Regulation of the leading edge motility by PI(4,5)P_2-dependent lipid microdomains

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Dekan

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Content

Summary

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The lipid second messenger PI(4,5)P_2 modulates actin dynamics, and its local accumulation at plasmalemmal microdomains (rafts) might mediate regulation of protrusive motility. However, how PI(4,5)P_2-rich rafts regulate surface motility is not well understood. In this study, we show that upon signals promoting cell surface motility, PI(4,5)P_2 directs the assembly of dynamic raft-rich plasmalemmal patches, which promote and sustain protrusive motility. The accumulation of PI(4,5)P_2 at rafts, together with Cdc42, promotes patch assembly through N-WASP. The patches exhibit locally regulated PI(4,5)P_2 turnover and reduced diffusion-mediated exchange with their environment. Patches capture microtubules (MTs) through IQGAP1, to stabilize MTs at the leading edge. Captured MTs in turn deliver PKA to patches, to promote higher order patch clustering through further PI(4,5)P_2 accumulation in response to cAMP. Patch clustering restricts, spatially confines and polarizes protrusive motility. Thus, PI(4,5)P_2-dependent raft-rich patches enhance local signaling for motility, and their assembly into clusters is regulated through captured MTs and PKA, coupling local regulation of motility to cell polarity and organization.

1. Introduction

1.1. Actin

1.1.1. Actin polymerization in cell motility

Directional motility is a fundamental cellular process essential for tissue development, wound healing, immune responses and embryonic development, to name but a few. Motility involves cycle of four steps: protrusion of the leading edge lamellipodium, adhesion to substrate, retraction of the trailing edge and de-adhesion. The leading lamellipodium of motile cells is a thin (0.1-0.2 um), sheet-like protrusion filled with actin filaments at high density . Actin, the most abundant protein in many eukaryotic cells, arranges its globular subunits head-to-tail to build double helical filaments with molecular polarity. The barbed end is favored for growth, and often points towards cell surface, whereas the opposite end is called pointed end (Small et al, 1978).

Pure actin filaments, at steady state *in vitro* grow very slowly and subunit addition at the barbed end is diffusion limited (Hagen et al, 1986). In contrast to *in vitro* rates of actin polymerization, rates *in vivo* are more then two orders of magnitude faster. The function of regulatory proteins is thus required to explain actin dynamics under physiological conditions. There is limited a limited number of actin-binding proteins that can reconstitute bacterial motility in purified system, and these are actin, ADF/cofilin, capping protein, WASp/Scar activator or Arp2/3 complex and profilin (Cooper and Schafer, 2000; Pollard and Beltzner, 2002; Weaver et al, 2003; Pollard and Borisy, 2003)
.
The interplay of these proteins in actin polymerization and depolymerization at the leading edge is explained by “dendritic nucleation treadmilling model”.

1.1.2. Treadmilling model

In quiescent cells, the actin system stably maintained, with pool of unpolymerized globular actin, bound to either profilin or to sequestering proteins such as tymosin-β4, and existing filaments capped by capping protein. Extracellular stimuli such as chemoattractants initiate signal transduction that leads to activation of nucleation-promoting factors such as the WASP/Scar family of proteins. These bind and activate Arp2/3 complex, which binds to the side of filaments, and initiates polymerization of a side-branch at a 70° angle which elongates until it gets capped by capping protein (Amann and Pollard, 2001; 3:306; . The filaments age through hydrolysis of bound ATP by actin, and dissociation of the γ phosphate (Blanchoin and Pollard, 2002; Carlier and Pantaloni , 1986). ADF/cofilin accelerates phosphate release, which is the rate limiting step for filament aging, and promotes severing and dissociation of ADP-subunits from the filament ends. ADF/cofilin also generates new barbed ends by severing filaments, thus further increasing actin dynamics (Zebda et al, 2000). ADF/cofilin is regulated negatively by LIM kinase, which phosphorylates cofilin and blocks its interaction with ADP-actin filaments (Arber et al, 1998), and positively through the phosphatase *slingshot*, which removes the inhibitory phosphate group (Niwa et al, 2002)
Profilin that has affinity for monomeric ADP-actin catalyzes the exchange of ADP for ATP, in that way replenishing the pool of ATP-actin monomer which serves as a substrate for polymerization.

1.1.3. Actin nucleating factors

1.1.3.1. Arp2/3

The Arp2/3 complex, discovered more then a decade ago, consists of two actin-related proteins Arp2 and Arp3, and five additional subunits, ARPC1-5, all of which are highly conserved during evolution (Machesky et al, 1994; Svitkina and Borisy, 1999; Robinson et al, 2001). It is especially important for cell motility, since the branched actin networks it creates provide the protruding force for leading edge lamellipodia in motile cells.

