after nocodazole washout, focal adhesions undergo

disassembly to their initial state (Ezratty, Partridge et al. 2005). Therefore monitoring changes in adhesion size upon nocodazole treatment and its subsequent wash-out can serve as an assay for identification of focal adhesion assembly and disassembly factors.

I adopted the nocodazole wash-out protocol from the Gunderson laboratory for the use with microarray siRNA platforms. HeLa cells were grown on the LabTeck slides in triplicate with spotted siRNA oligonucleotides. One of the three microarray replicas was fixed 50 hours after plating the cells. The two other replicas were treated with nocodazole after 1 hour serum starvation. One replica of the two was fixed following 4 hours incubation with nocodazole. The last slide was subjected to nocodazole wash-out for two hours and also fixed (Figure 7). All slides were stained with Hoechst 33342, an antibody against focal adhesion protein paxillin and phalloidin to label respectively nuclei, focal adhesions and the actin cytoskeleton, and images from each siRNA spot were automatically acquired. Parallel analysis of the three replicas facilitated comparison of the effect of the same siRNA gene knockdown on focal adhesion organization in standard conditions, on focal adhesion assembly stimulated by

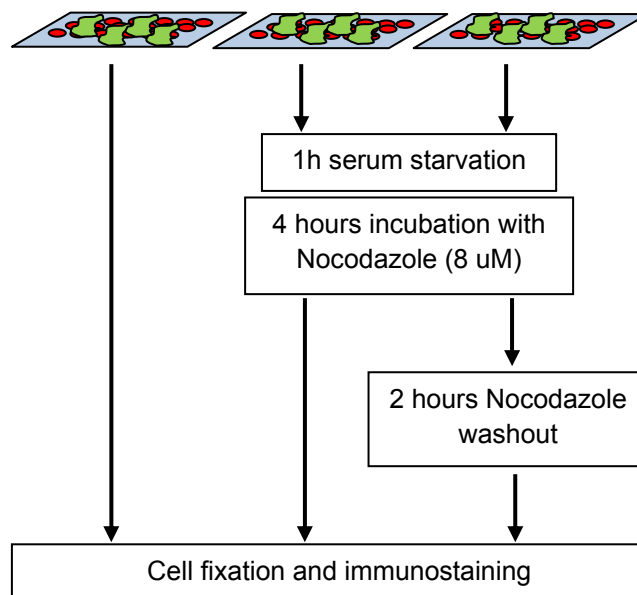


Figure 7 | Schematic representation of nocodazole assay. | HeLa cells were seeded onto three microarray replicas. After 50 hours incubation time only one replica was fixed. Two other replicas were exposed to 1 hour serum starvation following by 4 hours incubation with nocodazole (8 μ M). Afterward one of the replicas was fixed and the second one was subjected to nocodazole wash-out, incubated for 2 hours and also fixed. All microarrays were stained with anti-paxillin antibody, phalloidin and Hoechst 33342 to visualize focal adhesions, actin and nuclei respectively.

nocodazole, and on focal adhesion disassembly induced by nocodazole washout.

Visual inspection of the images revealed that cells from microarray spots with negative control siRNAs indeed showed a significant increase in focal adhesion size upon nocodazole addition accompanied by formation of prominent actin stress fibers. Nocodazole washout leads to the reversion of focal adhesions to their initial state and disappearance of stress fibers (Figure 8).

For the first screening round the nocodazole assay was repeated twice with two siRNA microarray platforms created in the previously described part of the project and containing siRNA oligonucleotides to knockdown 240 genes including RhoGTPases, their regulators and motor proteins (Supplementary tables 3, 4). A gene product was considered to be involved in focal adhesion assembly if upon knockdown nocodazole failed to produce enlargement of cellular adhesions. Conversely, a gene product was expected to participate in focal adhesion disassembly if its knockdown prevented reduction of focal adhesions to their initial size after nocodazole wash-out. A phenotype was scored if it was observed in both experiments.

Knockdown of only 4 genes lead to a strong inhibition of focal adhesion growth upon nocodazole treatment. Those genes were MYH9 (non-muscle Myosin IIa heavy chain), RhoB, ARHGDI and SH3BP1 (Figure 9). Focal adhesion assembly defects were reproduced using conventional siRNA transfection to knockdown MYH9 and RhoB (Figure 10). It is interesting to mention that among 240 examined genes suppression of 8 genes including

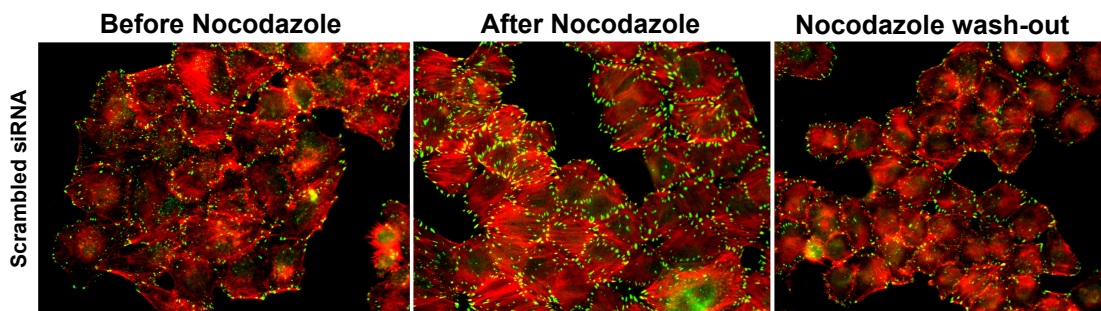


Figure 8 | Assembly of focal adhesions upon nocodazole treatment and disassembly to the initial state after nocodazole wash-out. | Presented images were acquired from the same scrambled siRNA spot on the three microarray replicas. First replica was fixed before treatment, second replica was fixed after 1 h starvation and 4 h incubation with nocodazole, third replica was fixed after 2 h nocodazole wash-out. Assembly of focal adhesions was accompanied by stress fibers formation that was reversible upon nocodazole wash-out. Focal adhesions were visualized by anti-paxillin antibody staining (green), actin was stained with phalloidin (red). Images were acquired at 25x magnification.

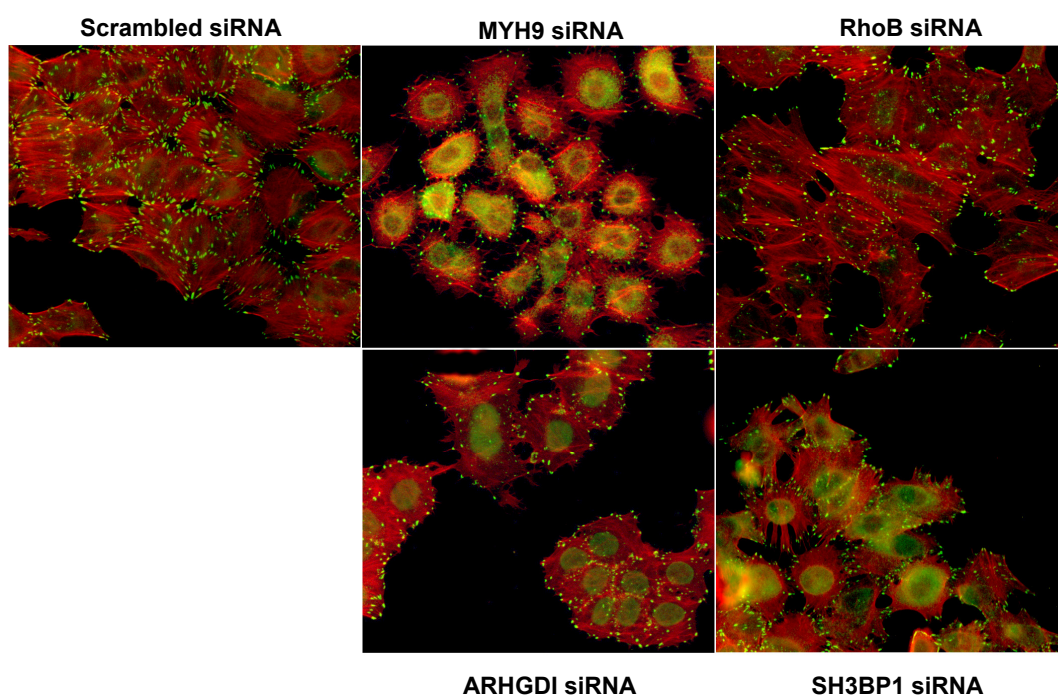


Figure 9 | Enlargement of focal adhesions upon nocodazole treatment was abrogated after siRNA knockdown of MYH9, RhoB, ARHGDI and SH3BP1. | Presented images were acquired from microarrays fixed after 4 h nocodazole treatment. Focal adhesions were visualized by anti-paxillin antibody staining (green), actin was stained with phalloidin (red). Images were acquired at 25x magnification.

MYH9 and RhoB (Table 1) caused defects in focal adhesion formation in standard conditions. However upon nocodazole addition only MYH9 and RhoB genes appeared to be essential for stimulated assembly of focal adhesions whereas the other 6 genes were not necessary for this process. This means that microtubule depolymerization by nocodazole activates focal adhesion assembly by bypassing proteins involved in a normal focal adhesion formation under standard conditions.

Interestingly ARHGDI and SH3BP1 were identified as regulators of focal adhesion assembly only after nocodazole treatment. Most likely these genes influence focal adhesion formation also in a standard situation but the effects of their depletion on focal adhesion size are compensated by other factors and therefore not detected. Stimulation of focal adhesion assembly by nocodazole apparently reduces the impact of other regulators and exposes the role of ARHGDI and SH3BP1 in this process.

The process of focal adhesion disassembly upon nocodazole wash out was less efficient and therefore difficult to score. Also cell populations on siRNA spots did not react uniformly to microtubule re-polymerization upon nocodazole removal. Figure 11 illustrates an example of evenly enlarged focal adhesions in nocodazole treated cells and heterogeneity in focal adhesion dimensions after wash out of the drug. This fact might indicate that adhesion disassembly induced by nocodazole washout is a more complex process with increased dependence on intracellular and environmental conditions.

In the course of nocodazole assay many cells were deattaching (cells marked with asterisks, Figure 11) probably due to a combination of several factors such as siRNA knockdown, serum starvation and microtubule depolymerization. The detached cells affected the performance of the autofocus program and led to the accumulation of out-of-focus images, adding on additional complication to this assay.

Nevertheless I demonstrated that the nocodazole assay may be exploited to identify some proteins required for focal adhesion assembly that would be missed in the standard control situation. However the assay was not robust enough for identification of disassembly regulators during microtubule re-

polymerization. Based on these conclusions the idea of performing a further large-scale siRNA screen on the basis of nocodazole assay was set aside.

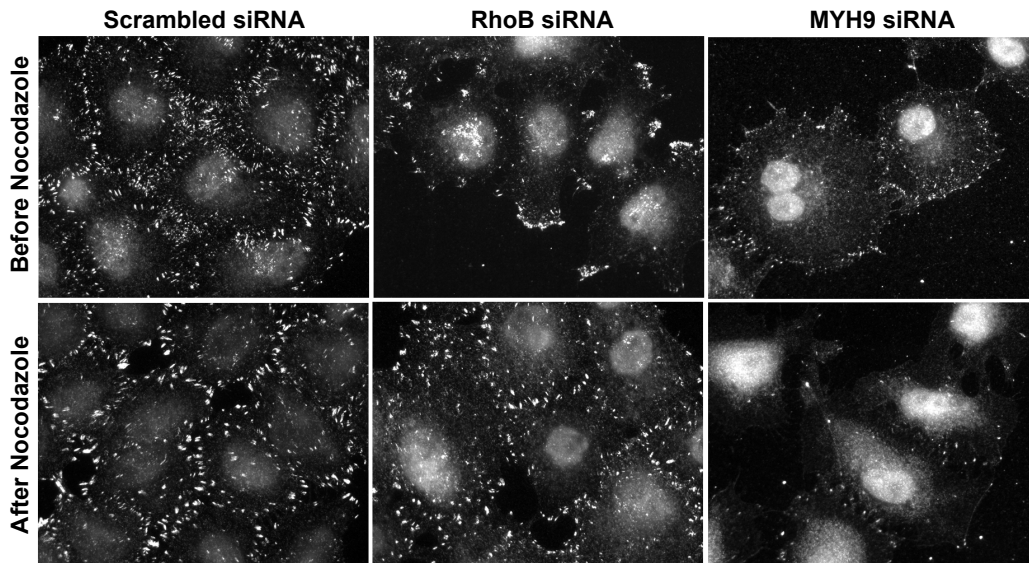


Figure 10 | Defects in focal adhesion assembly upon nocodazole addition were reproduced by “conventional” siRNA transfection. | Upon 50 h incubation after “conventional” siRNA transfection cells were fixed before and after 4 h treatment with nocodazole. Suppression of RhoB and MYH9 genes leads to the reduction in focal adhesion size already in the standard conditions and prevents focal adhesion assembly after nocodazole addition. Focal adhesions were stained with anti-paxillin antibody. Images were acquired at 63x magnification.

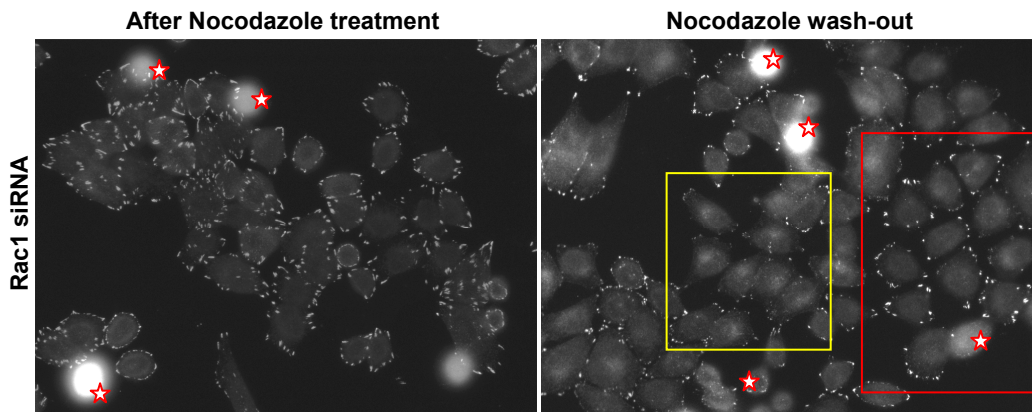


Figure 11 | Illustration of heterogeneity in focal adhesion disassembly upon nocodazole wash-out by example of Rac1 depleted cells. | Images were acquired from the Rac1 siRNA spot after 4 h nocodazole treatment and after 2 h upon nocodazole wash-out. The whole population of nocodazole-treated cells displays equally enlarged focal adhesions. However upon nocodazole wash-out cells exhibit different levels of focal adhesion disassembly. Yellow and red rectangles highlight group of cells with high and low adhesion disassembly rates. Asterisks mark cells that detached in the process of nocodazole assay. Focal adhesions were stained with anti-paxillin antibody. Images were acquired at 25x magnification.

Identification of factors involved in vesicular trafficking and affecting focal adhesion turnover

As already indicated the RNAi screen was carried out in close collaboration with Rainer Pepperkok's group in EMBL, Heidelberg. They have developed and performed a genome-wide siRNA microarray screen to systematically study proteins affecting the vesicular trafficking machinery in HeLa cells (Simpson, Cetin et al. 2007). Their assay was based upon the transport of a temperature sensitive viral transmembrane protein (tsO45G) from the ER, through the Golgi, to the cell surface. Automated image analysis with subsequent single-cell quantification of the protein transport efficiency allowed identification of the factors that are essential for the trafficking to plasma membrane. In total they scored around 700 genes involved in that process. To investigate the link between focal adhesion turnover and vesicular trafficking to the plasma membrane I decided to monitor focal adhesion changes using the microarray slides (Supplementary Table 6) containing trafficking hits kindly provided by Jez Simpson and Rainer Pepperkok.

The procedure of the screen was similar to the one described in the first chapter. Briefly, HeLa cells were plated onto siRNA microarrays, fixed after 50 hours incubation and stained to visualize focal adhesions, actin and nuclei. Images were acquired with the help of automated microscopy and subjected to manual inspection. The screen was carried out in duplicate. I selected hits according to the defects in focal adhesion size dividing them into two groups – with enlarged and reduced focal adhesions. In addition I monitored changes in actin cytoskeleton, paying attention to the formation of prominent stress fibers and loss of organized actin structure. The gene was scored as a hit if a similar defective phenotype was seen in both experiments.

I identified 87 genes whose knockdown caused a strong alteration in focal adhesion size (Table 2). Only 15% of phenotypes represented reduction in focal adhesion size whereas the majority of the phenotypes showed focal adhesion enlargement accompanied by the appearance of strong stress fibers in 25% of the cases.

Group	#	Gene	Focal Adhesions	Actin	siRNAs	Golgi
Cytoskeleton (Cyt)	1	iqsec1	Bigger		2 out of 3	
	2	kif19	Bigger	SF	1 out of 1	
	3	spnb2	Bigger		1 out of 1	
	4	tm4sf19	Bigger		1 out of 2	
	5	rap1gds1	Bigger		1 out of 3	
Trafficking (Trf)	6	arcn1	Bigger		1 out of 1	
	7	copb2	Bigger		1 out of 1	
	8	copg	Bigger		1 out of 1	
	9	copb	Bigger		1 out of 1	
	10	rabac1	Bigger		1 out of 1	
	11	skd3	Bigger		2 out of 2	
Transmembrane proteins, receptors (TmR)	12	agtrap	Bigger		1 out of 1	
	13	flt3lg	Bigger	SF	1 out of 1	
	14	glra1	Bigger		2 out of 3	
	15	il1f5	Bigger	SF	1 out of 1	
	16	sez6l2	Bigger	SF	1 out of 1	
	17	eps15l2	Bigger		1 out of 1	
	18	flt4	Bigger		2 out of 3	
	19	fprl2	Bigger		1 out of 1	
	20	grin2c	Bigger	SF	1 out of 1	
	21	ms4a6a	Bigger		1 out of 3	
	22	or10a4	Bigger		2 out of 3	
	23	or4p4	Bigger		1 out of 1	
	24	tyrobp	Bigger		2 out of 3	
	25	sgcg	Bigger	SF	1 out of 1	
	26	np_775772.1	Bigger		1 out of 1	
	27	ccdc51	Bigger		1 out of 1	
	28	loc653383	Bigger	SF	1 out of 1	
	29	reg4	Bigger		1 out of 1	
Kinases (K)	30	cdk5r1	Bigger		1 out of 1	
	31	khk	Bigger		1 out of 3	
	32	ppapdc1	Bigger	SF	1 out of 3	
	33	loc646643	Bigger		1 out of 1	
	34	mast3	Bigger		1 out of 2	
Transcription factors, nuclei proteins (Nuc)	35	nhp2l1	Bigger		1 out of 1	
	36	znf10	Bigger	SF	1 out of 1	
	37	bcorl2	Bigger		2 out of 3	
	38	dqx1	Bigger	SF	2 out of 3	
	39	hscarg	Bigger	SF	1 out of 2	
	41	mxd4	Bigger		1 out of 3	
	42	oa48-18	Bigger		1 out of 2	
	43	pml	Bigger	SF	2 out of 3	
	44	mcm7	Bigger		2 out of 2	
	45	polr3f	Bigger	SF	1 out of 3	
	46	tead1	Bigger		1 out of 1	
	47	ccdc124	Bigger		1 out of 1	

Table 2 | List of the genes whose knockdown caused vesicular trafficking and focal adhesion defects. | Genes were grouped according to known or predicted functions of corresponding proteins. The “Focal adhesion” column shows the effect of gene knockdown on focal adhesion size. The “Actin” column shows the effect of gene knockdown on the organization of the actin cytoskeleton (SF – formation of prominent stress fibers). “siRNAs” column indicates how many different siRNA oligonucleotides for each gene were spotted onto microarrays and how many of them gave similar defective phenotype (for example 2 siRNAs out of 2 spotted produced similar phenotype). The last column indicates (red boxes) about defects in Golgi apparatus organization revealed by staining with anti-GM130 antibody.

Group	#	Gene	Focal Adhesions	Actin	siRNAs	Golgi
Unknown function (UF)	48	iqcf1	Bigger		1 out of 2	
	49	notch2nl	Bigger		1 out of 1	
	50	loc348180	Bigger		1 out of 1	
	51	9430057o19rik	Bigger		1 out of 1	
	52	loc401565	Bigger		1 out of 1	
	53	spryd4	Bigger		1 out of 1	
	54	thap4	Bigger		1 out of 1	
	55	loc649917	Bigger		1 out of 1	
	56	znf167	Bigger		1 out of 3	
	57	q8n507_human	Bigger		1 out of 1	
	58	q8n779_human	Bigger		1 out of 1	
	59	q8wuc7_human	Bigger		1 out of 1	
	60	q96nc1_human	Bigger		1 out of 1	
	61	xp_370634.2	Bigger		1 out of 1	
	62	dkfzp434g072	Bigger	SF	2 out of 2	
	63	c19orf165	Bigger	SF	2 out of 2	
	64	c5orf17	Bigger		1 out of 2	
	65	flj10781	Bigger		1 out of 3	
	66	mgc20398	Bigger	SF	2 out of 2	
Diverse Functions (Df)	67	rplp2	Bigger	SF	1 out of 1	
	68	adam20	Bigger		1 out of 1	
	69	insl6	Bigger		1 out of 1	
	70	ipo8	Bigger		1 out of 1	
	71	npc1l1	Bigger		1 out of 1	
	72	sst	Bigger	SF	1 out of 2	
	73	npw	Bigger	SF	2 out of 2	
	74	ndnl2	Bigger		2 out of 3	
Cyt	75	itgav	Smaller		1 out of 1	
	76	itgb5	Smaller		1 out of 1	
	77	rhof	Smaller		1 out of 3	
Trf	78	c9orf19	Smaller		1 out of 3	
Tmr	79	fgf19	Smaller		1 out of 1	
	80	tva2_human	Smaller		1 out of 1	
	81	egfl6	Smaller		1 out of 3	
Nuc	82	hoxc9	Smaller		1 out of 1	
	83	rela	Smaller		1 out of 1	
	84	kiaa0363	Smaller		1 out of 1	
Uf	85	o15421_human	Smaller		1 out of 1	
Df	86	dnajc4	Smaller		1 out of 1	

Table 2 | List of the genes whose knockdown caused vesicular trafficking and focal adhesion defects. | Genes were grouped according to known or predicted functions of corresponding proteins. The “Focal adhesion” column shows the effect of gene knockdown on focal adhesion size. The “Actin” column shows the effect of gene knockdown on the organization of the actin cytoskeleton (SF – formation of prominent stress fibers). “siRNAs” column indicates how many different siRNA oligonucleotides for each gene were spotted onto microarrays and how many of them gave similar defective phenotype (for example 2 siRNAs out of 2 spotted produced similar phenotype). The last column indicates (red boxes) about defects in Golgi apparatus organization revealed by staining with anti-GM130 antibody.

For most of the genes there was only one siRNA oligonucleotide taken from the aforementioned genome-wide screen. For 34 genes additional oligonucleotides were designed by Ambion and Quiagen companies. For half of those genes (44%) two oligonucleotides with distinct sequences gave identical knockdown phenotype. However only 52% of the additionally synthesized siRNAs were efficient in gene depletion.

All identified genes were annotated with the help of Bioinformatic Harvester database (<http://harvester.fzk.de/harvester/>) and assigned to various functional groups based on the known or predicted functions of corresponding proteins: cytoskeleton related proteins, vesicular trafficking related proteins, receptors and transmembrane proteins, transcription factors and other nuclear proteins, proteins with unknown functions and with known miscellaneous functions including ribosomal proteins (rplp2, npc111), signaling peptides (sst, npw, insl6), metalloproteinase (adam20).

I decided to narrow the number of final hit-genes by performing an additional secondary screen. As a parameter for screening I chose the morphology of the Golgi apparatus, the central membrane structure in the secretory trafficking pathway. The Golgi complex in mammalian cells is organized in the form of continuous ribbon-like membrane structure positioned in the perinuclear area. An appropriate coordination of membrane traffic to and from the Golgi is essential to preserve its organization and function. Therefore an alteration in Golgi morphology could serve as a marker of trafficking defects. For example it was shown that siRNA knockdown of Golgi-localized protein golgin45 lead to the dispersal of Golgi ribbon into small vesicular fragments and at the same time caused an almost complete block in transport of VSVG protein (Short, Preisinger et al. 2001). Interference with the function of Golgi-localized serine/threonine kinase YSK1 by means of siRNA transfection also induced Golgi scattering. Notably blocking YSK1 function with a dominant-negative mutant abrogated cell migration into the wound (Preisinger, Short et al. 2004). Thereby there is a potential link not only between Golgi apparatus morphology and membrane trafficking, but also to cell migration and eventually focal adhesion turnover. However other cellular events can also influence Golgi

dispersal such as arrest in mitosis or defects in fatty acid and cholesterol biosynthesis (Bard, Casano et al. 2006), for this reason an interpretation of the results based on Golgi morphology should be made with care.

To monitor changes in Golgi morphology cells split onto microarrays were fixed after 48 hours incubation time and stained with antibody against Golgi peripheral membrane protein GM130. Automatically acquired images were manually scored for defects in Golgi morphology, such as fragmentation of Golgi ribbons into smaller elements or Golgi aggregation and swelling (Figure 12). The secondary screen was repeated twice and those genes that showed the same phenotype were considered. I found that 33 out of 86 genes affecting vesicular transport to plasma membrane and focal adhesion size were also necessary for the maintenance of Golgi architecture (Table 2).

Among the identified genes were several subunits of the coat protein complex COPI (copb, copb2, copg), their siRNA knockdown lead to a similar increase in focal adhesion size and aggregation of Golgi structure. COPI complex is an essential coat component of vesicles, participating in the anterograde transport within the Golgi and recycling to the ER. In the R. Pepperkokk laboratory siRNA oligonucleotides targeting beta-COP subunit (copb) has been previously validated and it has been shown that siRNA depletion of beta-COP severely reduced transport of the tsO45G protein to the cell surface (Erflé, Simpson et al. 2004). I decided to reproduce and investigate in more detail the influence of beta-COP knockdown on focal adhesion size and turnover.

I performed “conventional” transfection of HeLa cells with beta-COP siRNA and observed first of all swelling of the Golgi apparatus labeled with GM130 antibodies (not shown) and secondly formation of enlarged paxillin-stained adhesions at the cell periphery that mimicked the phenotype seen in the microarray screen (Figure 13). The focal adhesions were connected with prominent actin stress fibers aligned with the cell edge giving the cells a polygonal shape. The cell center of beta-COP depleted cells in contrast to cells transfected with scrambled siRNA was depleted of focal adhesions and actin

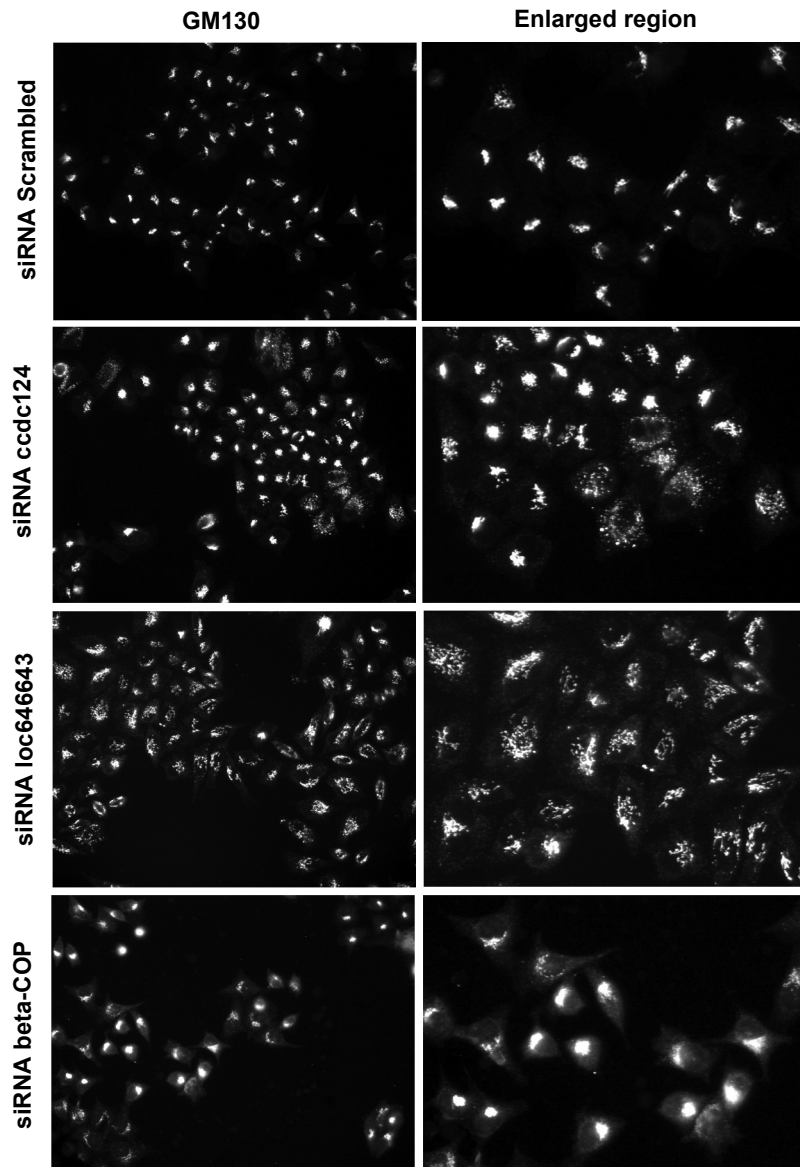


Figure 12| Defects in Golgi apparatus organization detected in microarray screen. | Left column represents examples of images acquired at 25x magnification during micro-array screen. Knockdown of *ccdc124* and *loc646643* genes caused fragmentation of the Golgi structure. Knockdown of *beta-COP* lead to the Golgi swelling. Images in the right column represent enlarged part of the image at the left. Golgi apparatus was visualized by anti-GM130 antibody staining.

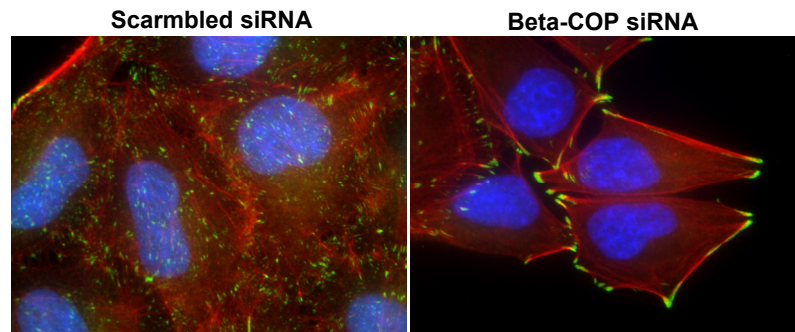


Figure 13 | siRNA knockdown of beta-COP produces enlargement of focal adhesions and formation of peripheral actin stress fibers. | HeLa cells were transfected with Scrambled or beta-COP siRNAs for 55 hours, fixed and stained with anti-paxillin antibody (green), phalloidin (red) and Hoechst 33342 (blue).

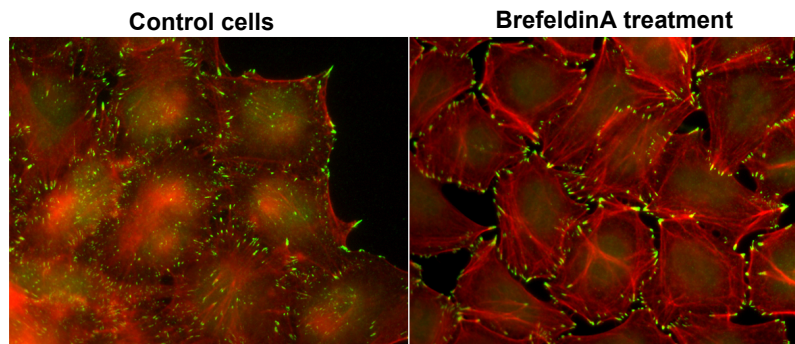


Figure 14 | Blocking of COPI- complex function with BrefeldinA lead to the enlargement of focal adhesions and formation of strong stress fibers. | HeLa cells were treated with 10 ug/ml BrefeldinA for 15 hours, fixed and stained with anti-paxillin antibody (green) and phalloidin (red).

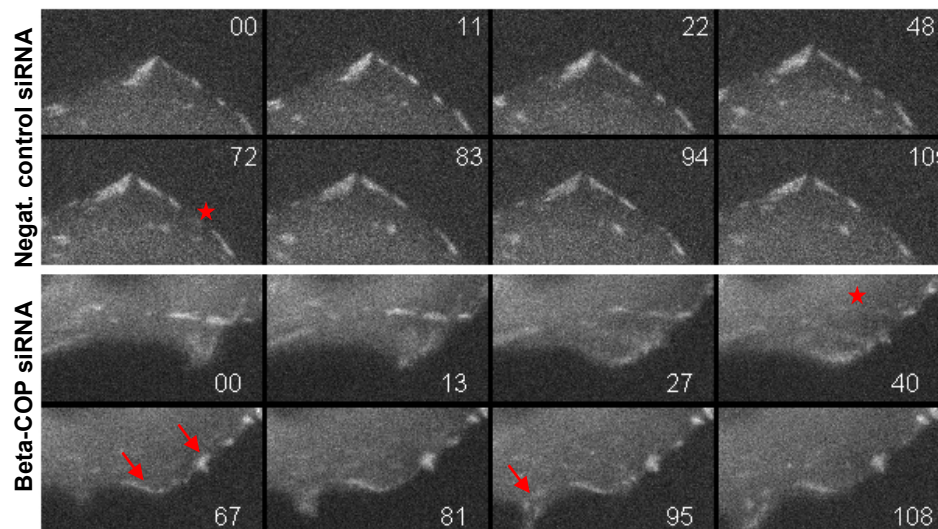


Figure 15 | beta-COP knockdown cells display increased focal adhesion turnover rate at the cell periphery. | HeLa cells with stable expression of GFP-Paxillin were transfected with negative control or beta-COP siRNAs for 55 hours and imaged for 120 minutes (1 frame per 30 sec). Arrows mark appearance of new focal adhesions at the cell edge of beta-COP depleted cells. Focal adhesions at the edge of negative control siRNA transfected cells remained mostly stable. Asterisks point to the disassembly of focal adhesions. Images are taken from Supplementary Movie 1.

filaments. Other focal adhesion proteins vinculin and zyxin were likewise associated with the more prominent peripheral focal adhesions in cells with reduced beta-COP expression (not shown).

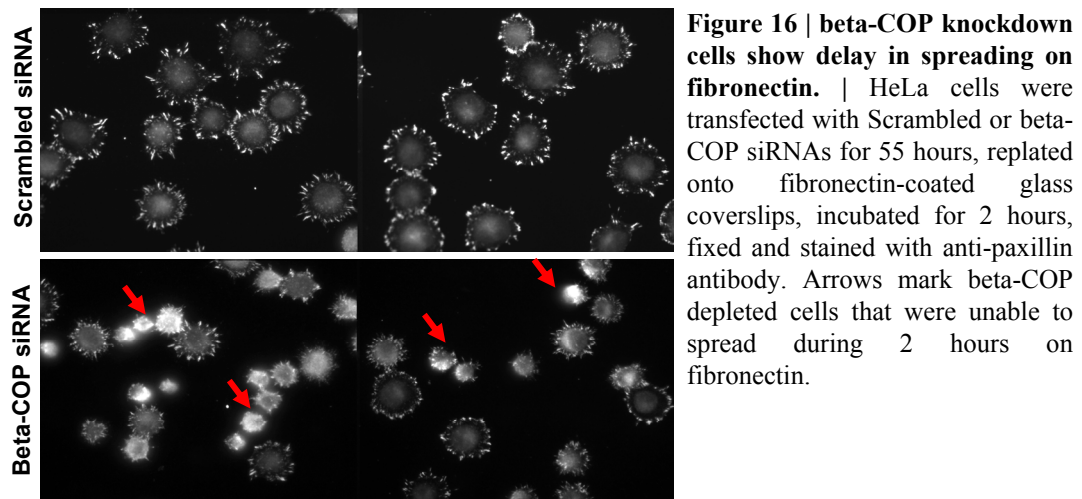
It is interesting to mention that prolonged treatment of cells with Brefeldin A, a compound that inhibits COPI complex formation and disrupts Golgi structure, also leads to the enlargement of focal adhesions at the cell periphery, disassembly of inner focal adhesions and assembly of strong stress fibers (Figure 14).

An explanation for the enlarged peripheral focal adhesions in beta-COP depleted cells might be a change in the kinetics of focal adhesion turnover. To determine whether focal adhesion dynamics was impaired upon beta-COP knockdown I performed live imaging of cells stably transfected with fluorescent focal adhesion protein GFP-Paxillin and depleted of beta-COP. During 100 minutes observation the formation and disassembly of focal adhesions at the cell periphery was markedly increased in beta-COP knockdown cells in comparison with negative control siRNA treated cells (Figure 15, Supplementary movie 1).

I also examined spreading of beta-COP depleted cells on fibronectin. Upon beta-COP siRNA knockdown HeLa cells showed delay in spreading after the first two hours (Figure 16). Collectively, these data for the first time provided the evidence of the involvement of beta-COP and thus the whole COPI complex in regulating focal adhesion organization and turnover as well as downstream processes such as cell spreading. Therefore the siRNA microarray method may be considered as a useful tool to identify a link between focal adhesion turnover and vesicular trafficking machinery.

Next I performed detailed bioinformatics and database analysis of the final hits with a focus on their domain architecture, homologues in other species and expression profile. I excluded genes that might affect membrane trafficking as well as focal adhesion state indirectly through their roles in transcription regulation, protein translation or basic metabolism. I was interested in the evolutionary conserved genes with ubiquitous expression, preferably with predicted transmembrane domains. Finally I selected three genes with unknown function for the further characterization: KIF19, CCDC51 and TM4SF19.

CCDC51 (Coiled-coil Domain Containing 51) is a 46kDa highly conserved and ubiquitously expressed protein with two predicted transmembrane domains. Kinesin family member KIF19 belongs to a N-8 kinesin subfamily with N-terminal motor domain and was shown to be expressed in brain, lung, ovary and testis (Miki, Okada et al. 2005). TM4SF19 is an uncharacterized ubiquitously expressed member of tetraspanin family with 4 transmembrane domains; the other members are involved in cell-substrate contact (Hemler 2005). Currently I am in the process of cloning the chosen genes with the further aim of determining their cellular localization and roles in vesicular transport and focal adhesion turnover.



Conclusions and Perspectives

siRNA microarray screening technique as a method to identify regulators of focal adhesion turnover

I have adapted the siRNA microarray screening approach to find genes involved in focal adhesion organization and turnover. A special candidate-set of siRNA oligonucleotides targeting genes with known or predicted roles in cell adhesion or migration was created. Using microarray platforms with spotted siRNAs from the candidate-set I have demonstrated that the microarray technique was suitable for detection of genes involved in focal adhesion turnover. However the number of identified genes was lower than I expected from the candidate set. Moreover an attempt to conduct systematical characterization of whole protein families (such as RhoGTPases or motor proteins) on the basis of their function in focal adhesion turnover was not successful. Below I will summarize and discuss the main caveats and limitations of the siRNA technique that could explain unsatisfactory efficiency of the performed screen.

First of all siRNAs containing 21-23 nucleotides are less efficient triggers of RNAi-based gene suppression than long dsRNAs that can be used in invertebrates but not in mammalian cells due to the mammalian-specific antiviral response. siRNAs with different nucleotide sequences but targeting the same gene could have different efficiency of gene knockdown. Thus the right choice of siRNA sequence is a major determinant of the effective gene suppression. Especially proper design of siRNA sequences is important in large-scale experiments such as microarray screens when a degree of gene knockdown could not be estimated. That is why most of the screens include multiple siRNAs targeting the same gene to increase the probability of obtaining efficient gene knockdown. Despite the use in my screen of 2 to 6 different siRNA sequences for each gene, only one of the siRNAs usually produced a knockdown phenotype and the proportion of the efficient siRNAs was only 30%. Therefore suboptimal siRNA oligonucleotide design might be one of the main reasons for low hit number.

Secondly I monitored loss-of-function phenotypes only at single time point (50 hours after siRNA transfection) that was determined by optimal cell

density. However the appearance of the RNAi phenotypes is dependent on the protein half-life that may significantly vary from protein to protein. In some cases even a small residual amount of protein is sufficient to maintain its function therefore knockdown of stable proteins usually requires more time. This means that the phenotypes developing only after prolonged incubation after siRNA treatment were left undetected in my assay.

Functional redundancy among proteins is the next issue that restricts identification of gene function by analysis of its knockdown phenotype (for review see (Thomas 1993)). For example only three motor proteins (two kinesins and one myosin) were identified in the screen; however more of them are expected to be involved in cytoskeleton and focal adhesion regulation. I suggest that in the absence of one particular motor protein other closely-related motors can transport its cargos. Combinatorial siRNA screen that would allow simultaneous knockdown of multiple motors in different combinations could be the next step toward uncovering motor proteins functions (for review see (Sahin, Loebke et al. 2007)).

A robust and unbiased assay is another important element that determines efficiency of the siRNA screen. Nocodazole wash-out assay is a valuable approach to dissect regulators of the focal adhesion disassembly (Ezratty, Partridge et al. 2005). But when applied to cells seeded onto microarray platforms it produced a highly variable response within a cell population and therefore was not considered as appropriate assay for large-scale experiments.