The most potent Arp2/3 activators are WASP/Scar family of proteins, which share a common C-terminal verprolin-homology acidic domain (VCA) that is responsible for binding and activation of Arp2/3 (Weaver et al, 2003). This is thought to induce a conformational change in the Arp2/3 complex upon filament binding, bringing it into a more "filament-like" conformation (Marchand et al, 2001).
WASP proteins are autoinhibited in resting state, and binding of signaling molecules such as Cdc42 and $PI(4,5)P_2$ (Weaver et al, 2003), or Grb2 and Nck in concert with $PI(4,5)P_2$, activates WASP proteins through cooperative mechanism. Scar was shown to be kept inactive in a complex with four other proteins PIR121/Sra-1, Nap125, HSPC300 and Abl interactor 2 (Abi 2) (Eden et al, 2002).

1.1.3.2. Formins

Interest in formins rose with several findings during the past few years: first, induction of actin stress fibers by Rho requires formin, mDia (Watanabe et al, 1999); second, certain actin structures in yeast do not require Arp2/3 for development, but do require a formin (Evangelista et al, 2002); third, actin filaments can be nucleated in vitro by incubation of G-actin with formin FH2 domain (Pruyne et al, 2002). It seems therefore that formins are capable of nucleating actin, just like Arp2/3. However, filaments nucleated through these two distinct mechanisms apper to serve different functions – formin nucleated filaments

might sustain tension for contraction, whereas Arp2/3 nucleated filaments might result in protrusion.
Like in the case of the Arp2/3 complex, formin-induced actin nucleation *in vivo* is much faster then measured in cell-free system, suggesting that additional factors accelerate nucleation (Pelham and Chang, 2002). Although the regulation of formins is still being actively investigated, it is already clear that RhoGTPases provide a main pathway for formin activation (Li and Higgs, 2003)

1.1.4. Capping proteins

Each filament only grows transiently, since abundant capping proteins such as CapZ and gelsolin terminate growth (Cooper and Schafer, 2000; Sun et al, 1999). Capping makes two important contributions to actin-driven motility: first, it limits the length of the growing branches leading to shorter filaments, which are stiffer and therefore more effective in pushing on the membrane (Pollard and Borisy, 2003); second, capping proteins are very important to control where actin filaments will "push". Furthermore, capping prevents non-productive usage of actin-subunits, concentrating them for focal polymerization (Carlier and Pantaloni, 1997; Cooper and Schafer, 2000). Capping proteins are subject to regulation by $PI(4,5)P_2$ which sequesters gelsolin and capping protein (Janmey and Stosssel, 1987; Schafer et al, 1996).

At suboptimal concentrations of capping proteins, movement of *Listeria* in "minimal motility medium" (ATP, Arp2/3, cofilin, capping protein, profilin and G-actin) is slow, and the actin tails exhibit a fishbone pattern, showing that branched filaments continue to grow away from the surface of bacterium until a capping protein stops their growth (Pantaloni et al, 2000). The length and life time of filaments is thus not determined only by severing proteins like cofilin, but also by capping proteins, which restrict filament growth to the sites where force has to be produced.

Furthermore, recent work from the laboratory of Gary Borisy, revealed a new role for capping proteins in determining whether cells produce lamellipodia or filopodia (Mejillano et al, 2004).

1.2. Microtubules (MT)

1.2.1. MT structure

Microtubules are tubular polymers made of α- and β-tubulin heterodimers, which are incorporated into a MT lattice, so that α-tubulin is exposed at the ”minus”-end, and β-tubulin at the growing “plus” end. Many cell types have a microtubule organizing center (MTOC), in which the minus end of MTs is embedded, while the plus-end undergoes dynamic transitions between shrinkage (termed “catastrophe”) and growth (“rescue”) (Howard and Hyman, 2003). Work in tissue culture has revealed that MTs growing from the MTOC initially exhibit similar dynamic instability properties as described in vitro (Perez et al, 1999). However, when MTs reach the cell periphery the stability of their plus-end changes dramatically, in that it shows much more frequent fluctuations between shrinking and growth (Komarova et al, 2002).
The plus end of the MTs might thus “explore” cytoplasmic space and if it makes productive interactions with other cellular structures (such as kinetochores on chromosomes) it can be captured and stabilized (Kirschner and Mitchison, 1986; Hayden et al, 1990).

The energy to drive MTs polymerization derives from GTP hydrolysis (Mitchison, 1993; Nogales et al, 1999). The resulting GDP-tubulin has a new bend conformation, which induces a destabilizing curvature in the MT lattice (Muller-Reichert et al, 1998; Arnal et al, 2000; Hyman et al, 1995). However, hydrolysis alone will not trigger depolymerization, and there are regulatory proteins that regulate MT dynamics by

causing further bending, and triggering depolymerization (Desai et al, 1999; Hunter et al, 2003).

1.2.2. Role of MTs

An important function of MTs is to serve as tracks for the movement of cellular structures (such as chromosomes, nucleus, organelles) inside cells. Transport is driven by motor proteins such as kinesin and dynein that interact with MTs and use energy from ATP hydrolysis for lateral movement along the surface of MTs (Hirokawa, 1998). However, the movement of chromosomes or nucleus does not relay on molecular motors. Thus, MTs themselves, in the absence of motors, can serve to move cellular structure around the cell by maintaining attachments as the grow and shrink, thereby moving the structure away or towards the "minus" end (Rieder and Salmon, 1998; Coue et al, 1991). In an important further function, remodeled MTs can direct polarized secretion, or the local delivery of factors essential for proper cell motility and polarization (Galjart and Perez, 2003).

1.2.3. Microbutule plus-end tracking proteins (+TIPs)

CLIP-170, the first TIP to be discovered, is added to the plus-ends of growing microtubules, but dissociate shortly thereafter, behind the region of new growth. Accordingly, although individual CLIP-170 molecules are stationary, the population of CLIP-170 molecules appears to surf on the growing ends of MTs. This mechanism is termed "treadmilling", and shrinking MTs are devoid of +TIPs (Perez et al, 1999; Komarova et al, 2002).

Other plus-end binding proteins do not bind MTs directly, but through other MTs binding proteins. One such example is APC, which is recruited onto MTs through EB1, which in turn treadmills along MTs by binding preferentially to the MT end, and dissociating from MT sides (Askham et al, 2000; Mimori-Kiyosue et al, 2000).

In addition to binding to treadmilling TIP, APC can also bind kinesin motors (KIF3) and in that way accumulate at MT plus-ends (Jimbo et al, 2002). Shrinking MTs will also be labeled by +TIPs that use motor-based mechanism (Kusch, 2002).

1.2.4. Role of TIPs

There is a substantial evidence supporting the notion that +TIPs are involved in the control of the MT plus-end dynamics. Recent observations argue for a role of +TIPs in the capture of MTs at the cell cortex, which might then be followed by deposition of proteins at the cell periphery (Komarova et al, 2002; Kusch, 2002; Carvalho et al, 2003). In mammalian cells, it has been shown that CLIP-170 interacts with IQGAP1, an effector of the small GTPases Rac1 and Cdc42, and is able to form a tripartite complex with activated Rac1/Cdc42. In this study the authors suggest that Rac1 and Cdc42 may mark specific cortical docking sites, where a IQGAP-CLIP170-MT complex is captured, leading to a polarized MT array (Fukata et al, 2002). More recent study from the same group further identified IQGAP1 binding to APC. IQGAP might mediate complex local stability at leading lamellae (Watanabe et al, 2004).

1.2.5. RhoGTPases as regulators of MT-cortex interactions

In their active GTP-bound state, RhoGTPases interact with and activate effectors, which directly or indirectly influence cortical capture of MTs. The earliest evidence came from the finding that Cdc42 was involved in MTOC reorientation during interactions between T cells and their targets (Stowers et al, 1995). This finding has now been extended, as it was shown that Cdc42 plays a similar role in migrating fibroblasts (Palazzo et al, 2001), astrocytes (Etienne-Manneville and Hall, 2001) and endothelial cells (Tzima et al, 2003).

Subsequently, RhoA was found to regulate a subset of stabilized MTs at the leading edge of migrating fibroblasts (Cook et al, 1998). A Rho effector domain screen identified the

formin mDia as the effector involved in the selective stabilization of MTs in migrating fibroblasts (Palazzo et al, 2001). MTs stabilized by mDia neither shrink nor grow and are thought to be capped on their plus ends to give them long term stability (>1hr) (Infante et al, 2000).

Finally, Rac1 was shown to regulate MTs not through regulation of their capture, but by activating its effector PAK to phosphorylate the MT-destabilizing protein stathmin (Daub et al, 2001).

1.2.6. Targeting of MTs

It has been clear since 1988 that during cell migration, stabilized microtubules are preferentially oriented towards the leading edge (Gundersen and Bulinski, 1988). Consistent with this notion, microtubules in protruding lamellipodia appear to spend more time growing than MTs in quiescent cells (Waterman-Storer et al, 2000). However, where exactly along the cell membrane at the leading MTs get captured and stabilized has remained unknown.