For the primary focal adhesion turnover screen I chose a simple assay based on the visual changes in focal adhesion size upon siRNA knockdown that were monitored by eye. The manual scoring procedure can not provide precise quantitative measurements and reveals only the most obvious phenotypes leaving minor changes undetected. Thus the visual analysis method could also be one of the reasons for the low number of scored hits. Development of an automated processing program extracting quantitative data from the images could improve identification of the weak phenotypes but from the other side it could also increase the rate of false-positive hits.

It was interesting to compare the results of my candidate screen with the similar siRNA-based screen performed by K. Simpson and colleagues (Simpson, Selfors et al. 2008). Using candidate siRNA library targeted genes with known or predicted roles in cell migration and adhesion they were looking for genes involved in epithelial cell migration into the wound. There were 118 common genes between their and mine siRNA libraries. Among those genes they scored 9 high confidence hits, 13 genes were picked up as hits in my screen, but only one gene was scored in both screens (Figure 17). The percentage of scored genes in Simpson's and my screen were similar, 7.6% and 11% respectively. These two screens could not be directly compared because the assays used and cell types were different. However based on the fact that cellular movement is dependent on focal adhesion turnover a higher overlap between two screens would be expected. In my opinion the problems of the siRNA knockdown method together with assay limitations compromise the outcome of the screens making them unsuitable for complete systematic analysis of gene functions. But on the other hand siRNA large-scale screens could be considered as one of the options for the fast identification of novel proteins involved in specific process.

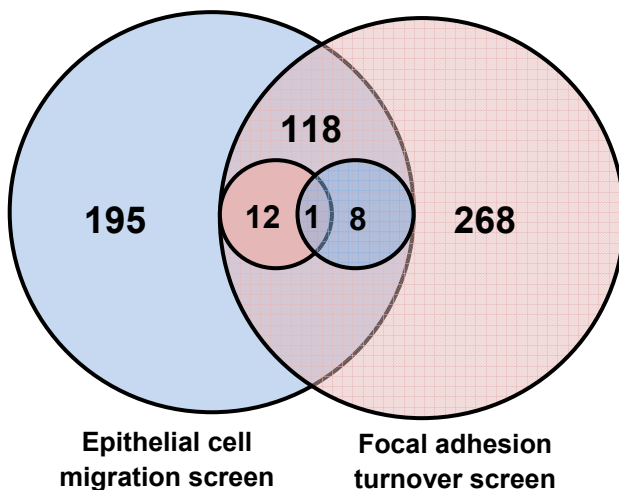


Figure 17 | Schematic comparison of screen results. | Epithelial cell migration screen was performed by Simpson and colleges (Simpson, Selfors et al. 2008) to identify inhibitors and accelerators of MCF7-cell migration into the wound (blue circles). Focal adhesion turnover screen detected regulators of focal adhesion organization in HeLa cells (pink circles). For 118 overlapped genes the hit-rates were around 10% in both screens. Only one gene was identified in both screens.

A link between vesicular trafficking and focal adhesion turnover as revealed by siRNA microarray screening

With the aim to uncover a possible connection between vesicular trafficking and focal adhesion turnover I have performed a siRNA microarray screen looking for gene products whose knockdown affects cellular trafficking machinery and at the same time causes focal adhesion defects. For the screen I used a siRNA library against subset of genes identified in a genome- wide siRNA screen as regulators of constitutive protein trafficking to the plasma membrane (kindly provided by J. Simpson and R. Pepperkok). As an outcome of the screen 33 genes important for the maintenance of focal adhesion organization as well as for the integrity of Golgi apparatus structure were detected. Disturbance of the Golgi structure was chosen as an indicator of vesicular trafficking alterations.

Among final hits there were several subunits (copb, copb2, copg) of the well-characterized COPI complex that is essential for the Golgi to the endoplasmic reticulum (ER) and intra-Golgi membrane transport. A blockade of the Golgi-to-ER retrograde transport causes perturbations of Golgi organization and subsequently of anterograde trafficking pathway including secretory protein transport. Knockdown of a beta-COP subunit (copb) was shown to reduce transport of the viral glycoprotein ts-O45-G to the plasma membrane (Erfle, Simpson et al. 2004). I have demonstrated that siRNA-induced beta-COP suppression leads to the defects in focal adhesion turnover, actin cytoskeleton organization and cell spreading. Therefore functional vesicular trafficking machinery seems to play a role in the regulation of focal adhesion dynamics. However suppression of the COPI complex influences focal adhesion organization rather indirectly through the general perturbation of the vesicular trafficking system and does not provide insight into the specific vesicular membrane pathway involved in focal adhesion turnover.

At the origin of the screen I hoped to identify genes that would highlight an element of the cellular transport machinery taking part in focal adhesion turnover. But the majority of the final hits turned out to be known or predicted transcription factors, DNA/RNA associated proteins, signaling peptides, receptors with rather general cell maintenance functions and unknown proteins. The role of some of those proteins especially transcription factors might be very important

for focal adhesion organization however their influence is not direct and they do not bring information about the core adhesion regulatory machinery. I chose three candidates (KIF19, TM4SF19, CCDC51) from the uncharacterized genes that are ubiquitously expressed, highly conserved and possess either motor or transmembrane domains. I intend in follow up studies to define their cellular localization and further characterize their roles in vesicular trafficking and focal adhesion turnover.

The low number of specific vesicular trafficking genes identified in my screen may be explained by the nature of the exploited siRNA library created on the basis of the wide-genome screen performed in Pepperkok laboratory. It included siRNAs for genes found to be involved in the secretory transport of a viral ts-O45-G protein (Simpson, Cetin et al. 2007). First of all the trafficking pathway utilized by ts-O45-G could be different from the one involved in focal adhesion regulation. Secondly, the wide-genome screen had its own limitations that probably lead to the loss of some important vesicular trafficking genes and accumulation of false-positive genes affecting general house-keeping pathways. Notably the compiled siRNA library did not target major genes of the cellular vesicular system coding components of clathrin and caveolin vesicular pathways, and many important regulators of endocytic pathway such as Rab GTPases (Rab4, Rab5, Rab9) and their regulators. That is why a better approach would be to compose a specific set of siRNAs targeting genes with known or predicted function in vesicular trafficking (similar to the candidate siRNA library that was created for screening of adhesion and migration related genes). Such a library screened for focal adhesion dynamic defects may provide more information regarding the role of vesicular trafficking pathway in focal adhesion turnover.

Future perspective

During the course of my work I noticed that a great part of loss-of-function experiments including individual siRNA knockdown studies as well as siRNA large-scale screens (Kiger, Baum et al. 2003; Simpson, Selfors et al. 2008) concentrated on the characterization of gene functions in very general processes such as cell migration or cell morphology. However identified defects in cell migration do not provide information about the particular cellular pathway in which a gene of interest is involved. I think that it would be very important in the future to dissect the roles of genes on a deeper level and look at the separate pathways of cell migration such as focal adhesion turnover.

The screen that I initiated here represents a first step toward identification of cellular pathways regulating focal adhesion assembly and disassembly. An improvement of siRNA oligonucleotide design as well as development of appropriate automated phenotype analysis software will help to increase efficiency of the screen and expand it to a genome-wide scale.

I have also demonstrated that vesicular trafficking pathway (likely different from ts-O45-G secretory route) is involved in focal adhesion turnover. In the future a siRNA screen with a candidate set of oligonucleotides may help to uncover the network of molecules linking these two processes.

PART II

IDENTIFICATION OF PAXILLIN VESICULAR RECYCLING PATHWAY

Introduction

As discussed earlier it has been suggested that the turnover of the main focal adhesion receptors integrins is dependent on a vesicular endocytotic pathway. I have demonstrated in the first part of my work that suppression of retrograde vesicular trafficking by knockdown of COPI complex or BrefeldinA treatment led to the alteration of focal adhesion organization and dynamics. However it is unclear whether the vesicular trafficking machinery is involved only in the transport of focal adhesion membrane components or also plays a role in the delivery of the adhesion's cytoplasmic scaffolding and regulatory proteins.

Several research groups have published evidence for a possible involvement of vesicular transport in the turnover of the focal adhesion protein paxillin. Moreover analysis of numerous paxillin immunolabelings during my siRNA screening project revealed an occasional appearance of distinct perinuclear vesicular structures. Therefore I focused on the role of vesicular transport in paxillin turnover.

Paxillin is a 68kDa protein that was first purified in 1990 and characterized as a direct binding partner of another focal adhesion protein vinculin (Turner, Glenney et al. 1990). The C-terminal part of paxillin contains four LIM domains with double zinc-finger motifs. The N-terminal half includes five leucine- and aspartate-rich LD modules with the conservative consensus

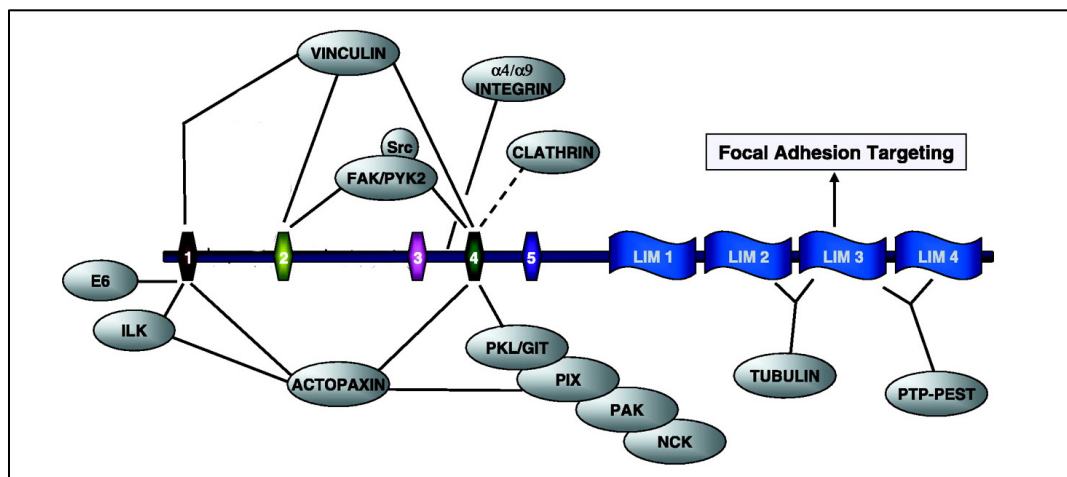


Figure 1 | Paxillin binding partners. | Paxillin contains multiple protein binding domains, including five N-terminal LD motifs and four C-terminal LIM domains. Represented binding partners were identified on the basis of various GST-pull down, coprecipitation and yeast two-hybrid experiments. Adapted from Brown&Turner, 2004.

sequence LDxLLxx. Both types of paxillin domains were shown to be implicated in protein-protein interactions (Figure 1). The LIM domains serve as binding sites for tubulin and PTP-PEST tyrosine phosphatase; the LD motifs bind vinculin, FAK and ILK kinases, actopaxin and GIT1/GIT2 RhoGTPase activating proteins (for review see (Brown and Turner 2004)). Consistent with its multidomain structure paxillin is a scaffolding molecule that mediates a signal cross-talk between regulatory and structural proteins at focal adhesions. A recent review by Deakin and Turner comprehensively summarizes paxillin functions in the regulation of Rho GTPase signaling in the proximity of adhesion sites highlighting its role as a key element in cell polarization and migration (Deakin and Turner 2008). The critical role of paxillin was confirmed by the early embryonic lethality in mice upon gene disruption (Hagel, George et al. 2002). Cultured paxillin-null fibroblasts displayed abnormal focal adhesions and defects in cell migration. This suggests that paxillin is not essential for the formation of focal adhesions but is important for their maintenance or turnover (Hagel, George et al. 2002). Similar defects in cell motility were observed in paxillin-null embryonic stem cells (Wade, Bohl et al. 2002).

Localization of paxillin to focal adhesions was shown to be dependent on the integrity of LIM2 and LIM3 domains (Brown, Perrotta et al. 1996), but the identity of the docking component at focal adhesion sites has not been determined. Paxillin was found to be one of the earliest proteins targeting to nascent adhesions at the cell edge that suggests its participation in the assembly of adhesions (Zaidel-Bar, Ballestrem et al. 2003; Digman, Brown et al. 2008). Conversely paxillin has also been demonstrated to contribute to focal adhesion disassembly. For example, overexpression of paxillin with mutations preventing its tyrosine phosphorylation at positions 31 and 118 impairs the disassembly of focal adhesions in migrating cells (Webb, Donais et al. 2004), implicating that a switch in paxillin phosphorylation is an important determinant of paxillin's influence on focal adhesion dynamics. Thus paxillin targeting to focal adhesions is a prerequisite of their efficient turnover and apparently requires fine temporal and spatial regulation.

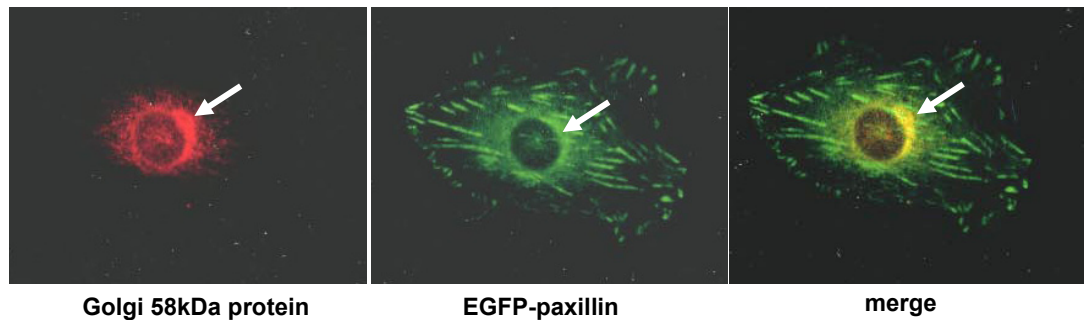


Figure 2 | Co-localization of paxillin with Golgi marker at the perinuclear area. | NIH3T3 cells were transfected with EGFP-paxillin (alpha-isoform) (red). Golgi was visualized by immunostaining with antibody recognizing 58kDa Golgi protein (green). Arrow highlight the area where EGFP-paxillin co-localize with Golgi marker. Adapted from Mazaki et al., 1998.

The pathway involved in paxillin targeting to focal adhesions remains to be clarified. In 1998 Mazaki and colleagues showed that overexpressed paxillin localized not only to focal adhesion plaques but also to perinuclear vesicular-like structures located in the Golgi area (Figure 2) (Mazaki, Uchida et al. 1998). Several years later the same authors published immunoelectron microscopy images confirming localization of a significant fraction of paxillin to Golgi-like structures and unidentified membrane structures in the perinuclear region of 3Y1 cells (Mazaki, Hashimoto et al. 2001). Based on these findings it was suggested for the first time that vesicular membrane trafficking was a possible mechanism of paxillin delivery to focal adhesions.

In parallel it was discovered that microinjection of a constitutively active form of ARF1, a GTPase involved in the assembly of coated vesicles at the Golgi and thereby in vesicular trafficking, induced redistribution of paxillin from the cytoplasmic fraction to focal adhesion structures (Norman, Jones et al. 1998). Moreover microinjection of a dominant-negative mutant of ARF1 ($\Delta 17$ ARF1) was found to prevent LPA-induced assembly of paxillin-containing focal adhesions. These data provided the first strong evidences of a role of ARF1 GTPase and hence the vesicular transport machinery in the recruitment of paxillin to focal adhesion structures.

Additional support for the involvement of vesicular trafficking in paxillin localization came from studies of G-protein-coupled receptor kinase interacting proteins (GIT1 and GIT2), which possess ArfGTPase-activating protein (ArfGAP) domains and control the activity of several ARF GTPases including ARF1 (for review see (Hoefen and Berk 2006)). GIT2 (ubiquitously expressed)

and the closely related GIT1 (expressed in specific cell types) were shown to interact directly with the paxillin LD4 motif (Turner, Brown et al. 1999; Zhao, Manser et al. 2000; West, Zhang et al. 2001). GIT proteins are also involved in the formation of a molecular complex that contains PAK-interacting exchange factors (α - and β -PIX) and p21-activated kinase (PAK). PIX proteins are guanine-nucleotide exchange factors (GEF) responsible for Rac1 and CDC42 activation (for review see (Rosenberger and Kutsche 2006)). PAK kinase is activated by Rac1 or Cdc42 and catalyzes disassembly of stress fibers through phosphorylation of LIM kinase and myosin-light-chain kinase (for review see (Bokoch 2003)). Localization of the GIT-PIX-PAK complex to focal adhesion sites is dependent on GIT binding to paxillin and essential for coordination of Rac1 signaling during cell spreading and migration (West, Zhang et al. 2001; Brown, Cary et al. 2005). Expression of a paxillin mutant without the LD4 sequence that is unable to recruit GIT-PIX-PAK complex leads to defects in focal adhesion turnover (Webb, Donais et al. 2004).

GIT1 protein was found not only at focal adhesion sites but also at the cytoplasmic vesicular-like structures that were motile and moved toward the cell periphery or toward the cell body in retracting regions of the cells (Manabe, Kovalenko et al. 2002). GIT1 cytoplasmic structures contained paxillin (Figure 3) as well as other proteins of the GIT-PIX-PAK signaling complex and thereby were proposed to play role in the delivery of components to and from focal adhesions. However Manabe and colleges did not consider those vesicular structures important for paxillin turnover.

Other experiments based on the overexpression of GIT proteins and their

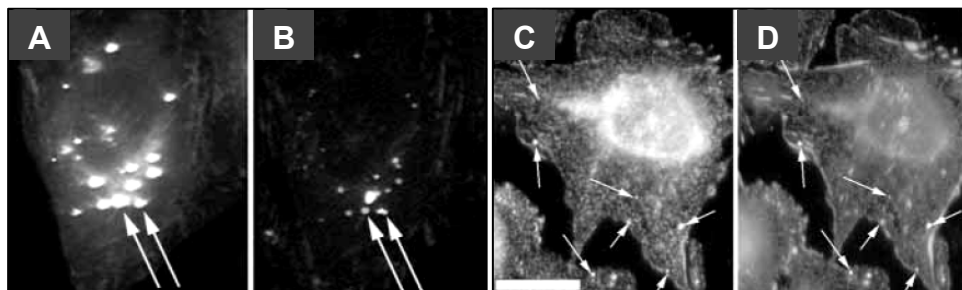


Figure 3 | GIT1 cytoplasmic complexes contain paxillin. | CHOK1 cells were transfected with EGFP-GIT1 (A) or stained with anti-GIT1 antibodies (C). Paxillin was visualized by immunostaining with anti-paxillin (B,D). Arrow highlight the cytoplasmic complexes where GIT1 and paxillin co-localize. Adapted from Manabe et al., 2002.

ArfGAP domain-mutants also demonstrated their ability to sequester paxillin from focal adhesions and recruit it to a vesicular-like cytoplasmic compartment (Di Cesare, Paris et al. 2000; Zhao, Manser et al. 2000; Mazaki, Hashimoto et al. 2001; Paris, Za et al. 2002). However the nature of the observed vesicular-like compartment remained ambiguous: de Curtis and co-workers identified it as Rab11-positive recycling endosomes (Matafora, Paris et al. 2001; Paris, Za et al. 2002), whereas Manabe and colleagues excluded the membrane nature of GIT1 and paxillin containing structures (Manabe, Kovalenko et al. 2002). Finally a hypothetical model was proposed according to which direct interaction of the GIT-complex with paxillin induces its re-localization away from focal adhesions and regulates its recycling back through a vesicular endocytic compartment to the cell periphery to newly formed focal adhesions (Figure 4, (Matafora, Paris et al. 2001)).

In addition, overexpression or downregulation of other ArfGAP family members (PAG3, ACAP1, ASAP1) also leads to the redistribution of paxillin from focal adhesions (Kondo, Hashimoto et al. 2000; Liu, Loijens et al. 2002; Liu, Yerushalmi et al. 2005). In summary, the role of ArfGAP proteins in paxillin localization highlights a possible connection between ARF-dependent vesicular trafficking and paxillin transport to focal adhesions.

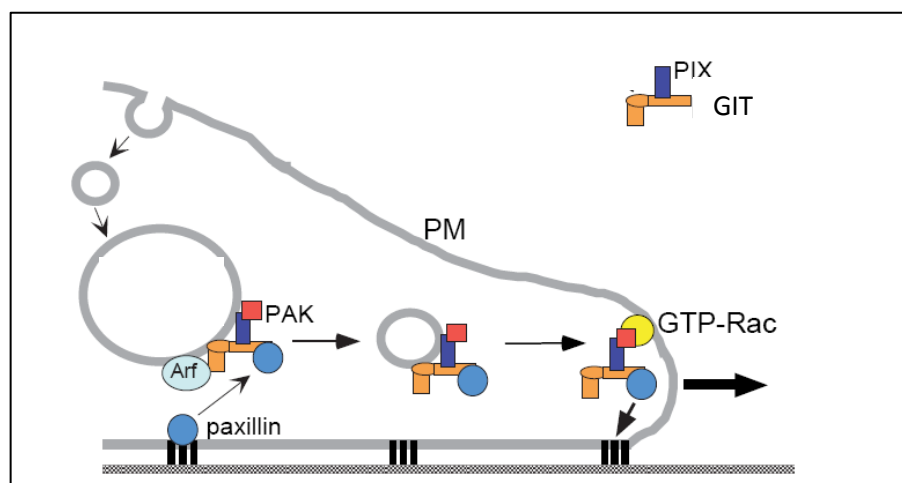


Figure 4 | Schematic model of paxillin vesicular recycling. | Paxillin interaction with GIT-PIX-PAK complex induces its re-localization away from focal adhesions. GIT and PIX proteins are responsible for the localization of paxillin to the recycling endocytic compartment. ARF-GAP domain of GIT is important for the regulation of paxillin vesicular transport back to the cell surface. Active form of Rac can be an important determinate of the translocation of paxillin and GIT complex from recycling vesicles to the cell membrane. Adapted from Matafora et al., 2001.

However the aforementioned data provide rather indirect evidences of paxillin vesicular recycling and do not reveal the exact mechanism. Further progress towards elucidating the paxillin trafficking process was considerably hampered by the inability to visualize paxillin-containing vesicular structures in living or fixed cells in standard conditions by conventional microscopy. Since the first report 10 years ago the existence of paxillin vesicular structures has not been confirmed.

I supposed that a block in paxillin targeting to focal adhesions might lead to its enhanced accumulation in putative vesicular structures which then might become visible by means of conventional microscopy. Therefore I created a series of paxillin mutants defective in focal adhesion targeting and demonstrated that those mutants indeed redistributed to the cytoplasmic vesicular-like structures which were clearly observed in live cells. Moreover I determined that the vesicular-like structures containing paxillin mutants belonged to the clathrin trafficking pathway. I also showed that wild type paxillin could localize to a subset of clathrin vesicles and interact with their components. As a conclusion from the performed experiments I suggest that accumulation of paxillin mutants defective in focal adhesion binding in the clathrin vesicles reflects the existence of a clathrin-dependent trafficking pathway involved in paxillin recycling and therefore in focal adhesion turnover. Using the RNAi approach I confirmed a role of a specific part of the clathrin pathway in focal adhesion organization.

Materials and methods

1. Antibodies and reagents

The following antibodies were used: mouse monoclonal antibody to mRFP (kindly provided by Dr. Jan Faix, Hannover Medical School, Germany), paxillin and clathrin heavy chain (BD Transduction Laboratories #P13520, #C43820); polyclonal rabbit antibody to GIT1 (Cell Signaling #2919) and EpsinR (ProteinTech Group, Chicago, #10470-1-AP). The secondary antibodies Alexa Fluor 568, Alexa Fluor 488, Alexa Fluor 350 were purchased from Molecular Probes, Invitrogen.

2. DNA Constructs

Human EGFP-Paxillin was provided by Ravi Salgia (Salgia, Li et al. 1995), EGFP-Clathrin Light Chain A was kindly provided by C. Rottner (HZI, Braunschweig), EGFP-fused Rab GTPases (Rab 1A, Rab 2A, Rab21, Rab 5A, Rab 11A, Rab 8A, Rab 9A, Rab7) were kindly provided by J. Simpson (EMBL, Heidelberg, Germany).

3. Plasmid Construction and Site-directed Mutagenesis

Coding sequence of the human paxillin was amplified from EGFP-Paxillin using primers incorporating EcoRI and KpnI restriction sites (Forward: GGA ATT CGG ATG GAC GAC CTC GAC GCC CTG; Reverse: GG GGT ACC CTA GCA GAA GAG CTT GAG GAA GCA) and ligated into mRFP-C3 vector (provided by R. Tsien, University of California, USA). The site-directed mutagenesis was performed according to the instruction manual using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers are indicated in the Table1.

To generate paxillin construct with deletion of the LIM3 domain (mRFP-Paxillin- Δ LIM3), the entire mRFP-Paxillin plasmid excluding only LIM3 sequence was amplified with primers containing SalI restriction site (Forward Δ LIM3: CAT GTC GAC TGT GAG GTG CAC TAC; Reverse Δ LIM3: GTA GTC GAC CTT GGG TGC GAA CAT). Amplification product was digested with SalI and re-ligated back into circular plasmid. Similar procedure with specific primers containing BclI restriction site was performed to create paxillin constructs with deletion of the LD4 domain (Forward Δ LD4: CAG ACT

	Forward primer
C465A	GGC ATC CTG AGG CCT TTG TGT GCC GGG AAT
C468A	CCT GAG GCC TTT GTG GCC CGG GAA TGC TTC ACG
C445A	C AAG TGT GGC GGC GCA GCC CGG GCC ATC CTG
C489A	GAC GGG CAG CCC TAC GCT GAG GTG CAC TAC C
ΔLIM3	CAT GTC GAC TGT GAG GTG CAC TAC
ΔLD4	CAG ACT AGT ACA GGG AGC AGC TCA C
	Reverse primer
C465A	CAT TCC CGG CAC ACA AAG GCC TCA GGA TG
C468A	CGT GAA GCA TTC CCG GGC CAC AAA GGC CTC AGG
C445A	CAG GAT GGC CCG GGC TGC GCC GCC ACA CTT G
C489A	G GTA GTG CAC CTC AGC GTA GGG CTG CCC GTC
ΔLIM3	GTA GTC GAC CTT GGG TGC GAA CAT
ΔLD4	GTC ACT AGT CCT GGT GGC AGA GGA G

Table 1 | Primers for site-directed mutagenesis

AGT ACA GGG AGC AGC TCA C; ReverseΔLD4: GTC ACT AGT CCT GGT GGC AGA GGA G).

To construct a prokaryotic expression vector encoding the GST-GFP fusion protein, the sequence of the EGFP protein was amplified from EGFP-C3 vector (BD Bioscience) with primers containing EcoRI and XhoI sites (Forward: C GAA TTC TTT AGT GAA CCG TCA GATC; Reverse: CTT GAG CTC GAG ATC TGA GTA CTT G). The amplification product was introduced into pGEX-4T-1 vector (GE Healthcare) using EcoRI and XhoI restriction enzymes. Afterward sequence of the paxillin or paxillin-2C/A mutant was amplified using primers with EcoRI and NheI restriction sites and introduced into the previously created GST-GFP-vector between GST and GFP sequences (Forward: A GAA TTC ATT CGG GGG ATG GAC G; Reverse: GGC TAG CGA GCA GAA GAG CTT GAG).

4. Cell culture and transfection

Hela cells (gift of Yuko Kiyosue, KaN Research Institute, Kyoto) were maintained in DMEM (Gibco #31885-023) supplemented with 10% Fetal Calf Serum (PAA Laboratories) and penicillin/streptomycin (Gibco #15140-122). RPE cells (hTERT RPE-1) were cultured in DMEM F12 (Sigma), 10% Fetal Bovine Serum (Sigma), 7.5% Sodium bicarbonate, L-glutamine and penicillin/streptomycin. HT1080 (gift of Dr. J. Schwarzbauer, Princeton University) were cultured in DMEM supplemented with 10% FBS (Hyclone), L-

glutamine and penicillin/streptomycin. HeLa cells were transfected using Superfect (Quiagen) according to the manufacture protocol. RPE and HT1080 cells were transfected with FuGENE6 (Roche) according to the manufacture protocol.

5. Immunostaining

For immunostaining cells were split on coverslips coated with fibronectin (10 ug/ml, Roche). Cells were fixed in 4% PFA for 20 minutes. For the staining cells were permeabilized with 0.05% Triton X-100 in 1xPBS for 5 minutes. After 15 minutes wash (3 times 5 minutes with 1XPBS) coverslips were incubated for 1 hour with primary antibody, washed with 1xPBS (3 times 10 minutes with 1XPBS) and incubated for 1 hour with secondary antibody. Cell nuclei were stained with 1 ug/ml Hoechst 33342 (Sigma-Aldrich) in 1xPBS for 10 minutes. For immunostaining of focal adhesions and actin cytoskeleton cells were fixed in a mixture of 3% Triton X-100 and 3% PFA for 3 minutes followed by fixation in 3%PFA for 35 minutes and standard incubation with antibodies. Actin was labeled with Alexa Fluor 568-Phalloidin (Molecular Probes, Invitrogen). Coverslips were subjected to immunostaining or upon transfection were directly mounted on the slides with Prolong Gold (Molecular Probes) and dried at room temperature over night.

6. siRNA transfection

HeLa cells were seeded in 12-well plates ($1,0 \times 10^5$ cells per well) 24 hours before transfection. siRNA oligonucleotides targeting human clathrin heavy chain (UAAUCCAAUUCGAAGACCAAU, Motley, Bright et al., 2003), human EpsinR (AAUACAGAU AUGGUCCAGAAA, Hirst, Miller et al., 2004) and also Silencer Negative Control #2 siRNA (catalog # 4613) were produced by Ambion and stored at -20°C as 50 uM working stocks. Mixtures of 6 ul Oligofectamine (Invitrogen) with 24 ul OPTIMEM (Gibco) and 3 ul siRNA with 100 ul OPTIMEM were prepared separately and incubated at room temperature for 7 minutes. Solutions were combined by gentle pipetting up and down and left for 20 minutes at room temperature. Culture medium was changed to the medium without antibiotics. The transfection mix was carefully added to the center of each well. On the next day cells were split on coverslips coated with fibronectin

(10 ug/ml, Roche). Fixation and immunostaining procedures were performed as described. siRNA knockdown of EpsinR was examined after 72 hours. For Clathrin Heavy Chain siRNA two transfections were performed two days apart, and experiments were carried out 2 days after the second knockdown.

7. Transferrin Uptake assay

Cells were starved for 45 minutes in DMEM with 0.2% BSA, incubated with 25 ug/ml Alexa Fluor 568 transferrin (Molecular Probes, Invitrogen) in the same medium for 15 minutes at 37°C, washed with 1xPBS and fixed as described for immunostaining.

8. Transwell migration assay

The Transwell cell migration assay was performed using transwell 12-well plates with the membrane pores of 8 um in diameter (Greiner). Cells were starved in serum-free medium with addition of 0.2% BSA overnight. Afterward $3-4 \times 10^5$ cells were plated in the serum-free medium to the upper well. The lower compartment was filled with 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 deattached from the membrane by gentle shaking of the plate, the inserts were removed and cells left in the wells were counted with hemocytometer.

9. Immunoprecipitation

Confluent cells from a 10 cm culture dish were harvested by trypsination and lysed on ice for 20 minutes in 250 ul of lysis buffer LBII (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 75 mM NaCl, 1% Triton X100, 10% Glycerol, 0.05 mM DTT supplemented with complete protease inhibitors (Roche)). The lysate was cleared by centrifugation (at 13000xg for 15 minutes at 4°C). Protein G-Sepharose beads (40 ul, GE Healthcare) were pre-coupled with anti-mRFP antibody (4 ug) in 500ul 0.01% TritonX-100 in 1xPBS during 1 hour at 4°C. The beads were washed twice with 1 ml of IP buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% Triton X100 supplemented with protease cocktail inhibitors tablets (Roche)). Thereafter beads were incubated for 2 hours at 4°C with 250 ul of crude lysate or 250 ul of particle fraction adjusted to 500 ul with IP buffer. Subsequently the beads were washed three times with IP buffer (30 minutes) and

resuspended in 50 ul of 2xSDS protein loading buffer, boiled for 10 minutes and centrifuged for 3 minutes at 10000xg. Proteins were detected by immunoblotting using appropriate antibodies. For mass spectrometry analysis immunoprecipitates were eluted with 0.1 M Glycine-HCl buffer pH 2.5. Beads were incubated twice with 50 ul of the buffer for 5 minutes, eluates were combined and immediately adjusted to a physiological pH by addition of 1.0 M Tris pH 7.5 (10 ul for 100 ul of eluate volume).

10. Cell fractionation

Protocol was adopted from Guillemin et al., 2005. Confluent cells from a 10 cm culture dish were harvested by trypsination and resuspended for 5 minutes on ice in 500 ul CLB buffer (10 mM HEPES, 10mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 5mM EDTA, 1 mM CaCl₂, 0.5 mM MgCl₂). Homogenization was performed by applying 12 strokes with a syringe (26G needle). Then 50 ul of 2.5 M sucrose was added to restore isotonic conditions. The first round of centrifugation was performed at 6300xg for 12 minutes in a tabletop centrifuge. Supernatant was centrifuged at 107000xg for 30 minutes using S100AT 4-463 rotor (Sorvall, RC-M150 GX ultracentrifuge). The resulting pellet representing the particle fraction with vesicular membranes was resuspended in 100 ul or 250 ul of the LBII buffer.

11. GST-Pull down assay

Cellular lysates were prepared as described for immunoprecipitation. Glutathione-Sepharose 4B beads (GE Healthcare) were incubated first for 1 hour at 4°C with 10 ug of the recombinant GST-proteins in 500 ul of 0.01% Triton X100 in 1xPBS. Beads were washed twice with 1 ml of the IP buffer and incubated with 250 ul of the cell lysate adjusted to a final volume of 500 ul with IP buffer. Thereafter beads were washed three times for 30 minutes, boiled in 50 ul of 2xSDS protein loading buffer for 10 minutes and centrifuged for 3 minutes at 10000xg. Proteins were detected by immunoblotting with specific antibodies.

12. Purification of GST-Paxillin-GFP fusion proteins

GST-GFP and wild type and mutant GST-Paxillin-GFP were expressed in *Escherichia coli* BL21 and affinity purified from the crude lysates with Glutathione-Sepharose 4B according to the specifications of the manufacture (GE

Healthcare). Briefly, 500 ml exponentially growing cultures were induced with 0.1 mM IPTG at 16°C overnight. Cells were harvested by centrifugation and sonicated in 25 ml of 1xPBS on ice in short bursts. Triton-X100 was added to the lysate to a final concentration of 1% and incubated for 30 minutes at 4°C. After centrifugation (10 minutes 12000xg) the supernatant was incubated with 500 ul 50% Gluthatione-Sepharose 4B slurry for 30 minutes at room temperature. The GST-fusion proteins were eluted with the help of disposable columns using 250 ul of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione (Sigma) pH 8.0). Four eluate fractions were collected and combined. The integrity of the purified proteins was controlled by SDS-PAGE electrophoresis and Coomassie Blue staining. Eluted proteins were extensively dialyzed against Microinjection buffer (2mM Tris-acetate pH 7.0, 50 mM KCl, 0.1 mM DTT) using Slida-A-lyzer Dialysis Cassette (Pierce). Recombinant proteins were concentrated with Vivaspın columns (Sartorius stedim biotech). Concentration of the proteins was adjusted to 1.6 mg/ml with microinjection buffer.

13. Live microscopy and microinjection

Live-cell videos were acquired on a Zeiss Axiovert 200M inverted microscope equipped with a rear-illuminated, cooled CCD camera (Micromax or Cascade, Roper), halogen lamp, and filter wheel and shutters controlled with Metamorph software. Images were collected in one or two fluorescent channels with 100x1.46 NA Plan-Apochroma objective (designed for TIRF). During imaging cells were maintained in an open heating chamber (Warner instruments) at 37°C in the complete medium supplemented with 15 mM HEPES.

Injectıons were performed with sterile Femtotips (Eppendorf) held in a micromanipulator with a pressure supply from an Eppendorf Microinjector 5242. Cells were injected with a continuous outflow mode from the needle under a constant pressure of between 20 and 40 hPa.

Results and Discussions

Paxillin mutant defective in focal adhesion targeting accumulates in the vesicular-like cytoplasmic structures

The domain structure of the paxillin molecule is shown in Figure 1. The LIM domains of paxillin are unique cysteine-rich motifs that form a double zinc finger structure with the consensus sequence $CX_2CX_{16-23}(C/H)X_2CX_2CX_{16-21}CXX(D/H/C)$ and are found in a variety of proteins including transcription factors and other focal adhesion components (Hic5, Zyxin) (Michelsen, Schmeichel et al. 1993; Schmeichel and Beckerle 1994; Konrat, Weiskirchen et al. 1997). Each zinc finger is stabilized through the chelation of one molecule of zinc by a combination of four cysteine, histidine, or aspartate amino acid residues. In 1996 Brown and colleagues demonstrated that point mutations of zinc chelating cysteines affecting the formation of the first and second zinc-fingers of LIM3 as well as deletion of the entire LIM3 domain eliminated paxillin targeting to focal adhesions (Brown, Perrotta et al. 1996). Based on the studies of the similar mutations the LIM2 domain was suggested to influence the efficiency of the paxillin binding to adhesions. Recently Kaverina and co-workers demonstrated that separate GST-fused LIM2 or LIM3 domains injected into cells localized only weakly to focal adhesions, whereas a GST-LIM2-LIM3 construct was localized strongly (Efimov, Schiefermeier et al. 2008). Therefore cooperativity between LIM2 and LIM3 domains is essential and sufficient for effective localization of paxillin to focal adhesions.

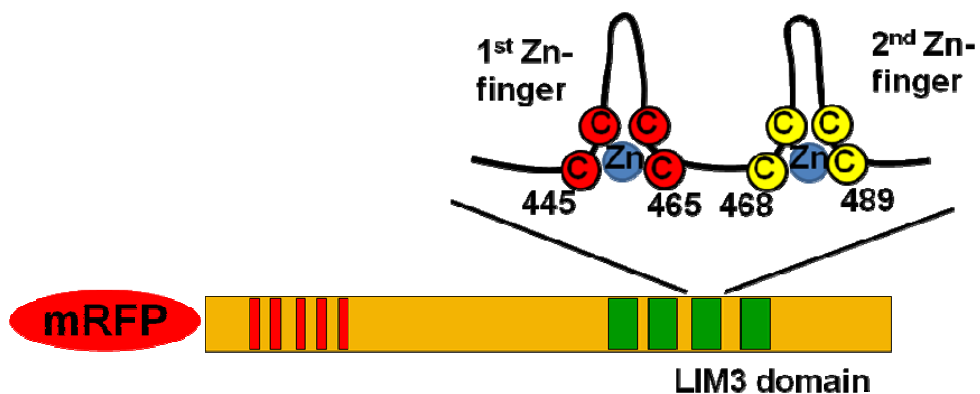


Figure 1 | Schematic representation of mRFP-Paxillin molecule with detailed structure of LIM3 domain . | Paxillin LIM3 domain forms a double zinc-finger structure. Each zinc finger is stabilized through the chelation of one molecule of zinc by a combination of four cysteine residues.

I proposed that paxillin defective in focal adhesion binding might accumulate in putative vesicular structures involved in its recycling. In order to check my hypothesis I generated a paxillin mutant with amino-acid substitutions of two chelating cysteins in the LIM3 domain and fused it to mRFP (monomeric red fluorescent protein) at the C-terminal end (mRFP-Paxillin-C465A/C468A later named as mRFP-Paxillin-2C/A). Upon transfection into different cell lines (HeLa, RPE, HT1080) this paxillin mutant localized only residually to focal adhesions compared to the mRFP-Paxillin-Wild Type (WT) construct and concentrated instead in numerous cytoplasmic vesicular-like structures (Figure 2). Similar vesicular-like structures were observed upon overexpression of the identical mutant fused to EGFP that excluded the possibility of unspecific protein aggregation caused by the mRFP-tag (data not shown). Upon prolonged overexpression Paxillin-2C/A induced the appearance of abnormally large “vesicles”, therefore all subsequent experiments were performed after 10-12 hours upon transfection (Figure 2, D).

Upon live imaging, stable as well as fast moving mRFP-Paxillin-2C/A-containing structures were detected. The movement of the vesicular-like structures was abolished by nocodazole and was thus dependent on an intact microtubule network (Figure 3, Supplementary movie 2). In addition, the mRFP-Paxillin-2C/A punctate structures underwent apparent fusion and budding events. On the basis of the described observations I concluded that mRFP-Paxillin-2C/A structures were not protein aggregates but likely functional vesicles transported along microtubules.

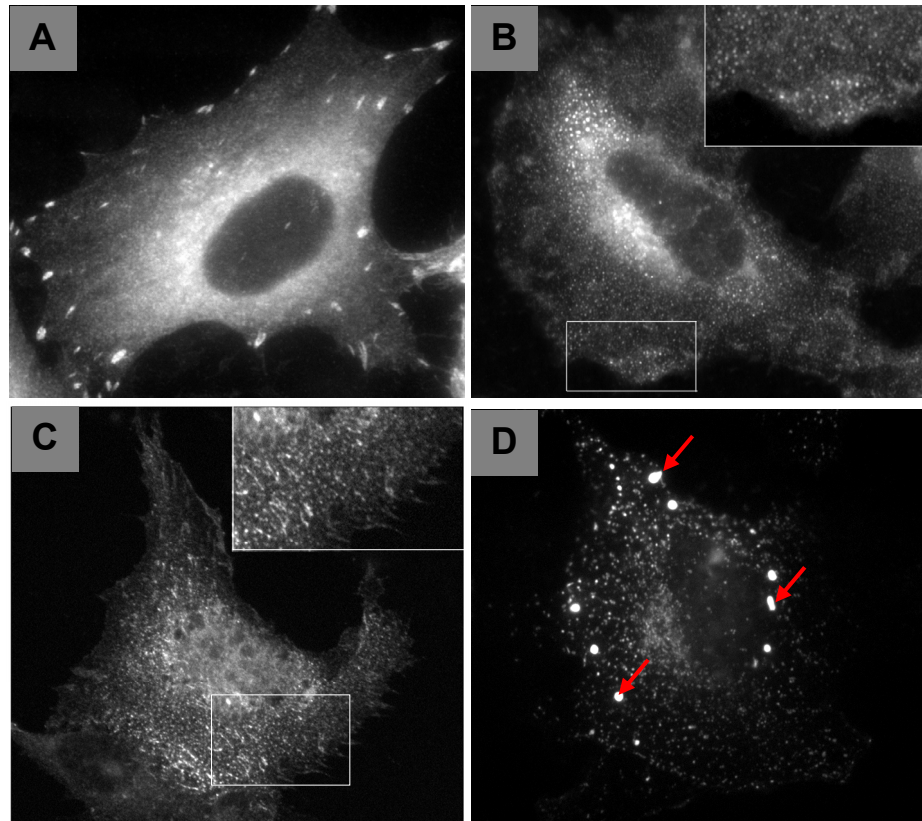


Figure 2 | Localization of *mRFP-Paxillin-2C/A* mutant to intracellular vesicular-like structures. | *mRFP-Paxillin-Wild Type* (WT) efficiently localized to focal adhesions upon transfection in HeLa cells (A). *mRFP-Paxillin-2C/A* concentrated at the cytoplasmic vesicular-like structures upon transfection in HeLa (B) and RPE cells (C). Prolonged overexpression time of *mRFP-Paxillin-2C/A* led to the formation of abnormal large vesicular-like structures marked by arrows (D, RPE cell). Cells were subjected to fluorescent microscopy directly after fixation.

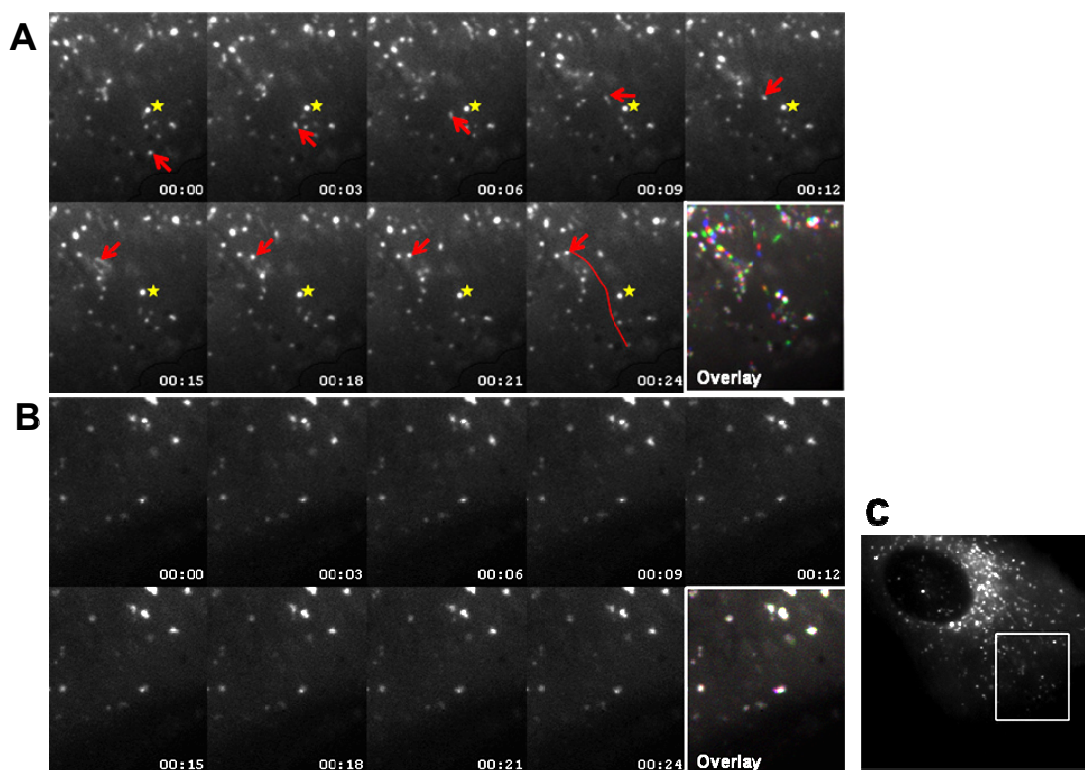


Figure 3 | Movement of *mRFP-Paxillin-2C/A* structures is dependent on intact microtubule network. | HT1080 cells were transfected with *mRFP-Paxillin-2C/A* and imaged at 3 s interval before and after Nocodazole addition (A, B, Supplementary movie 2). Overlays of the frames (1,4,7 frames in green, 2,5,8 – in red; 3,6,9 – in blue) demonstrate complete inhibition of vesicular movement upon nocodazole treatment. The arrow indicates example of fast moving *mRFP-Paxillin-2C/A* vesicle, the asterisk marks one of the stable vesicles. Image of the analyzed cell with examined rectangular region (C).

Paxillin accumulation in the vesicular-like structures is reversely dependent on the focal adhesion binding efficiency

To exclude the possibility that mutations of the two particular cysteines (C465A and C468A, Figure 1) caused unspecific binding of paxillin to vesicular-like structures, additional paxillin mutants with alternative mutations in the LIM3 domain were created: mRFP-Paxillin-C445A with disruption of only the first zinc-finger, mRFP-Paxillin-C489A with disruption of only the second zinc-finger, and mRFP-Paxillin-ΔLIM3 with deletion of the entire LIM3 domain. Upon transfection into HeLa cells all paxillin mutants showed enhanced accumulation in the similar vesicular-like structures (data not shown). However within the same cell population each of the paxillin mutants was observed not only in the vesicular-like structures, but also in focal adhesions or diffusively distributed in the cytoplasm (Figure 4). Cytoplasmic labeling appeared particulate in nature but the vesicular structures could not be clearly resolved (Figure 4C, enlarged rectangle). In the majority of cells the paxillin constructs displayed predominant localization either in the vesicular-like structures or in focal adhesions. Other cells showed simultaneous localization of the paxillin mutants in vesicular-like structures and focal adhesions (Figure 4D). In this case focal adhesion binding was less efficient as suggested by a weaker intensity of adhesion labeling.

I calculated the percentage of cells exhibiting predominant vesicular, focal adhesion or cytoplasmic localization of each transfected mutant or wild type paxillin. The histogram (Figure 4E) shows that wild type paxillin localized to focal adhesion sites in more than 90% of cells examined. Notably a fraction of the wild type paxillin was also found in the cytoplasm with concentration in the perinuclear area consistent with the previous studies demonstrating existence of the paxillin pool in the perinuclear Golgi region (as discussed in the Introduction, (Mazaki, Uchida et al. 1998), 1998, Figure 4A). The paxillin cytoplasmic fraction seemed to have a vesicular nature (Figure 4A, enlarged rectangle).

The integrity of the first but not the second LIM3 zinc finger was more important for paxillin binding to focal adhesions, as reflected by the smaller percentage of cells with focal adhesion localization of mRFP-Paxillin-C445A in comparison with mRFP-Paxillin-C489A (16% and 31% respectively). Disruption

of both zinc-fingers in the LIM3 domain (mRFP-Paxillin-2C/A) almost completely eliminated paxillin binding to focal adhesions and led to its accumulation in vesicular-like structures in 50% of the cells.

Surprisingly, deletion of the entire LIM3 domain did not have such a crucial impact on paxillin adhesion localization and mRFP-Paxillin- Δ LIM3 was found at focal adhesions in 32% of cells. This data is in contrast with the original studies of Brown and colleagues demonstrating that deletion of the LIM3 domain abrogated paxillin targeting to focal adhesions completely (Brown, Perrotta et al. 1996). However they expressed chicken paxillin in hamster CHO.K1 cells and analyzed paxillin localization by immunostaining with polyclonal antiserum specifically recognizing the chicken isoform of paxillin. Their method was likely not sensitive enough to detect residual binding of paxillin mutants to focal adhesions or to paxillin-containing vesicular structures. In my experiments I utilized a more direct method and observed living cells or cells fixed without any further manipulations.

First, as a conclusion from the localization analysis of different LIM3 mutants I suggest that paxillin targeting to focal adhesions does not depend exclusively on the LIM3 domain. In the absence of the intact LIM3 domain it may be mediated by another motif, likely the LIM2 domain as supported by the previous studies (Brown, Perrotta et al. 1996; Efimov, Schiefermeier et al. 2008). Secondly paxillin redistribution to vesicular-like structures is not a consequence of specific mutations but is observed for each paxillin mutant that failed to localize efficiently to focal adhesions. Moreover wild-type paxillin is also likely localized to the vesicular-like structures in the perinuclear area but they can not be clearly resolved by conventional microscopy.

And finally there is an inverse correlation between the ability of paxillin to target to focal adhesions or vesicular structures. Accordingly I hypothesize that the presence of paxillin mutants in vesicular structures reflects the existence of trafficking vesicles involved in paxillin transport that can't be resolved by conventional fluorescent microscopy under standard conditions. When paxillin is defective in focal adhesion binding it is retained at the putative transporting

vesicles and leads to their stabilization and enlargement and thereby makes them distinctly visible.

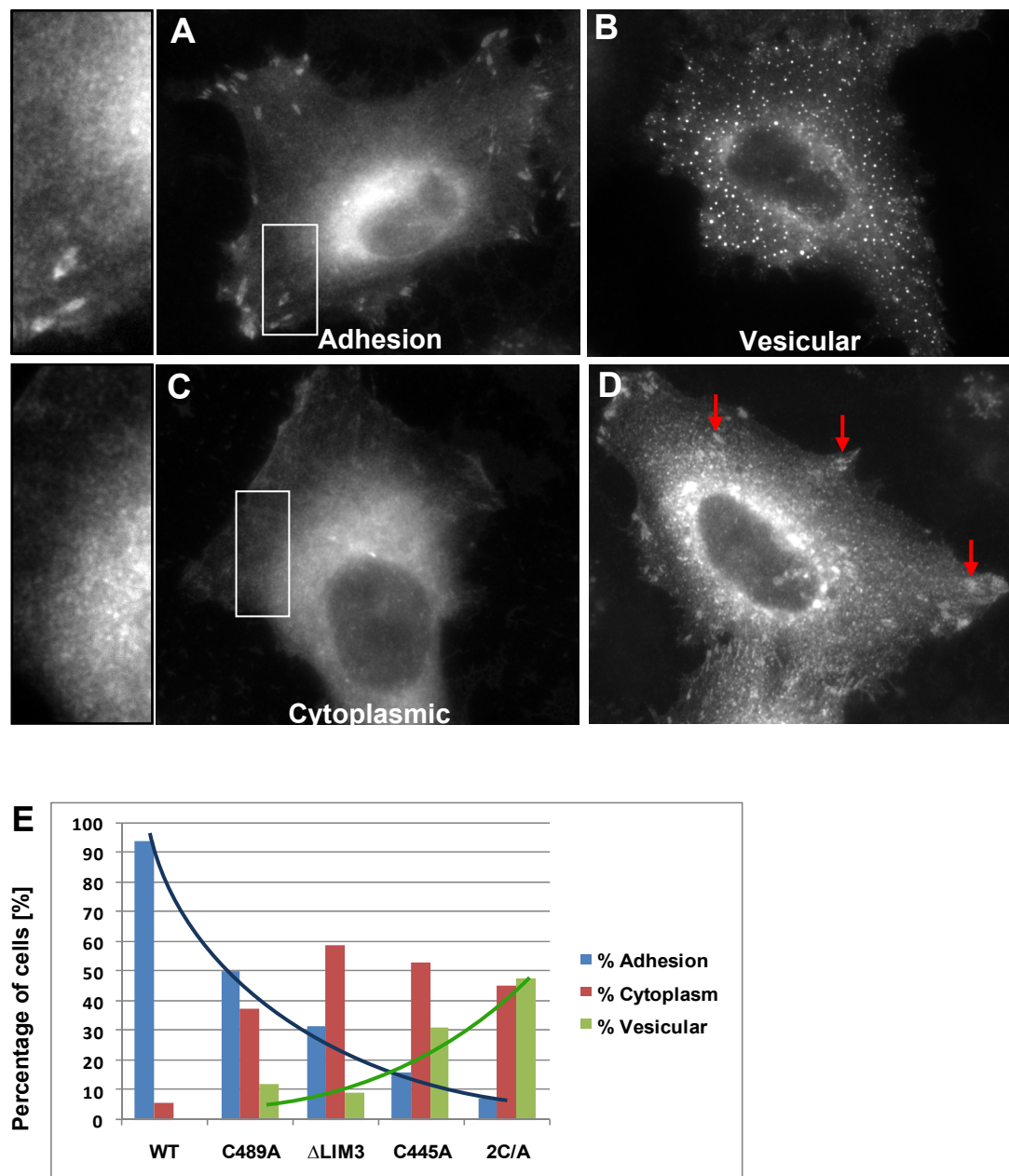


Figure 4 | Heterogeneous localization of expressed paxillin mutants. | Upon expression in HeLa cells paxillin mutants showed focal adhesion (A), vesicular (B) or diffuse cytoplasmic (C) localization in different cells of the same population. Enlarged rectangular regions at the left demonstrate putative particle nature of the cytoplasmic staining. (D) In some cells paxillin mutants were located at the vesicular-like structures and at focal adhesions simultaneously (mRFP-Paxillin-C489A expression, arrows mark weak focal adhesion labeling). Histogram shows percentage of cells exhibiting adhesion, vesicular or cytoplasmic localization of the expressed mutant (E). The blue curve highlights the efficiency of focal adhesion binding of different mutants. The green curve highlights the efficiency of their vesicular localization. HeLa cells were transfected with the following constructs: mRFP-Paxillin-WT, mRFP-Paxillin-C489A, mRFP-Paxillin- Δ LIM3, mRFP-Paxillin-C445A, mRFP-Paxillin-2C/A. More than 200 cells were analyzed upon transfection of each paxillin construct.

Identification of the putative vesicular compartment associated with paxillin mutant proteins

To determine the nature of the vesicular-like structures containing paxillin defective in focal adhesion targeting I examined their co-localization with various GFP-tagged Rab GTPases – molecules involved in vesicular membrane fusion and budding. Different Rab proteins function in distinct parts of the trafficking pathway and provide convenient markers of certain vesicular compartments. The tested Rab GTPases included Rab1A and Rab2A which are specifically involved in ER-to-Golgi trafficking, Rab21 and Rab5A which are markers of early endosomes, Rab11A and Rab8A - markers of recycling endosomes and Rab9A and Rab7 - markers of late endosomes (for the review see (Zerial and McBride 2001). However none of the aforementioned RabGTPases showed significant co-localization with mRFP-containing vesicular structures (Figure 5).

In addition the paxillin mutant did not co-localize with vesicles containing internalized fluorescent transferrin (Figure 6). Transferrin is a ligand of the transferrin receptor that is constitutively internalized from the cell surface in clathrin-coated vesicles, delivered to the sorting or recycling endosomes and transported back to the plasma membrane. Transferrin recycling was shown to be dependent on Rab5, Rab4 and Rab11 GTPases. Therefore the absence of the overlap between paxillin mutant and transferrin signals was in agreement with the lack of its co-localization with Rab5 and Rab11 in the previous experiment.

Since I was unable to co-localize paxillin mutant-containing structures with any of the available vesicular pathway markers I adopted a biochemical approach. I purified the cellular particle fraction enriched in vesicular membrane components using standard ultracentrifugation. The amount of the expressed mRFP-Paxillin-2C/A found in the particle fraction was significantly higher in comparison with mRFP-Paxillin-WT, whereas their expression levels in crude lysates were similar (Figure 7A). Afterwards I performed immunoprecipitation of the mutant and wild-type paxillin proteins from the particle fraction using an anti-mRFP antibody followed by mass spectrometry analysis of the immunoprecipitates. As demonstrated in Figure 7B the recovery of the mutant protein from the particle fraction was more efficient than recovery of the wild-

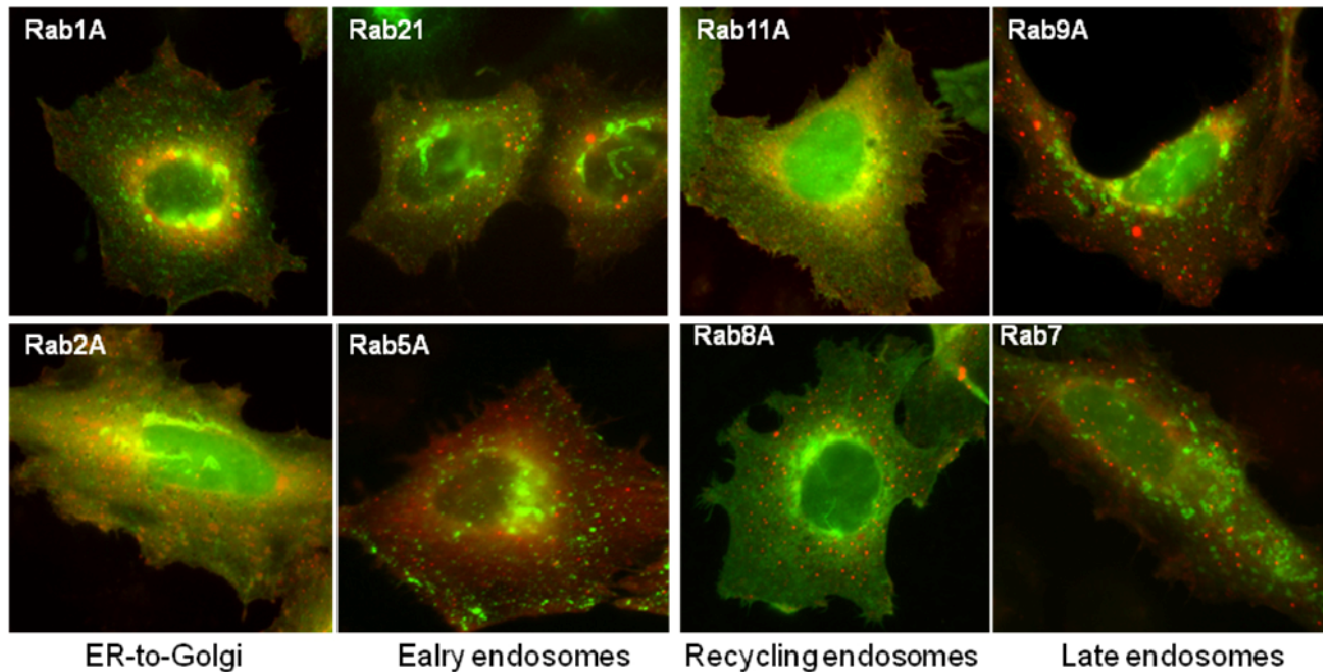


Figure 5 | *mRFP-Paxillin-2C/A* structures did not contain any of the tested RabGTPases. | HeLa cells were co-transfected with *mRFP-Paxillin-2C/A* (in red) and one of the following GFP-tagged RabGTPases (in green): Rab1A and Rab2A (markers of ER-to-Golgi trafficking pathway), Rab21 and Rab5A (markers of early endosomes), Rab11A and Rab8A (markers of recycling endosomes), Rab9A and Rab7 (markers of late endosomes). There is no significant overlap between green- and red-colored structures on the merge images. Cells were fixed and subjected to fluorescent microscopy.

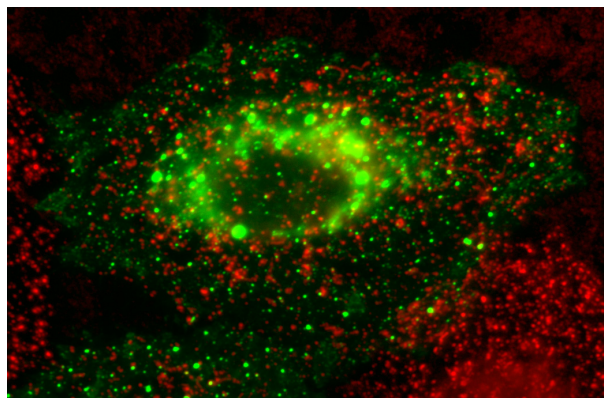


Figure 6| GFP-Paxillin-2C/A vesicular-like structures do not co-localize with endocytotic vesicles containing transferrin. | HeLa cells transfected with GFP-Paxillin-2C/A (in green) were incubated for 30 minutes at 37°C with Alexa568-labeled transferrin (in red). The cells were then washed and fixed.

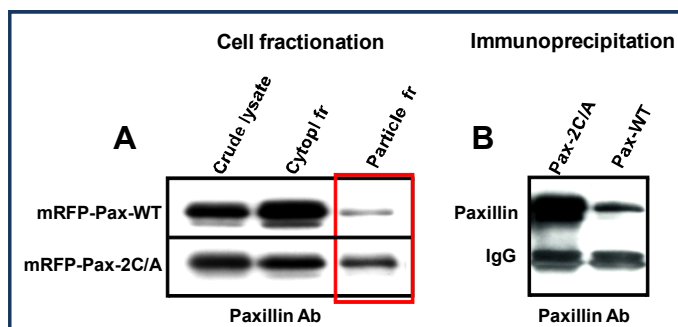


Figure 7 | Immunoprecipitation of expressed paxillin proteins from cellular particle fraction. | (A) Immunoblot showing enrichment of mRFP-Paxillin-2C/A in particle fraction in comparison with mRFP-Paxillin-WT. (B) Immunoblot showing mRFP-Paxillin-2C/A and mRFP-Paxillin-WT precipitated from the particle fraction using anti-mRFP antibody. Particle fraction was purified according to the protocol described in Material and Methods.

type paxillin. The low level of the wild type paxillin in the immunoprecipitate did not allow its identification by mass spectrometry analysis. Mass spectrometry analysis of the mRFP-Paxillin-2C/A precipitate detected paxillin itself and number of its binding partners presented in Table 1. Mass spectrometry analysis was repeated twice for two independent experiments and proteins found in the negative control immunoprecipitate from untransfected cells were excluded from the final results.

As shown in Table 1 mass spectrometry analysis identified the well-known paxillin binding partners - GIT1, GIT2 and beta-PIX proteins as well as tubulin. GIT1 and GIT2 proteins were demonstrated to bind both paxillin and beta-PIX directly, forming a signaling complex involved in the regulation of cell protrusion and migration (as discussed in the Introduction). Tubulin was identified as a direct paxillin binding partner in a yeast two-hybrid screen (Herreros, Rodriguez-Fernandez et al. 2000) and later on confirmed by GST-pull down experiments to bind to the LIM2 and LIM3 domains of paxillin (Brown and Turner 2002). Paxillin binding to tubulin was shown to play a role in the regulation of microtubule dynamics, particularly catastrophe events at focal adhesion sites (Efimov, Schiefermeier et al. 2008). Detection of tubulin and components of the GIT-signaling complex confirmed that I was able to isolate paxillin-binding partners combining immunoprecipitation from the enriched particle fraction with mass spectrometry analysis.

Notably several components of clathrin vesicles emerged as binding

Components of clathrin vesicles	Signaling complex	PXN	paxillin
		GIT1	ARF-GTPase activating protein GIT1
		GIT2	ARF-GTPase activating protein GIT2
		ARHGEF7	Rho-guanine nucleotide exchange factor beta-PIX
	clathrin vesicles	CLTC	clathrin heavy chain 1
		CLTA	Clathrin light chain A
		CLTB	Clathrin light chain B
		CLINT1	Clathrin interacting protein, Epsin R
		PIK3C2A	Phosphoinositide 3 kinase, class 2, alpha
		TUBB1	Tubulin beta-1 chain
		HSPB1	Heat shock 27 kDa protein
		CAD	Aspartate Carbomoyltransferase

Table 1 | Binding partners of mRFP-Paxillin-2C/A detected by mass spectrometry analysis. | Identified binding partners of mRFP-Paxillin-2C/A included components of the well-known GIT-PIX signaling complex as well as several components of clathrin vesicles.

partners of mRFP-Paxillin-2C/A (Table 1): clathrin heavy chain, two clathrin light chains, the clathrin adaptor protein EpsinR and phosphoinositide 3-kinase C2alpha (PIK3C2A). Clathrin heavy chain and light chains form the lattice-like coat of the vesicles that are endocytosed from the plasma membrane or bud from the intracellular trans-Golgi network (TGN). When coating membranes, clathrin does not bind to the membrane directly but does so via adaptor proteins. One of those adaptor proteins is EpsinR that simultaneously binds to clathrin and phosphoinositides at the membrane (preferentially PtdIns(4)P) and has been localized to the TGN and to the endosomal structures (Kalthoff, Groos et al. 2002; Wasiak, Legendre-Guillemain et al. 2002; Hirst, Motley et al. 2003; Mills, Praefcke et al. 2003; Saint-Pol, Yélamos et al. 2004). Phosphoinositide 3-kinase C2alpha (PIK3C2A) was detected in a subset of clathrin-coated vesicles at the plasma membrane and also at the intracellular vesicular structures in the TGN (Gaidarov, Smith et al. 2001). It has been shown to interact specifically with the terminal domain of the clathrin heavy chain (Gaidarov, Zhao et al. 2005). PIK3C2A was suggested to participate in the production of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ that accumulates at the leading edge of motile cells (Rickert, Weiner et al. 2000).

The identification of the clathrin chains, EpsinR and PIK3C2A in the mRFP-Paxillin-2C/A immunoprecipitate suggests that the vesicular-like structures observed upon overexpression of paxillin mutants defective in focal adhesion targeting are clathrin vesicles. Indeed mRFP-Paxillin-2C/A was localized to a subset of clathrin vesicles labeled with anti-clathrin antibody (not shown) or GFP-Clathrin Light Chain A (Figure 8) in HeLa cells. The fact that only a distinct population of clathrin vesicles contained the paxillin mutant indicated the presence of a specific factor determining paxillin mutant binding to clathrin structures. I therefore conclude that the paxillin mutants most likely bind clathrin heavy and light chains indirectly or in cooperation with an additional adaptor protein. The other three paxillin mutants defective in focal adhesion targeting (mRFP-Paxillin-C445A, mRFP-Paxillin-C489A, mRFP-Paxillin-ΔLIM3) were also found to localize to a subset of clathrin vesicles (data not shown).

I confirmed the interaction of paxillin mutants as well as wild type protein with clathrin heavy chain and EpsinR using immunoprecipitation from both the particle fraction (data not shown) and crude lysates by Western-blot analysis (Figure 9). Interestingly paxillin wild type protein was able to bind both clathrin heavy chain and EpsinR in immunoprecipitation experiments but upon co-transfection of mRFP-Paxillin-WT with GFP-Clathrin Light Chain A these two proteins localized to focal adhesions and vesicular structures respectively and no co-localization was observed (Figure 10). However when GFP-Paxillin-WT was expressed together with mRFP-Paxillin-2C/A, the wild type protein localized together with the mutant protein in vesicular structures, in addition to focal adhesions (Figure 11). Those structures were identified as clathrin vesicles by immunostaining with clathrin heavy chain antibodies (Figure 12). Immunoprecipitation did not reveal an interaction between expressed mRFP-Paxillin-2C/A and endogenous wild type paxillin and therefore excluded the role of oligomerization in the wild type paxillin localization to the vesicular structures induced by the mutant (data not shown). Accumulation of the wild type paxillin in clathrin vesicles induced by overexpression of its mutant is consistent with the interaction of wild type paxillin with clathrin demonstrated by immunoprecipitation.

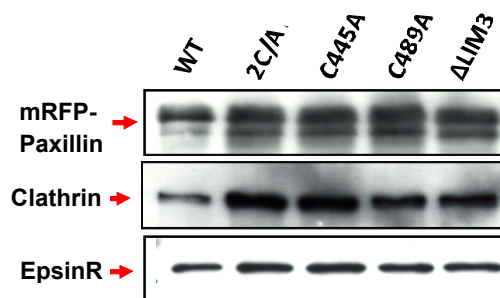
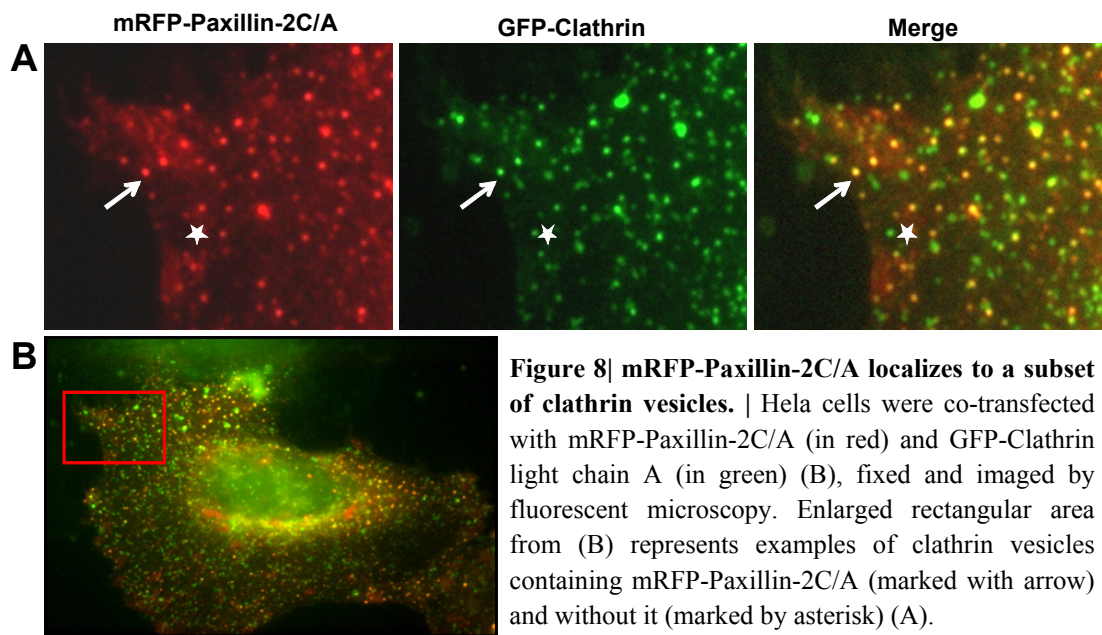


Figure 9| Binding of paxillin wild type and mutant proteins to Clathrin Heavy Chain and EpsinR as confirmed by immunoprecipitation. | Immunoprecipitation was performed with anti-mRFP antibody from crude lysates of HeLa cells transfected with one of the paxillin constructs (RFP-Paxillin-WT, RFP-Paxillin-2C/A, RFP-Paxillin-C445A, RFP-Paxillin-C489A, RFP-Paxillin-ΔLIM3). Blots were probed with anti-paxillin, anti-CHC and anti-EpsinR antibodies. Negative control immunoprecipitation from untransfected cells did not show any signal (not shown).

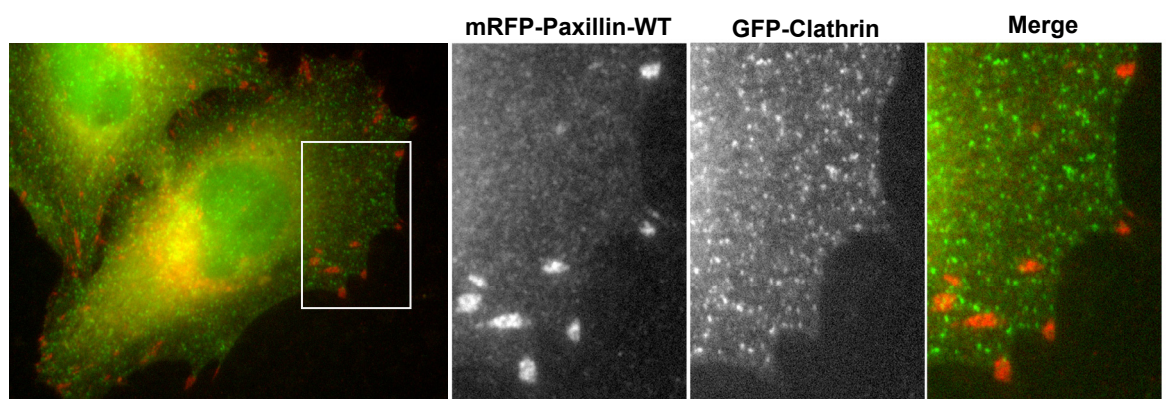
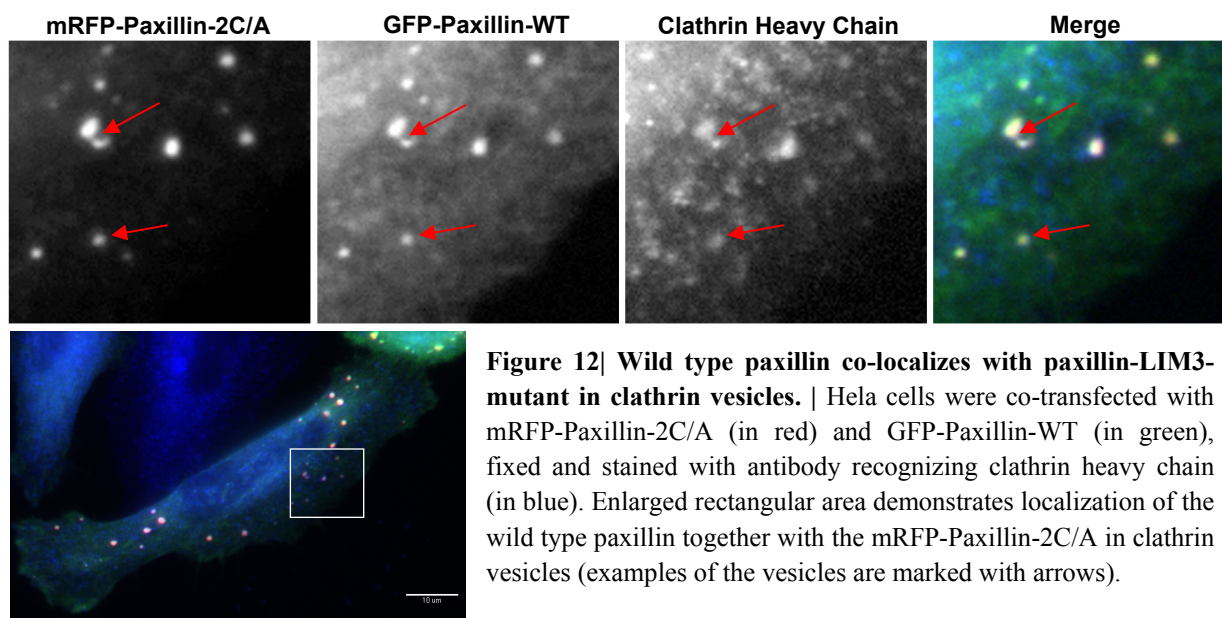
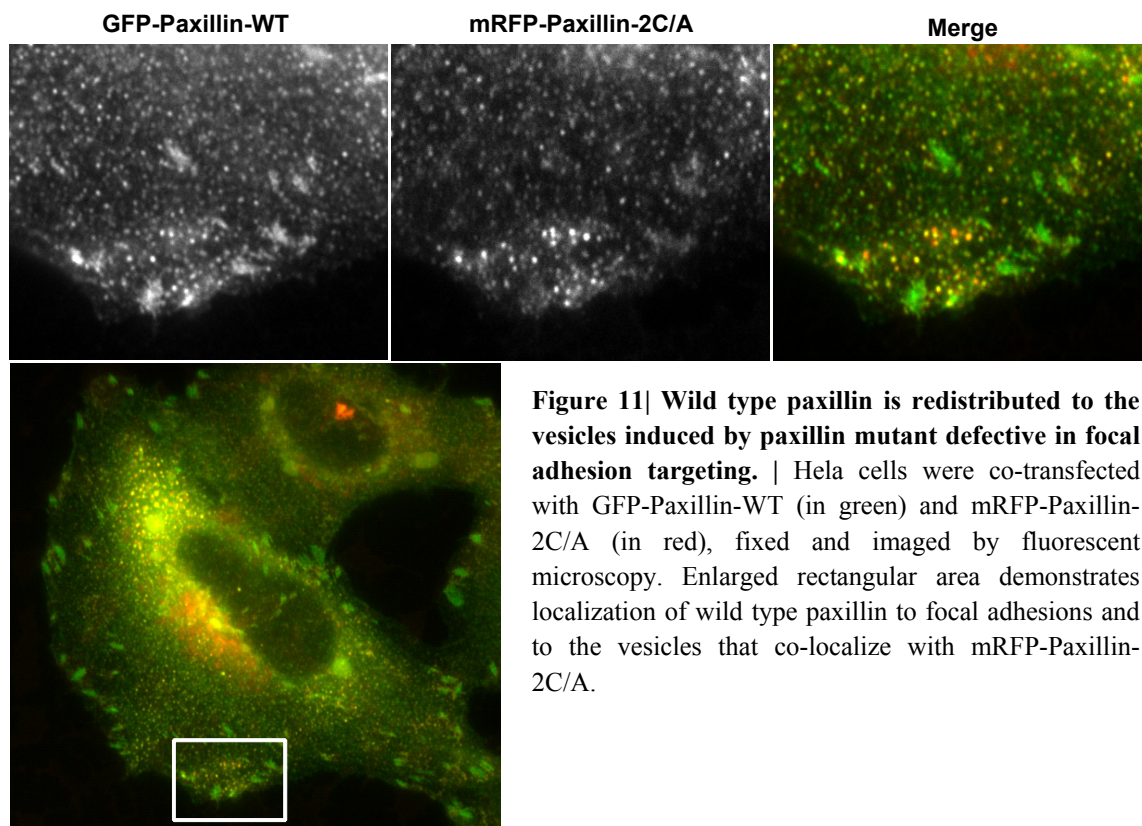


Figure 10| mRFP-Paxillin-WT was not detected at clathrin vesicles. | HeLa cells were co-transfected with mRFP-Paxillin-WT (in red) and GFP-Clathrin light chain A (in green), fixed and imaged by fluorescent microscopy. Enlarged rectangular area demonstrates paxillin localization to focal adhesions and clathrin light chain localization to vesicles without overlap between the two structures.



Direct observation of GFP-tagged wild type and mutant paxillin molecules upon microinjection into live cells

The standard approach for observation of protein localization and dynamics in live cells is overexpression of a protein of interest fused to a fluorescent molecule. However protein overexpression is a gradual process taking at least several hours after introduction of a coding plasmid into the cells. During this time increasing intracellular concentration of the expressed protein can lead to the readjustment of the cellular processes as well as to the protein accumulation in unspecific compartments such as lysosomes. Microinjection of the fluorescently-labeled protein into live cells is an alternative to the overexpression method and allows immediate analysis of protein intracellular dynamics and localization.

With the aim to study paxillin dynamics upon microinjection I created constructs coding wild type paxillin and paxillin-2C/A-mutant proteins fused to the glutathione S-transferase (GST) at the C-terminus and to the EGFP protein at the N-terminus. The GST moiety allows affinity purification of the proteins upon bacterial expression; the EGFP tag enables visualization of the hybrid proteins directly after injection into cultured cells. In addition EGFP is useful for monitoring and optimizing recombinant protein expression in bacteria. Fusion of the EGFP to the recombinant proteins was also suggested to enhance their stability and solubility (Rücker, Schneider et al. 2001). The GST-Paxillin-WT-GFP, GST-Paxillin-2C/A-GFP and GST-GFP hybrid proteins were efficiently expressed in *Escherichia coli* BL21 and affinity-purified using Glutathione-Sepharose 4B. SDS-PAGE analysis revealed major bands of approximately 117 kDa for recombinant paxillin proteins and 56 kDa for GST-GFP hybrid that were recognized by anti-GFP antibodies (Figure 14 B, A).

Dynamics of GST-GFP hybrid paxillin proteins as well as control GST-GFP molecule were monitored by time-lapse microscopy directly after injection into HeLa cells, followed by cell fixation and subsequent fluorescent microscopy analysis. Injection of the control GST-GFP protein produced a homogeneous cytoplasmic signal observed by live microscopy and imaging of fixed cells (Figure 13, Supplementary movie 3). Recombinant Paxillin-WT started to concentrate at focal adhesion sites already 30 seconds after injection (time

required to switch from a manual microinjection mode to image acquisition). Figure 15 demonstrates the dynamics of paxillin accumulation in two focal adhesion regions in one of the injected HeLa cells (Supplementary movie 4, small rectangular area). Analysis of similar regions adjacent to focal adhesions did not show an increase in paxillin signal intensity. Therefore it was concluded that targeting of recombinant paxillin to focal adhesions was specific.

Interestingly appearance of small vesicular structures was clearly observed upon injection of the wild type paxillin in some of the cells (Figure 15 D, Supplementary movie 4, large rectangular area). Those structures exhibited fast movement in the perinuclear area as well as around focal adhesions. However the speed of the time-lapse microscopy imaging did not allow detailed analysis of their dynamics.

Imaging of the fixed cells confirmed GST-Paxillin-WT-GFP localization to the focal adhesions and to the vesicular structures (Figure 13). Paxillin vesicular structures contained clathrin in the perinuclear region of the cells as was shown by co-localization with mRFP-Clathrin Light Chain and by immunostaining with anti-CHC antibody (Figure 17).

Surprisingly distribution of the injected recombinant paxillin was not uniform within focal adhesions. Paxillin was found to localize in distinct clusters that were often concentrated only at the focal adhesion periphery (Figure 16).