Although, there was a report that MTs target and destablize focal adhesions, (Kaverina et al, 1998; Kaverina et al, 1999; Kaverina et al, 2000; Krylyshkina et al, 2003), this is thought to be more important for the disassembly of focal adhesions at the rear of the cell, where "old" focal adhesions must be disassembled in order for the cell to move forward (Ballestrem et al 2000).

1.3. Lipid microdomains - rafts

1.3.1. Existence and functions of rafts

The Singer-Nicholson fluid mosaic model of the plasma membrane, which proposes that proteins can move freely in a two-dimentional lipid solvent is proving to be oversimplified. Thus, at the end of the 80' scientists found that lipids exist in lipid bilayers in distinct phases: liquid-ordered and liquid-disordered phase. In the liquid-ordered phase, phospholipids with saturated hydrocarbon chains pack tightly with cholesterol and sphingolipids, staying mobile in the plane of the membrane (Sankaram and Thompson,1990). The lipid raft model proposes that cholesterol and sphingolipids of the plasma membrane are not evenly distributed, but rather accumulate locally into lipid ordered domains that float in a lipid disordered bilayer (Schroeder et al, 1994; Brown, 1998; Rietveld and Simons, 1998)
The raft concept has been controversial for a number of years, because of the difficulty of proving the existence of rafts in living cells, mainly due to their small size, which is bellow resolution limit of standard light microscopy (app. 50nm) (Pralle et al, 2000, Munro S, 2003). Although, improved methodology has dispelled most of these doubts, the concept of raft clustering and patching has remained controversial.

Rafts have been implicated in a number of cellular processes, including signal transduction, sorting at the trans-Golgi network (Simons and Ikonen 1997; Benting et al, 1999), sorting in the endocytic pathway (Mallet and Maxfield,1999), integrin function (Smart et al, 1999; Green at al, 1999; Krauss and Altevogt,1999) and polarization in migrating cells (Manes et al, 1999). In addition rafts can serve as docking sites for certain pathogens and toxins (Fivaz et al, 1999), and they've been implicated in abberant amyloid precursor protein processing that contributes to Alzheimer disease (Kurzchalia and Parton,1999; Golub et al, 2004; Ledesma et al, 2003)

1.3.2. Raft composition

Lipid rafts are dynamic assemblies of cholesterol and sphingolipid-rich microdomains that can selectively incorporate or exclude proteins, in that way influencing protein-protein and protein-lipid interactions. Proteins attracted to rafts include GPI-anchored proteins (outer leaflet), and doubly acetylated proteins (inner leaflet) (Simons and Toomre, 2000). The number of proteins in each raft unit depends on packing density, but is probably not more than 10-30 proteins. Whether or not proteins are randomly distributed among distinct rafts remains to be elucidated. There are, however, some studies that support non-random distribution (Varma and Mayor, 1998; Friedrichson and Kurzchalia,1998).

The size of a raft unit is also controversial and varies from 5 nm (Sharma et al, 2004) to 50-100 nm (Varma and Mayor, 1998; Pralle et al, 2000; Zacharias et al, 2002; Prior et al, 2003).

The lipid composition of the two sides of the bilayer are very different: sphingolipids are present in the outer leaflet, whereas some glycerophospholipids (such as phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine) are restricted to the inner leaflet. Cholesterol has a substantial rate of spontaneous flipping between the two leaflets, and is therefore present at comparable concentrations in the outer and inner leaflet.

Apart from the plasma membrane, where rafts are most abundant, rafts can also be found in the biosynthetic and endocytic pathways.

1.3.3. Methods to detect rafts

Detergent extraction

Sphingolipid-cholesterol rafts are insoluble in non-ionic detergents at 4°C degrees, and because of their high lipid content they float to a low density during centrifugation. Initial studies used Triton X-100 as a detergent, but subsequent studies also introduced Triton X-114, CHAPS, Brij 96 and Lubrol WX. Interestingly, Roeper and colleagues (Roper et al, 2000) provided evidence for differential solubility of raft components as a function of a detergent used, and suggested that this reflected differential spatial distribution of the components at the membrane surface.

The drawback of this method is that the original subcellular localization of Detergent Resistant Membranes (DRMs) remains unknown. Furthermore, some proteins may be linked to the cytoskeleton and hence not float, or their association with rafts may be too weak affinity to survive the extraction procedure.

Immunofluorescence microscopy

Staining for raft components (such as GPI-linked proteins) has shown to be trickier then anticipated, since they often exhibit uniform distribution on the cell surface when detected by light. Specific fixation protocols, using PFA as fixative and mild permeabilization with saponin, are necessary to obtain patchy staining patterns (Laux et al, 2000). A further complications of detection methods in the fixed cells is that clustering of one raft marker (with antibodies or other reagents) can cause redistribution of other markers, even when the two are unlikely to interact directly (Viola et al, 1999; Schutz et al, 2000).

While this was until recently the most widely used method to identify putative raft associations (Harder et al, 1998; Janes et al, 1999; Caroni, 2000), methods to monitor rafts in living cells are increasingly substituting those involving fixed preparations.

Monitoring rafts in living cells

Techniques for raft visualization in living cells include the use of GFP- labeled double acylated proteins, GFP fusion proteins including the PH domain of PLCδ1 that will visualize $PI(4,5)P_2$ (Tall et al, 2000), fluorescence recovery after photobleaching (Kenworthy et al, 2000), photonic force microscopy (Pralle et al, 2000) , single molecule microscopy (Schutz et al, 2000), and fluorescence resonance energy tranfer (FRET) (Varma and Mayor, 1998; Kenworthy et al, 2000; Glebov and Nichols, 2004)
The results from these studies suggest that rafts are relatively small, in the range od 50 nm diameter, although considerable variations exist between reported values.

1.4. Trafficking

1.4.1. Endocytosis

Endoyctic membrane trafficking is the aspect of intracellular trafficking that has been implicated most conclusively with lipid rafts. Cellular processes involving endocytosis include nutrient uptake, synaptic vesicle recycling, the regulation of signaling receptors levels at the cell surfaces, remodeling of the plasma membrane, and the generation of cell polarity. There are several pathways for internalization of molecules from the cell surface. Receptor-triggered clathrin-mediated endocytosis might be the most widespread mechanism and involves internalization of the receptor and its bound ligand through clathrin-coated pits. Many of the ligands are then degraded in lysosomes, while others are recycled back to the surface. These recycling pathways are essential to maintain the composition of appropriate compartments (like for example in polarized cells; Wang et al, 2000), and to return essential molecules to the cell surface. In addition to keeping the homeostatic regulation of compartment composition, the rates of membrane trafficking can be altered to increase or decrease surface expression of components, in response to signals (Maxfield and Mc Graw, 2004).

The nomenclature for endocytic systems has not been completely standardized, but the main components of the endocytic route are: early endosomes (sorting endosomes and endocytic recycling compartment – ERC; Sheff et al, 1999), late endosomes, and lysosomes (Maxfield and Mc Graw, 2004).

1.4.1.1. Maturation of endocytic vesicles

Clathrin-coated pits are the best understood entery points for endocytic vesicles. Although the maturation steps for endocytic vesicles are derived from research on clathrin-coated pits, it is often assumed that similar steps might be valid for other forms of endocytosis, such as caveolae-mediated endocytosis, and/or raft-dependent endocytosis. In fact, it was shown that some molecules (like GPI-linked proteins), which get internalized via caveolae, are targeted to early endosomes that fuse with early endosomes of the clathrin pathway (Sharma et al, 2003).

After pinching off from the plasma membrane, and shedding off the clathrin coat, new endosomes fuse with one another, and with pre-existing sorting endosomes beneath the plasma membrane. This step is not fully understood, but it is known to be controlled in part by Rab5, EEA1 (early endosome antigen 1), and SNAREs (Clague, 1999; Woodman, 2000). The subsequent steps from early to late endosome require microtubules (Gruenberg et al, 1989).
In the case of signaling receptors, delivery of receptors to late endosomes/lysosomes functions to terminate signaling, and to render cells irresponsive to further signaling input until a new set of receptors has been synthesized (Gruenberg, 2001).

Membrane proteins can be delivered back to the plasma directly, or through ERC (endocytic recycling compartment), a collection of tubules which can sort molecules to the plasma membrane or the trans-golgi network (TGN; Hopkins, 1983).

1.4.1.2. Non-clathrin dependent endocytosis pathways

Molecular machinery specifically involved in clathrin-independent endocytosis pathways, includes caveolin, ARF6, dynamin, ankyrin/spectrin and actin. These markers have all been associated with lipid rafts. In contrast, typical markers for clathrin-mediated endocytosis, such as transferrin receptor and the low-density lipoprotein (LDL) receptor, are excluded from rafts.

1.4.1.2.1. Caveolae-mediated endocytosis

Caveolae have been identified almost 50 years ago as ca.60 nm diameter smooth-surfaced flask-shaped pits covering the surface of many mammalian cells (e.g. adipocytes, endothelial cells, muscle cells). Caveolins (-1,-2 and –3) are the major constituent proteins of those structures.