GFP-fused Paxillin-2C/A mutant did not localize to focal adhesion sites upon microinjection consistent with its previously shown defect in focal adhesion binding. Imaging of the injected cells after fixation revealed labeling of numerous small vesicles concentrating at the perinuclear area distinct from the diffuse cytoplasmic localization of the control GST-GFP protein (Figure 13). Time-lapse microscopy failed to resolve the same vesicular structures in live cells probably due to their tiny size and fast movement. In several cells blurred perinuclear motility of the vesicular-like structures was observed whereas the majority of the cells displayed cytoplasmic signal (Figure 18A, Supplementary movie 5).

The size of the vesicles labeled by recombinant mRFP-Paxillin-2C/A was smaller and more uniform compared with vesicles induced by overexpression of the same paxillin mutant. However injected GST-Paxillin-2C/A-GFP co-localized

with expressed mRFP-Paxillin-2C/A in the enlarged vesicular structures (Figure 18 B, Supplementary movie 6), indicating that small vesicles visualized by GST-Paxillin-2C/A-GFP and bigger vesicles observed upon mRFP-Paxillin-2C/A overexpression belong to the same vesicular compartment. This fact further supports the previously proposed idea that paxillin mutant redistribution from focal adhesions to the vesicles leads to their stabilization and increase in size. The time after microinjection was too short to induce changes in the vesicles dimensions upon Paxillin-2C/A binding. Therefore it is likely that small vesicular structures labeled by recombinant GST-Paxillin-2C/A-GFP (Figure 13) demonstrate physiological size of the putative vesicles related to paxillin trafficking.

I also confirmed the ability of GST-Paxillin-WT-GFP and GST-Paxillin-2C/A-GFP to bind clathrin heavy chain and EpsinR *in vitro* by GST-pull downs from cellular particle fractions (Figure 14). Both proteins also showed an interaction with the known paxillin-binding partner GIT1 providing supplementary evidence of their functionality upon affinity purification and bacterial expression.

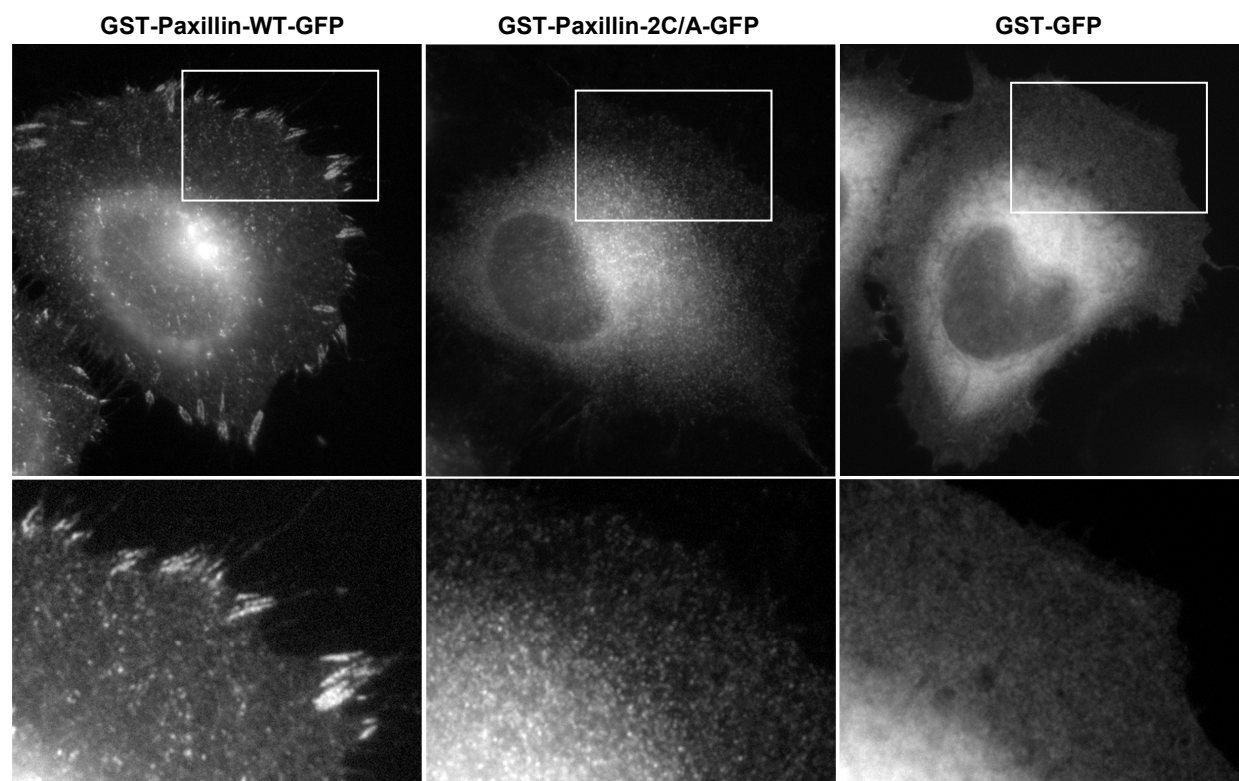


Figure 13 | Localization of the recombinant GFP-tagged wild type and mutant paxillin proteins upon microinjection. | HeLa cells were injected with GST-Paxillin-WT-GFP, GST-Paxillin-2C/A-GFP or GST-GFP as a control, fixed and imaged by fluorescent microscopy. Enlarged rectangular areas demonstrate localization of the wild type paxillin to the focal adhesions and vesicular structures, of the mutant paxillin defective in focal adhesion targeting to the population of small numerous vesicles. Injected GST-GFP produced diffused cytoplasmic staining.

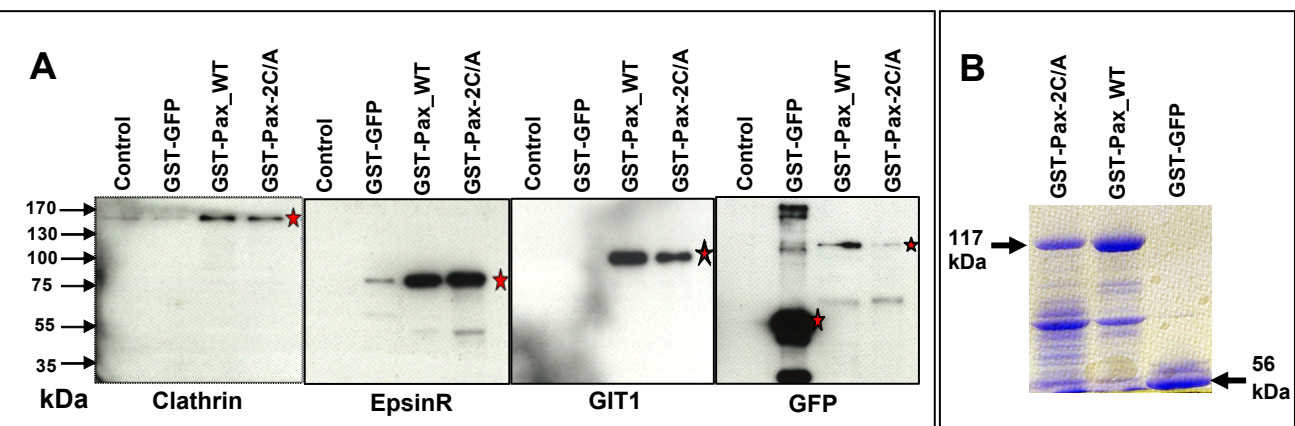


Figure 14 | Recombinant wild type and mutant paxillin proteins interact with clathrin, EpsinR and GIT1. | (A) GST-Paxillin-WT-GFP, GST-Paxillin-2C/A-GFP and GST-GFP recombinant proteins were coupled to Gluthatione-Sepharose 4B and used in pull-down assay from a particle fraction of HeLa cells. Control experiment contained only Gluthatione-Sepharose. Bound proteins were analyzed by Western Blot with antibodies against Clathrin Heavy Chain, EpsinR, GIT1 and GFP. Anti-GFP antibody recognized recombinant GST-GFP (56 kDa) and paxillin proteins (117 kDa). (B) Coomassie staining of the purified recombinant proteins used in the experiments.

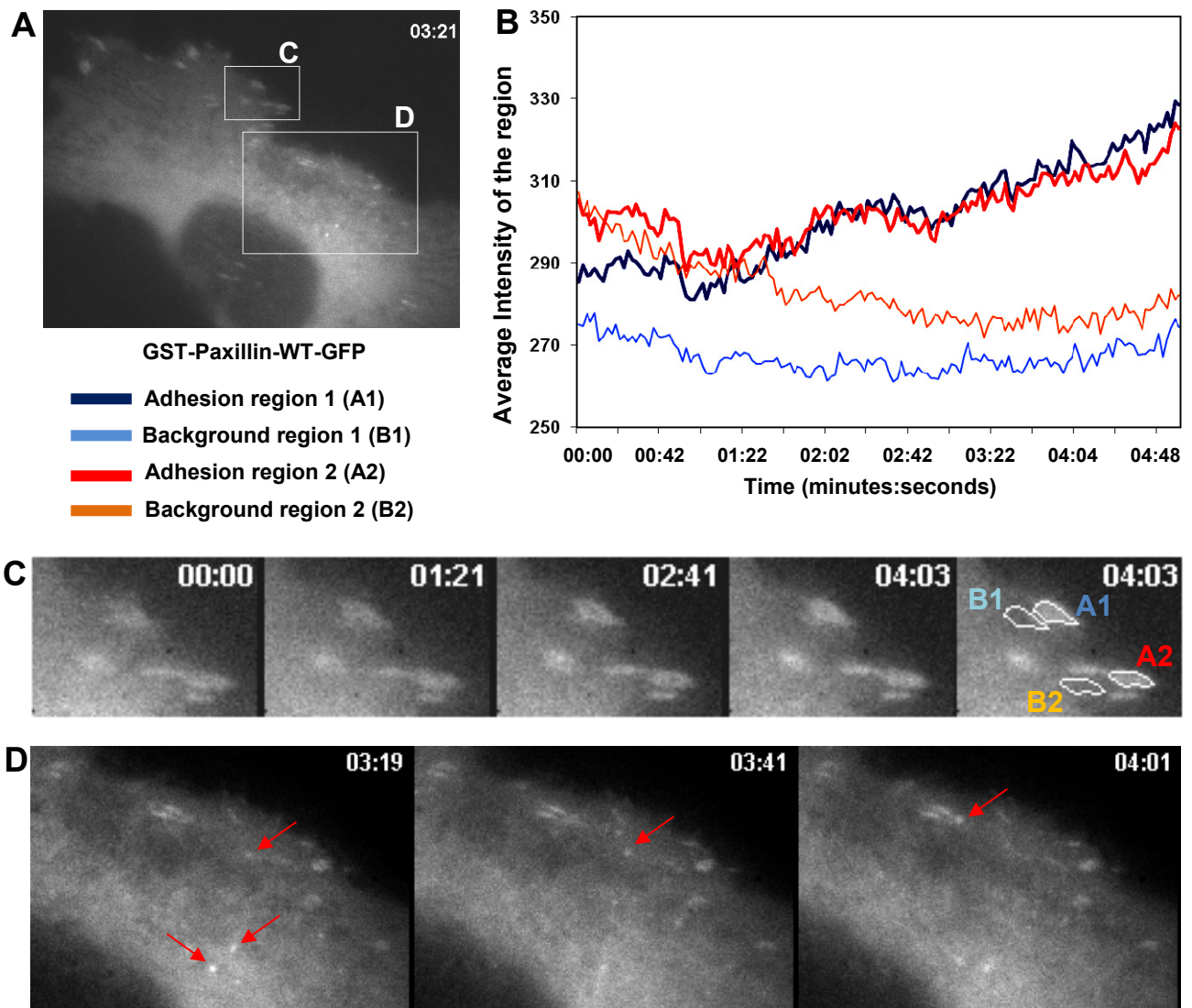


Figure 15 | Recombinant wild type paxillin localized to focal adhesions and vesicular structures upon injection into cultured cells. | (A) The HeLa cell shown was injected with GST-Paxillin-WT-GFP and directly imaged by time-lapse microscopy for 5 minutes with 1 sec interval. White rectangles highlight regions analyzed in (C) and (D) (Supplementary movie 4). (B, C) Dynamics of paxillin accumulation was analyzed in two focal adhesion regions (A1 and A2) and compared with similar control regions nearby (B1 and B2). Montage of the time-lapse frames (C) and graphs (B) demonstrate a gradual increase in the intensity of the paxillin signal at the focal adhesion sites and slight decrease at the adjacent cytoplasmic regions. (D) Frames of the time-lapse movie representing paxillin vesicles (marked by arrows) observed in the perinuclear area and around focal adhesions.

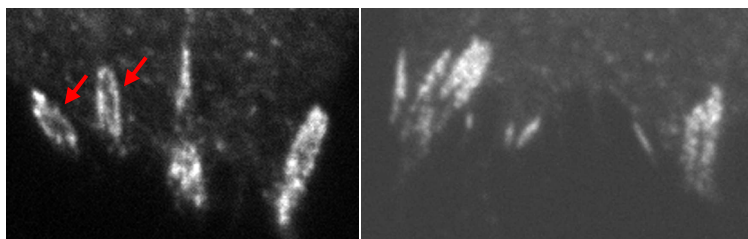


Figure 16 | Recombinant wild type paxillin formed clusters at the focal adhesions. | HeLa cells were injected with GST-Paxillin-WT-GFP, fixed and imaged by fluorescent microscopy. Arrows mark focal adhesions where paxillin distinctly concentrated in clusters at the periphery.

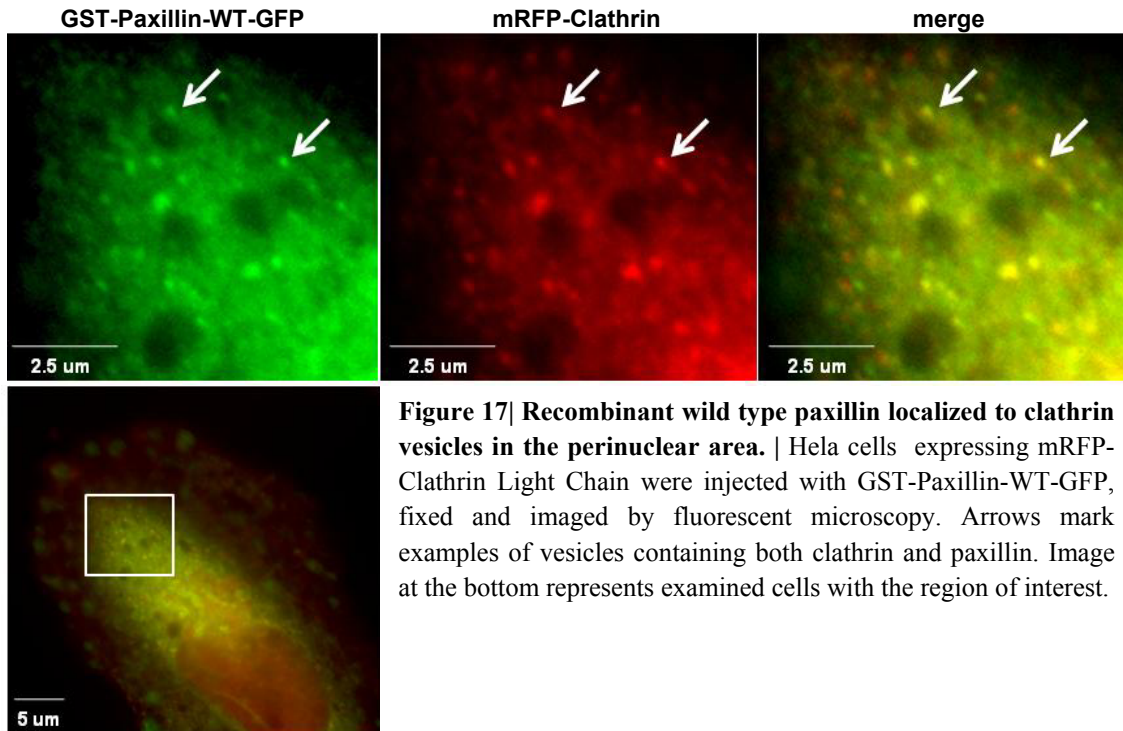


Figure 17| Recombinant wild type paxillin localized to clathrin vesicles in the perinuclear area. | HeLa cells expressing mRFP-Clathrin Light Chain were injected with GST-Paxillin-WT-GFP, fixed and imaged by fluorescent microscopy. Arrows mark examples of vesicles containing both clathrin and paxillin. Image at the bottom represents examined cells with the region of interest.

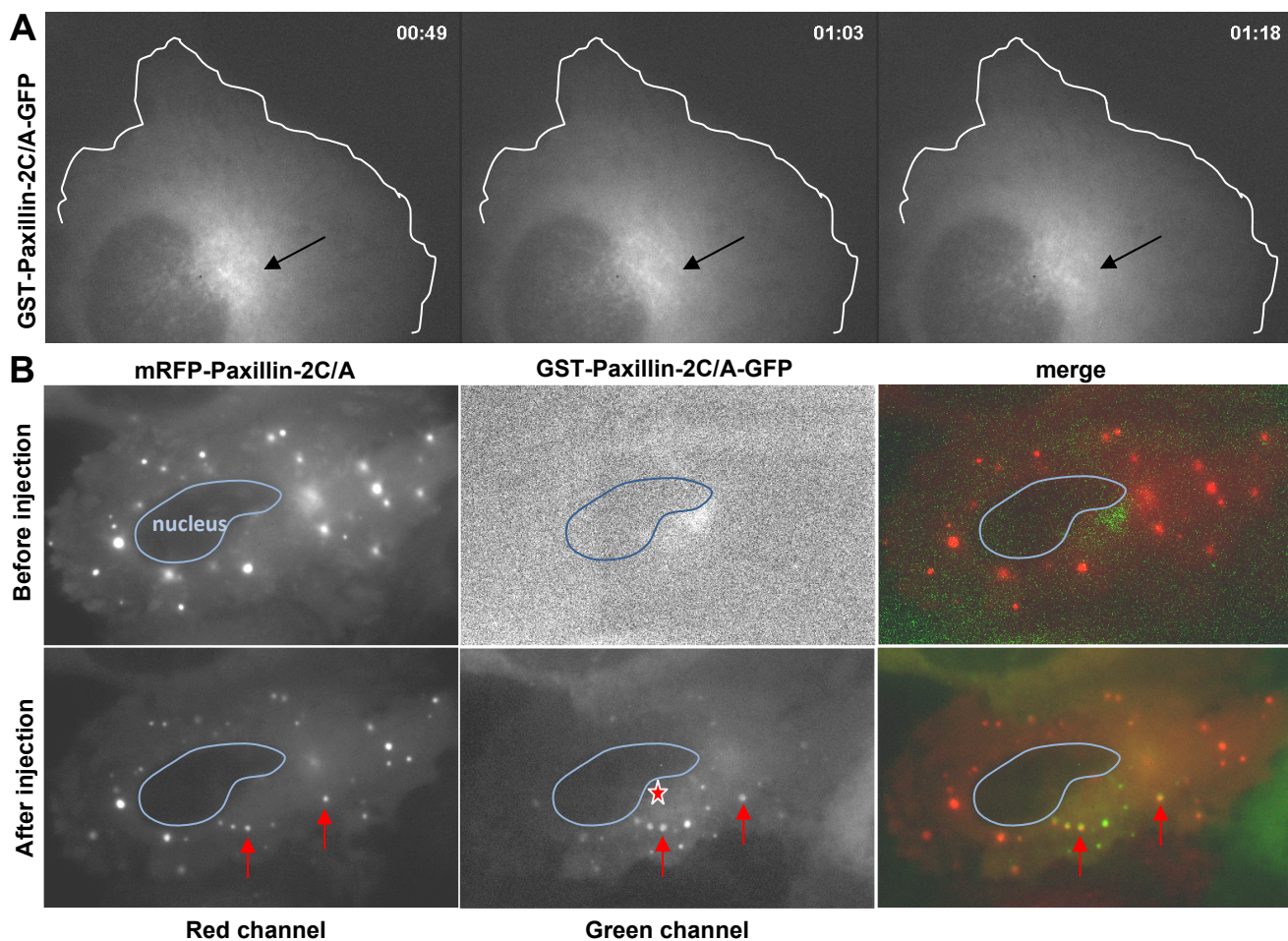


Figure 18| Live observations of the recombinant paxillin defective in focal adhesion targeting upon injection into cultured cells. | (A) HeLa cell was injected with GST-Paxillin-2C/A-GFP and directly imaged by time-lapse microscopy for 5 minutes with 1 sec interval. Several frames of the time-lapse movie show blurred vesicular-like structures in the perinuclear area (Supplementary movie 5). (B) HeLa cell was transfected with mRFP-Paxillin-2C/A, incubated overnight, injected with GST-Paxillin-2C/A-GFP and directly imaged by time-lapse microscopy (Supplementary movie 6). Upon injection GST-Paxillin-2C/A-GFP redistributed to the enlarged vesicles containing mRFP-Paxillin-2C/A at first in the proximity of the injection site (labeled by asterisk). Arrows mark example of the vesicles with the signal in both red and green channels.

Role of the LD4 domain in paxillin interaction with clathrin

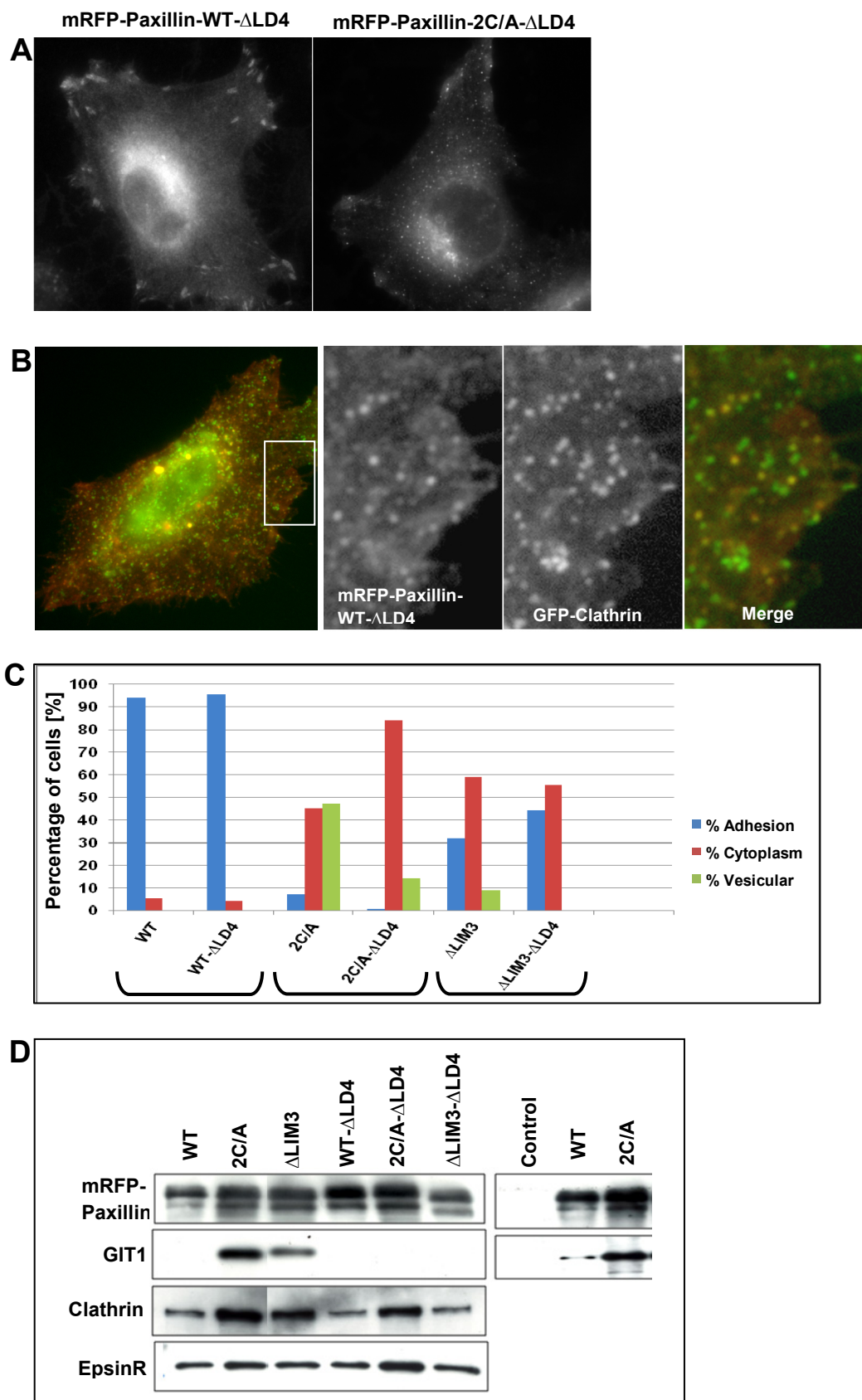
C. Turner and colleagues previously reported binding of the clathrin heavy chain to the recombinant LD4 domain of paxillin (Turner, Brown et al. 1999). However neither clathrin interaction with endogenous paxillin nor the role of the LD4 motif in this interaction was under investigation. In the same study direct interaction between paxillin LD4 domain and GIT1 protein was identified. Deletion of the LD4 domain was shown to abrogate GIT1 binding to paxillin and subsequent recruitment of GIT-PIX-PAK complex to focal adhesion sites (Turner, Brown et al. 1999; West, Zhang et al. 2001).

I aimed to examine a role of the LD4 motif in the paxillin interaction with clathrin using localization of the Paxillin-2C/A mutant to clathrin vesicles as a model. I generated constructs coding mRFP-Paxillin-WT, mRFP-Paxillin-2C/A and mRFP-Paxillin- Δ LIM3 with additional deletion of the LD4 domain and analyzed their localization upon transfection in cultured cells. The percentage of cells exhibiting distinct vesicular, focal adhesion or diffused cytoplasmic labeling upon transfection of the each construct was calculated (Figure 19C). Deletion of the LD4 domain did not influence the ability of the wild type paxillin to localize to focal adhesion sites, consistent with previously reported data (West, Zhang et al. 2001) (Figure 19 A, C). Paxillin-2C/A- Δ LD4 mutant exhibited predominant diffuse cytoplasmic localization however in 15% of the transfected cells it was still detected in vesicular structures containing clathrin (Figure 19 A, B, C). Before deletion of the LD4 domain mRFP-Paxillin-2C/A mutant localized to the clathrin vesicles in 45% of the transfected cells, therefore the LD4 motif probably determines the efficiency of the paxillin accumulation in the clathrin vesicles. Inline with this hypothesis, deletion of the LD4 motif in the Paxillin- Δ LIM3 construct completely eliminated appearance of the visible paxillin-containing vesicles.

Immunoprecipitation analysis demonstrated that deletion of the LD4 domain abrogated paxillin binding to GIT1 but not to clathrin heavy chain and EpsinR (Figure 19D). This fact together with mRFP-Paxillin-2C/A- Δ LD4 localization to the clathrin vesicles suggests that paxillin binding to clathrin is not dependent on its LD4 domain and interaction with GIT1. Identification of the

clathrin-binding sites within paxillin molecule will bring valuable insights into connection between clathrin trafficking and paxillin recycling.

Figure 19 | Role of the LD4 motif in paxillin binding to clathrin. | **(A)** Upon transfection into HeLa cells mRFP-Paxillin-WT and mRFP-Paxillin-2C/A lacking LD4 motif localized to focal adhesions and to vesicular-like structures respectively. **(B)** mRFP-Paxillin-2C/A-ΔLD4 (in red) localized to a subset of clathrin vesicles labeled by GFP-Clathrin Light Chain A (in green). **(C)** Histogram shows percentage of the cells exhibiting adhesion, vesicular or diffused localization of the paxillin proteins before and after deletion of LD4 domain. HeLa cells were transfected with the following constructs: mRFP-Paxillin-WT, mRFP-Paxillin-2C/A, mRFP-Paxillin-ΔLIM3 with or without LD4 domain. Number of analyzed transfected cells was higher than 200 for each case. **(D)** Deletion of LD4 domain abrogates interaction of paxillin constructs with GIT1 but not with Clathrin Heavy Chain (CHC) or EpsinR. Immunoprecipitation was performed with anti-mRFP antibody from crude lysates of HeLa cells transfected with one of the paxillin constructs (RFP-Paxillin-WT, RFP-Paxillin-2C/A, RFP-Paxillin-ΔLIM3 with or without LD4 domain). Blots were probed with anti-paxillin, anti-GIT1, anti-CHC and anti-EpsinR antibodies. The negative control with lysates from untransfected cells did not show any signal (not shown). Additional image at the right demonstrates weak binding of GIT1 to Paxillin-WT in comparison with Paxillin-2C/A.



Role of the clathrin adaptor EpsinR in the regulation of focal adhesion dynamics

Clathrin-coated vesicles take part in the diverse trafficking pathways including endocytosis from the plasma membrane and retrograde trafficking from the TGN membranes and endosomes. As already mentioned EpsinR is a clathrin adaptor protein that was found in a distinct subset of clathrin vesicles and shown to play a role in cargo-selection and vesicle budding from the TGN/endosomes rather than from the plasma membrane (Hirst, Motley et al. 2003). EpsinR has a specific function in the clathrin trafficking pathway that is distinct from the other clathrin adaptors. For example EpsinR mediates retrograde transport of Shiga toxin from endosomes to TGN whereas another abundant clathrin adaptor AP1 is not required for this process (Saint-Pol, Yélamos et al. 2004). Depletion of EpsinR by means of RNAi does not cause such a dramatic effect as clathrin knockdown which produces complete loss of clathrin-coated membranes and aggregation of the post-Golgi compartments (Motley, Bright et al. 2003). EpsinR depleted cells have a normal post-Golgi morphology with clathrin-coated budding membranes though their amount is reduced (Hirst, Miller et al. 2004).

Identification of EpsinR as an interaction partner of paxillin in the previous experiments led to the hypothesis that it may be a specific adaptor connecting paxillin to a subpopulation of clathrin vesicles. Therefore I investigated more closely the influence of EpsinR on paxillin turnover and focal adhesion organization. In order to analyze EpsinR function in focal adhesion dynamics I applied an RNAi approach. Hirst and colleagues have already successfully exploited siRNA knockdown of EpsinR to study its cellular functions in HeLa cells (Hirst, Miller et al. 2004). Therefore I used a siRNA oligonucleotide from their studies that was proven to be effective and specific for EpsinR depletion. To compare the consequences of the EpsinR knockdown with suppression of the entire clathrin machinery I performed in parallel knockdown of clathrin heavy chain (CHC).

Using siRNA transfection I effectively reduced levels of EpsinR and CHC at least by 70-80% (Figure 20 D). First I examined transferrin internalization rate and showed that it was affected by knockdown of CHC but not EpsinR (Figure 20 B). Inhibition of the transferrin uptake upon clathrin

suppression has been earlier described and therefore proved the efficiency of the clathrin knockdown (Hinrichsen, Harborth et al. 2003; Motley, Bright et al. 2003). Depletion of EpsinR caused re-distribution of the internalized transferrin vesicles from the perinuclear area to the cell periphery as compared with control cells. The different effects of EpsinR and CHC siRNA knockdowns on transferrin internalization confirmed a specific role of EpsinR in the clathrin trafficking pathway.

Secondly siRNA depletion of EpsinR and CHC induced an increase in size of focal adhesions containing paxillin and the formation of prominent actin stress fibers at the cell periphery (Figure 20A). Enlargement of focal adhesions upon EpsinR knockdown was more significant than upon CHC siRNA depletion. Moreover CHC and EpsinR siRNA-treated cells showed a 60% and 50% reduction in cell migration rate respectively in a Transwell migration assay. The observed changes in focal adhesion dimensions together with migration defects would be inline with an imbalance in focal adhesion turnover.

In summary, the obtained data revealed a role of the clathrin trafficking pathway, specifically its EpsinR-dependent part in focal adhesion organization and cell migration.

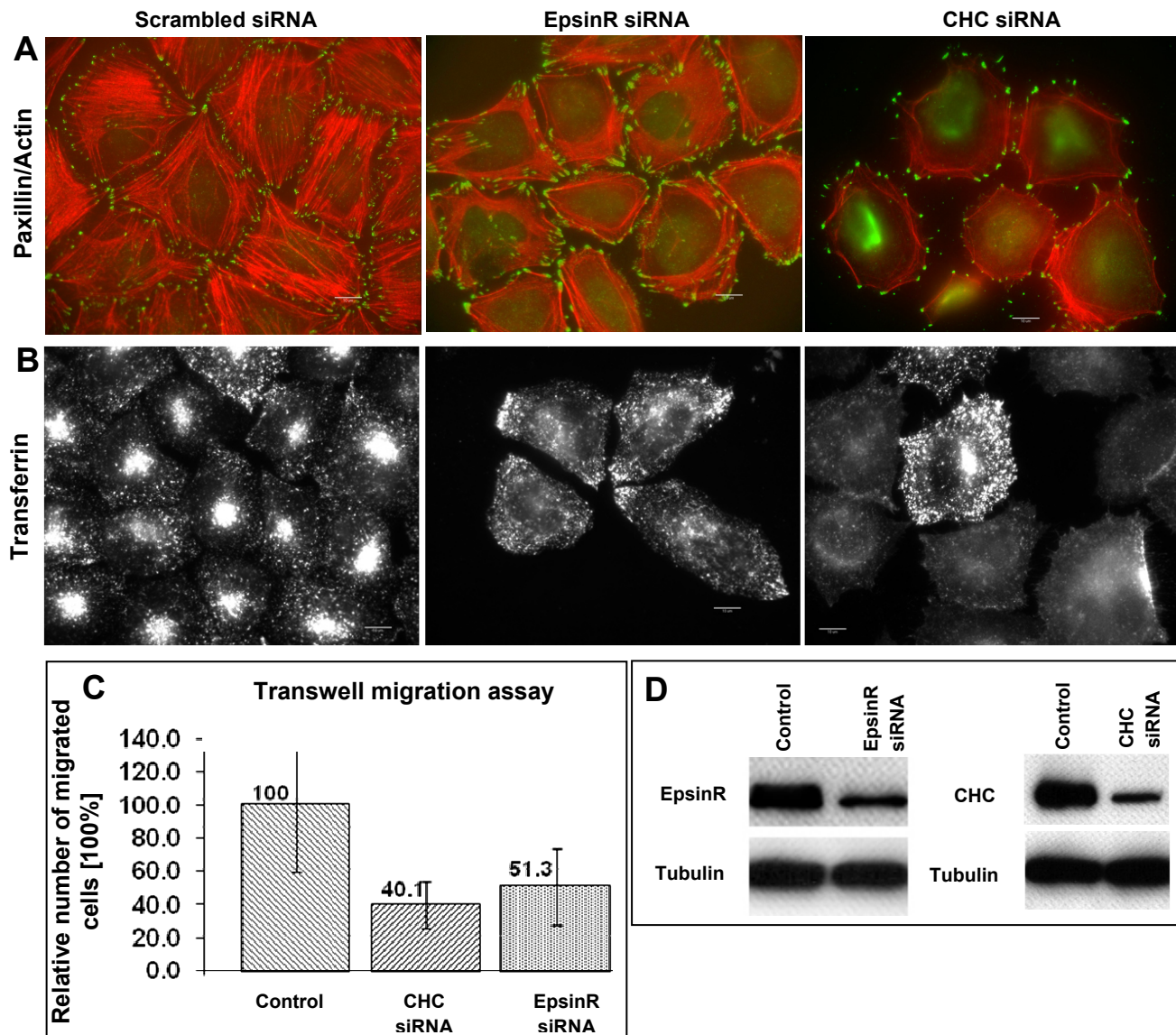


Figure 20| siRNA knockdown of EpsinR leads to the enlargement of focal adhesions and reduced cell migration. | (A) HeLa cells treated with Scrambled, EpsinR or Clathrin Heavy Chain (CHC) siRNAs were immunostained with phalloidin 568 to visualize actin (in red) and anti-paxillin antibody labeling focal adhesions (in green). (B) HeLa cells treated with Scrambled, EpsinR or CHC siRNAs were incubated for 15 minutes at 37°C with Alexa568-Transferrin, fixed and the efficiency of Transferrin endocytotic uptake judged by intensity of the intracellular vesicular labeling. Asterisk marks a cell that was not transfected with CHC siRNA and showed unaffected transferrin endocytosis similar to the control cells treated with Scrambled siRNA. (C) HeLa cells treated with Scrambled, CHC and EpsinR siRNAs were incubated for 48 hours and subjected to a Transwell-migration assay overnight. Relative number of migrated cells in each case was calculated. (D) Efficiency of the EpsinR and CHC siRNA knockdowns was confirmed by immunoblotting of crude cellular lysates with anti-EpsinR and anti-CHC antibodies and anti-tubulin antibody as a control for equal protein loading. All presented experiments were performed by Martin Breuss.

Conclusions and Perspectives

The existence of a vesicular trafficking pathway involved in the recycling of focal adhesion protein paxillin has been proposed previously. However the nature of this putative vesicular pathway remained elusive. From the studies of the paxillin mutants defective in focal adhesion binding I conclude that clathrin trafficking pathway is the route involved in paxillin recycling.

Mutations of the LIM3 domain reduced efficiency of paxillin binding to focal adhesions.

Paxillin localization to focal adhesions was shown to be dependent on the double zinc finger LIM3 domain (Brown, Perrotta et al. 1996). In contrast to previous data I found that deletion or structural alterations of LIM3 did not eliminate focal adhesion localization of paxillin completely but rather reduced its efficiency, suggesting that other motifs are also involved in paxillin binding to adhesions. Recent microinjection experiments of Kaverina and co-workers supported my findings and demonstrated that a LIM3 domain alone was not able to localize to focal adhesions as opposed to the combination of the LIM3 with LIM2 domains (Efimov, Schiefermeier et al. 2008). Interestingly focal adhesion localization of another LIM domain-protein zyxin was also found to be dependent on multiple LIM domains. None of the recombinant proteins harboring only one of the three zyxin LIM domains was accumulated in focal adhesions in contrast to the constructs combining at least two domains (LIM1 and LIM2 or LIM2 and LIM3) (Nix, Fradelizi et al. 2001). Therefore stable focal adhesion association of paxillin by analogy with zyxin might require tandem LIM domains (most likely LIM2 and LIM3).

I also showed that interference with the structure of the first LIM3 zinc finger affected paxillin localization to focal adhesions stronger than disruption of the second zinc finger, and disruption of both zinc fingers had a greater effect than deletion of the entire domain. Probably the altered structure of the LIM3 zinc fingers prevents binding of the adjacent LIM2 domain to focal adhesions. Accordingly disruption of the first LIM3 zinc finger is more crucial than of the

second one since it is located closer to LIM2. And deletion of LIM3 does not affect LIM2 binding to focal adhesions.

Accumulation of paxillin defective in focal adhesion binding in clathrin vesicles.

I demonstrated that the efficiency of paxillin binding to focal adhesions is inversely correlated with its accumulation in cytoplasmic vesicular-like structures. I suggest when paxillin is not able to bind to focal adhesions it is retained in the putative recycling vesicles that leads to their stabilization and enlargement and makes them readily observed by conventional microscopy. Under standard conditions vesicles containing wild type paxillin cannot be detected, probably due to their small size or transient nature. Therefore the observed vesicles containing paxillin defective in focal adhesion binding provide only indirect evidence for the existence of the paxillin vesicular recycling pathway.

It is interesting to compare my findings with studies of the paxillin yeast ortholog Pxl1p (Paxillin-like protein-1). Pxl1p contains two LIM domains (highly similar to the LIM3 and LIM4 domains of mammalian paxillin) that are responsible for protein targeting to the sites of polarized growth, at the tip of the bud and bud neck. At those sites the active reorganization of actin cytoskeleton occurs in response to localized RhoGTPase signaling, thereby they might be considered as analogs of mammalian focal adhesion sites. It was shown that deletion of the LIM domains led to the Pxl1p relocalization to the intracellular vesicular-like structures similar to the Golgi compartment (Mackin, Sousou et al. 2004). Therefore the LIM domains likely share evolutionary conserved function of protein targeting to specific cellular compartments such as mammalian focal adhesions or yeast bud tips. I suggest that the putative vesicular trafficking route involved in paxillin recycling may be also evolutionary conserved since both mammalian and yeast paxillin proteins accumulate in vesicular structures upon disruption of the LIM domains.

Additionally the mammalian paxillin homologue Hic-5 was also found redistributed to vesicular-like structures from focal adhesion plaques upon

structural alterations in the LIM3 domain (Nishiya, Iwabuchi et al. 1999). Unfortunately Nishiya and colleagues did not comment on possible implications of Hic-5 accumulation in vesicular structures. However their findings suggest that vesicular trafficking could be a common mechanism for the recycling of all paxillin family members.

Enhanced binding of the paxillin-LIM3-mutants to the recycling vesicular-like structures allowed identification of their components using mRFP-Paxillin-2C/A protein as a bait. The identified components included clathrin heavy chain, two light chains, clathrin-interacting protein EpsinR and PIK3C2A. Therefore I assigned those vesicular structures to the clathrin-trafficking pathway. Subsequent immunoprecipitation and co-localization experiments confirmed interaction of the paxillin mutants defective in focal adhesion binding with Clathrin Heavy Chain, clathrin adaptor EpsinR and their localization to a subpopulation of fast moving clathrin vesicles.

Localization of the wild type paxillin to the clathrin vesicles.

Accumulation of the paxillin-LIM3-mutants in clathrin vesicles suggest the existence of the clathrin trafficking route involved in paxillin recycling. Therefore I examined localization of the wild type paxillin to clathrin vesicles.

The first indication that paxillin interacts with clathrin came from studies of Turner and colleagues when they discovered that the LD4 domain of paxillin binds Clathrin Heavy Chain (CHC) in GST pulldown experiments *in vitro* (Turner, Brown et al. 1999). However significance of this finding was not further investigated.

At first I demonstrated that wild type paxillin binds CHC and EpsinR in the immunoprecipitation and GST-pull down experiments. However despite the detected interactions, overexpressed wild type paxillin did not localize to distinct clathrin vesicles. Therefore I undertook a different approach for localizing paxillin using microinjection of recombinant wild type paxillin fused to the GFP molecule. Recombinant paxillin demonstrated more distinct vesicular localization with reduced cytoplasmic background as compared with overexpression, probably due to the lower concentration of the introduced protein. In the

perinuclear area of the injected cells vesicular structures containing recombinant wild type paxillin co-localized with clathrin.

Additionally upon co-transfection of the wild type paxillin with one of the paxillin-LIM3-mutants both co-localized in the enlarged clathrin vesicles. This confirmed the ability of the wild type protein to localize to the clathrin structures.

In summary, wild type paxillin was demonstrated not only to bind to clathrin vesicle components but also to localize to clathrin vesicles. However paxillin localization to the clathrin vesicles could not be detected under standard conditions by conventional fluorescence microscopy because of the reasons suggested above (small size and transient nature of the structures). The size of clathrin vesicles can be as small as 60 nm in diameter and the speed of movement can reach 5-20 $\mu\text{m/s}$ under certain conditions (Zhao, Gaidarov et al. 2007), that would be beyond the detection of standard time-lapse microscopy resolution. I believe that this was the reason why the putative paxillin recycling vesicles still have not been observed in live cells. Therefore further studies of clathrin-dependent paxillin recycling may require the development of high resolution microscopy techniques. Attempts to perform super-resolution imaging of paxillin in focal adhesions of live cells using photoactivated localization microscopy (PALM) has been recently presented (Shroff, Galbraith et al. 2008). PALM allows the collection of images at spatial resolution close to 60 nm but with low frame rates ($\sim 25\text{s/frame}$). Interestingly, that PALM revealed nano-particles of paxillin inside focal adhesions that form a diffuse background when viewed by conventional diffraction-limited microscopy. Notably, I also observed clusters of the paxillin inside focal adhesions upon injection of the recombinant wild type paxillin but not after overexpression. Unfortunately, temporal resolution of the PALM technique is not sufficient yet to examine the fast dynamics of paxillin particles in respect of clathrin trafficking pathway. I suggest that the combination of microinjection with low amounts of labeled paxillin with fast time-lapse microscopy could provide an alternative approach to observe paxillin dynamics and in particular its recycling through clathrin pathway.

EpsinR-dependent clathrin trafficking pathway plays a role in focal adhesion turnover.

siRNA knockdown of clathrin heavy chain (CHC) and EpsinR caused dramatic enlargement of focal adhesions, formation of strong peripheral actin stress fibers and led to reduction in cell migration rate. Therefore clathrin trafficking pathway and specifically its EpsinR-dependent part likely play a role in focal adhesion turnover and cell migration.

As discussed before EpsinR is a clathrin adaptor, which is enriched in the TGN and endosomal compartments and does not overlap with AP2, the main endocytic clathrin adaptor at the plasma membrane. Therefore EpsinR is suggested to be responsible for intracellular trafficking rather than for endocytosis (Kalthoff, Groos et al. 2002; Wasiak, Legendre-Guillemain et al. 2002; Hirst, Motley et al. 2003; Mills, Praefcke et al. 2003; Saint-Pol, Yélamos et al. 2004). I have also demonstrated that EpsinR is actually not required for transferrin uptake. Therefore in respect of focal adhesion turnover, EpsinR might regulate rather recycling of focal adhesion components toward plasma membrane then internalization.

On the basis of the identified interaction between paxillin, clathrin and EpsinR it is tempting to speculate that the EpsinR-dependent clathrin trafficking pathway might be involved in paxillin recycling to focal adhesions. Enlargement of focal adhesions upon EpsinR knockdown could be explained by defects in paxillin turnover. However at this stage I cannot exclude possible the involvement of EpsinR in the trafficking of other focal adhesion components such as integrins.

As discussed in the main introduction it has been recently shown that integrins undergo clathrin-dependent internalization (Nishimura and Kaibuchi 2007). At present there are no direct studies demonstrating involvement of the clathrin-dependent recycling in the integrin delivery to the plasma membrane. However a GTPase-activating protein ACAP1 was found to regulate recycling of β 1-integrin (Li, Ballif et al. 2005) and recruitment of clathrin to the recycling endosomes (Li, Peters et al. 2007). This indicates that clathrin is involved not only in the internalization but also in the recycling of certain integrins.

Therefore it will be important to determine a role of the EpsinR-dependent clathrin trafficking in the recycling of not only paxillin but also other focal adhesion proteins including integrins. In addition it will be interesting to define the contribution of the paxillin recycling to the focal adhesion turnover separately from integrin recycling. For this purpose siRNA-mediated depletion of EpsinR and especially CHC would not be suitable because it blocks the entire recycling pathway. To dissect the specific impact of paxillin recycling on focal adhesion turnover it would be necessary to identify the mechanism of paxillin recruitment to clathrin vesicles and develop an approach to interfere only with paxillin trafficking.

SUMMARY

Speculation on the role of vesicular trafficking in focal adhesion turnover

Taken together the studies described above bring new insights into understanding of the link between vesicular trafficking and focal adhesion turnover.

I have demonstrated that paxillin defective in focal adhesion binding accumulates in a subpopulation of clathrin vesicles. This finding led me to a proposal that paxillin recycling might occur through clathrin-dependent trafficking pathway. As indirect evidence I show that the part of the clathrin-dependent pathway associated with the adaptor protein EpsinR is indeed involved in the regulation of focal adhesions. Further experiments toward understanding of how paxillin binds to clathrin vesicles and what factors regulate its turnover are required.

Theoretically, the clathrin machinery is a good candidate for the polarized transport of paxillin and other adhesion components including integrins. First of all, clathrin vesicles are quite small (up to 60 nm in diameter), can move as fast as 5-20 $\mu\text{m/s}$ under certain conditions (Zhao, Gaidarov et al. 2007), their movement is dependent on the microtubule tracks (Rappoport, Bushra et al. 2003; Zhao, Gaidarov et al. 2007) and their assembly is tightly regulated by a number of specific clathrin-adaptor proteins. Therefore clathrin vesicles can provide a fast but controlled delivery of the necessary components towards polarized cell edge along microtubule tracks. Secondly clathrin trafficking pathway was found to contribute to the polarized delivery of basolateral membrane proteins in epithelial cells (Deborde, Perret et al. 2008) in a possibly analogous process.

The involvement of the microtubules in vesicular trafficking of cell adhesion components remains an open question. On the one hand migrating cells form array of stable microtubules oriented toward the leading edge which might determine polarization of the vesicular transport (Gundersen and Bulinski 1988). On the other hand cells have been shown to be able to spread and form focal adhesions even in the absence of a microtubule network. My preliminary experiments also demonstrated that upon microinjection recombinant paxillin redistributed to focal adhesions even after microtubule disassembly. However in

the absence of microtubules paxillin bound first to focal adhesions close to the microinjection site, whereas in a control situation it evenly accumulated in all focal adhesions. I suggest that vesicular trafficking is dispensable for the localization of focal adhesion components, which than may be distributed by means of cytoplasmic diffusion. However polarized vesicular transport of focal adhesion components along microtubules becomes essential during directional cell migration.

Interestingly the formation of tight junctions was also found to be dependent on regulated membrane traffic (see review (Yu and Turner 2008)). The tight junctions form intercellular connections between epithelial cells and similar to focal adhesions consist of transmembrane proteins, plaque proteins and cytoskeletal components including actin. Therefore vesicular transport is likely a common mechanism underlying the polarized and controlled delivery of the components to different cellular plasma membrane structures. Notably tight junction components were shown to pre-assemble at the vesicular membranes prior to recycling to the plasma membrane (see review (Yu and Turner 2008)). Whether focal adhesion molecules are also transported as a pre-formed complex remains to be established. In the future it will be interesting to determine whether the clathrin trafficking pathway plays a role only in paxillin recycling or also influence turnover of the integrins and other focal adhesion proteins.

In conclusion, Figure 1 demonstrates a model of the possible involvement of clathrin trafficking pathway in the turnover of focal adhesion components (integrins and paxillin) in a migrating cell. Clathrin machinery and specifically endocytic clathrin-adaptor AP2 plays a role in the integrin internalization (Nishimura and Kaibuchi 2007). Upon internalization integrins are directed to a recycling endosomal compartment in the perinuclear area. I suggest that paxillin is transported to the recycling compartment in complex with integrins or alternatively, recruited there directly from the cytoplasm via unknown factor. Trafficking of paxillin and integrins from recycling compartment to the cellular leading edge depends on the clathrin vesicles containing adaptor EpsinR and on the polarized array of microtubules.

I think that the future application of evolving high resolution microscopy techniques in combination with selective dissection of the clathrin pathway should shed more light on the involvement of vesicular trafficking in cytoskeletal dynamics and cell motility.

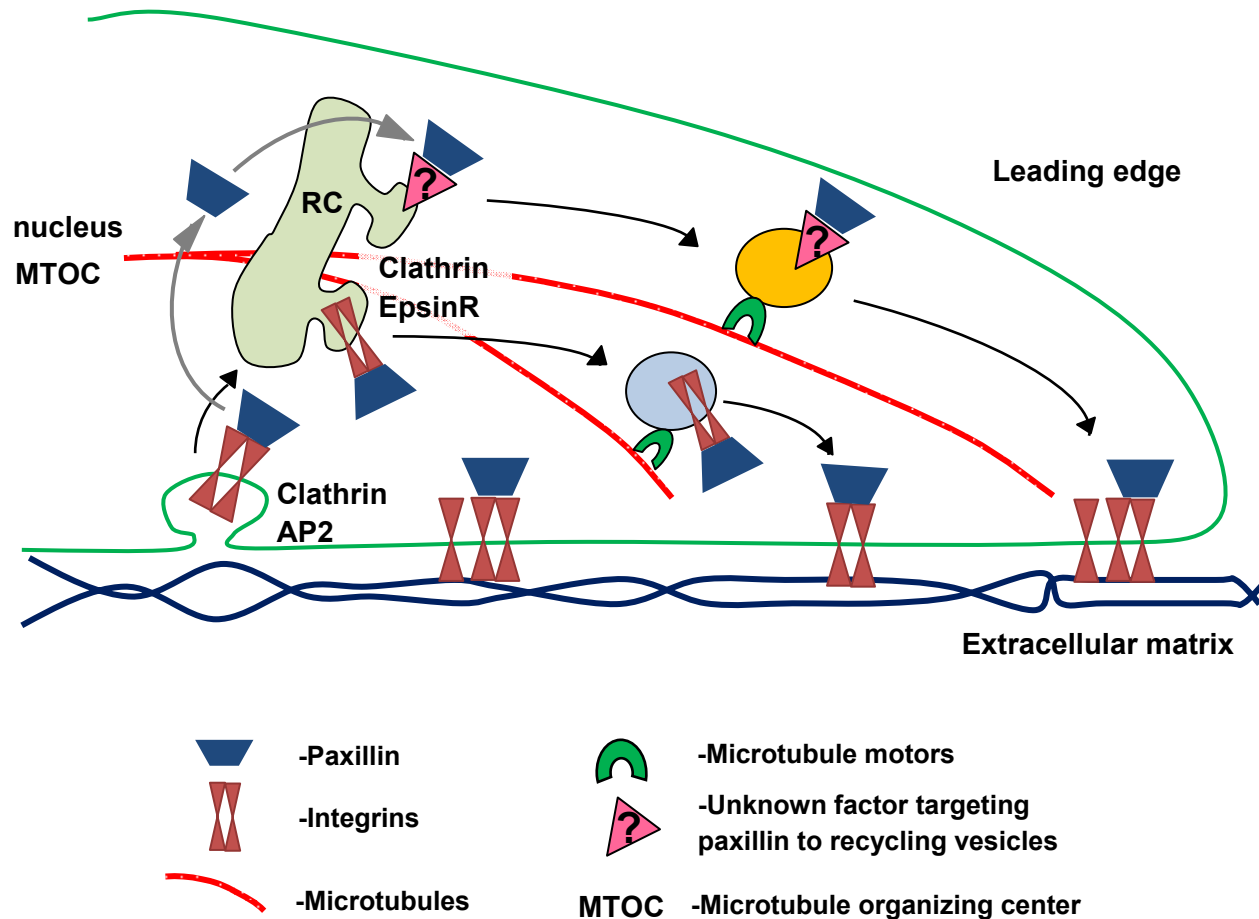


Figure 1 | Scheme demonstrating a role of the clathrin trafficking pathway in the turnover of focal adhesion components. | Integrins are internalized via a mechanism that is dependent on clathrin and its adaptor AP2 (Nishimura and Kaibuchi 2007). Paxillin might be transported to the recycling compartment (RC) in a complex with integrins. Alternatively paxillin might be recruited to the RC directly from the cytoplasm via unknown component. Delivery of paxillin (jointly or apart from the integrins) to the leading edge of the cell from the RC involves clathrin vesicles containing the EpsinR adaptor. Transport of the paxillin-containing recycling vesicles occurs along microtubules polarized toward the cell front.

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SUPPLEMENTARY MATERIAL

Supplementary movies

Movie 1. Dynamics of focal adhesions in control (at the bottom) and beta-COP depleted (on the top) HeLa cells with stable expression of EGFP-Paxillin. Time-lapse movie with 1 minute interval between frames.

Movie 2. Dynamics of the vesicular structures containing mRFP-Paxillin-2C/A in HT1080 cell. Movement of the vesicles is inhibited upon microtubule disassembly after nocodazole addition. Time-lapse movie with 3 sec between frames.

Movie 3. Cytoplasmic labeling upon microinjection of the recombinant GST-GFP protein into HeLa cell. Time-lapse movie with 1 second interval between frames. The movie was acquired starting 30 seconds after injection.

Movie 4. Microinjection of the GST-Paxillin-WT-GFP protein into HeLa cell. The injected protein localized to focal adhesions (as observed in the small rectangle) and to vesicular structures (as observed in the big rectangle). Time-lapse movie with 1 second interval between frames. The movie was acquired starting 30 seconds after injection.

Movie 5. Dynamics of blurred vesicular-like structures in the perinuclear area upon injection of the GST-Paxillin-2C/A-GFP protein into HeLa cell. Time-lapse movie with 1 second interval between frames. The movie was acquired starting 30 seconds after injection.

Movie 6. Microinjected GST-Paxillin-2C/A-GFP protein (acquired in the green channel) localized to the vesicular-like structures containing overexpressed mRFP-Paxillin-2C/A (acquired in the red channel) in HeLa cell. Time-lapse movie with 3.5 seconds between frames. The movie was acquired starting 30 seconds after injection.

Supplementary tables

Table 1 | siRNA oligonucleotides for validation experiments of the candidate siRNA microarray screen.

Gene	Ambion siRNA ID	siRNA sense sequence 5'-3'	siRNA antisense sequence 5'-3'
AKAP13	136052	CCAACAAAGAUGCCUAUACtt	GUAUAGGCAUCUUUGUUGGtg
TM4SF6	138892	GCCAUGCAGUAGACAAGAUtt	AUCUUGUCUACUGCAUGGctt
PAK2	110776	GGAUCUGUUAAGCUCACUGtt	CAGUGAGCUUAAACAGAUCCtt
PAK4	288585	CCACUGCCUGAAGAACCAGtt	CUGGUUCUUCAGGCAGUGGtt
TLN1	5467	GGAGGAAUAACAGGGACctt	GGUCCCUGUUAUUUCCUCctt
CDC42	10418	GGAUUAUGACAGAUUACGAtt	UCGUAAUCUGUCAUAAUCctc
Rac1	120600	CCUUUGUACGCUUUGCUCAtt	UGAGCAAAGCGUACAAAGGtt
RhoB	41889	GAACUAUGUGGCCGACAUUtt	AAUGUCGGCCACAUAGUUCtc
KIF1A	14392	GGGAUACAACGUGUGCAUCtt	GAUGCACACGUUGUAUCCctc
MYH9	11605	GGUGAAGGUGAACAAGGAUtt	AUCCUUGUUCACCUUCACctt
MACF1	258846	CCAAAGUGACUUGAAGGAUtt	AUCCUUCAAGUCACUUUGGtt
AP1B1	9431	GGAGAAGAAGAAGGAGGCAtt	UGCCUCCUUCUUCUUCUCctt
ITGB5	11108	GGUCUCAACAU AUGCACUAtt	UAGUGCAU AUGUUGAGACctg
MLPH	42410	GAUGAGUCCUGCUCAGAGAtt	UCUCUGAGCAGGACUCAUCtg
PTK2	103596	GGGAGAAGUAUGAGCUUGCtt	GCAAGCUCAUACUUCUCCctc

Table 2 | Cell migration-related proteins (including focal adhesion scaffold and regulatory proteins) selected for the candidate siRNA screen.

Gene	RefSeq	Gene	RefSeq	Gene	RefSeq
ITGB1	NM_002211	NCK2	NM_003581	GIT1	NM_014030
ITGB3	NM_000212	BCAR1	NM_014567	GIT2	NM_057169
ITGB4	NM_000213	PAX	NM_002859	ANXA2	NM_001002858
ITGB5	NM_002213	LIMS1	NM_004987	CFL1	NM_005507
ITGB6	NM_000888	SORBS1	NM_006434	EVL	NM_016337
CD151	NM_004357	PPFIA1	NM_177423	GSN	NM_000177
CD81	NM_004356	GNB2L1	NM_006098	DIAPH1	NM_005219
TM4SF6	NM_003270	NUDT16L1	NM_032349	DIAPH2	NM_007309
TM4SF7	NM_001025237	SDCBP	NM_005625	DIAPH3	NM_030932
TRPM7	NM_017672	SORBS3	NM_005775	NEXN	NM_144573
LRP1	NM_002332	ABL1	NM_007313	PFN1	NM_005022
PTPNS1	NM_080792	ABL2	NM_005158	PFN2	NM_053024
CALR	NM_004343	CSK	NM_004383	PARVA	NM_018222
CANX	NM_001746	PTK2	NM_153831	PARVB	NM_001003828
CIB	NM_006384	FYN	NM_002037	TNS	NM_022648
ITGB3BP	NM_014288	SRC	NM_005417	TENC1	NM_015319
ICAP1	NM_004763	YES1	NM_005433	TPM1	NM_001018004
ACTB	NM_001101	MAPK3	NM_002746	TPM2	NM_003289
K-alpha-1	NM_006082	MAPK1	NM_002745	TPM3	NM_152263
TUBB	NM_178014	ILK	NM_004517	TPM4	NM_003290
VIM	NM_003380	LIMK1	NM_002314	VASP	NM_003370
ACTN1	NM_001102	LIMK2	NM_001031801	PPIA	NM_021130
ACTN4	NM_004924	MAP3K1	NM_005921	GAPDH	NM_002046
VIL2	NM_003379	MYLK	NM_053025		
FLNA	NM_001456	PAK1	NM_002576		
FLNB	NM_001457	PAK2	NM_002577		
FLNC	NM_001458	PAK4	NM_005884		
NF2	NM_000268	PKCA	NM_002737		
PLEKHC1	NM_006832	PKCE	NM_005400		
MIGFILIN	NM_017556	PRKCM	NM_002742		
MSN	NM_002444	ROCK1	NM_005406		
MYLIP	NM_013262	ROCK2	NM_004850		
PALLD	NM_016081	PIK3CA	NM_006218		
RDX	NM_002906	PIK3CB	NM_006219		
TLN1	NM_006289	PI3KCG	NM_002649		
TLN2	NM_015059	PIK3R1	NM_181523		
TES	NM_015641	PIP5K1C	NM_012398		
VCL	NM_014000	PTEN	NM_000314		
ZYX	NM_003461	PTPN1	NM_002827		
CAV1	NM_001753	PTPN12	NM_002835		
CAV2	NM_001233	PTPRF	NM_002840		
CBL	NM_005188	PTPN11	NM_002834		
CRK	NM_005206	PTPRA	NM_002836		
CRKL	NM_005207	CAPN1	NM_005186		
CSRP1	NM_004078	CAPN2	NM_001748		
GRB2	NM_002086	CAPNS1	NM_001749		
GRB7	NM_005310	RNF5	NM_006913		
TGFB1I1	NM_015927	ARF1	NM_001024227		
LASP1	NM_006148	DDEF1	NM_018482		
LIMS2	NM_017980	DNM1	NM_004408		
NCK1	NM_006153	DNM2	NM_001005360		

Table 3 | RhoGTPases and their regulators selected for the candidate siRNA screen.

Gene	RefSeq	Gene	RefSeq	Gene	RefSeq
RhoGTPases		<i>ARHGEF10</i>	NM_014629	GDI	
<i>ARHA</i>	NM_001664	<i>ARHGEF10L</i>	NM_018125	<i>ARHGDIA</i>	NM_004309
<i>ARHB</i>	NM_004040	<i>ARHGEF17</i>	NM_014786	<i>ARHGDIB</i>	NM_001175
<i>ARHC</i>	NM_175744	<i>ABR</i>	NM_021962	<i>ARHGDIG</i>	NM_001176
<i>RAC1</i>	NM_006908	<i>BCR</i>	NM_004327	RhoGAPs	
<i>RAC2</i>	NM_002872	<i>ALSIN</i>	NM_020919	<i>ARHGAP23</i>	XM_290799
<i>RAC3</i>	NM_005052	<i>VAV2</i>	NM_003371	<i>ARHGAP10</i>	NM_024605
<i>ARHG</i>	NM_001665	<i>VAV3</i>	NM_006113	<i>Chimerin 1</i>	NM_001822
<i>CDC42</i>	NM_001791	<i>VAV1</i>	NM_005428	<i>Chimerin 2</i>	NM_004067
<i>ARHQ</i>	NM_012249	<i>OBSCN</i>	NM_052843	<i>ARHGAP5</i>	NM_001030055
<i>ARHJ</i>	NM_020663	<i>ARFGEF6</i>	NM_004840	<i>GRLF1</i>	NM_004491
<i>WRCH1</i>	NM_021205	<i>ARHGEF7</i>	NM_003899	<i>OPHN1</i>	NM_002547
<i>RHOV</i>	NM_133639	<i>PREX1</i>	NM_022835	<i>GRAF</i>	NM_015071
<i>RND1</i>	NM_014470	<i>ARHGEF4</i>	NM_020820	<i>ARHGAP11A</i>	NM_014783
<i>ARHN</i>	NM_005440	<i>ARHGEF9</i>	NM_015320	<i>STARD8</i>	NM_014725
<i>ARHE</i>	NM_005168	<i>SPATA13</i>	NM_153023	<i>STARD13</i>	NM_178006
<i>ARHD</i>	NM_014578	<i>TIAM2</i>	NM_012454	<i>DLC1</i>	NM_182643
<i>RHOF</i>	NM_019034	<i>TIAM1</i>	NM_003253	<i>ARHGAP6</i>	NM_013427
<i>RHOH</i>	NM_004310	<i>PLEKHG4</i>	NM_015432	<i>ARHGAP19</i>	NM_032900
<i>RHOBTB1</i>	NM_004310	<i>HAPIP</i>	NM_001024660	<i>ARHGAP28</i>	NM_001010000
<i>RHOBTB2</i>	NM_004310	<i>TRIO</i>	NM_007118	<i>ARHGAP18</i>	NM_033515
RhoGEFs (Dbl family)		<i>FLJ32854</i>	AK057416	<i>ARHGAP20</i>	NM_020809
<i>ARHGEF19</i>	NM_014448	<i>GEFT</i>	NM_182947	<i>ARHGAP8</i>	NM_001017526
<i>SGEF</i>	NM_015595	<i>MCF2L2</i>	NM_015078	<i>ARHGAP1</i>	NM_004308
<i>ARHGEF15</i>	NM_173728	<i>MCF2</i>	NM_005369	<i>ARHGAP4</i>	NM_001666
<i>ARHGEF5</i>	NM_005435	<i>MCF2L</i>	NM_024979	<i>SRGAP3</i>	NM_014850
<i>NGEF</i>	NM_019850	<i>FLJ20184</i>	NM_017700	<i>SRGAP1</i>	NM_020762
<i>ARHGEF2</i>	NM_004723	<i>DNMBP</i>	NM_015221	<i>FNBP2</i>	NM_015326
<i>AKAP13</i>	NM_006738	<i>SOS1</i>	NM_005633	<i>SH3BP1</i>	NM_018957
<i>ARHGEF18</i>	NM_015318	<i>SOS2</i>	NM_006939	<i>RICH2</i>	NM_014859
<i>RGNEF</i>	NM_001080479	<i>ECT2</i>	NM_018098	<i>RICH1</i>	NM_001006634
<i>ARHGEF12</i>	NM_015313	<i>PLEKHG5</i>	NM_020631	<i>GMIP</i>	NM_016573
<i>ARHGEF11</i>	NM_014784	<i>PLEKHG6</i>	NM_018173	<i>ARHGAP29</i>	NM_004815
<i>ARHGEF1</i>	NM_199002	RhoGEFs (Dock family)		<i>HA1</i>	NM_012292
<i>ITSN1</i>	NM_003024	<i>DOCK1</i>	NM_001380	<i>PIK3R1</i>	NM_181523
<i>ITSN2</i>	NM_006277	<i>DOCK2</i>	NM_004946	<i>PIK3R2</i>	NM_005027
<i>Net1</i>	NM_005863	<i>DOCK5</i>	NM_024940	<i>OCRL1</i>	NM_000276
<i>ARHGEF3</i>	NM_019555	<i>DOCK3</i>	NM_004947	<i>C5ORF5</i>	NM_016603
<i>FARP1</i>	NM_005766	<i>DOCK4</i>	NM_014705	<i>KIAA1688</i>	NM_025251
<i>FARP2</i>	NM_014808	<i>DOCK6</i>	NM_020812	<i>ARHGAP24</i>	NM_001025616
<i>FGD6</i>	NM_018351	<i>DOCK7</i>	NM_033407	<i>ARHGAP25</i>	NM_001007231
<i>FGD2</i>	NM_173558	<i>DOCK8</i>	NM_203447	<i>MYO9B</i>	NM_004145
<i>FGD4</i>	NM_139241	<i>Zizimin1</i>	NM_015296	<i>MYO9A</i>	NM_006901
<i>FGD1</i>	NM_004463	<i>DOCK10</i>	NM_014689	<i>SYDE1</i>	NM_033025
<i>FGD3</i>	NM_033086	<i>DOCK11</i>	NM_144658	<i>CENTD2</i>	NM_139181
<i>RasGRF1</i>	NM_002891			<i>CENTD1</i>	NM_015230
<i>RasGRF2</i>	NM_006909			<i>CENTD3</i>	NM_022481

Table 3 (ending) RhoGTPases and their regulators selected for the candidate siRNA screen.

Gene	RefSeq	Gene	RefSeq	Gene	RefSeq
RhoGAPs					
<i>CDGAP</i>	NM 020754				
<i>SNX26</i>	NM 052948				
<i>RACGAP1</i>	NM 013277				
<i>RALBP1</i>	NM 006788				
<i>BCR</i>	NM 004327				
<i>ABR</i>	NM 021962				
<i>ARHGAP9</i>	NM 032496				
<i>ARHGAP12</i>	NM 018287				
<i>ARHGAP27</i>	NM 199282				
<i>ARHGAP15</i>	NM 018460				
<i>ARHGAP22</i>	NM 021226				
<i>RICS</i>	NM 014715				
<i>IQGAP1</i>	NM 003870				

Table 4 | Motor and microtubule +TIP proteins selected for the candidate siRNA screen.

Gene	RefSeq	Gene	RefSeq	Gene	RefSeq
Kinesin family		Myosin family		Microtubule Tip Proteins	
KIF1A	NM 004321	MYO1A	NM 005379	CLIP170	NM 002956
KIF1B	NM 015074	MYO1B	NM 012223	CLIP115	NM 003388
KIF1C	NM 006612	MYO1C	NM 033375	Dynactin1	NM 004082
KIF2A	NM 004520	MYO1D	NM 015194	EB1	NM 012325
KIF2B	NM 032559	MYO1E	NM 004998	EB2	NM 014268
KIF2C	NM 006845	MYO1F	NM 012335	EB3	NM 012326
KIF3A	NM 007054	MYO1G	NM 033054	CLASP1	NM 015282
KIF3B	NM 004798	MYH1	NM 005963	CLASP2	NM 015097
KIF3C	NM 002254	MYH2	NM 017534	LIS1	NM 000430
KIF4A	AF071592	MYH3	NM 002470	APC	NM 000038
KIF4B	AF241316	MYH4	NM 017533	APC2	NM 005883
KIF5A	NM 004984	MYH6	NM 002471	ACF7	NM 012090
KIF5B	NM 004521	MYH7	NM 000257	XMAP215	NM 001008938
KIF5C	NM 004522	MYH8	NM 002472	melanophilin	NM 024101
KIF6	NM 145027	MYH9	NM 002473		
KIF7	NM 198525	MYH10	NM 005964		
KIF9	NM 022342	MYH11	NM 002474		
KIF10	NM 001813	MYH13	NM 003802		
KIF11	NM 004523	KIAA1512	AB040945		
KIF12	NM 138424	KIAA1000	AB023217		
KIF13A	NM 022113	MYO3A	NM 017433		
KIF13B	NM 015254	MYO3B	NM 138995		
KIF14	NM 014875	MYO5A	NM 000259		
KIF15	NM 020242	MYO5B	AY274809		
KIF16A	AK122666	MYO5C	NM 018728		
KIF16B	BX647572	MYO6	NM 004999		
KIF17	NM 020816	MYO7A	NM 000260		
KIF18A	NM 031217	MYO7B	XM 291001		
KIF18B	XM 085634	MYO9A	NM 006901		
KIF19A	NM 153209	MYO9B	NM 004145		
KIF20A	NM005733	MYO10	NM 012334		
KIF20B	NM 016195	MYO15A	NM 016239		
KIF21A	NM 017641	MYO15B	BC027875		
KIF21B	BC078676	MYO16	AB020672		
KIF22	NM 007317	MYO18A	NM 203318		
KIF23	NM 004856	MYO18B	NM 032608		
KIF24	NM 018278	Dynein family			
KIF25	NM 005355	DYNC1H1	NM 001376		
KIF26A	XM 050278	DYNC1I1	NM 004411		
KIF26B	BC035896	DYNC1I2	NM 001378		
KIF27	NM 017576	DYNC1LI1	NM 016141		
KIF28	XM 497745	DYNC1LI2	NM 006141		
KIFC1	NM 002263	DYNLT1	NM 006519		
KIFC2	NM 145754	DYNLT3	NM 006520		
KIFC3	NM 005550	DYNLRB1	NM 014183		
KAP3	NM 014970	DYNLRB2	NM 130897		
beta1-	NM 145730	DYNLL1	NM 003746		
JIP-1	NM 005456	DYNLL2	NM 080677		
JIP-2	NM 139124	DYNC2H1	HSU53531		
JIP-3	NM 033392	DYNC2LI1	NM 015522		

Table 5 | Structure of the siRNA microarray chip #160. Each table cell contains [number of the siRNA spot- -number of the siRNA oligonucleotide- -gene name]. siRNAs were designed and produced by Ambion Europe, Ltd. Empty spots did not contain any siRNA.

001--28431--INCENP	052--empty--empty	103--147798--ARHGAP22	154--empty--empty
002--219541--CDGAP	053--empty--empty	104--288549--ABR	155--empty--empty
003--18693--RALBP1	054--empty--empty	105--empty--empty	156--empty--empty
004--empty--empty	055--242584--GSN	106--empty--empty	157--empty--empty
005--empty--empty	056--33026--DIAPH3	107--empty--empty	158--empty--empty
006--empty--empty	057--118353--TPM1	108--empty--empty	159--35874--GIT2
007--132552--ARHGAP15	058--empty--empty	109--empty--empty	160--147287--ANXA2
008--29772--ABR	059--empty--empty	110--empty--empty	161--143784--PFN2
009--empty--empty	060--empty--empty	111--22840--CENTD1	162--empty--empty
010--empty--empty	061--empty--empty	112--35730--SNX26	163--empty--empty
011--empty--empty	062--empty--empty	113--empty--empty	164--empty--empty
012--empty--empty	063--scramble--scramble	114--empty--empty	165--43886--TPM3
013--empty--empty	064--35700--GIT2	115--empty--empty	166--112126--PPIA
014--empty--empty	065--45010--PFN1	116--empty--empty	167--empty--empty
015--scramble--scramble	066--empty--empty	117--empty--empty	168--empty--empty
016--219542--CDGAP	067--empty--empty	118--empty--empty	169--5366--CFL1
017--empty--empty	068--empty--empty	119--empty--empty	170--empty--empty
018--empty--empty	069--46535--TPM3	120--empty--empty	171--empty--empty
019--empty--empty	070--13105--VASP	121--34278--ARHGAP9	172--empty--empty
020--empty--empty	071--44902--GAPD	122--empty--empty	173--134050--PARVB
021--empty--empty	072--empty--empty	123--empty--empty	174--22677--TENC1
022--empty--empty	073--288568--ANXA2	124--empty--empty	175--8523--TPM1
023--empty--empty	074--empty--empty	125--empty--empty	176--empty--empty
024--empty--empty	075--empty--empty	126--empty--empty	177--empty--empty
025--18602--RALBP1	076--empty--empty	127--empty--empty	178--empty--empty
026--empty--empty	077--20312--PARVB	128--empty--empty	179--288634--TPM3
027--empty--empty	078--136884--TENC1	129--empty--empty	180--103856--PPIA
028--empty--empty	079--8333--TPM1	130--empty--empty	181--242566--DIAPH1
029--empty--empty	080--empty--empty	131--empty--empty	182--26445--ENAH
030--empty--empty	081--empty--empty	132--empty--empty	183--11862--PFN2
031--empty--empty	082--empty--empty	133--29210--ARHGAP22	184--empty--empty
032--empty--empty	083--43982--TPM3	134--288548--ABR	185--empty--empty
033--empty--empty	084--13195--VASP	135--empty--empty	186--empty--empty
034--empty--empty	085--8127--GSN	136--empty--empty	187--20220--PARVB
035--empty--empty	086--32930--DIAPH3	137--empty--empty	188--136886--TENC1
036--empty--empty	087--15713--PFN1	138--empty--empty	189--empty--empty
037--26915--ARHGAP15	088--empty--empty	139--empty--empty	190--empty--empty
038--29678--ABR	089--empty--empty	140--empty--empty	191--empty--empty
039--empty--empty	090--empty--empty	141--empty--empty	192--empty--empty
040--empty--empty	091--134049--PARVB	142--empty--empty	193--22649--CENTD1
041--empty--empty	092--136885--TENC1	143--empty--empty	194--20189--RACGAP1
042--empty--empty	093--42838--GAPD	144--empty--empty	195--26692--ARHGAP12
043--empty--empty	094--empty--empty	145--35791--GIT2	196--empty--empty
044--empty--empty	095--empty--empty	146--147285--ANXA2	197--empty--empty
045--empty--empty	096--empty--empty	147--5455--CFL1	198--empty--empty
046--empty--empty	097--128701--CENTD1	148--empty--empty	199--21983--RICS
047--empty--empty	098--35819--SNX26	149--empty--empty	200--147331--BCR
048--empty--empty	099--34374--ARHGAP9	150--empty--empty	201--empty--empty
049--28431--INCENP	100--empty--empty	151--15997--DIAPH1	202--empty--empty
050--138619--GIT2	101--empty--empty	152--132359--ENAH	203--empty--empty
051--147286--ANXA2	102--empty--empty	153--118354--TPM1	204--empty--empty

Table 5 (continued) | Structure of the siRNA microarray chip #160.

205--empty--empty	264--empty--empty	323--empty--empty	382--empty--empty
206--empty--empty	265--24216--EVL	324--empty--empty	383--empty--empty
207--22745--CENTD1	266--empty--empty	325--13799--IQGAP1	384--empty--empty
208--20283--RACGAP1	267--empty--empty	326--1332--BCR	
209--empty--empty	268--empty--empty	327--empty--empty	
210--empty--empty	269--30149--TNS	328--empty--empty	
211--empty--empty	270--8429--TPM1	329--empty--empty	
212--empty--empty	271--13149--TPM2	330--empty--empty	
213--empty--empty	272--empty--empty	331--empty--empty	
214--empty--empty	273--empty--empty	332--empty--empty	
215--empty--empty	274--empty--empty	333--24546--COPB	
216--empty--empty	275--13059--TPM4	334--empty--empty	
217--128737--ARHGAP12	276--7523--PPIA	335--empty--empty	
218--empty--empty	277--18657--DIAPH2	336--empty--empty	
219--empty--empty	278--132358--ENAH	337--288631--M_014776	
220--empty--empty	279--11684--PFN2	338--scramble--scramble	
221--empty--empty	280--empty--empty	339--8031--GSN	
222--empty--empty	281--empty--empty	340--empty--empty	
223--empty--empty	282--empty--empty	341--empty--empty	
224--empty--empty	283--30244--TNS	342--empty--empty	
225--empty--empty	284--288545--TPM1	343--18467--DIAPH2	
226--empty--empty	285--empty--empty	344--37341--NEXN	
227--empty--empty	286--empty--empty	345--118375--TPM2	
228--empty--empty	287--empty--empty	346--empty--empty	
229--21792--RICS	288--empty--empty	347--empty--empty	
230--147330--BCR	289--29944--ARAP3	348--empty--empty	
231--empty--empty	290--scramble--scramble	349--empty--empty	
232--empty--empty	291--142018--ARHGAP27	350--empty--empty	
233--empty--empty	292--empty--empty	351--288632--GIT2	
234--empty--empty	293--empty--empty	352--scramble--scramble	
235--empty--empty	294--empty--empty	353--26357--PARVA	
236--empty--empty	295--13894--IQGAP1	354--empty--empty	
237--empty--empty	296--1427--BCR	355--empty--empty	
238--empty--empty	297--empty--empty	356--empty--empty	
239--empty--empty	298--empty--empty	357--13013--VASP	
240--empty--empty	299--empty--empty	358--122515--PPIA	
241--138618--GIT2	300--empty--empty	359--empty--empty	
242--288566--ANXA2	301--empty--empty	360--empty--empty	
243--24121--EVL	302--empty--empty	361--8218--GSN	
244--empty--empty	303--30036--ARAP3	362--empty--empty	
245--empty--empty	304--scramble--scramble	363--empty--empty	
246--empty--empty	305--empty--empty	364--empty--empty	
247--146690--DIAPH2	306--empty--empty	365--22868--TENC1	
248--26351--ENAH	307--empty--empty	366--118352--TPM1	
249--12962--TPM2	308--empty--empty	367--13058--TPM2	
250--empty--empty	309--empty--empty	368--empty--empty	
251--empty--empty	310--empty--empty	369--empty--empty	
252--empty--empty	311--empty--empty	370--empty--empty	
253--empty--empty	312--empty--empty	371--288562--VASP	
254--empty--empty	313--142019--ARHGAP27	372--288645--PPIA	
255--138620--GIT2	314--empty--empty	373--146689--DIAPH2	
256--288567--ANXA2	315--empty--empty	374--37246--NEXN	
257--11776--PFN2	316--empty--empty	375--26545--PARVA	
258--empty--empty	317--empty--empty	376--empty--empty	
259--empty--empty	318--empty--empty	377--empty--empty	
260--empty--empty	319--empty--empty	378--empty--empty	
261--12963--TPM4	320--empty--empty	379--22773--TENC1	

Table 5 (continued) | Structure of the siRNA microarray chip #161. Each table cell contains [number of the siRNA spot- -number of the siRNA oligonucleotide- -gene name]. siRNAs were designed and produced by Ambion Europe, Ltd. Empty spots did not contain any siRNA.