Caveolae play a role in endocytosis by budding off from the membrane to form endocytic vesicles. The GTPase dynamin is found at the neck of caveolae, and is necessary for plasma membrane detachment of caveolae in the same way as it is required for the formation of clathrin-coated pits (Oh et al,1998; Henley et al, 1998).

A Src kinase-mediated tyrosine phosphorylation cascade induces phosphorylation of caveolin-1, and association of dynamin with the neck of caveolae, triggering endocytosis (Ahn et al, 2002; Parton and Richards, 2003).

Although caveolae are rich in cholesterol and GM1, and were for some time equalized with lipid rafts, it has been shown that cells that do not contain caveolin or caveolae still exhibit cholesterol-dependent endocytosis, indicating that rafts can act as sites for endocytosis independently of caveolae. Recent findings in fact suggest that caveolae might act as negative regulators of raft-mediated uptake. Thus, one study showed that overexpression of caveolin-1 in NIH3T3 cells decreases the rate of internalization of AMF-R (Le et al, 2002), and another study showed that cell-surface caveolae are largely static (Thomsen et al, 2002), suggesting that they probably don’t play a role in constitutive endocytosis. Accordingly, a current model proposes that to allow caveolae

budding in response to a specific stimulus (e.g. SV40 binding; Harder et al, 1997; Pelkmans et al, 2001), the inhibitory effect of caveolin has to be overcome, through phosphorylation of cav-1, and recruitment of other proteins such as dynamin.

1.4.1.2.2. Rafts in endocytosis

Some GPI-anchored proteins (e.g. folate receptor) traffic to the ERC, but unlike transferrin which returns to the cell surface with a half life of about t=10 min, exhibit return with half-lives of about 30 min. If cellular cholesterol levels are experimentally reduced by 30%, then recycling rates increase and match to those measured for transferrin (Mayor et al, 1998) suggesting a role for lipid microdomains in ERC sorting.

The same molecular events that happen during caveolar endocytosis, including activation of src-family kinases, localized tyrosine phosphorylation, and F-actin accumulation, also take place in lymphocytes which neither express caveolin-1 nor exhibit cell surface caveolae (Harder and Simons, 1999). Althought this endocytosis pathway is clathrin-independent, it is nevertheless inhibited by the loss-of-function dynamin-mutant K22A (Lamaze et al, 2001).
New studies have provided evidence that clathrin-dependent endosomes and raft-dependent endosomes signal differentially (Di Guglielmo et al, 2003).
How exactly do raft-dependent and clathrin-dependent endocytosis interface remains to be elucidated, but in their excellent review L. Johannes and C. Lamaze speculate that differential sorting at the plasma membrane might predispose the intracellular fate of a given molecule (Johannes and Lamaze, 2002). Moreover, a review by J. Gruenberg suggests that the different endocytic routes all connect to the same early endosome, which serves as a distribution station inside the cell (Gruenberg, 2001).

1.4.2. Rafts in chemotaxis and cell polarity

Many important biological events such as wound repair, axon guidance and immune responses, involve persistent cell movement towards a directional signal, a process termed chemotaxis. To achieve directed movement, cells must acquire and maintain functional and spatial cell polarity, a process that requires raft function. Thus, in polarization, either during cell spreading, migration or differentiation, there is a need for a spatial redistribution of signaling components involved in the regulation of cell shape change and movement.

Among the first studies that connected rafts to polarization, that by Gomez-Mouton and colleagues (Gomez-Mouton et al, 2001), showed an asymmetric redistribution of GM1 and GM3 rafts at uropod (trailing edge) and leading edge of migrating neutrophils. A later study on migrating of leukocytes expanded the findings of raft marker polarization by showing asymmetric recruitment and activation of the signaling molecule PI3K on the cell edge facing the chemoattractant (Gomez-Mouton et al, 2004).

However, the reported GM1 vs GM3 distribution varies in different reports, and in their brief review Manes and Martinez point out that this could be attributed to differences among cell types analyzed or among modes of cell migration (Manes and Martinez-A, 2004).

Finally, an important recent study on growth cone guidance showed how raft integrity is necessary for growth cones to turn in response to BDNF, netrin-1 and Sema3A, but not glutamate. They also showed that rafts accumulated asymmetrically towards a BDNF-gradient, suggesting that the turning response may be result of a spatial rearrangement of lipid rafts on the surface of growth cones (Guirland et al, 2004).

1.5. Phosphoinositides

Phosphoinositides (PIs) constitute less then 10% of total cellular phospholipids. Nevertheless, they are key components of cell membranes, involved not only as second messengers, but also as components that can bind and specifically localize certain

cytosolic molecules to the membrane, or alter protein functions through binding (Clarke, 2003). The unique features of PIs that enable them to carry out their unique roles are that they can repeatedly undergo phospho/deposphorylation cycles at positions 3', 4' and 5' of their inositol headgroup without leaving the membrane, and that every organelle is equipped with distinct sets of PI kinases and PI phosphatases, giving rise to different intracellular distributions of PIs (De Matteis and Godi, 2004). The recruitment of PI-metabolizing enzymes are only partially understood but in some cases they involve small GTPases: for example Rac recruits synaptojanin (PI(4,5)P_2-phosphatase) to the plasma membrane (Malecz et al, 2000), and Arf1 recruits PI4K to the Golgi complex (Godi et al, 1999).

1.5.1. Subcellular localization of PIs

Studies using phosphoinositide-binding motifs such as PH domains fused to GFP protein (GFP; Tall et al, 2000) and specific anti-PI antibodies (Fukami et al, 1988) have revealed patchy distribution of PIs at the plasma membrane that co-localize with cholesterol-rich lipid microdomains (Laux et al, 2000), and specific accumulation at the leading edge of migrating cells (Gomez-Mouton et al, 2004; Manes et al, 1999).

Localization of enzymes that produce phosphoinositides at sites in the membrane where actin polymerization occurs supports the idea that PI(4,5)P_2 and PI(3,4,5,)P_3 are synthesized locally (Ling et al, 2002; Doughman et al, 2003). Along the same lines, PI5K is recruited to active sites of cytoskeleton assembly at membrane ruffles (Honda et al, 1999), at sites of phagosome formation (Botelho et al, 2000), and to Golgi membranes (Godi et al, 1999; Jones et al, 2000).

Finally, the PI(4,5)P_2-binding PH domain of phospholipase Cδ1 (PLCδ1) was shown to transiently accumulate at active sites of phagocytosis (Bajno et al, 2000; Botelho et al, 2000; Vieira et all, 2001)

1.5.2. Phosphatidylinositol (4,5) bisphosphate – PI(4,5)P_2

$PI(4,5)P_2$ plays a major role as a second messenger that gets hydrolyzed by phospholipase C into diacylglycerol (DAG) and inositol triphosphate (IP_3), leading to the activation of protein kinase C (PKC), and to release of Ca^{2+} from intracellular stores. $PI(4,5)P_2$, however, has several additional cellular functions, which include a major role in the regulation of actin cytoskeleton assembly.

1.5.3. PI(4,5)P_2 and actin assembly

Targets of phosphoinositides that are responsible for actin-cytoskeleton regulation mainly include actin binding proteins (ABPs) which are either activated or inhibited by $PI(4,5)P_2$ binding. $PI(4,5)P_2$ can bind and inactivate ABPs that sever or depolymerize actin, such as profilin (Lassing and Linderberg, 1985), cofilin (Ojala et al, 2001), CapZ (Schafer et al, 1996), and gelsolin (Janmey and Lindberg, 2004).

The net effect of this regulations is that increasing levels of $PI(4,5P)_2$ promote actin assembly, whereas reduced levels of $PI(4,5)P_2$ tend to promote actin disassembly (Janmey and Lindberg, 2004).

Consistent with these observations, $PI(4,5)P_2$ vesicles in can induce actin polymerization that will induce propulsion of the vesicle through the medium, if added to appopriate cell extract (Ma et al, 1998). Furthermore, overexpression of PI5K causes endogenous vesicles to move (Rozelle et al, 2000).

1.6. Goal of the study

The existence of cholesterol- and sphingolipid-enriched lipid microdomains, and their importance in cell trafficking and motility are well established. However, whether and in

what ways lipid rafts can organize into higher order domains at defined sites on the cell surface, and how this influences signaling, had remained controversial.
With respect to actin and motility regulation, it has been known that disruption of rafts prevents sustained activation of actin dynamics in cellular responses such as membrane ruffling and pinocytosis, but how exactly rafts contribute to local control of signaling at the cell surface, leading to cell surface motility had remained unclear.
Finally, with respect to neurite outgrowth and synapse remodeling, growth-associated proteins such as GAP43 and CAP23 had been shown to promote actin-based motility at the cell surface through an interaction with the lipid second messenger $PI(4,5)P_2$ at rafts. $PI(4,5)P_2$ is a major regulator of actin dynamics, but the mechanisms through which it orchestrates dynamics and motility were poorly understood.

This study aimed at elucidating mechanisms of $PI(4,5)P_2$-rich raft assembly, and the roles of these mechanisms in the regulation of cell surface motility. The study paid particular attention to how mechanisms of raft assembly and regulation might mediate temporal and spatial regulation of motility important for directed cell migration and neuronal growth cone navigation.