001--28431--INCENP	052--28431--INCENP	103--19755--RHOQ	154--22278--KIAA0672
002--120602--RAC1	053--202491--ARHGAP8	104--21389--RND1	155--122574--PARG1
003--43342--RAC2	054--138476--SRGAP2	105--15256--ARHGEF2	156--15718--PIK3R2
004--28431--INCENP	055--129201--FGD4	106--258560--FLJ20220	157--20459--ARHGAP6
005--145822--DOCK1	056--120493--RASGRF2	107--9553--ARHGDIG	158--121663--ARHGAP6
006--21785--DOCK4	057--38124--ARHGEF7	108--10622--CHN1	159--133783--ITSN2
007--242555--CDC42	058--27556--SH3BP1	109--2835--MCF2	160--27760--ARHGEF3
008--36445--RHOV	059--108901--GMIP	110--136834--DNMBP	161--29582--ABR
009--135247--NGEF	060--288635--PIK3R1	111--2808--RHOA	162--35378--ARHGAP18
010--22765--DOCK9	061--20636--ARHGAP6	112--27408--RAC1	163--14477--ARHGAP1
011--9552--ARHGDIB	062--121662--ARHGAP6	113--27796--RHOF	164--288609--SRGAP2
012--146035--CHN1	063--scramble--scramble	114--217273--KIAA0720	165--22731--ARHGEF9
013--119310--KIAA0861	064--16981--NET1	115--146562--DOCK2	166--4716--TIAM1
014--25195--FLJ20184	065--21946--ARHGEF17	116--133245--DOCK6	167--13846--HAPIP
015--scramble--scramble	066--288619--FLJ10312	117--43583--ARHGEF18	168--261264--KIAA1688
016--242587--RAC1	067--213201--LOC553158	118--119417--ARHGEF11	169--22148--FARP2
017--21613--RHOD	068--138477--SRGAP2	119--12546--ITSN1	170--31650--DLC1
018--26070--ECT2	069--119317--ARHGEF4	120--288678--p190-A	171--288605--ARHGAP6
019--145823--DOCK1	070--258555--TIAM2	121--41918--RAC3	172--20550--ARHGAP6
020--21881--DOCK4	071--14029--HAPIP	122--288684--FLJ32854	173--28809--ALS2
021--103506--AKAP13	072--135118--C5orf5	123--144005--MCF2	174--251364--OBSCN
022--214646--ARHGEF11	073--138078--FARP1	124--136835--DNMBP	175--148850--CLG
023--119420--ARHGEF1	074--scramble--scramble	125--44031--RHOBTB1	176--22182--KIAA0672
024--147081--ARHGAP5	075--288604--ARHGAP6	126--122079--ARHGEF15	177--22105--KIAA0053
025--43438--RAC2	076--288607--ARHGAP6	127--15160--ARHGEF2	178--128708--CENTD2
026--scramble--scramble	077--110844--BCR	128--258559--FLJ20220	179--22826--ARHGEF9
027--22402--KIAA0861	078--2839--VAV1	129--21847--ARHGAP11A	180--138877--TIAM1
028--25387--FLJ20184	079--38295--ARHGEF7	130--131863--STARD13	181--14738--FGD1
029--43848--RHOBTB1	080--27462--SH3BP1	131--43400--ARHGEF18	182--21727--ARHGEF10
030--121751--SGEF	081--22201--KIAA0053	132--119418--ARHGEF11	183--147813--ABR
031--135246--NGEF	082--34994--7h3	133--19665--RHOQ	184--128827--ARHGAP18
032--22669--DOCK9	083--22774--ARHGEF4	134--21295--RND1	185--122573--PARG1
033--140197--ARHGAP11A	084--20229--TIAM2	135--27700--RHOF	186--15906--PIK3R2
034--202336--STARD13	085--129200--FGD4	136--217272--KIAA0720	187--133298--ALS2
035--136052--AKAP13	086--120492--RASGRF2	137--9458--ARHGDIG	188--131675--OBSCN
036--21848--ARHGEF11	087--21850--ARHGEF17	138--10528--CHN1	189--124683--HAPIP
037--242554--CDC42	088--32847--FLJ10312	139--138465--RHOBTB1	190--288618--KIAA1688
038--120713--RHOV	089--24794--GMIP	140--122080--ARHGEF15	191--288610--KIAA0053
039--21520--RHOD	090--118085--PIK3R1	141--142608--ITSN1	192--22844--CENTD2
040--146755--ECT2	091--1237--BCR	142--288677--p190-A	193--120361--RHOB
041--9457--ARHGDIB	092--2933--VAV1	143--21943--ARHGAP11A	194--120600--RAC1
042--146034--CHN1	093--137405--HAPIP	144--288666--M 052851	195--45990--RHOG
043--138464--RHOBTB1	094--135119--C5orf5	145--17648--ITSN2	196--36435--GEFT
044--22998--SGEF	095--22297--KIAA0053	146--135243--ARHGEF3	197--31982--DOCK5
045--214561--ARHGEF1	096--125786--7h3	147--22052--FARP2	198--147974--DOCK7
046--147082--ARHGAP5	097--120211--RHOA	148--31555--DLC1	199--28644--RHOJ
047--140198--ARHGAP11A	098--45358--RAC1	149--14383--ARHGAP1	200--16082--ARHN
048--288668--M 178007	099--42005--RAC3	150--22273--SRGAP2	201--103341--AKAP13
049--28431--INCENP	100--288683--FLJ32854	151--14826--FGD1	202--129250--DOCK11
050--16886--NET1	101--146561--DOCK2	152--138290--ARHGEF10	203--272051--ARHGAP23
051--16818--FARP1	102--133244--DOCK6	153--148851--CLG	204--146298--CHN2

Table 5 (continued) | Structure of the siRNA microarray chip #161.

205--32098--MCF2L	256--16914--FARP1	307--146565--DOCK3	358--845--HAPIP
206--16561--SOS1	257--147815--ABR	308--149462--DOCK8	359--empty--empty
207--41889--RHOB	258--148782--ARHGAP20	309--119311--ARHGEF12	360--33369--ARHGAP24
208--120601--RAC1	259--10340--ARHGAP4	310--119422--ARHGEF1	361--41034--FGD2
209--5062--RHOH	260--148767--SRGAP1	311--empty--empty	362--213191--DLC1
210--288617--KIAA0720	261--39762--SPATA13	312--22398--ARHGAP26	363--scramble--scramble
211--130801--DOCK5	262--288681--FLJ00068	313--10324--CDC42	364--32759--FLJ10312
212--147975--DOCK7	263--849--TRIO	314--36623--GEFT	365--17378--VAV3
213--288688--RGNEF	264--33188--ARHGAP24	315--scramble--scramble	366--38215--ARHGEF7
214--5491--ARHGEF1	265--26743--FLJ11183	316--143049--SOS2	367--22869--ARHGEF4
215--12452--ITSN1	266--202494--DLC1	317--42188--ARHGEF16	368--132262--ARHGAP17
216--11646--OPHN1	267--288606--ARHGAP6	318--2841--ARHGEF5	369--18770--MYO9A
217--10243--RHOG	268--34874--ARHGAP19	319--136053--AKAP13	370--empty--empty
218--141956--GEFT	269--13014--VAV2	320--119907--ARHGDIA	371--258556--TIAM2
219--137007--MCF2L	270--119232--ARHGEF6	321--202362--STARD13	372--13939--HAPIP
220--142883--SOS1	271--28779--PREX1	322--empty--empty	373--12181--RASGRF1
221--136812--RHOBTB2	272--132261--ARHGAP17	323--22673--ARHGEF12	374--26377--FLJ10521
222--121538--ARHGEF5	273--14116--MYO9B	324--5309--ARHGEF1	375--214833--BCR
223--1569--AKAP13	274--128706--CENTD2	325--29108--RHOU	376--213040--LOC553158
224--37496--DOCK11	275--129604--SPATA13	326--15891--ARHE	377--118086--PIK3R1
225--21799--STARD8	276--288682--FLJ00068	327--43944--RHOBTB1	378--8189--OCRL
226--35407--STARD13	277--131558--FGD3	328--26321--FLJ10665	379--107954--VAV3
227--288687--RGNEF	278--26281--FLJ10521	329--31229--ARHGAP10	380--119396--ARHGEF7
228--119421--ARHGEF1	279--147814--ABR	330--9456--ARHGAP5	381--24546--COPB
229--28549--RHOJ	280--148781--ARHGAP20	331--42260--ARHGEF16	382--148941--ARHGAP24
230--16252--ARHN	281--147474--HA-1	332--288577--ARHGEF5	383--18866--MYO9A
231--120805--RHOH	282--104448--OCRL	333--24546--COPB	384--24546--COPB
232--123225--KIAA0720	283--138981--VAV2	334--22590--ARHGAP26	
233--272050--ARHGAP23	284--5326--ARHGEF6	335--35584--STARD13	
234--14167--CHN2	285--103343--TRIO	336--24546--COPB	
235--22631--RHOBTB2	286--148939--ARHGAP24	337--133782--ITSN2	
236--3027--ARHGEF5	287--14207--MYO9B	338--scramble--scramble	
237--12635--ITSN1	288--128707--CENTD2	339--40846--FGD2	
238--143673--OPHN1	289--44189--RHOC	340--46352--DLC1	
239--138366--STARD8	290--scramble--scramble	341--22081--SRGAP2	
240--35499--STARD13	291--10418--CDC42	342--261179--SRGAP2	
241--17743--ITSN2	292--36531--GEFT	343--120291--RASGRF1	
242--215120--FARP1	293--146564--DOCK3	344--123058--FLJ10521	
243--26648--FLJ11183	294--149461--DOCK8	345--119318--ARHGEF4	
244--17367--DLC1	295--29197--RHOU	346--26031--ARHGAP17	
245--10244--ARHGAP4	296--15798--ARHE	347--118087--PIK3R1	
246--148766--SRGAP1	297--1475--AKAP13	348--104450--M 001587	
247--131559--FGD3	298--14384--ARHGDIA	349--scramble--scramble	
248--123057--FLJ10521	299--127413--ARHGAP10	350--32666--FLJ10312	
249--121870--PREX1	300--9551--ARHGAP5	351--17834--ITSN2	
250--132263--ARHGAP17	301--scramble--scramble	352--scramble--scramble	
251--147475--HA-1	302--143048--SOS2	353--147329--BCR	
252--114042--OCRL	303--44091--RHOC	354--213042--LOC553158	
253--121664--ARHGAP6	304--scramble--scramble	355--138478--SRGAP2	
254--35051--ARHGAP19	305--138466--RHOBTB1	356--261180--SRGAP2	
255--133784--ITSN2	306--140527--FLJ10665	357--258554--TIAM2	

Table 5 (continued) | Structure of the siRNA microarray chip #162. Each table cell contains [number of the siRNA spot- -number of the siRNA oligonucleotide- -gene name]. siRNAs were designed and produced by Ambion Europe, Ltd. Empty spots did not contain any siRNA.

001--28431--INCENP	052--28431--INCENP	103--15501--KIF5A	154--empty--empty
002--14762--KIF2	053--empty--empty	104--118514--LOC374654	155--empty--empty
003--118447--KIF3A	054--empty--empty	105--24036--MPHOSPH1	156--empty--empty
004--28431--INCENP	055--261145--CLASP2	106--288671--MYH	157--13539--DNCL1
005--11788--MYH3	056--258848--MACF1	107--288685--MYO5B	158--215817--D2LIC
006--11604--MYH8	057--empty--empty	108--263744--MYO7B	159--142517--RSN
007--288680--KIF4B	058--empty--empty	109--22516--MAPK8IP3	160--146318--DCTN1
008--37893--C6orf102	059--empty--empty	110--118410--MYO1C	161--empty--empty
009--16618--KIF20A	060--empty--empty	111--14392--KIF1A	162--empty--empty
010--13674--MYH13	061--118342--DNCL2B	112--34309--LOC84643	163--empty--empty
011--8174--MYO5A	062--288696--DYNC2H1	113--36707--KIF12	164--empty--empty
012--8175--MYO7A	063--scramble--scramble	114--221472--MYO1G	165--empty--empty
013--20076--MAPK8IP2	064--43533--CYLN2	115--24994--MYH4	166--empty--empty
014--148408--MYO1B	065--42410--MLPH	116--11701--MYH9	167--empty--empty
015--scramble--scramble	066--empty--empty	117--118501--KIF23	168--empty--empty
016--14669--KIF2	067--empty--empty	118--288689--KIF26B	169--20987--MAPRE2
017--14765--KIF11	068--empty--empty	119--118504--KIFC2	170--17983--TCTEL1
018--19899--MYO1F	069--empty--empty	120--118400--MYO15A	171--118315--DNCL1
019--11602--MYH3	070--empty--empty	121--15207--KIF3B	172--124933--D2LIC
020--11700--MYH8	071--empty--empty	122--22364--KIFAP3	173--empty--empty
021--118450--KIF22	072--empty--empty	123--136770--MAPK8IP3	174--empty--empty
022--263710--KIF26A	073--3708--MAPRE1	124--35347--MYO1C	175--empty--empty
023--43827--KIFC1	074--scramble--scramble	125--118474--KNSL7	176--empty--empty
024--19898--MYO10	075--36233--DNCL2B	126--118492--KIF18A	177--empty--empty
025--19038--KIF3A	076--288695--DYNC2H1	127--118465--MPHOSPH1	178--empty--empty
026--scramble--scramble	077--empty--empty	128--288673--MYH	179--empty--empty
027--19889--MAPK8IP2	078--empty--empty	129--118407--MYO18B	180--empty--empty
028--148409--MYO1B	079--empty--empty	130--24008--DNCL1I	181--8383--PAFAH1B1
029--22099--KIF14	080--empty--empty	131--36749--KIF23	182--258847--MACF1
030--118477--KIF17	081--empty--empty	132--288690--KIF26B	183--empty--empty
031--288582--KIF20A	082--empty--empty	133--118435--KIF5A	184--empty--empty
032--13581--MYH13	083--empty--empty	134--118513--LOC374654	185--empty--empty
033--147986--MYO18A	084--empty--empty	135--118498--KIF12	186--empty--empty
034--9741--DNCL2	085--261144--CLASP2	136--221471--MYO1G	187--empty--empty
035--19353--KIF22	086--258846--MACF1	137--288686--MYO5B	188--empty--empty
036--263711--KIF26A	087--42493--MLPH	138--263743--MYO7B	189--empty--empty
037--288679--KIF4B	088--empty--empty	139--28246--KNSL7	190--empty--empty
038--37797--C6orf102	089--empty--empty	140--32973--KIF18A	191--empty--empty
039--14672--KIF11	090--empty--empty	141--38128--KIFC2	192--empty--empty
040--118394--MYO1F	091--empty--empty	142--24065--MYO15A	193--22400--KIF1B
041--8081--MYO5A	092--empty--empty	143--34346--MYO18B	194--18738--KIF2C
042--8082--MYO7A	093--empty--empty	144--23822--DNCL1I	195--118421--KIF3C
043--22195--KIF14	094--empty--empty	145--142516--RSN	196--9431--AP1B1
044--118478--KIF17	095--empty--empty	146--242560--DCTN1	197--11603--MYH6
045--43923--KIFC1	096--empty--empty	147--136574--MAPRE2	198--147398--MYH10
046--19992--MYO10	097--147322--KIF1A	148--139625--TCTEL1	199--14763--KIF5B
047--147987--MYO18A	098--118495--LOC84643	149--empty--empty	200--30070--KIF9
048--9836--DNCL2	099--118432--KIF3B	150--empty--empty	201--118472--KIF21A
049--28431--INCENP	100--118456--KIFAP3	151--8478--PAFAH1B1	202--1486--MYO3A
050--43346--CYLN2	101--24899--MYH4	152--288620--MACF1	203--27397--MYO5C
051--3891--MAPRE1	102--11605--MYH9	153--empty--empty	204--144275--MYO9A

Table 5 (continued) | Structure of the siRNA microarray chip #162.

205--144012--MYO1A	256--14081--DCTN1	307--8172--MYH7	358--empty--empty
206--251683--MYO1D	257--empty--empty	308--43338--MYH11	359--empty--empty
207--118460--KIF1B	258--empty--empty	309--118490--KIF25	360--empty--empty
208--18553--KIF2C	259--empty--empty	310--271492--LOC441930	361--136867--CLASP1
209--29745--KIF13A	260--empty--empty	311--empty--empty	362--279207--DNCL2A
210--17208--MYH1	261--empty--empty	312--288670--MYO16	363--scramble--scramble
211--11699--MYH6	262--empty--empty	313--288675--KIF4A	364--empty--empty
212--147399--MYH10	263--empty--empty	314--16356--MAPK8IP1	365--empty--empty
213--26777--KIF24	264--empty--empty	315--scramble--scramble	366--empty--empty
214--118468--KIF27	265--19890--MAPRE3	316--15509--MYO1E	367--empty--empty
215--118438--KIFC3	266--18077--TCTE1L	317--288694--KIF16B	368--empty--empty
216--251256--MYO15B	267--35969--Dlc2	318--39789--FLJ37300	369--empty--empty
217--11118--KIF3C	268--288672--MYH	319--288692--KIF21B	370--empty--empty
218--9527--AP1B1	269--empty--empty	320--1137--MYO3B	371--empty--empty
219--16050--MYO1A	270--empty--empty	321--14712--DNCL1	372--empty--empty
220--222682--MYO1D	271--empty--empty	322--empty--empty	373--16896--APC2
221--251250--STARD9	272--empty--empty	323--32833--KIF25	374--122703--ch-TOG
222--251223--LOC146909	273--empty--empty	324--271493--LOC441930	375--empty--empty
223--118471--KIF21A	274--empty--empty	325--14850--KIF5C	376--empty--empty
224--131930--MYO3A	275--empty--empty	326--121337--CENPE	377--empty--empty
225--118309--DNCH1	276--empty--empty	327--22848--KIF13B	378--empty--empty
226--118326--DNCL12	277--42812--APC	328--118403--MYH2	379--empty--empty
227--26683--KIF24	278--21827--ch-TOG	329--15510--MYO6	380--empty--empty
228--118469--KIF27	279--empty--empty	330--14293--MYO9B	381--24546--COPB
229--14670--KIF5B	280--empty--empty	331--288693--KIF16B	382--empty--empty
230--118509--KIF9	281--empty--empty	332--39883--FLJ37300	383--empty--empty
231--29651--KIF13A	282--empty--empty	333--24546--COPB	384--24546--COPB
232--17114--MYH1	283--empty--empty	334--288669--MYO16	
233--118404--MYO5C	284--empty--empty	335--14617--DNCL1	
234--18960--MYO9A	285--empty--empty	336--24546--COPB	
235--251251--STARD9	286--empty--empty	337--139011--CYLN2	
236--251224--LOC146909	287--empty--empty	338--scramble--scramble	
237--16324--KIFC3	288--empty--empty	339--136866--CLASP1	
238--251257--MYO15B	289--18210--KIF1C	340--279206--DNCL2A	
239--118310--DNCH1	290--scramble--scramble	341--empty--empty	
240--17395--DNCL12	291--288676--KIF4A	342--empty--empty	
241--12224--RSN	292--16261--MAPK8IP1	343--16991--APC2	
242--146317--DCTN1	293--8079--MYH7	344--22017--ch-TOG	
243--136502--MAPRE3	294--118370--MYH11	345--empty--empty	
244--17984--TCTE1L	295--14764--KIF5C	346--empty--empty	
245--empty--empty	296--10520--CENPE	347--empty--empty	
246--empty--empty	297--288691--KIF21B	348--empty--empty	
247--44755--APC	298--1138--MYO3B	349--scramble--scramble	
248--21923--ch-TOG	299--15603--MYO6	350--empty--empty	
249--empty--empty	300--143862--MYO9B	351--43442--CYLN2	
250--empty--empty	301--scramble--scramble	352--scramble--scramble	
251--empty--empty	302--15693--MYO1E	353--empty--empty	
252--empty--empty	303--18304--KIF1C	354--empty--empty	
253--118341--Dlc2	304--scramble--scramble	355--empty--empty	
254--288674--MYH	305--22657--KIF13B	356--empty--empty	
255--12317--RSN	306--24900--MYH2	357--empty--empty	

Table 5 (continued) | Structure of the siRNA microarray chip #163. Each table cell contains [number of the siRNA spot- number of the siRNA oligonucleotide- gene name]. siRNAs were designed and produced by Ambion Europe, Ltd. Empty spots did not contain any siRNA.

001--28431--INCENP	052--28431--INCENP	103--9041--ITGB6	154--4366--PTPRF
002--109878--ITGB1	053--11974--PIK3CG	104--12768--TM4SF6	155--114285--PTPRA
003--106001--ITGB3	054--136520--PIP5K1C	105--21093--ITGB3BP	156--10475--CAPNS1
004--28431--INCENP	055--12193--RDX	106--1442--FYN	157--288585--PAK4
005--16824--SCAM-1	056--23307--TES	107--142304--MAPK3	158--42854--PRKCA
006--146413--CSK	057--288576--GRB7	108--145116--ILK	159--288641--NF2
007--11108--ITGB5	058--104588--PTPRF	109--107195--PPFIA1	160--108962--FBLP-1
008--14501--CD81	059--4363--PTPRA	110--16651--SDCBP	161--10297--CAV1
009--17808--CIB1	060--105571--CAPNS1	111--109879--ITGB1	162--110865--ROCK2
010--144829--FYN	061--288584--PAK4	112--288621--ITGB1	163--288639--PIK3R1
011--691--YES1	062--288587--PAK4	113--288558--TM4SF7	164--45927--PTEN
012--288570--ILK	063--scramble--scramble	114--288581--SDCBP	165--215169--NCK2
013--13394--PPFIA1	064--136120--PLEKHC1	115--147447--ABL1	166--12157--PXN
014--34040--SDOS	065--13145--ZYX	116--103698--PTK2	167--22801--SORBS1
015--scramble--scramble	066--142844--ROCK1	117--42217--K-ALPHA-1	168--10333--ARF1
016--288625--ITGB1	067--11879--PIK3CG	118--15476--ACTN4	169--11593--MSN
017--288557--TM4SF7	068--136521--PIP5K1C	119--9965--FLNC	170--110779--PAK2
018--288580--SDCBP	069--17402--NCK1	120--1653--LIMK2	171--288591--PAK4
019--16920--SCAM-1	070--21515--BCAR1	121--288542--ITGB4	172--300--PRKCA
020--146414--CSK	071--136917--SORBS1	122--288599--SORBS1	173--146745--CRK
021--9415--ACTB	072--10237--ARF1	123--13311--PPFIA1	174--144866--GRB2
022--9416--ACTN1	073--288615--FBLP-1	124--121551--SDCBP	175--23481--TGFB1I1
023--9964--FLNB	074--scramble--scramble	125--12434--SDC4	176--4454--PTPRF
024--143455--LIMK1	075--288590--PAK4	126--14494--CALR	177--14711--DNM1
025--8055--ITGB3	076--288593--PAK4	127--21001--ITGB3BP	178--146559--DNM2
026--scramble--scramble	077--2827--CBL	128--144828--FYN	179--13296--NCK2
027--13472--PPFIA1	078--14079--CSRP1	129--103376--MYLK	180--12253--PXN
028--34136--SDOS	079--2834--GRB7	130--288626--MYLK	181--5379--TLN1
029--288554--TM4SF7	080--4274--PTPRF	131--42130--K-ALPHA-1	182--108453--VCL
030--112326--PTPNS1	081--14616--DNM1	132--15570--ACTN4	183--145951--CAV1
031--17715--CIB1	082--146560--DNM2	133--8947--inbeta6	184--595--ROCK2
032--144827--FYN	083--17498--NCK1	134--138892--TM4SF6	185--104566--PTPRA
033--263692--MAP3K1	084--21608--BCAR1	135--12769--TM4SF7	186--10388--CAPNS1
034--103460--MYLK	085--142472--RDX	136--121550--SDCBP	187--6472--CRK
035--9511--ACTB	086--134609--TES	137--214749--MAPK3	188--4292--GRB2
036--9512--ACTN1	087--13054--ZYX	138--1556--ILK	189--288597--SORBS1
037--11289--ITGB5	088--681--ROCK1	139--12529--SDC4	190--288550--ARF1
038--14407--CD81	089--4271--PTPRA	140--14400--CALR	191--146449--DNM1
039--138896--TM4SF7	090--10293--CAPNS1	141--10060--FLNC	192--288574--DNM2
040--16742--SDCBP	091--2921--CBL	142--144048--LIMK2	193--112291--ITGB1
041--690--YES1	092--14172--CSRP1	143--288627--MYLK	194--35488--ITGB1
042--145117--ILK	093--288596--SORBS1	144--288628--MYLK	195--2981--ITGB4
043--288561--TM4SF7	094--10426--ARF1	145--202368--NF2	196--136919--SORBS1
044--43659--PTPNS1	095--14804--DNM1	146--279294--FBLP-1	197--147450--ABL2
045--10059--FLNB	096--288573--DNM2	147--143572--MSN	198--103649--PTK2
046--143456--LIMK1	097--109877--ITGB1	148--110776--PAK2	199--288629--CD151
047--263693--MAP3K1	098--35575--ITGB1	149--288636--PIK3R1	200--288555--TM4SF7
048--1510--MYLK	099--2888--ITGB4	150--142044--PTEN	201--137693--ITGB1BP1
049--28431--INCENP	100--22895--SORBS1	151--5467--TLN1	202--103417--SRC
050--136119--PLEKHC1	101--1241--ABL1	152--20570--VCL	203--1449--MAPK1
051--288613--FBLP-1	102--103596--PTK2	153--23577--TGFB1I1	204--145118--ILK

Table 5 (continued) | Structure of the siRNA microarray chip #163

205--3664--GNB2L1	256--288614--FBLP-1	307--103430--ABL2	358--22706--SORBS1
206--121549--SDCBP	257--9575--CAV2	308--1627--FYN	359--empty--empty
207--112292--ITGB1	258--2960--PIK3CA	309--13019--VIM	360--43082--DDEF1
208--288622--ITGB1	259--288640--PIK3R1	310--144674--FLNA	361--23784--KIAA0992
209--288559--TM4SF7	260--4264--PTPN1	311--empty--empty	362--288589--PAK4
210--12968--TRIP6	261--13379--NCK2	312--144049--LIMK2	363--scramble--scramble
211--103346--ABL2	262--15503--LIMS1	313--288544--ITGB4	364--103397--PRKCM
212--142859--PTK2	263--136918--SORBS1	314--288601--SORBS1	365--2828--CRKL
213--120764--OK-SW-	264--214733--ARF1	315--scramble--scramble	366--2928--GRB7
214--13018--VIL2	265--20179--MYLIP	316--288579--SDCBP	367--132221--LIMS2
215--202366--NF2	266--288588--PAK4	317--111141--LRP1	368--104563--PTPRA
216--144050--LIMK2	267--288592--PAK4	318--10385--CANX	369--15581--DNM2
217--288543--ITGB4	268--679--PRKCE	319--137692--ITGB1BP1	370--empty--empty
218--288600--SORBS1	269--146743--CRK	320--103333--SRC	371--288564--NCK2
219--3757--GNB2L1	270--4196--GRB2	321--144402--MYLK	372--288595--SORBS1
220--288578--SDCBP	271--17398--LASP1	322--empty--empty	373--23211--TES
221--1490--TRPM7	272--4361--PTPN11	323--13111--VIM	374--115435--ZYX
222--10290--CANX	273--15488--DNM2	324--9963--FLNA	375--145680--CAV2
223--44218--ITGB1BP1	274--20586--GIT1	325--43538--CD151	376--144253--PIK3CB
224--142855--SRC	275--288563--NCK2	326--138895--TM4SF7	377--10292--CAPN2
225--144403--MYLK	276--15596--LIMS1	327--288553--TM4SF7	378--147188--ARF1
226--143703--PAK1	277--5622--TLN2	328--17014--SCAM-1	379--2922--CRKL
227--120844--OK-SW-	278--108454--VCL	329--1544--MAPK1	380--3020--GRB7
228--13110--VIL2	279--9481--CAV2	330--1318--LIMK1	381--24546--COPB
229--43352--CD151	280--144250--PIK3CA	331--11342--LRP1	382--42901--DDEF1
230--46050--TM4SF7	281--15805--CAPN1	332--145945--CANX	383--288572--DNM2
231--12860--TM4SF7	282--46173--RNF5	333--24546--COPB	384--24546--COPB
232--13155--TRIP6	283--146744--CRK	334--1469--LIMK2	
233--142301--MAPK1	284--106680--GRB2	335--1692--MYLK	
234--1461--ILK	285--288598--SORBS1	336--24546--COPB	
235--212797--TRPM7	286--288551--ARF1	337--202367--NF2	
236--10472--CANX	287--15673--DNM2	338--scramble--scramble	
237--8090--NF2	288--20497--GIT1	339--23879--KIAA0992	
238--1563--LIMK2	289--35396--ITGB1	340--733--PAK4	
239--144401--MYLK	290--scramble--scramble	341--288638--PIK3R1	
240--249--PAK1	291--2793--ITGB4	342--142400--PTPN12	
241--8183--NF2	292--288594--SORBS1	343--134610--TES	
242--279293--FBLP-1	293--1478--ABL2	344--139068--ZYX	
243--118397--MYLIP	294--1537--FYN	345--25885--LIMS2	
244--732--PAK4	295--43447--CD151	346--104569--PTPRA	
245--288637--PIK3R1	296--288556--TM4SF7	347--10387--CAPN2	
246--104533--PTPN1	297--44998--ITGB1BP1	348--147189--ARF1	
247--5717--TLN2	298--288644--SRC	349--scramble--scramble	
248--20480--VCL	299--1634--MAPK1	350--103310--PRKCM	
249--17494--LASP1	300--1413--LIMK1	351--288642--NF2	
250--4449--PTPN11	301--scramble--scramble	352--scramble--scramble	
251--15898--CAPN1	302--16556--SDCBP	353--145681--CAV2	
252--18776--RNF5	303--112293--ITGB1	354--17439--PIK3CB	
253--288586--PAK4	304--scramble--scramble	355--203811--M 181524	
254--677--epsilon	305--288560--TM4SF7	356--4270--PTPN12	
255--8273--NF2	306--126654--SCAM-1	357--13457--NCK2	

Table 6 | Structure of the microarray chips containing genes identified in a genome-wide siRNA screen as regulators of constitutive protein trafficking to the plasma membrane (kindly provided by J. Simpson and R. Pepperkok). In total there were 7 chips numbered from 305 to 311.

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
305	1	incenp		305	51	akap3	ENSG00000111254
305	2	aadat	ENSG00000109576	305	52	akap5	ENSG00000179841
305	3	aak1	ENSG00000115977	305	53	akap9	ENSG00000127914
305	4	abat	ENSG00000183044	305	54	akr1cl1	ENSG00000196326
305	5	abca1	ENSG00000165029	305	55	akt1	ENSG00000142208
305	6	abca13	ENSG00000179869	305	56	aldh16a1	ENSG00000161618
305	7	abcc10	ENSG00000124574	305	57	aldh1a1	ENSG00000165092
305	8	abcc6	ENSG00000091262	305	58	aldh1a2	ENSG00000128918
305	9	abhd1	ENSG00000143994	305	59	aldh9a1	ENSG00000143149
305	10	abhd13	ENSG00000139826	305	60	alpk2	ENSG00000198796
305	11	abhd5	ENSG00000011198	305	61	alpp	ENSG00000163283
305	12	incenp		305	62	als2cl	ENSG00000178038
305	13	ablim1	ENSG00000099204	305	63	rab1a	
305	14	copb		305	64	ambp	ENSG00000106927
305	15	ablim2	ENSG00000163995	305	65	ank1	ENSG00000029534
305	16	acaa2	ENSG00000167315	305	66	ankfn1	ENSG00000153930
305	17	acat2	ENSG00000120437	305	67	ankh	ENSG00000154122
305	18	acbd6	ENSG00000135847	305	68	ankrd11	ENSG00000167522
305	19	ache	ENSG00000087085	305	69	ankrd35	ENSG00000198483
305	20	scrambled		305	70	scrambled	
305	21	acox2	ENSG00000168306	305	71	ankrd5	ENSG00000132623
305	22	acoxl	ENSG00000153093	305	72	ankrd9	ENSG00000156381
305	23	actl6b	ENSG00000077080	305	73	anks6	ENSG00000165138
305	24	actr3	ENSG00000115091	305	74	anpep	ENSG00000166825
305	25	acvr1b	ENSG00000135503	305	75	ap2s1	ENSG00000042753
305	26	acvr2b	ENSG00000114739	305	76	cpeb1	ENSG00000103723
305	27	acvr1l	ENSG00000139567	305	77	ap3s1	ENSG00000177879
305	28	adam11	ENSG00000073670	305	78	ap4s1	ENSG00000100478
305	29	adam12	ENSG00000148848	305	79	copb	
305	30	gosr1		305	80	apbb1ip	ENSG00000077420
305	31	adam20	ENSG00000134007	305	81	apoa5	ENSG00000110243
305	32	adam21	ENSG00000139985	305	82	apobec3b	ENSG00000179750
305	33	adam30	ENSG00000134249	305	83	ar	ENSG00000169083
305	34	adamts14	ENSG00000138316	305	84	arcn1	ENSG00000095139
305	35	adamts7	ENSG00000136378	305	85	arf3	ENSG00000134287
305	36	adamts9	ENSG00000163638	305	86	arfip1	ENSG00000164144
305	37	adar	ENSG00000160710	305	87	arhgap12	ENSG00000165322
305	38	adfp	ENSG00000147872	305	88	scrambled	
305	39	scrambled		305	89	arhgap24	ENSG00000138639
305	40	adk	ENSG00000156110	305	90	arhgef18	ENSG00000104880
305	41	adora1	ENSG00000163485	305	91	arid1a	ENSG00000117713
305	42	adora2a	ENSG00000128271	305	92	arih1	ENSG00000166233
305	43	adra2a	ENSG00000150594	305	93	arl14	ENSG00000179674
305	44	aebp1	ENSG00000106624	305	94	arl5a	ENSG00000162980
305	45	agpat2	ENSG00000169692	305	95	gosr1	
305	46	rab1a		305	96	arpc1b	ENSG00000130429
305	47	agpat4	ENSG00000026652	305	97	arrdc1	ENSG00000197070
305	48	agtrap	ENSG00000177674	305	98	ars2	ENSG00000087087
305	49	agxt2l2	ENSG00000175309	305	99	arsf	ENSG00000062096
305	50	aim1l	ENSG00000176092	305	100	art4	ENSG00000111339

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
305	101	asah3l	ENSG00000177076	305	151	c10orf33	ENSG00000119943
305	102	asb12	ENSG00000198881	305	152	c10orf61	ENSG00000119977
305	103	asb15	ENSG00000146809	305	153	c10orf73	ENSG00000172538
305	104	asb18	ENSG00000182177	305	154	c10orf79	ENSG00000197748
305	105	ascc2	ENSG00000100325	305	155	c10orf9	ENSG00000108100
305	106	asf1a	ENSG00000111875	305	156	c10orf99	ENSG00000188373
305	107	astn2	ENSG00000148219	305	157	c11orf55	ENSG00000196559
305	108	atp2c2	ENSG00000064270	305	158	bc024479	ENSG00000120458
305	109	atad3a	ENSG00000197785	305	159	copb	
305	110	gosr1		305	160	c11orf64	ENSG00000181995
305	111	atf7ip	ENSG00000171681	305	161	c11orf71	ENSG00000180425
305	112	atg4a	ENSG00000101844	305	162	c12orf34	ENSG00000139438
305	113	atg4b	ENSG00000168397	305	163	c12orf44	ENSG00000123395
305	114	atg5	ENSG00000057663	305	164	c13orf18	ENSG00000102445
305	115	atg9b	ENSG00000181652	305	165	1200003c05rik	ENSG00000050130
305	116	scrambled		305	166	scrambled	
305	117	atp6v1b2	ENSG00000147416	305	167	c14orf102	ENSG00000119720
305	118	atp6v1d	ENSG00000100554	305	168	c14orf126	ENSG00000129480
305	119	atrx	ENSG00000085224	305	169	c14orf162	ENSG00000181492
305	120	avpi1	ENSG00000119986	305	170	c14orf178	ENSG00000197734
305	121	axl	ENSG00000167601	305	171	c17orf56	ENSG00000167302
305	122	b3gat2	ENSG00000112309	305	172	c17orf76	ENSG00000181350
305	123	b3gat3	ENSG00000149541	305	173	c17orf85	ENSG00000074356
305	124	b4galt5	ENSG00000158470	305	174	c19orf10	ENSG00000074842
305	125	b4galt6	ENSG00000118276	305	175	gosr1	
305	126	rab1a		305	176	c19orf35	ENSG00000188305
305	127	baiap2l1	ENSG00000006453	305	177	c19orf4	ENSG00000105696
305	128	bccip	ENSG00000107949	305	178	2310036o22rik	ENSG00000123144
305	129	bcl11b	ENSG00000127152	305	179	c19orf57	ENSG00000132016
305	130	bcor	ENSG00000183337	305	180	c19orf7	ENSG00000130749
305	131	bcr	ENSG00000186716	305	181	c1orf109	ENSG00000116922
305	132	kiaa1446	ENSG00000183092	305	182	c1orf151	ENSG00000173436
305	133	pmf1	ENSG00000160783	305	183	c1orf157	ENSG00000176754
305	134	bhlhb9	ENSG00000198908	305	184	scrambled	
305	135	scrambled		305	185	c1orf191	ENSG00000198711
305	136	birc8	ENSG00000180152	305	186	c1orf201	ENSG00000001460
305	137	bmp3	ENSG00000152785	305	187	c1orf32	ENSG00000143195
305	138	brd1	ENSG00000100425	305	188	rd3	ENSG00000198570
305	139	brd4	ENSG00000141867	305	189	c1orf42	ENSG00000169509
305	140	brd7	ENSG00000166164	305	190	c1orf50	ENSG00000164008
305	141	btbd14a	ENSG00000148411	305	191	rab1a	
305	142	copb		305	192	c1orf54	ENSG00000118292
305	143	btbd7	ENSG00000011114	305	193	c1orf85	ENSG00000198715
305	144	btn3a1	ENSG00000026950	305	194	c1qa	ENSG00000173372
305	145	bub1b	ENSG00000156970	305	195	c1s	ENSG00000182326
305	146	bub3	ENSG00000154473	305	196	c20orf133	ENSG00000172264
305	147	bud31	ENSG00000106245	305	197	c20orf175	ENSG00000042062
305	148	bxdc5	ENSG00000117133	305	198	c22orf34	ENSG00000188511
305	149	loc151579	ENSG00000082153	305	199	c2orf32	ENSG00000119865
305	150	c10orf137	ENSG00000107938	305	200	c2orf43	ENSG00000118961

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
305	201	c2orf47	ENSG00000162972	305	251	ccbl1	ENSG00000171097
305	202	c3orf18	ENSG00000088543	305	252	ccdc107	ENSG00000159884
305	203	c3orf26	ENSG00000184220	305	253	ccdc112	ENSG00000164221
305	204	c3orf32	ENSG00000125046	305	254	ccdc124	ENSG00000007080
305	205	c3orf38	ENSG00000179021	305	255	gosr1	
305	206	rab1a		305	256	ccdc126	ENSG00000169193
305	207	c3orf52	ENSG00000114529	305	257	ccdc16	ENSG00000198783
305	208	c3orf60	ENSG00000178057	305	258	ccdc43	ENSG00000180329
305	209	c4orf14	ENSG00000084092	305	259	ccdc46	ENSG00000154240
305	210	c4orf17	ENSG00000138813	305	260	ccdc51	ENSG00000164051
305	211	c5orf24	ENSG00000181904	305	261	ccdc68	ENSG00000166510
305	212	scrambled		305	262	scrambled	
305	213	c6	ENSG00000039537	305	263	ccdc93	ENSG00000125633
305	214	c6orf115	ENSG00000146386	305	264	ccnk	ENSG00000090061
305	215	c6orf167	ENSG00000146263	305	265	ccr4	ENSG00000183813
305	216	c6orf170	ENSG00000146350	305	266	ccr5	ENSG00000160791
305	217	c6orf190	ENSG00000172673	305	267	fgfr1op	ENSG00000112486
305	218	c6orf81	ENSG00000157343	305	268	cct8	ENSG00000156261
305	219	c6orf96	ENSG00000155906	305	269	cd300lg	ENSG00000161649
305	220	c7orf25	ENSG00000136197	305	270	cd40	ENSG00000101017
305	221	c8b	ENSG00000021852	305	271	rab1a	
305	222	copb		305	272	cd40lg	ENSG00000102245
305	223	c8orf40	ENSG00000176209	305	273	cdc37	ENSG00000105401
305	224	c8orf51	ENSG00000183610	305	274	cdc42bpb	ENSG00000198752
305	225	c8orf54	ENSG00000181171	305	275	cdc42ep1	ENSG00000128283
305	226	c9orf103	ENSG00000148057	305	276	cdh24	ENSG00000139880
305	227	c9orf105	ENSG00000175768	305	277	cdh26	ENSG00000124215
305	228	c9orf116	ENSG00000160345	305	278	cdk5r1	ENSG00000176749
305	229	c9orf19	ENSG00000122694	305	279	cdkn2c	ENSG00000123080
305	230	c9orf25	ENSG00000164970	305	280	scrambled	
305	231	scrambled		305	281	cdkn2d	ENSG00000129355
305	232	c9orf46	ENSG00000107020	305	282	cdkn3	ENSG00000100526
305	233	c9orf48	ENSG00000186638	305	283	cecr5	ENSG00000069998
305	234	c9orf58	ENSG00000126878	305	284	cenpi	ENSG00000102384
305	235	c9orf80	ENSG00000148153	305	285	cenpm	ENSG00000100162
305	236	c9orf98	ENSG00000165695	305	286	centa1	ENSG00000105963
305	237	cabc1	ENSG00000163050	305	287	copb	
305	238	gosr1		305	288	cflar	ENSG00000003402
305	239	cacna1a	ENSG00000141837	305	289	cfr	ENSG00000001626
305	240	cacna2d3	ENSG00000157445	305	290	cgrf1	ENSG00000100532
305	241	calm3	ENSG00000160014	305	291	chchd1	ENSG00000172586
305	242	calm1	ENSG00000198668	305	292	chchd3	ENSG00000106554
305	243	calm2	ENSG00000143933	305	293	chchd6	ENSG00000159685
305	244	camk2n1	ENSG00000162545	305	294	chi3l1	ENSG00000133048
305	245	camta2	ENSG00000108509	305	295	chid1	ENSG00000177830
305	246	casc1	ENSG00000118307	305	296	chmp2b	ENSG00000083937
305	247	casz1	ENSG00000130940	305	297	chma4	ENSG00000101204
305	248	cblb	ENSG00000114423	305	298	chmb4	ENSG00000117971
305	249	cbln4	ENSG00000054803	305	299	chmd	ENSG00000135902
305	250	cbx6	ENSG00000183741	305	300	chst6	ENSG00000183196

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
305	301	cirbp	ENSG00000099622	311	351	rab1a	ENSG00000138069
305	302	copb		311	352	empty	
305	303	cirh1a	ENSG00000141076	311	353	empty	
305	304	tbc1b	ENSG00000105254	311	354	empty	
305	305	ckap2	ENSG00000136108	311	355	empty	
305	306	clca2	ENSG00000137975	311	356	empty	
305	307	clcn4	ENSG00000073464	311	357	empty	
305	308	scrambled		311	358	scramble	
305	309	cldn22	ENSG00000177300	311	359	empty	
305	310	clcc2a	ENSG00000188393	311	360	empty	
305	311	clcc2	ENSG00000155962	311	361	empty	
305	312	clcc4	ENSG00000169504	311	362	empty	
305	313	clk1	ENSG00000013441	311	363	empty	
305	314	clk3	ENSG00000179335	311	364	empty	
305	315	clpb	ENSG00000162129	311	365	empty	
305	316	clstn3	ENSG00000139182	311	366	empty	
305	317	clu	ENSG00000120885	311	367	copb	ENSG00000129083
305	318	gosr1		311	368	empty	
305	319	cmtm4	ENSG00000183723	311	369	empty	
305	320	ngdn	ENSG00000129460	311	370	empty	
305	321	cndp1	ENSG00000150656	311	371	empty	
305	322	cnksr1	ENSG00000142675	311	372	empty	
305	323	cnnm1	ENSG00000119946	311	373	incenp	ENSG00000149503
305	324	cnr2	ENSG00000188822	311	374	empty	
305	325	cntfr	ENSG00000122756	311	375	empty	
305	326	col11a1	ENSG00000060718	311	376	scramble	
305	327	scrambled		311	377	empty	
305	328	commd4	ENSG00000140365	311	378	empty	
305	329	copb2	ENSG00000184432	311	379	empty	
305	330	copg	ENSG00000181789	311	380	empty	
305	331	cova1	ENSG00000165675	311	381	empty	
305	332	cox5a	ENSG00000178741	311	382	empty	
305	333	cpeb3	ENSG00000107864	311	383	gosr1	ENSG00000108587
305	334	rab1a		311	384	incenp	ENSG00000149503
305	335	cpm	ENSG00000135678				
305	336	cpne8	ENSG00000139117				
305	337	cpsf3l	ENSG00000127054				
305	338	cpxm1	ENSG00000088882				
305	339	creb5	ENSG00000146592				
305	340	crmp1	ENSG00000072832				
305	341	crtc3	ENSG00000140577				
305	342	crygn	ENSG00000127377				
305	343	csad	ENSG00000139631				
305	344	csmd1	ENSG00000183117				
305	345	cst9	ENSG00000173335				
305	346	cstb	ENSG00000160213				
305	347	ctgf	ENSG00000118523				
305	348	ctsg	ENSG00000100448				
305	349	cul4a	ENSG00000139842				
305	350	cutc	ENSG00000119929				

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
306	1	incenp		306	51	eed	ENSG00000074266
306	2	dera	ENSG00000023697	306	52	eef1a2	ENSG00000101210
306	3	dgkd	ENSG00000077044	306	53	eef2k	ENSG00000103319
306	4	dgke	ENSG00000153933	306	54	efna3	ENSG00000143590
306	5	dgkq	ENSG00000145214	306	55	egfl6	ENSG00000198759
306	6	dguok	ENSG00000114956	306	56	egln2	ENSG00000171570
306	7	dhx35	ENSG00000101452	306	57	egr2	ENSG00000122877
306	8	dhx37	ENSG00000150990	306	58	eif1ax	ENSG00000173674
306	9	dicer1	ENSG00000100697	306	59	eif2b1	ENSG00000111361
306	10	diras1	ENSG00000176490	306	60	eif2s2	ENSG00000125977
306	11	dkk3	ENSG00000050165	306	61	eif3s3	ENSG00000147677
306	12	incenp		306	62	eif5b	ENSG00000158417
306	13	dlgap3	ENSG00000116544	306	63	rab1a	
306	14	copb		306	64	elac1	ENSG00000141642
306	15	dmbt1	ENSG00000187908	306	65	elavl2	ENSG00000107105
306	16	dnajc15	ENSG00000120675	306	66	elovl1	ENSG00000066322
306	17	dnal1	ENSG00000119661	306	67	elovl5	ENSG00000012660
306	18	dnase1l1	ENSG00000013563	306	68	emilin1	ENSG00000138080
306	19	dnase1l3	ENSG00000163687	306	69	emilin2	ENSG00000132205
306	20	scrambled		306	70	scrambled	
306	21	dnd1	ENSG00000183403	306	71	enpp3	ENSG00000154269
306	22	dnhd1	ENSG00000132283	306	72	enpp6	ENSG00000164303
306	23	dnm1	ENSG00000106976	306	73	loc644511	ENSG00000148713
306	24	dok3	ENSG00000146094	306	74	loc730167	ENSG00000172760
306	25	dok5	ENSG00000101134	306	75	loc729662	ENSG00000173609
306	26	dpep1	ENSG00000015413	306	76	fsip2	ENSG00000180922
306	27	bbs1	ENSG00000174483	306	77	loc440585	ENSG00000186973
306	28	dpp9	ENSG00000142002	306	78	loc644334	ENSG00000188179
306	29	dppa4	ENSG00000121570	306	79	copb	
306	30	gosr1		306	80	loc285193	ENSG00000188542
306	31	dqx1	ENSG00000144045	306	81	mgc59937	ENSG00000197191
306	32	dr1	ENSG00000117505	306	82	loc652708	ENSG00000198848
306	33	drd2	ENSG00000149295	306	83	epb41l4b	ENSG00000095203
306	34	drg1	ENSG00000185721	306	84	epdr1	ENSG00000086289
306	35	dsp	ENSG00000096696	306	85	epha3	ENSG00000044524
306	36	dst	ENSG00000151914	306	86	ephb3	ENSG00000182580
306	37	dusp13	ENSG00000079393	306	87	eps8l3	ENSG00000198758
306	38	dusp15	ENSG00000149599	306	88	scrambled	
306	39	scrambled		306	89	erc2	ENSG00000187672
306	40	dydc1	ENSG00000170788	306	90	ercc3	ENSG00000163161
306	41	dyrk3	ENSG00000143479	306	91	ergic3	ENSG00000125991
306	42	ebi3	ENSG00000105246	306	92	ervwe1	ENSG00000197604
306	43	ecd	ENSG00000122882	306	93	etnk1	ENSG00000139163
306	44	ecel1	ENSG00000171551	306	94	ets2	ENSG00000157557
306	45	ecgf1	ENSG00000025708	306	95	gosr1	
306	46	rab1a		306	96	ext1	ENSG00000182197
306	47	echdc2	ENSG00000121310	306	97	extl1	ENSG00000158008
306	48	edc3	ENSG00000179151	306	98	extl2	ENSG00000162694
306	49	edf1	ENSG00000107223	306	99	f2r	ENSG00000181104
306	50	ednra	ENSG00000151617	306	100	f5	ENSG00000198734

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
306	101	f7	ENSG00000057593	306	151	gabra6	ENSG00000145863
306	102	fabp4	ENSG00000170323	306	152	gabrr1	ENSG00000146276
306	103	fam101b	ENSG00000183688	306	153	galr3	ENSG00000128310
306	104	fam109b	ENSG00000177096	306	154	ganab	ENSG00000089597
306	105	c030004a17rik	ENSG00000197798	306	155	gapdhs	ENSG00000105679
306	106	fam3d	ENSG00000198643	306	156	gbp2	ENSG00000162645
306	107	fam43b	ENSG00000183114	306	157	gcnt4	ENSG00000176928
306	108	fam55a	ENSG00000095110	306	158	gdf6	ENSG00000156466
306	109	fam63a	ENSG00000143409	306	159	copb	
306	110	gosr1		306	160	gdf8	ENSG00000138379
306	111	fam63b	ENSG00000128923	306	161	gfra1	ENSG00000151892
306	112	fam81b	ENSG00000153347	306	162	gimap2	ENSG00000106560
306	113	fam98a	ENSG00000119812	306	163	gimap6	ENSG00000133561
306	114	fam9b	ENSG00000177138	306	164	git1	ENSG00000108262
306	115	fastkd3	ENSG00000124279	306	165	glra1	ENSG00000145888
306	116	scrambled		306	166	scrambled	
306	117	fbln2	ENSG00000163520	306	167	glrb	ENSG00000109738
306	118	fbln5	ENSG00000140092	306	168	glrx2	ENSG00000023572
306	119	fbxl8	ENSG00000135722	306	169	gmfb	ENSG00000197045
306	120	fbxo45	ENSG00000174013	306	170	gmppa	ENSG00000144591
306	121	fbxw5	ENSG00000159069	306	171	gnai3	ENSG00000065135
306	122	fbxw9	ENSG00000132004	306	172	gnb2	ENSG00000172354
306	123	fcer2	ENSG00000104921	306	173	gng10	ENSG00000059769
306	124	fcgr1a	ENSG00000150337	306	174	gng13	ENSG00000127588
306	125	fcgrt	ENSG00000104870	306	175	gosr1	
306	126	rab1a		306	176	gng2	ENSG00000186469
306	127	fcmd	ENSG00000106692	306	177	gng8	ENSG00000167414
306	128	fech	ENSG00000066926	306	178	golga	ENSG00000140478
306	129	fer	ENSG00000151422	306	179	gon4l	ENSG00000116580
306	130	ffar2	ENSG00000126262	306	180	gosr1	ENSG00000108587
306	131	fgf19	ENSG00000162344	306	181	gosr2	ENSG00000108433
306	132	fgr	ENSG00000000938	306	182	gp5	ENSG00000178732
306	133	fhl1	ENSG00000022267	306	183	gpatc2	ENSG00000092978
306	134	flt3lg	ENSG00000090554	306	184	scrambled	
306	135	scrambled		306	185	gpatc3	ENSG00000198746
306	136	flt4	ENSG00000037280	306	186	gbbp1	ENSG00000062194
306	137	flywch1	ENSG00000059122	306	187	gpc2	ENSG00000197093
306	138	foxr1	ENSG00000176302	306	188	gpc3	ENSG00000147257
306	139	fpri2	ENSG00000187474	306	189	gpnmb	ENSG00000136235
306	140	frmpd3	ENSG00000147234	306	190	tmem181	ENSG00000146433
306	141	fscn2	ENSG00000186765	306	191	rab1a	
306	142	copb		306	192	gpr31	ENSG00000120436
306	143	fsd1l	ENSG00000106701	306	193	gpr35	ENSG00000178623
306	144	fst	ENSG00000134363	306	194	gpr37	ENSG00000170775
306	145	fuk	ENSG00000157353	306	195	gpr45	ENSG00000135973
306	146	fut7	ENSG00000180549	306	196	gpr50	ENSG00000102195
306	147	fuz	ENSG00000010361	306	197	gps2	ENSG00000132522
306	148	fxyd5	ENSG00000089327	306	198	gpt	ENSG00000167701
306	149	gabaprl2	ENSG00000034713	306	199	grb14	ENSG00000115290
306	150	gabra5	ENSG00000186297	306	200	grb7	ENSG00000141738

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
306	201	grem1	ENSG00000166923	306	251	hsd17b1	ENSG00000108786
306	202	grik2	ENSG00000164418	306	252	hsd17b4	ENSG00000133835
306	203	grik5	ENSG00000105737	306	253	hsd17b6	ENSG00000025423
306	204	grin1	ENSG00000176884	306	254	hsd3b2	ENSG000000203859
306	205	grin2b	ENSG00000150086	306	255	gosr1	
306	206	rab1a		306	256	hsf5	ENSG00000176160
306	207	grin2c	ENSG00000161509	306	257	hsp90b1	ENSG00000166598
306	208	grin3a	ENSG00000198785	306	258	hspa4	ENSG00000170606
306	209	grina	ENSG00000178719	306	259	hspb8	ENSG00000152137
306	210	grk5	ENSG00000198873	306	260	htr3c	ENSG00000178084
306	211	gsg2	ENSG00000177602	306	261	htr3e	ENSG00000186038
306	212	scrambled		306	262	scrambled	
306	213	gstcd	ENSG00000138780	306	263	htr6	ENSG00000158748
306	214	gstp1	ENSG00000084207	306	264	htra2	ENSG00000115317
306	215	gtf2h3	ENSG00000111358	306	265	hyal4	ENSG00000106302
306	216	gtf2ird1	ENSG00000006704	306	266	ifih1	ENSG00000115267
306	217	gtpbp5	ENSG00000101181	306	267	ifnar1	ENSG00000142166
306	218	gucy2d	ENSG00000132518	306	268	ift88	ENSG00000032742
306	219	gyg1	ENSG00000163754	306	269	igf1r	ENSG00000140443
306	220	gys2	ENSG00000111713	306	270	igfbp2	ENSG00000115457
306	221	gzma	ENSG00000145649	306	271	rab1a	
306	222	copb		306	272	ihpk3	ENSG00000161896
306	223	h1fnt	ENSG00000187166	306	273	il15ra	ENSG00000134470
306	224	hadha	ENSG00000084754	306	274	il17rd	ENSG00000144730
306	225	haghl	ENSG00000103253	306	275	il18r1	ENSG00000115604
306	226	has3	ENSG00000103044	306	276	il1f5	ENSG00000136695
306	227	hcfc2	ENSG00000111727	306	277	il1rap	ENSG00000196083
306	228	hcn3	ENSG00000143630	306	278	il20ra	ENSG00000016402
306	229	hdgfl1	ENSG00000112273	306	279	il21	ENSG00000138684
306	230	hebp1	ENSG00000013583	306	280	scrambled	
306	231	scrambled		306	281	il23r	ENSG00000162594
306	232	herc6	ENSG00000138642	306	282	il2rb	ENSG00000100385
306	233	hexa	ENSG00000140488	306	283	il9r	ENSG00000124334
306	234	hgd	ENSG00000113924	306	284	impdh2	ENSG00000178035
306	235	hif1a	ENSG00000100644	306	285	inadl	ENSG00000132849
306	236	hif3a	ENSG00000124440	306	286	loc727773	ENSG00000168395
306	237	hipk1	ENSG00000163349	306	287	copb	
306	238	gosr1		306	288	insl6	ENSG00000120210
306	239	hisppd1	ENSG00000145725	306	289	ipo8	ENSG00000133704
306	240	hist1h2ac	ENSG00000180573	306	290	iqce	ENSG00000106012
306	241	hist1h2ae	ENSG00000168274	306	291	iqcf1	ENSG00000173389
306	242	hmbox1	ENSG00000147421	306	292	iqgap3	ENSG00000183856
306	243	hnrap1	ENSG00000135486	306	293	iqsec1	ENSG00000144711
306	244	hnrap2b1	ENSG00000122566	306	294	itgav	ENSG00000138448
306	245	hoxc9	ENSG00000180806	306	295	itgb5	ENSG00000082781
306	246	hoxd11	ENSG00000128713	306	296	itgb6	ENSG00000115221
306	247	hp	ENSG00000197711	306	297	itih1	ENSG00000055957
306	248	hpgd	ENSG00000164120	306	298	itpa	ENSG00000125877
306	249	hpn	ENSG00000105707	306	299	itpr2	ENSG00000123104
306	250	hs6st2	ENSG00000171004	306	300	ivd	ENSG00000128928

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
306	301	jag1	ENSG00000101384	306	351	rab1a	
306	302	copb		306	352	larp5	ENSG00000107929
306	303	jak2	ENSG00000096968	306	353	lcn6	ENSG00000204003
306	304	jazf1	ENSG00000153814	306	354	ldb2	ENSG00000169744
306	305	jmjd1b	ENSG00000120733	306	355	ldhb	ENSG00000111716
306	306	josd2	ENSG00000161677	306	356	leng4	ENSG00000125505
306	307	visa	ENSG00000088888	306	357	leo1	ENSG00000166477
306	308	scrambled		306	358	scrambled	
306	309	kcnmb2	ENSG00000197584	306	359	letm1	ENSG00000168924
306	310	kcnn4	ENSG00000104783	306	360	lgals14	ENSG00000006659
306	311	kcnq2	ENSG00000075043	306	361	lgicz1	ENSG00000186919
306	312	kcnq4	ENSG00000117013	306	362	lhfp15	ENSG00000197753
306	313	kctd4	ENSG00000180332	306	363	lhx9	ENSG00000143355
306	314	khk	ENSG00000138030	306	364	lipc	ENSG00000166035
306	315	kiaa0133	ENSG00000135763	306	365	lmo3	ENSG00000048540
306	316	kiaa0240	ENSG00000112624	306	366	lnx2	ENSG00000139517
306	317	kiaa0372	ENSG00000198677	306	367	copb	
306	318	gosr1		306	368	lrfn3	ENSG00000126243
306	319	kiaa0423	ENSG00000198718	306	369	lrp4	ENSG00000134569
306	320	kiaa0907	ENSG00000132680	306	370	lrrc21	ENSG00000148602
306	321	kiaa1018	ENSG00000198690	306	371	lrrc23	ENSG00000010626
306	322	kiaa1161	ENSG00000164976	306	372	lrrc27	ENSG00000148814
306	323	kiaa1840	ENSG00000104133	306	373	incenp	
306	324	kif15	ENSG00000163808	306	374	lrrc31	ENSG00000114248
306	325	kif19	ENSG00000196169	306	375	lrrc39	ENSG00000122477
306	326	kif1c	ENSG00000129250	306	376	scrambled	
306	327	scrambled		306	377	lrrc3b	ENSG00000179796
306	328	kif23	ENSG00000137807	306	378	lrrc46	ENSG00000141294
306	329	kif26a	ENSG00000066735	306	379	lsm14a	ENSG00000105216
306	330	kif2c	ENSG00000142945	306	380	lsr	ENSG00000105699
306	331	kifap3	ENSG00000075945	306	381	ltbp1	ENSG00000049323
306	332	kir2dl4	ENSG00000189013	306	382	ltk	ENSG00000062524
306	333	kirrel	ENSG00000183853	306	383	gosr1	
306	334	rab1a		306	384	incenp	
306	335	kit	ENSG00000157404				
306	336	kl	ENSG00000133116				
306	337	klhl17	ENSG00000187961				
306	338	klhl21	ENSG00000162413				
306	339	phospho2	ENSG00000144362				
306	340	klhl4	ENSG00000102271				
306	341	klk2	ENSG00000167751				
306	342	kng1	ENSG00000113889				
306	343	kpna6	ENSG00000025800				
306	344	kremen1	ENSG00000183762				
306	345	smarce1	ENSG00000073584				
306	346	krt6b	ENSG00000185479				
306	347	krt8	ENSG00000170421				
306	348	l1cam	ENSG00000198910				
306	349	lair1	ENSG00000167613				
306	350	lamp3	ENSG00000078081				

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
307	1	incenp		307	51	mgst1	ENSG00000008394
307	2	luzp1	ENSG00000169641	307	52	mid2	ENSG00000080561
307	3	ly75	ENSG00000054219	307	53	minpp1	ENSG00000107789
307	4	lyn	ENSG00000147507	307	54	mlf1ip	ENSG00000151725
307	5	lypla3	ENSG00000103066	307	55	mlh1	ENSG00000076242
307	6	lyplal1	ENSG00000143353	307	56	mll	ENSG00000118058
307	7	lyst	ENSG00000143669	307	57	mlph	ENSG00000115648
307	8	lztr2	ENSG00000120341	307	58	mlstd2	ENSG00000197601
307	9	madd	ENSG00000110514	307	59	mlxipl	ENSG00000009950
307	10	mafg	ENSG00000197063	307	60	mme	ENSG00000196549
307	11	magea4	ENSG00000147381	307	61	mmp14	ENSG00000157227
307	12	incenp		307	62	mmp16	ENSG00000156103
307	13	mageb10	ENSG00000177689	307	63	rab1a	
307	14	copb		307	64	mmp2	ENSG00000087245
307	15	magi3	ENSG00000081026	307	65	mmp21	ENSG00000154485
307	16	mansc1	ENSG00000111261	307	66	mmp28	ENSG00000129270
307	17	map3k15	ENSG00000180815	307	67	mnd1	ENSG00000121211
307	18	mapk11	ENSG00000185386	307	68	mnt	ENSG00000070444
307	19	mapk12	ENSG00000188130	307	69	mosc2	ENSG00000117791
307	20	scrambled		307	70	scrambled	
307	21	mapk15	ENSG00000181085	307	71	mpv17	ENSG00000115204
307	22	mapkap1	ENSG00000119487	307	72	mrpre	ENSG00000184350
307	23	mapkapk2	ENSG00000162889	307	73	mrpl41	ENSG00000182154
307	24	mapkbp1	ENSG00000137802	307	74	mrps2	ENSG00000122140
307	25	mark2	ENSG00000072518	307	75	mrps33	ENSG00000090263
307	26	mast2	ENSG00000086015	307	76	mrps35	ENSG00000061794
307	27	mast3	ENSG00000099308	307	77	mrps5	ENSG00000144029
307	28	matr3	ENSG00000015479	307	78	ms4a6a	ENSG00000110077
307	29	maz	ENSG00000103495	307	79	copb	
307	30	gosr1		307	80	msr1	ENSG00000038945
307	31	mbi2	ENSG00000165471	307	81	mtfmt	ENSG00000103707
307	32	jtv1	ENSG00000106305	307	82	mtnr1b	ENSG00000134640
307	33	mchr1	ENSG00000128285	307	83	muc2	ENSG00000198788
307	34	mcm10	ENSG00000065328	307	84	mutyh	ENSG00000132781
307	35	mcm7	ENSG00000166508	307	85	mxd4	ENSG00000123933
307	36	mdn1	ENSG00000112159	307	86	myb	ENSG00000118513
307	37	med28	ENSG00000118579	307	87	mybpc1	ENSG00000196091
307	38	mrp1	ENSG00000134138	307	88	scrambled	
307	39	scrambled		307	89	mybph	ENSG00000133055
307	40	mesdc2	ENSG00000117899	307	90	myh10	ENSG00000133026
307	41	metrnl	ENSG00000176845	307	91	myh7	ENSG00000092054
307	42	mettl3	ENSG00000165819	307	92	myl1	ENSG00000168530
307	43	mettl4	ENSG00000101574	307	93	myoc	ENSG00000034971
307	44	mfap3l	ENSG00000198948	307	94	myom2	ENSG00000036448
307	45	mfge8	ENSG00000140545	307	95	gosr1	
307	46	rab1a		307	96	myot	ENSG00000120729
307	47	mfng	ENSG00000100060	307	97	myoz3	ENSG00000164591
307	48	mfsd5	ENSG00000182544	307	98	nalp14	ENSG00000158077
307	49	mga	ENSG00000174197	307	99	nat13	ENSG00000121579
307	50	mgat4b	ENSG00000161013	307	100	nat8	ENSG00000144035

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
307	101	nav3	ENSG00000067798	307	151	larp7	ENSG00000174720
307	102	ndnl2	ENSG00000185115	307	152	tug1	ENSG00000182457
307	103	ndst2	ENSG00000166507	307	153	flj20628	ENSG00000171103
307	104	ndufa12l	ENSG00000164182	307	154	mreg	ENSG00000118242
307	105	ndufs2	ENSG00000158864	307	155	np_060529.2	ENSG00000157927
307	106	nedd4	ENSG00000069869	307	156	flj10490	ENSG00000104865
307	107	nek2	ENSG00000117650	307	157	flj10781	ENSG00000182013
307	108	neurog1	ENSG00000181965	307	158	mettl2b	ENSG00000165055
307	109	nfatc4	ENSG00000100968	307	159	copb	
307	110	gosr1		307	160	bc021381	ENSG00000146067
307	111	nfkbie	ENSG00000146232	307	161	kiaa1505	ENSG00000135205
307	112	nfkbil2	ENSG00000160949	307	162	kiaa1530	ENSG00000163945
307	113	nfyb	ENSG00000120837	307	163	9430057o19rik	ENSG00000084693
307	114	nhp2l1	ENSG00000100138	307	164	p53aip1	ENSG00000120471
307	115	nipbl	ENSG00000164190	307	165	flj21865	ENSG00000167280
307	116	scrambled		307	166	scrambled	
307	117	nle1	ENSG00000073536	307	167	ccdc134	ENSG00000100147
307	118	eg238564	ENSG00000145949	307	168	flj14154	ENSG00000122390
307	119	nmral1	ENSG00000153406	307	169	flj21687	ENSG00000017621
307	120	nmu	ENSG00000109255	307	170	flj22374	ENSG00000106121
307	121	nol12	ENSG00000100101	307	171	flj23356	ENSG00000185900
307	122	nol3	ENSG00000140939	307	172	kiaa1729	ENSG00000178163
307	123	nol4	ENSG00000101746	307	173	loc653383	ENSG00000187713
307	124	nolc1	ENSG00000166197	307	174	hel308	ENSG00000163312
307	125	nono	ENSG00000147140	307	175	gosr1	
307	126	rab1a		307	176	xtp7	ENSG00000130201
307	127	notch2nl	ENSG00000163386	307	177	slc25a46	ENSG00000164209
307	128	notum	ENSG00000185269	307	178	loc113386	ENSG00000166718
307	129	nox4	ENSG00000086991	307	179	mgc29891	ENSG00000143458
307	130	spink5l2	ENSG00000196800	307	180	dact3	ENSG00000197380
307	131	ttmb	ENSG00000187975	307	181	flj38663	ENSG00000130921
307	132	flj90757	ENSG00000175901	307	182	mgc45438	ENSG00000153446
307	133	flj40194	ENSG00000177369	307	183	flj36004	ENSG00000152936
307	134	loc136242	ENSG00000165076	307	184	scrambled	
307	135	scrambled		307	185	flj31196	ENSG00000180638
307	136	loc348180	ENSG00000174177	307	186	flj39501	ENSG00000171954
307	137	loc388965	ENSG00000182814	307	187	flj25791	ENSG00000177214
307	138	np_001013713.1	ENSG00000197332	307	188	glb1l3	ENSG00000166105
307	139	loc51149	ENSG00000161010	307	189	flj40288	ENSG00000183470
307	140	loc286076	ENSG00000181097	307	190	flj90650	ENSG00000172901
307	141	c1d	ENSG00000197223	307	191	rab1a	
307	142	copb		307	192	flj12949	ENSG00000129347
307	143	hspc159	ENSG00000119862	307	193	dnhd2	ENSG00000163669
307	144	rics	ENSG00000134909	307	194	agbl3	ENSG00000146856
307	145	kiaa0738	ENSG00000198420	307	195	ostbeta	ENSG00000186198
307	146	kiaa0672	ENSG00000006740	307	196	aasdh	ENSG00000157426
307	147	kiaa0523	ENSG00000179314	307	197	gddr	ENSG00000183607
307	148	cgi-09	ENSG00000089195	307	198	np_919270.1	ENSG00000188676
307	149	crop	ENSG00000108848	307	199	hspc268	ENSG00000164898
307	150	loc51252	ENSG00000168754	307	200	loc196264	ENSG00000160588

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
307	201	mgc12965	ENSG00000198048	307	251	or4p4	ENSG00000181927
307	202	loc284361	ENSG00000161671	307	252	or51a7	ENSG00000176895
307	203	loc150763	ENSG00000186281	307	253	or51e1	ENSG00000180785
307	204	np_997271.1	ENSG00000167934	307	254	or51f2	ENSG00000176925
307	205	flj45244	ENSG00000198411	307	255	gosr1	
307	206	rab1a		307	256	or51m1	ENSG00000184698
307	207	flj45256	ENSG00000197672	307	257	or51s1	ENSG00000176922
307	208	mgc48628	ENSG00000184305	307	258	or52e4	ENSG00000180974
307	209	ai593442	ENSG00000185742	307	259	or52k2	ENSG00000181963
307	210	npc1l1	ENSG00000015520	307	260	or5a2	ENSG00000172324
307	211	npepps	ENSG00000141279	307	261	or5as1	ENSG00000181785
307	212	scrambled		307	262	scrambled	
307	213	npm1	ENSG00000181163	307	263	or5au1	ENSG00000169327
307	214	npw	ENSG00000183971	307	264	or5b2	ENSG00000172365
307	215	nr1i3	ENSG00000143257	307	265	or5bf1	ENSG00000177174
307	216	nr2c1	ENSG00000120798	307	266	or5t3	ENSG00000172489
307	217	nr2f6	ENSG00000160113	307	267	or6c2	ENSG00000179695
307	218	nr4a2	ENSG00000153234	307	268	or6c70	ENSG00000184954
307	219	nr6a1	ENSG00000148200	307	269	or6k6	ENSG00000180433
307	220	csde1	ENSG00000009307	307	270	or7a10	ENSG00000127515
307	221	nrp1	ENSG00000180530	307	271	rab1a	
307	222	copb		307	272	or7g1	ENSG00000161807
307	223	nrp2	ENSG00000053702	307	273	osbp	ENSG00000110048
307	224	nt5c	ENSG00000125458	307	274	otud7a	ENSG00000169918
307	225	nt5c2	ENSG00000076685	307	275	ovch1	ENSG00000187950
307	226	nt5c3	ENSG00000122643	307	276	oxct1	ENSG00000083720
307	227	nudt16	ENSG00000198585	307	277	oxsr1	ENSG00000172939
307	228	nudt4	ENSG00000173598	307	278	oxtr	ENSG00000180914
307	229	nudt9	ENSG00000170502	307	279	p2rx7	ENSG00000089041
307	230	nup188	ENSG00000095319	307	280	scrambled	
307	231	scrambled		307	281	pacrg	ENSG00000112530
307	232	nup35	ENSG00000163002	307	282	pacs1	ENSG00000175115
307	233	nxn	ENSG00000167693	307	283	paep	ENSG00000122133
307	234	kiaa0363	ENSG00000136274	307	284	pag1	ENSG00000076641
307	235	ocel1	ENSG00000099330	307	285	pak7	ENSG00000101349
307	236	odz3	ENSG00000109716	307	286	palm	ENSG00000099864
307	237	ogfr	ENSG00000060491	307	287	copb	
307	238	gosr1		307	288	pank2_predicted	ENSG00000125779
307	239	ogg1	ENSG00000114026	307	289	paqr4	ENSG00000162073
307	240	olfml3	ENSG00000116774	307	290	parvg	ENSG00000138964
307	241	oma1	ENSG00000162600	307	291	pax4	ENSG00000106331
307	242	omg	ENSG00000126861	307	292	paxip1	ENSG00000157212
307	243	opr1	ENSG00000125510	307	293	pbxip1	ENSG00000163346
307	244	or10a4	ENSG00000170782	307	294	pcbd2	ENSG00000132570
307	245	or10a7	ENSG00000179919	307	295	pcd1b10	ENSG00000120324
307	246	or10ag1	ENSG00000174970	307	296	pcd1b12	ENSG00000120328
307	247	or10j5	ENSG00000184155	307	297	pcd1b18	ENSG00000146001
307	248	or11h4	ENSG00000176198	307	298	pcn12	ENSG00000135749
307	249	or13c3	ENSG00000204246	307	299	pcsk4	ENSG00000115257
307	250	or13f1	ENSG00000186881	307	300	pdcd10	ENSG00000114209

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
307	301	pdgfa	ENSG00000197461	307	351	rab1a	
307	302	copb		307	352	polh	ENSG00000170734
307	303	pdp2	ENSG00000172840	307	353	polr3f	ENSG00000132664
307	304	pdxk	ENSG00000160209	307	354	polr3g	ENSG00000113356
307	305	intu	ENSG00000164066	307	355	polr3gl	ENSG00000121851
307	306	pea15	ENSG00000162734	307	356	pou6f1	ENSG00000184271
307	307	pecam1	ENSG00000198802	307	357	ppapdc1	ENSG00000147535
307	308	scrambled		307	358	scrambled	
307	309	pikfb4	ENSG00000114268	307	359	ppfia1	ENSG00000131626
307	310	pgam5	ENSG00000176894	307	360	ppie	ENSG00000084072
307	311	ercc6	ENSG00000032514	307	361	ppox	ENSG00000143224
307	312	pgc	ENSG00000096088	307	362	ppp1r14c	ENSG00000198729
307	313	pglyrp1	ENSG00000008438	307	363	ppp1r16a	ENSG00000160972
307	314	pgm5	ENSG00000154330	307	364	ppp2cb	ENSG00000104695
307	315	phf11	ENSG00000136147	307	365	dock5	ENSG00000147459
307	316	phf2	ENSG00000197724	307	366	ppp2r2d	ENSG00000175470
307	317	phf7	ENSG00000010318	307	367	copb	
307	318	gosr1		307	368	ppp4r1	ENSG00000154845
307	319	phkg2	ENSG00000156873	307	369	ppp4r2	ENSG00000163605
307	320	phlpp	ENSG00000081913	307	370	pric285	ENSG00000130589
307	321	phlpp1	ENSG00000040199	307	371	prdm5	ENSG00000138738
307	322	phtf1	ENSG00000116793	307	372	prdx3	ENSG00000165672
307	323	pi5pa	ENSG00000185133	307	373	incenp	
307	324	picalm	ENSG00000073921	307	374	prep	ENSG00000085377
307	325	pigb	ENSG00000069943	307	375	prg1	ENSG00000122862
307	326	pigh	ENSG00000100564	307	376	scrambled	
307	327	scrambled		307	377	prg3	ENSG00000156575
307	328	pigr	ENSG00000162896	307	378	prkab2	ENSG00000131791
307	329	pik3r4	ENSG00000196455	307	379	prkod	ENSG00000163932
307	330	pilra	ENSG00000085514	307	380	proc	ENSG00000115718
307	331	pitx3	ENSG00000107859	307	381	prpf8	ENSG00000174231
307	332	piwil4	ENSG00000134627	307	382	prr4	ENSG00000111215
307	333	pkp1	ENSG00000081277	307	383	gosr1	
307	334	rab1a		307	384	incenp	
307	335	pla2g12b	ENSG00000138308				
307	336	pla2g4c	ENSG00000105499				
307	337	plcb2	ENSG00000137841				
307	338	plch1	ENSG00000114805				
307	339	plcz1	ENSG00000139151				
307	340	tnfrsf25	ENSG00000171680				
307	341	plekhm2	ENSG00000116786				
307	342	plscr4	ENSG00000114698				
307	343	plunc	ENSG00000198183				
307	344	pmaip1	ENSG00000141682				
307	345	pml	ENSG00000140464				
307	346	pms2	ENSG00000122512				
307	347	pnldc1	ENSG00000146453				
307	348	pnliprp2	ENSG00000165862				
307	349	pnma2	ENSG00000171362				
307	350	pnma5	ENSG00000198883				

Table 6 (continued)|

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
308	1	incenp		308	51	flj31033	ENSG00000181381
308	2	prrc1	ENSG00000164244	308	52	loc90113	ENSG00000145198
308	3	prrg2	ENSG00000126460	308	53	loc152217	ENSG00000179823
308	4	prrt3	ENSG00000163704	308	54	q9h052_human	ENSG00000184405
308	5	prss22	ENSG000000005001	308	55	loc642452	ENSG00000182794
308	6	prtn3	ENSG00000196415	308	56	flj13305	ENSG00000170264
308	7	psat1	ENSG00000135069	308	57	dkfzp434i0714	ENSG00000170009
308	8	pscd2	ENSG00000105443	308	58	loc388554	ENSG00000104983
308	9	psd	ENSG00000059915	308	59	qrich1	ENSG00000198218
308	10	psg7	ENSG00000124435	308	60	dnm2	ENSG00000079805
308	11	pskh2	ENSG00000147613	308	61	rab11a	ENSG00000103769
308	12	incenp		308	62	rab11b	ENSG00000185236
308	13	psmb1	ENSG00000008018	308	63	rab1a	
308	14	copb		308	64	rab18	ENSG00000099246
308	15	psmc3ip	ENSG00000131470	308	65	rab8b	ENSG00000166128
308	16	psmc4	ENSG00000013275	308	66	rabac1	ENSG00000105404
308	17	pstpip1	ENSG00000140368	308	67	ramp3	ENSG00000122679
308	18	ptafr	ENSG00000169403	308	68	ranbp6	ENSG00000137040
308	19	ptbp1	ENSG00000011304	308	69	rap1gds1	ENSG00000138698
308	20	scrambled		308	70	scrambled	
308	21	ptcd3	ENSG00000132300	308	71	rasl11b	ENSG00000128045
308	22	ptdsr	ENSG00000070495	308	72	rassf2	ENSG00000101265
308	23	ptdss1	ENSG00000156471	308	73	rbbp6	ENSG00000122257
308	24	ptdss2	ENSG00000174915	308	74	rbbp9	ENSG00000089050
308	25	ptov1	ENSG00000104960	308	75	rbm15b	ENSG00000179837
308	26	ptpn12	ENSG00000127947	308	76	rbm41	ENSG00000089682
308	27	ptpn21	ENSG00000070778	308	77	rbm5	ENSG00000003756
308	28	ptpn22	ENSG00000134242	308	78	rbp4	ENSG00000138207
308	29	ptpn9	ENSG00000169410	308	79	copb	
308	30	gosr1		308	80	rbpsuhl	ENSG00000124232
308	31	ptpm	ENSG00000054356	308	81	rccd1	ENSG00000166965
308	32	pus3	ENSG00000110060	308	82	rdh12	ENSG00000139988
308	33	pvalb	ENSG00000100362	308	83	rdh16	ENSG00000139547
308	34	pvr	ENSG00000073008	308	84	rdm1	ENSG00000187456
308	35	pycr2	ENSG00000143811	308	85	reep3	ENSG00000165476
308	36	pzp	ENSG00000126838	308	86	reep6	ENSG00000115255
308	37	q5jss6_human	ENSG00000197889	308	87	reg4	ENSG00000134193
308	38	mgc10334	ENSG00000187488	308	88	scrambled	
308	39	scrambled		308	89	rela	ENSG00000173039
308	40	plch2	ENSG00000149527	308	90	reerg	ENSG00000134533
308	41	ovos2	ENSG00000177359	308	91	rev1	ENSG00000135945
308	42	q6p462_human	ENSG00000189316	308	92	rftn2	ENSG00000162944
308	43	loc401565	ENSG00000188163	308	93	rfx3	ENSG00000080298
308	44	flj38596	ENSG00000176753	308	94	rfx5	ENSG00000143390
308	45	flj35880	ENSG00000172752	308	95	gosr1	
308	46	rab1a		308	96	rfoxp1	ENSG00000133111
308	47	flj34651	ENSG00000178803	308	97	rgn	ENSG00000130988
308	48	loc283507	ENSG00000168852	308	98	rgs19	ENSG00000171700
308	49	loc113230	ENSG00000141854	308	99	rhbd11	ENSG00000103269
308	50	ubqlnl	ENSG00000175518	308	100	rhoc	ENSG00000155366

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
308	101	rhof	ENSG00000139725	308	151	sepp1	ENSG00000211446
308	102	rhoj	ENSG00000126785	308	152	sepx1	ENSG00000198736
308	103	rho1	ENSG00000126858	308	153	serpinf2	ENSG00000167711
308	104	rhoh	ENSG00000116574	308	154	sez6l2	ENSG00000174938
308	105	ribc1	ENSG00000158423	308	155	sf3b4	ENSG00000143368
308	106	riok3	ENSG00000101782	308	156	sfrmbt1	ENSG00000163935
308	107	rin2	ENSG00000107014	308	157	sfrs1	ENSG00000136450
308	108	mase2	ENSG00000169385	308	158	sfrs6	ENSG00000124193
308	109	mf11	ENSG00000123091	308	159	copb	
308	110	gcsr1		308	160	sfxn4	ENSG00000183605
308	111	mf130	ENSG00000113269	308	161	sgcg	ENSG00000102683
308	112	mf133	ENSG00000188050	308	162	sgk	ENSG00000118515
308	113	mf207	ENSG00000189429	308	163	sh2b1	ENSG00000178188
308	114	rrp9	ENSG00000114767	308	164	sh3glb2	ENSG00000148341
308	115	robo3	ENSG00000154134	308	165	shank3	ENSG00000099882
308	116	scrambled		308	166	scrambled	
308	117	ros1	ENSG00000047936	308	167	shb	ENSG00000107338
308	118	rpgr1	ENSG00000092200	308	168	sipa1	ENSG00000173442
308	119	rpl32	ENSG00000144713	308	169	sirt2	ENSG00000068903
308	120	rpl35a	ENSG00000182899	308	170	six2	ENSG00000170577
308	121	rplp2	ENSG00000177600	308	171	ski	ENSG00000157933
308	122	rpp14	ENSG00000163684	308	172	skiv2l2	ENSG00000039123
308	123	rpp40	ENSG00000124787	308	173	loc681062	ENSG00000163950
308	124	rprc1	ENSG00000116871	308	174	slc12a8	ENSG00000163848
308	125	rps26	ENSG00000197728	308	175	gcsr1	
308	126	rab1a		308	176	slc20a1	ENSG00000144136
308	127	rps27l	ENSG00000185088	308	177	slc25a30	ENSG00000174032
308	128	rrad	ENSG00000166592	308	178	slc25a4	ENSG00000151729
308	129	rtn4r	ENSG00000040608	308	179	slc35b1	ENSG00000121073
308	130	rufy3	ENSG00000018189	308	180	slc36a2	ENSG00000186335
308	131	runx1t1	ENSG00000079102	308	181	slc36a3	ENSG00000186334
308	132	rusc1	ENSG00000160753	308	182	slc39a6	ENSG00000141424
308	133	ruvbl1	ENSG00000175792	308	183	slc3a2	ENSG00000168003
308	134	rxra	ENSG00000186350	308	184	scrambled	
308	135	scrambled		308	185	slc5a1	ENSG00000100170
308	136	rxrg	ENSG00000143171	308	186	slc6a14	ENSG00000087916
308	137	ryr1	ENSG00000196218	308	187	slc6a3	ENSG00000142319
308	138	s100a10	ENSG00000197747	308	188	slit1	ENSG00000187122
308	139	samd3	ENSG00000164483	308	189	slk	ENSG00000065613
308	140	samd4a	ENSG00000020577	308	190	hltf	ENSG00000071794
308	141	scgb2a2	ENSG00000110484	308	191	rab1a	
308	142	copb		308	192	smarcd1	ENSG00000066117
308	143	scgb3a1	ENSG00000161055	308	193	sms	ENSG00000102172
308	144	scn3b	ENSG00000166257	308	194	smyd2	ENSG00000143499
308	145	scn5a	ENSG00000183873	308	195	snap29	ENSG00000099940
308	146	scn7a	ENSG00000136546	308	196	snaip	ENSG00000064692
308	147	scyl1	ENSG00000142186	308	197	snx13	ENSG00000071189
308	148	sele	ENSG00000007908	308	198	snx19	ENSG00000120451
308	149	sema4b	ENSG00000185033	308	199	soat1	ENSG00000057252
308	150	sema4f	ENSG00000135622	308	200	sorbs3	ENSG00000120896

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
308	201	sorcs1	ENSG00000108018	308	251	taldo1	ENSG00000177156
308	202	sos2	ENSG00000100485	308	252	tanc1	ENSG00000115183
308	203	sox9	ENSG00000125398	308	253	tanc2	ENSG00000170921
308	204	spag6	ENSG00000077327	308	254	tbc1d17	ENSG00000104946
308	205	spata5	ENSG00000145375	308	255	gosr1	
308	206	rab1a		308	256	tbc1d8b	ENSG00000133138
308	207	spata5l1	ENSG00000171763	308	257	tbca	ENSG00000171530
308	208	spcs3	ENSG00000129128	308	258	tbx2	ENSG00000121068
308	209	spdy1	ENSG00000163806	308	259	tbx6	ENSG00000149922
308	210	sphk1	ENSG00000176170	308	260	tcp11	ENSG00000124678
308	211	spry3	ENSG00000168939	308	261	tead1	ENSG00000187079
308	212	scrambled		308	262	scrambled	
308	213	spry4	ENSG00000176422	308	263	tead3	ENSG00000007866
308	214	spnb2	ENSG00000115306	308	264	tecta	ENSG00000109927
308	215	srms	ENSG00000125508	308	265	tgfb3	ENSG00000069702
308	216	ssbp1	ENSG00000106028	308	266	tgm7	ENSG00000159495
308	217	sssc1	ENSG00000173465	308	267	thap4	ENSG00000176946
308	218	sst	ENSG00000157005	308	268	thap7	ENSG00000184436
308	219	st3gal5	ENSG00000115525	308	269	thop1	ENSG00000172009
308	220	st5	ENSG00000166444	308	270	thpo	ENSG00000090534
308	221	st6gal1	ENSG00000073849	308	271	rab1a	
308	222	copb		308	272	thumpd1	ENSG00000066654
308	223	st8sia1	ENSG00000111728	308	273	tigd5	ENSG00000179886
308	224	st8sia2	ENSG00000140557	308	274	timeless	ENSG00000111602
308	225	stac2	ENSG00000141750	308	275	tim9	ENSG00000100575
308	226	stam2	ENSG00000115145	308	276	tkl1	ENSG00000007350
308	227	brdg1	ENSG00000035720	308	277	tle4	ENSG00000106829
308	228	stard3nl	ENSG0000010270	308	278	tlr3	ENSG00000164342
308	229	stc1	ENSG00000159167	308	279	tlr5	ENSG00000187554
308	230	stk40	ENSG00000196182	308	280	scrambled	
308	231	scrambled		308	281	tlr7	ENSG00000196664
308	232	stox2	ENSG00000173320	308	282	mgc33212	ENSG00000145107
308	233	stt3b	ENSG00000163527	308	283	tmcc3	ENSG00000057704
308	234	stx17	ENSG00000136874	308	284	tmco4	ENSG00000162542
308	235	stx19	ENSG00000178750	308	285	tmem1	ENSG00000160218
308	236	stx1b2	ENSG00000099365	308	286	tmem116	ENSG00000198270
308	237	suhw2	ENSG00000198477	308	287	copb	
308	238	gosr1		308	288	tmem119	ENSG00000183160
308	239	suox	ENSG00000139531	308	289	tmem135	ENSG00000166575
308	240	sybl1	ENSG00000124333	308	290	tmem15	ENSG00000175283
308	241	syk	ENSG00000165025	308	291	tmem166	ENSG00000115363
308	242	dbn2	ENSG00000198641	308	292	tmem169	ENSG00000163449
308	243	syt1	ENSG00000067715	308	293	tmem16a	ENSG00000131620
308	244	syt6	ENSG00000134207	308	294	tmem176b	ENSG00000106565
308	245	syt7	ENSG00000011347	308	295	tmem38b	ENSG00000095209
308	246	syt9	ENSG00000170743	308	296	tmem46	ENSG00000180730
308	247	syt15	ENSG00000147041	308	297	tmem5	ENSG00000118600
308	248	tacc1	ENSG00000147526	308	298	tmem53	ENSG00000126106
308	249	tada2l	ENSG00000108264	308	299	tmem61	ENSG00000143001
308	250	taf7l	ENSG00000102387	308	300	tmem64	ENSG00000180694

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
308	301	tmprss2	ENSG00000184012	308	351	rab1a	
308	302	copb		308	352	ube2d3	ENSG00000109332
308	303	tnmc1	ENSG00000133687	308	353	ube2d2	ENSG00000131508
308	304	tnfrsf10d	ENSG00000173530	308	354	ube2e1	ENSG00000170142
308	305	tnfrsf19	ENSG00000127863	308	355	ube2m	ENSG00000130725
308	306	tnfrsf8	ENSG00000120949	308	356	ube2q2	ENSG00000140367
308	307	tnfrsf9	ENSG00000049249	308	357	ube2t	ENSG00000077152
308	308	scrambled		308	358	scrambled	
308	309	tnfsf11	ENSG00000120659	308	359	ube2u	ENSG00000177414
308	310	tnks	ENSG00000173273	308	360	ube2v2	ENSG00000169139
308	311	tnnt2	ENSG00000118194	308	361	ubl5	ENSG00000198258
308	312	tnrc5	ENSG00000137161	308	362	ufc1	ENSG00000143222
308	313	tomm20	ENSG00000173726	308	363	ufm1	ENSG00000120686
308	314	tor1a	ENSG00000136827	308	364	ugp2	ENSG00000169764
308	315	tp73l	ENSG00000073282	308	365	uqcrq	ENSG00000164405
308	316	tram2	ENSG00000065308	308	366	urod	ENSG00000126088
308	317	trappc4	ENSG00000196655	308	367	copb	
308	318	gosr1		308	368	mcds032	ENSG00000053501
308	319	trappc5	ENSG00000181029	308	369	usp1	ENSG00000162607
308	320	trem13	ENSG00000184106	308	370	usp12	ENSG00000152484
308	321	trim29	ENSG00000137699	308	371	usp19	ENSG00000172046
308	322	trim41	ENSG00000146063	308	372	usp34	ENSG00000115464
308	323	trim72	ENSG00000177238	308	373	incenp	
308	324	trip10	ENSG00000125733	308	374	usp35	ENSG00000118369
308	325	trip13	ENSG00000071539	308	375	usp5	ENSG00000111667
308	326	trpc5	ENSG00000072315	308	376	scrambled	
308	327	scrambled		308	377	ust	ENSG00000111962
308	328	trpt1	ENSG00000149743	308	378	utp18	ENSG00000011260
308	329	tsc1	ENSG00000165699	308	379	uts2r	ENSG00000181408
308	330	tshz2	ENSG00000182463	308	380	vipr1	ENSG00000114812
308	331	tspan1	ENSG00000117472	308	381	best2	ENSG00000039987
308	332	tspan2	ENSG00000134198	308	382	vmn3	ENSG00000093134
308	333	tspan32	ENSG00000064201	308	383	gosr1	
308	334	rab1a		308	384	incenp	
308	335	ttbk1	ENSG00000146216				
308	336	ttc12	ENSG00000149292				
308	337	ttf1	ENSG00000125482				
308	338	ttf2	ENSG00000116830				
308	339	ttll1	ENSG00000100271				
308	340	tulp2	ENSG00000104804				
308	341	tusc3	ENSG00000104723				
308	342	twist1	ENSG00000122691				
308	343	txnl6	ENSG00000171773				
308	344	tym5	ENSG00000176890				
308	345	tyro3	ENSG00000092445				
308	346	tyrobp	ENSG00000011600				
308	347	loc51035	ENSG00000162191				
308	348	uaca	ENSG00000137831				
308	349	triad3	ENSG00000011275				
308	350	ube2a	ENSG00000077721				

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
309	1	incenp		309	51	zic2	ENSG00000043355
309	2	vps37b	ENSG00000139722	309	52	znf10	ENSG00000090612
309	3	vps72	ENSG00000163159	309	53	znf100	ENSG00000197020
309	4	vsig1	ENSG00000101842	309	54	znf133	ENSG00000125846
309	5	wbp2	ENSG00000132471	309	55	znf167	ENSG00000196345
309	6	wbscr22	ENSG00000071462	309	56	znf185	ENSG00000147394
309	7	wdr27	ENSG00000184465	309	57	znf205	ENSG00000122386
309	8	wdr40a	ENSG00000198876	309	58	znf207	ENSG00000010244
309	9	wdr49	ENSG00000174776	309	59	znf222	ENSG00000159885
309	10	wdr52	ENSG00000206530	309	60	znf287	ENSG00000141040
309	11	wdr75	ENSG00000115368	309	61	znf306	ENSG00000189298
309	12	incenp		309	62	znf32	ENSG00000169740
309	13	wfikkn1	ENSG00000127578	309	63	rab1a	
309	14	copb		309	64	znf334	ENSG00000198185
309	15	wnk3	ENSG00000196632	309	65	znf350	ENSG00000171032
309	16	xg	ENSG00000124343	309	66	znf407	ENSG00000166540
309	17	loc127099	ENSG00000174092	309	67	znf446	ENSG00000083838
309	18	loc730647	ENSG00000173568	309	68	znf447	ENSG00000121413
309	19	loc646643	ENSG00000187550	309	69	znf451	ENSG00000112200
309	20	scrambled		309	70	scrambled	
309	21	rps6p1	ENSG00000173322	309	71	znf473	ENSG00000142528
309	22	xpo5	ENSG00000124571	309	72	znf485	ENSG00000198298
309	23	xylb	ENSG00000093217	309	73	znf503	ENSG00000165655
309	24	kiaa0143	ENSG00000132294	309	74	znf512	ENSG00000198522
309	25	y0574_human	ENSG00000104059	309	75	znf607	ENSG00000198182
309	26	yipf7	ENSG00000177752	309	76	znf649	ENSG00000198093
309	27	cted	ENSG00000090238	309	77	znf684	ENSG00000117010
309	28	ythdf2	ENSG00000198492	309	78	znf705a	ENSG00000196946
309	29	yy2	ENSG00000198767	309	79	copb	
309	30	gosr1		309	80	znf71	ENSG00000197951
309	31	zadh1	ENSG00000140043	309	81	znf799	ENSG00000196466
309	32	zbed4	ENSG00000100426	309	82	znf800	ENSG00000048405
309	33	zbed5	ENSG00000129586	309	83	znf85	ENSG00000105750
309	34	zbtb25	ENSG00000089775	309	84	znhit1	ENSG00000106400
309	35	zbtb8	ENSG00000160062	309	85	zscan1	ENSG00000152467
309	36	zc3h8	ENSG00000144161	309	86	empty	
309	37	zochc17	ENSG00000121766	309	87	empty	
309	38	zod2	ENSG00000145354	309	88	scrambled	
309	39	scrambled		309	89	empty	
309	40	zcwpw1	ENSG00000078487	309	90	empty	
309	41	loc649917	ENSG00000188818	309	91	empty	
309	42	zdhhc13	ENSG00000177054	309	92	empty	
309	43	hszfp36	ENSG00000197933	309	93	empty	
309	44	zfhx1b	ENSG00000169554	309	94	empty	
309	45	zfp14	ENSG00000142065	309	95	gosr1	
309	46	rab1a		309	96	empty	
309	47	zfp36	ENSG00000128016	309	97	empty	
309	48	zfp1	ENSG00000162300	309	98	empty	
309	49	zfyve20	ENSG00000131381	309	99	empty	
309	50	zhx2	ENSG00000178764	309	100	empty	

Table 6 (continued)|

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
309	101	empty		309	151	empty	
309	102	empty		309	152	empty	
309	103	empty		309	153	empty	
309	104	empty		309	154	empty	
309	105	empty		309	155	empty	
309	106	empty		309	156	empty	
309	107	empty		309	157	empty	
309	108	empty		309	158	empty	
309	109	empty		309	159	copb	
309	110	gosr1		309	160	empty	
309	111	empty		309	161	empty	
309	112	empty		309	162	empty	
309	113	empty		309	163	empty	
309	114	empty		309	164	empty	
309	115	empty		309	165	empty	
309	116	scrambled		309	166	scrambled	
309	117	empty		309	167	empty	
309	118	empty		309	168	empty	
309	119	empty		309	169	empty	
309	120	empty		309	170	empty	
309	121	empty		309	171	empty	
309	122	empty		309	172	empty	
309	123	empty		309	173	empty	
309	124	empty		309	174	empty	
309	125	empty		309	175	gosr1	
309	126	rab1a		309	176	empty	
309	127	empty		309	177	empty	
309	128	empty		309	178	empty	
309	129	empty		309	179	empty	
309	130	empty		309	180	empty	
309	131	empty		309	181	empty	
309	132	empty		309	182	empty	
309	133	empty		309	183	empty	
309	134	empty		309	184	scrambled	
309	135	scrambled		309	185	empty	
309	136	empty		309	186	empty	
309	137	empty		309	187	empty	
309	138	empty		309	188	empty	
309	139	empty		309	189	empty	
309	140	empty		309	190	empty	
309	141	empty		309	191	rab1a	
309	142	copb		309	192	empty	
309	143	empty		309	193	empty	
309	144	empty		309	194	empty	
309	145	empty		309	195	empty	
309	146	empty		309	196	empty	
309	147	empty		309	197	empty	
309	148	empty		309	198	empty	
309	149	empty		309	199	empty	
309	150	empty		309	200	empty	

Table 6 (continued)|

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
309	201	empty		309	251	empty	
309	202	empty		309	252	empty	
309	203	empty		309	253	empty	
309	204	empty		309	254	empty	
309	205	empty		309	255	gosr1	
309	206	rab1a		309	256	empty	
309	207	empty		309	257	empty	
309	208	empty		309	258	empty	
309	209	empty		309	259	empty	
309	210	empty		309	260	empty	
309	211	empty		309	261	empty	
309	212	scrambled		309	262	scrambled	
309	213	empty		309	263	empty	
309	214	empty		309	264	empty	
309	215	empty		309	265	empty	
309	216	empty		309	266	empty	
309	217	empty		309	267	empty	
309	218	empty		309	268	empty	
309	219	empty		309	269	empty	
309	220	empty		309	270	empty	
309	221	empty		309	271	rab1a	
309	222	copb		309	272	empty	
309	223	empty		309	273	empty	
309	224	empty		309	274	empty	
309	225	empty		309	275	empty	
309	226	empty		309	276	empty	
309	227	empty		309	277	empty	
309	228	empty		309	278	empty	
309	229	empty		309	279	empty	
309	230	empty		309	280	scrambled	
309	231	scrambled		309	281	empty	
309	232	empty		309	282	empty	
309	233	empty		309	283	empty	
309	234	empty		309	284	empty	
309	235	empty		309	285	empty	
309	236	empty		309	286	empty	
309	237	empty		309	287	copb	
309	238	gosr1		309	288	empty	
309	239	empty		309	289	empty	
309	240	empty		309	290	empty	
309	241	empty		309	291	empty	
309	242	empty		309	292	empty	
309	243	empty		309	293	empty	
309	244	empty		309	294	empty	
309	245	empty		309	295	empty	
309	246	empty		309	296	empty	
309	247	empty		309	297	empty	
309	248	empty		309	298	empty	
309	249	empty		309	299	empty	
309	250	empty		309	300	empty	

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
309	301	empty		309	351	rab1a	
309	302	copb		309	352	empty	
309	303	empty		309	353	empty	
309	304	empty		309	354	empty	
309	305	empty		309	355	empty	
309	306	empty		309	356	empty	
309	307	empty		309	357	empty	
309	308	scrambled		309	358	scrambled	
309	309	empty		309	359	empty	
309	310	empty		309	360	empty	
309	311	empty		309	361	empty	
309	312	empty		309	362	empty	
309	313	empty		309	363	empty	
309	314	empty		309	364	empty	
309	315	empty		309	365	empty	
309	316	empty		309	366	empty	
309	317	empty		309	367	copb	
309	318	gosr1		309	368	empty	
309	319	empty		309	369	empty	
309	320	empty		309	370	empty	
309	321	empty		309	371	empty	
309	322	empty		309	372	empty	
309	323	empty		309	373	incenp	
309	324	empty		309	374	empty	
309	325	empty		309	375	empty	
309	326	empty		309	376	scrambled	
309	327	scrambled		309	377	empty	
309	328	empty		309	378	empty	
309	329	empty		309	379	empty	
309	330	empty		309	380	empty	
309	331	empty		309	381	empty	
309	332	empty		309	382	empty	
309	333	empty		309	383	gosr1	
309	334	rab1a		309	384	incenp	
309	335	empty					
309	336	empty					
309	337	empty					
309	338	empty					
309	339	empty					
309	340	empty					
309	341	empty					
309	342	empty					
309	343	empty					
309	344	empty					
309	345	empty					
309	346	empty					
309	347	empty					
309	348	empty					
309	349	empty					
309	350	empty					

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
310	1	incenp		310	51	q5t7b9_human	ENSG00000170590
310	2	ad13_human	ENSG00000196097	310	52	q6kf84_human	ENSG00000180574
310	3	bcorl2	ENSG00000197840	310	53	q6p575_human	ENSG00000169325
310	4	c17orf59	ENSG00000196892	310	54	q7z4h1_human	ENSG00000198676
310	5	centg2	ENSG00000157985	310	55	q7z4v3_human	ENSG00000189054
310	6	cox1_human	ENSG00000198804	310	56	q8izm0_human	ENSG00000182992
310	7	ctage1	ENSG00000184546	310	57	q8n0t1_human	ENSG00000176731
310	8	cu122_human	ENSG00000183821	310	58	q8n1g8_human	ENSG00000181508
310	9	dnajc4	ENSG00000110011	310	59	q8n214_human	ENSG00000182109
310	10	eml3	ENSG00000149499	310	60	q8n2a4_human	ENSG00000180156
310	11	ensg00000125881	ENSG00000125881	310	61	q8n507_human	ENSG00000180769
310	12	incenp		310	62	q8n666_human	ENSG00000168913
310	13	ensg00000172666	ENSG00000172666	310	63	rab1a	
310	14	copb		310	64	q8n675_human	ENSG00000180840
310	15	ensg00000176243	ENSG00000176243	310	65	q8n6v7_human	ENSG00000177112
310	16	ensg00000177083	ENSG00000177083	310	66	q8n779_human	ENSG00000175789
310	17	ensg00000177835	ENSG00000177835	310	67	q8n7f4_human	ENSG00000177527
310	18	ensg00000185036	ENSG00000185036	310	68	q8n7s0_human	ENSG00000176833
310	19	ensg00000186459	ENSG00000186459	310	69	q8n8b3_human	ENSG00000180935
310	20	scrambled		310	70	scrambled	
310	21	ensg00000187175	ENSG00000187175	310	71	q8n8e8_human	ENSG00000196888
310	22	ensg00000197502	ENSG00000197502	310	72	q8n8z0_human	ENSG00000181558
310	23	ensg00000197559	ENSG00000197559	310	73	q8naj1_human	ENSG00000180642
310	24	eps15l2	ENSG00000176657	310	74	q8nam3_human	ENSG00000180838
310	25	id2b	ENSG00000182801	310	75	q8nan4_human	ENSG00000176078
310	26	ighg3	ENSG00000130076	310	76	q8nas9_human	ENSG00000177797
310	27	lpp	ENSG00000145012	310	77	q8nat4_human	ENSG00000175892
310	28	lv1g_human	ENSG00000182843	310	78	q8nbe0_human	ENSG00000178193
310	29	ncbp2l	ENSG00000170935	310	79	copb	
310	30	gosr1		310	80	q8ne65_human	ENSG00000172687
310	31	neil1	ENSG00000140398	310	81	q8nej6_human	ENSG00000186756
310	32	np_001004355.1	ENSG00000197152	310	82	q8nf02_human	ENSG00000185136
310	33	np_001013687.1	ENSG00000188185	310	83	q8ngm6_human	ENSG00000180909
310	34	np_001013691.1	ENSG00000177596	310	84	q8tch9_human	ENSG00000182424
310	35	np_001020362.1	ENSG00000172764	310	85	q8te90_human	ENSG00000182335
310	36	np_113604.1	ENSG00000132204	310	86	q8wuc7_human	ENSG00000184091
310	37	np_775772.1	ENSG00000185716	310	87	q8wus3_human	ENSG00000198596
310	38	np_775946.1	ENSG00000175753	310	88	scrambled	
310	39	scrambled		310	89	q8wvj8_human	ENSG00000170807
310	40	np_852607.2	ENSG00000186105	310	90	q8wyg7_human	ENSG00000177107
310	41	np_997351.1	ENSG00000197520	310	91	q8wyw9_human	ENSG00000177221
310	42	np_997353.1	ENSG00000197886	310	92	q8wz02_human	ENSG00000185370
310	43	nu4lm_human	ENSG00000198868	310	93	q969h1_human	ENSG00000182136
310	44	o15421_human	ENSG00000132075	310	94	q96fp4_human	ENSG00000184474
310	45	o94914_human	ENSG00000175483	310	95	gosr1	
310	46	rab1a		310	96	q96hg9_human	ENSG00000198672
310	47	or4fe_human	ENSG00000198615	310	97	q96kt8_human	ENSG00000178223
310	48	p1768_human	ENSG00000140031	310	98	q96lv3_human	ENSG00000177806
310	49	q16370_human	ENSG00000177962	310	99	q96mt0_human	ENSG00000182418
310	50	q562t6_human	ENSG00000204443	310	100	q96nc1_human	ENSG00000182341

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
310	101	q9bsl4_human	ENSG00000177620	310	151	empty	
310	102	q9bsy8_human	ENSG00000176120	310	152	empty	
310	103	q9btr0_human	ENSG00000198664	310	153	empty	
310	104	q9bvq6_human	ENSG00000180924	310	154	empty	
310	105	q9h1s0_human	ENSG00000171738	310	155	empty	
310	106	q9h669_human	ENSG00000182505	310	156	empty	
310	107	q9h752_human	ENSG00000128345	310	157	empty	
310	108	q9ha39_human	ENSG00000185517	310	158	empty	
310	109	q9hbf5_human	ENSG00000180953	310	159	copb	
310	110	gosr1		310	160	empty	
310	111	q9npd2_human	ENSG00000188362	310	161	empty	
310	112	q9nps2_human	ENSG00000176173	310	162	empty	
310	113	q9ntt1_human	ENSG00000149345	310	163	empty	
310	114	q9p132_human	ENSG00000197791	310	164	empty	
310	115	q9p146_human	ENSG00000196232	310	165	empty	
310	116	scrambled		310	166	scrambled	
310	117	q9p1i9_human	ENSG00000180639	310	167	empty	
310	118	q9ud07_human	ENSG00000198857	310	168	empty	
310	119	q9uhu7_human	ENSG00000180441	310	169	empty	
310	120	q9uhz5_human	ENSG00000177320	310	170	empty	
310	121	rbm27	ENSG00000091009	310	171	empty	
310	122	tp53ap1	ENSG00000182165	310	172	empty	
310	123	tva2_human	ENSG00000129563	310	173	empty	
310	124	unc84a	ENSG00000164828	310	174	empty	
310	125	vdac4	ENSG00000198696	310	175	gosr1	
310	126	rab1a		310	176	empty	
310	127	xp_370634.2	ENSG00000173727	310	177	empty	
310	128	xp_371480.1	ENSG00000198639	310	178	empty	
310	129	xp_372632.3	ENSG00000205454	310	179	empty	
310	130	yg02_human	ENSG00000196813	310	180	empty	
310	131	znf283	ENSG00000176232	310	181	empty	
310	132	znf29	ENSG00000108452	310	182	empty	
310	133	znf716	ENSG00000182111	310	183	empty	
310	134	empty		310	184	scrambled	
310	135	scrambled		310	185	empty	
310	136	empty		310	186	empty	
310	137	empty		310	187	empty	
310	138	empty		310	188	empty	
310	139	empty		310	189	empty	
310	140	empty		310	190	empty	
310	141	empty		310	191	rab1a	
310	142	copb		310	192	empty	
310	143	empty		310	193	empty	
310	144	empty		310	194	empty	
310	145	empty		310	195	empty	
310	146	empty		310	196	empty	
310	147	empty		310	197	empty	
310	148	empty		310	198	empty	
310	149	empty		310	199	empty	
310	150	empty		310	200	empty	

Table 6 (continued)|

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
310	201	empty		310	251	empty	
310	202	empty		310	252	empty	
310	203	empty		310	253	empty	
310	204	empty		310	254	empty	
310	205	empty		310	255	gosr1	
310	206	rab1a		310	256	empty	
310	207	empty		310	257	empty	
310	208	empty		310	258	empty	
310	209	empty		310	259	empty	
310	210	empty		310	260	empty	
310	211	empty		310	261	empty	
310	212	scrambled		310	262	scrambled	
310	213	empty		310	263	empty	
310	214	empty		310	264	empty	
310	215	empty		310	265	empty	
310	216	empty		310	266	empty	
310	217	empty		310	267	empty	
310	218	empty		310	268	empty	
310	219	empty		310	269	empty	
310	220	empty		310	270	empty	
310	221	empty		310	271	rab1a	
310	222	copb		310	272	empty	
310	223	empty		310	273	empty	
310	224	empty		310	274	empty	
310	225	empty		310	275	empty	
310	226	empty		310	276	empty	
310	227	empty		310	277	empty	
310	228	empty		310	278	empty	
310	229	empty		310	279	empty	
310	230	empty		310	280	scrambled	
310	231	scrambled		310	281	empty	
310	232	empty		310	282	empty	
310	233	empty		310	283	empty	
310	234	empty		310	284	empty	
310	235	empty		310	285	empty	
310	236	empty		310	286	empty	
310	237	empty		310	287	copb	
310	238	gosr1		310	288	empty	
310	239	empty		310	289	empty	
310	240	empty		310	290	empty	
310	241	empty		310	291	empty	
310	242	empty		310	292	empty	
310	243	empty		310	293	empty	
310	244	empty		310	294	empty	
310	245	empty		310	295	empty	
310	246	empty		310	296	empty	
310	247	empty		310	297	empty	
310	248	empty		310	298	empty	
310	249	empty		310	299	empty	
310	250	empty		310	300	empty	

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
310	301	empty		310	351	rab1a	
310	302	copb		310	352	empty	
310	303	empty		310	353	empty	
310	304	empty		310	354	empty	
310	305	empty		310	355	empty	
310	306	empty		310	356	empty	
310	307	empty		310	357	empty	
310	308	scrambled		310	358	scrambled	
310	309	empty		310	359	empty	
310	310	empty		310	360	empty	
310	311	empty		310	361	empty	
310	312	empty		310	362	empty	
310	313	empty		310	363	empty	
310	314	empty		310	364	empty	
310	315	empty		310	365	empty	
310	316	empty		310	366	empty	
310	317	empty		310	367	copb	
310	318	gosr1		310	368	empty	
310	319	empty		310	369	empty	
310	320	empty		310	370	empty	
310	321	empty		310	371	empty	
310	322	empty		310	372	empty	
310	323	empty		310	373	incenp	
310	324	empty		310	374	empty	
310	325	empty		310	375	empty	
310	326	empty		310	376	scrambled	
310	327	scrambled		310	377	empty	
310	328	empty		310	378	empty	
310	329	empty		310	379	empty	
310	330	empty		310	380	empty	
310	331	empty		310	381	empty	
310	332	empty		310	382	empty	
310	333	empty		310	383	gosr1	
310	334	rab1a		310	384	incenp	
310	335	empty					
310	336	empty					
310	337	empty					
310	338	empty					
310	339	empty					
310	340	empty					
310	341	empty					
310	342	empty					
310	343	empty					
310	344	empty					
310	345	empty					
310	346	empty					
310	347	empty					
310	348	empty					
310	349	empty					
310	350	empty					

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
311	1	incenp	ENSG00000149503	311	51	ensg00000125458	ENSG00000125458
311	2	aadat	ENSG00000109576	311	52	ensg00000125458	ENSG00000125458
311	3	aadat	ENSG00000109576	311	53	ensg00000133704	ENSG00000133704
311	4	aadat	ENSG00000109576	311	54	ensg00000133704	ENSG00000133704
311	5	ap2s1	ENSG00000042753	311	55	ensg00000145107	ENSG00000145107
311	6	ap2s1	ENSG00000042753	311	56	ensg00000145107	ENSG00000145107
311	7	axl	ENSG00000167601	311	57	ensg00000148814	ENSG00000148814
311	8	axl	ENSG00000167601	311	58	ensg00000148814	ENSG00000148814
311	9	bcorl2	ENSG00000197840	311	59	ensg00000157005	ENSG00000157005
311	10	bcorl2	ENSG00000197840	311	60	ensg00000157005	ENSG00000157005
311	11	c10orf33	ENSG00000119943	311	61	ensg00000163935	ENSG00000163935
311	12	incenp	ENSG00000149503	311	62	ensg00000163935	ENSG00000163935
311	13	c10orf33	ENSG00000119943	311	63	rab1a	ENSG00000138069
311	14	copb	ENSG00000129083	311	64	ensg00000166508	ENSG00000166508
311	15	c9orf19	ENSG00000122694	311	65	ensg00000166508	ENSG00000166508
311	16	c9orf19	ENSG00000122694	311	66	ensg00000168274	ENSG00000168274
311	17	c9orf55	ENSG00000137145	311	67	ensg00000168274	ENSG00000168274
311	18	c9orf55	ENSG00000137145	311	68	ensg00000168913	ENSG00000168913
311	19	cbln4	ENSG00000054803	311	69	ensg00000168913	ENSG00000168913
311	20	scramble		311	70	scramble	
311	21	cbln4	ENSG00000054803	311	71	ensg00000173598	ENSG00000173598
311	22	cflar	ENSG00000003402	311	72	ensg00000173598	ENSG00000173598
311	23	cflar	ENSG00000003402	311	73	ensg00000176833	ENSG00000176833
311	24	chmb4	ENSG00000117971	311	74	ensg00000176833	ENSG00000176833
311	25	chmb4	ENSG00000117971	311	75	ensg00000177752	ENSG00000177752
311	26	ctgf	ENSG00000118523	311	76	ensg00000177752	ENSG00000177752
311	27	ctgf	ENSG00000118523	311	77	ensg00000177797	ENSG00000177797
311	28	dgkd	ENSG00000077044	311	78	ensg00000177797	ENSG00000177797
311	29	dgkd	ENSG00000077044	311	79	copb	ENSG00000129083
311	30	gosr1	ENSG00000108587	311	80	ensg00000181789	ENSG00000181789
311	31	diras1	ENSG00000176490	311	81	ensg00000181789	ENSG00000181789
311	32	diras1	ENSG00000176490	311	82	ensg00000181995	ENSG00000181995
311	33	dkfzp434g072	ENSG00000138813	311	83	ensg00000181995	ENSG00000181995
311	34	dkfzp434g072	ENSG00000138813	311	84	ensg00000182335	ENSG00000182335
311	35	dkfzp761c169	ENSG00000062194	311	85	ensg00000182335	ENSG00000182335
311	36	dkfzp761c169	ENSG00000062194	311	86	ensg00000182418	ENSG00000182418
311	37	dnm2	ENSG00000079805	311	87	ensg00000182418	ENSG00000182418
311	38	dnm2	ENSG00000079805	311	88	scramble	
311	39	scramble		311	89	ensg00000183971	ENSG00000183971
311	40	dqx1	ENSG00000144045	311	90	ensg00000183971	ENSG00000183971
311	41	dqx1	ENSG00000144045	311	91	ensg00000185136	ENSG00000185136
311	42	eef2k	ENSG00000103319	311	92	ensg00000185136	ENSG00000185136
311	43	eef2k	ENSG00000103319	311	93	flj10324	ENSG00000157927
311	44	egfl6	ENSG00000198759	311	94	flj10324	ENSG00000157927
311	45	egfl6	ENSG00000198759	311	95	gosr1	ENSG00000108587
311	46	rab1a	ENSG00000138069	311	96	flj10781	ENSG00000182013
311	47	ensg00000099308	ENSG00000099308	311	97	flj10781	ENSG00000182013
311	48	ensg00000099308	ENSG00000099308	311	98	flj12644	ENSG00000198093
311	49	ensg00000118369	ENSG00000118369	311	99	flj12644	ENSG00000198093
311	50	ensg00000118369	ENSG00000118369	311	100	flj12949	ENSG00000129347

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
311	101	flj12949	ENSG00000129347	311	151	loc90529	ENSG00000001460
311	102	flj23221	ENSG00000118292	311	152	loc90529	ENSG00000001460
311	103	flj23221	ENSG00000118292	311	153	ly75	ENSG000000054219
311	104	flj23259	ENSG00000114248	311	154	ly75	ENSG000000054219
311	105	flj23259	ENSG00000114248	311	155	mgc20398	ENSG00000198783
311	106	flj25333	ENSG00000153347	311	156	mgc20398	ENSG00000198783
311	107	flj25333	ENSG00000153347	311	157	minpp1	ENSG00000107789
311	108	flj45244	ENSG00000198411	311	158	minpp1	ENSG00000107789
311	109	flj45244	ENSG00000198411	311	159	copb	ENSG00000129083
311	110	gosr1	ENSG00000108587	311	160	mmp14	ENSG00000157227
311	111	flj90650	ENSG00000172901	311	161	mmp14	ENSG00000157227
311	112	flj90650	ENSG00000172901	311	162	mrps5	ENSG00000144029
311	113	flt4	ENSG00000037280	311	163	mrps5	ENSG00000144029
311	114	flt4	ENSG00000037280	311	164	ms4a6a	ENSG00000110077
311	115	glra1	ENSG00000145888	311	165	ms4a6a	ENSG00000110077
311	116	scramble		311	166	scramble	
311	117	glra1	ENSG00000145888	311	167	mxd4	ENSG00000123933
311	118	grin2b	ENSG00000150086	311	168	mxd4	ENSG00000123933
311	119	grin2b	ENSG00000150086	311	169	myb	ENSG00000118513
311	120	gstp1	ENSG00000084207	311	170	myb	ENSG00000118513
311	121	gstp1	ENSG00000084207	311	171	ndnl2	ENSG00000185115
311	122	gzma	ENSG00000145649	311	172	ndnl2	ENSG00000185115
311	123	gzma	ENSG00000145649	311	173	ndufs2	ENSG00000158864
311	124	hscarg	ENSG00000153406	311	174	ndufs2	ENSG00000158864
311	125	hscarg	ENSG00000153406	311	175	gosr1	ENSG00000108587
311	126	rab1a	ENSG00000138069	311	176	oa48-18	ENSG00000108848
311	127	htpap	ENSG00000147535	311	177	oa48-18	ENSG00000108848
311	128	htpap	ENSG00000147535	311	178	or10a4	ENSG00000170782
311	129	ifih1	ENSG00000115267	311	179	or10a4	ENSG00000170782
311	130	ifih1	ENSG00000115267	311	180	pecam1	ENSG00000198802
311	131	iqsec1	ENSG00000144711	311	181	pecam1	ENSG00000198802
311	132	iqsec1	ENSG00000144711	311	182	piib5pa	ENSG00000185133
311	133	khk	ENSG00000138030	311	183	piib5pa	ENSG00000185133
311	134	khk	ENSG00000138030	311	184	scramble	
311	135	scramble		311	185	pml	ENSG00000140464
311	136	kiaa0052	ENSG00000039123	311	186	pml	ENSG00000140464
311	137	kiaa0052	ENSG00000039123	311	187	pms2	ENSG00000122512
311	138	kiaa0626	ENSG00000198948	311	188	pms2	ENSG00000122512
311	139	kiaa0626	ENSG00000198948	311	189	polr3f	ENSG00000132664
311	140	kiaa0672	ENSG00000006740	311	190	polr3f	ENSG00000132664
311	141	kiaa0672	ENSG00000006740	311	191	rab1a	ENSG00000138069
311	142	copb	ENSG00000129083	311	192	proc	ENSG00000115718
311	143	kiaa0703	ENSG00000064270	311	193	proc	ENSG00000115718
311	144	kiaa0703	ENSG00000064270	311	194	rap1gds1	ENSG00000138698
311	145	kiaa1145	ENSG00000057704	311	195	rap1gds1	ENSG00000138698
311	146	kiaa1145	ENSG00000057704	311	196	rhof	ENSG00000139725
311	147	loc51035	ENSG00000162191	311	197	rhof	ENSG00000139725
311	148	loc51035	ENSG00000162191	311	198	rhoh	ENSG00000116574
311	149	loc58486	ENSG00000129586	311	199	rhoh	ENSG00000116574
311	150	loc58486	ENSG00000129586	311	200	rics	ENSG00000134909

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
311	201	rics	ENSG00000134909	311	251	empty	ENSG00000108587
311	202	rxra	ENSG00000186350	311	252	empty	
311	203	rxra	ENSG00000196940	311	253	empty	
311	204	sema4f	ENSG00000135622	311	254	empty	
311	205	sema4f	ENSG00000135622	311	255	gosr1	
311	206	rab1a	ENSG00000138069	311	256	empty	
311	207	sepp1	ENSG00000198865	311	257	empty	
311	208	sepp1	ENSG00000198865	311	258	empty	
311	209	sirt2	ENSG00000068903	311	259	empty	
311	210	sirt2	ENSG00000068903	311	260	empty	
311	211	skd3	ENSG00000162129	311	261	empty	ENSG00000138069
311	212	scramble		311	262	scramble	
311	213	skd3	ENSG00000162129	311	263	empty	
311	214	smarcd1	ENSG00000066117	311	264	empty	
311	215	smarcd1	ENSG00000066117	311	265	empty	
311	216	stx1b2	ENSG00000099365	311	266	empty	
311	217	stx1b2	ENSG00000099365	311	267	empty	
311	218	syt7	ENSG00000011347	311	268	empty	
311	219	syt7	ENSG00000011347	311	269	empty	
311	220	tbx2	ENSG00000121068	311	270	empty	
311	221	tbx2	ENSG00000121068	311	271	rab1a	ENSG00000129083
311	222	copb	ENSG00000129083	311	272	empty	
311	223	ttid	ENSG00000120729	311	273	empty	
311	224	ttid	ENSG00000120729	311	274	empty	
311	225	tyrobp	ENSG00000011600	311	275	empty	
311	226	tyrobp	ENSG00000011600	311	276	empty	
311	227	yy2	ENSG00000198767	311	277	empty	
311	228	yy2	ENSG00000198767	311	278	empty	
311	229	znf167	ENSG00000196345	311	279	empty	
311	230	znf167	ENSG00000196345	311	280	scramble	
311	231	scramble		311	281	empty	ENSG00000129083
311	232	znf334	ENSG00000198185	311	282	empty	
311	233	znf334	ENSG00000198185	311	283	empty	
311	234	znf71	ENSG00000197951	311	284	empty	
311	235	znf71	ENSG00000197951	311	285	empty	
311	236	empty		311	286	empty	
311	237	empty		311	287	copb	
311	238	gosr1	ENSG00000108587	311	288	empty	
311	239	empty		311	289	empty	
311	240	empty		311	290	empty	
311	241	empty		311	291	empty	
311	242	empty		311	292	empty	
311	243	empty		311	293	empty	
311	244	empty		311	294	empty	
311	245	empty		311	295	empty	
311	246	empty		311	296	empty	
311	247	empty		311	297	empty	
311	248	empty		311	298	empty	
311	249	empty		311	299	empty	
311	250	empty		311	300	empty	

Table 6 (continued)

Plate	Position	Gene	ENSEMBL ID	Plate	Position	Gene	ENSEMBL ID
311	301	empty	ENSG00000129083	311	351	rab1a	ENSG00000138069
311	302	copb		311	352	empty	ENSG00000129083
311	303	empty		311	353	empty	
311	304	empty		311	354	empty	
311	305	empty		311	355	empty	
311	306	empty		311	356	empty	
311	307	empty		311	357	empty	
311	308	scramble		311	358	scramble	
311	309	empty		311	359	empty	
311	310	empty		311	360	empty	
311	311	empty		311	361	empty	
311	312	empty		311	362	empty	
311	313	empty		311	363	empty	
311	314	empty		311	364	empty	
311	315	empty		311	365	empty	
311	316	empty		311	366	empty	
311	317	empty	ENSG00000108587	311	367	copb	ENSG00000129083
311	318	gosr1		311	368	empty	ENSG00000149503
311	319	empty		311	369	empty	
311	320	empty		311	370	empty	
311	321	empty		311	371	empty	
311	322	empty		311	372	empty	
311	323	empty		311	373	incenp	
311	324	empty		311	374	empty	
311	325	empty		311	375	empty	
311	326	empty		311	376	scramble	
311	327	scramble		311	377	empty	
311	328	empty		311	378	empty	
311	329	empty		311	379	empty	
311	330	empty		311	380	empty	
311	331	empty		311	381	empty	
311	332	empty	ENSG00000138069	311	382	empty	ENSG00000108587 ENSG00000149503
311	333	empty		311	383	gosr1	
311	334	rab1a		311	384	incenp	
311	335	empty					
311	336	empty					
311	337	empty					
311	338	empty					
311	339	empty					
311	340	empty					
311	341	empty					
311	342	empty					
311	343	empty					
311	344	empty					
311	345	empty					
311	346	empty					
311	347	empty					
311	348	empty					
311	349	empty					
311	350	empty					