2. Results

2.1. Spatial and temporal control of signaling through lipid rafts

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[1]equal contributions

2.1.1. Summary

Sphingolipid- and cholesterol-dependent microdomains (rafts) order proteins at biological membranes and have been implicated in most signaling processes at the cell surface, but the principles and mechanisms through which lipid rafts influence signaling are not well understood. Recent studies have revealed how lipid rafts are rapidly redistributed and assembled locally in response to extracellular signals, and how components of raft-based signaling domains undergo rapid and regulated rearrangements influencing signal quality, duration and strength. These findings highlight the exquisitely dynamic properties of signaling domains based on lipid rafts, and suggest that processes of raft trafficking and assembly play central roles in mediating spatial and temporal control of signaling.

Abbreviations:

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APP	amyloid precursor protein
BACE-1	beta-secretase
ECM	extracellular matrix
ERM	ezrin-radixin-moesin
FAK	focal adhesion kinase
GDNF	glia cell derived neurotrophic factor
GPI	glycosylphosphatidylinositol
LAT	linker of activated T-cells
Lck	Src-like kinase Lck
NCAM	neuronal cell adhesion protein
MAG	myelin associated glycoprotein

MHC	major histocompatibility complex
NMDA	N-methyl-D-aspartate
PI3K	PI(3,4,5)P3 kinase
PKC	protein kinase C
PSD	postsynaptic density
PI(4,5)P2	phoshoinositol-4,5-bisphosphate
Smad	protein components of of TGFβ signal transduction pathways
TCR	T-cell receptor
TGF	transforming growth factor
VGCC	voltage-gated calcium channel

2.1.2. Introduction

The membrane systems in any type of cell exhibit substantial and specific differences in their lipid compositions. In addition, specific differences exist between the two leaflets of most bilayers, and lipids in individual leaflets are not distributed homogeneously in the plane of the membrane. Mainly due to their distinct biophysical properties, sphingolipids and cholesterol play a predominant role in generating microdomains in biological membranes [Simons and Toomre, 2000; van Meer, 2002]. These sphingolipid- and cholesterol-dependent microdomains are also designated as lipid rafts. Lipid rafts are first assembled at the Golgi, and play major roles in specific trafficking of proteins and lipids to and from cellular compartments [Lai, 2003]. Different types of cells at distinct developmental stages can differ substantially in their raft contents, and association with lipid rafts influences signaling and the assembly of cellular structures in specific ways [Simons and Toomre, 2000].

The existence and functional significance of lipid rafts are well established. However, issues such as the molecular nature (e.g. domains versus lipid shells) and half-lifes of rafts, as well as the states of assembly of raft-based platforms in situ have remained controversial [Glebov and Nichols, 2004; Ikonen, 2001]. As a consequence, while much progress is being made in elucidating the roles of lipid rafts in cell trafficking and signaling, it is not yet clear how exactly rafts contribute to signaling at the molecular level.

This review focuses on recent developments of how lipid rafts influence spatial and temporal control of signaling in neurons. We elaborate on the notion that lipid rafts reflect ordering mechanisms to reliably ensure that defined components come in close vicinity within microdomains of membranes, in specific ways, at defined sites, and at defined times. Because many of the conceptual developments have emerged from studies of non-neuronal cells, we also discuss some of those studies in the review, elaborating on how the results have guided studies of related processes in the nervous system. More in depth discussions about the nature and roles of lipid rafts in trafficking and signaling, as well as their emerging roles in nervous system diseases such as Alzheimer's and Prion diseases can be found in several recent reviews [e.g. Simons and Toomre, 2000; van Meer, 2002; Simons and Ehehalt, 2002].

In the following sections we first discuss basic principles of how rafts can influence signaling, and then turn to the ways in which processes of raft recuitment and assembly contribute to spatial and temporal specificity of signaling in neurons.

2.1.2.1. Signal-induced recruitment and assembly of raft-dependent platforms

Extracellular ligands can initiate raft recruitment and assembly, which in turn can affect the quality, strength and duration of intracellular signaling. Lipid microdomains are thought to enhance the efficacy and reliability of signaling by locally concentrating selected protein components at specific sites on membranes. This principle is nicely illustrated in T-cell activation, where a requirement for raft association of MHC molecules can be bypassed by raising the concentration of these molecules on the surface of antigen presenting cells [Hiltbold et al, 2003].

The mechanisms through which extracellular ligands initiate raft recruitment are best understood in T- and B-cell activation, but mechanisms in neurons are thought to be similar. In T-cells, the critical non-receptor-type tyrosine kinase Lck is present in both lipid and non-lipid fractions. Initial signaling is raft-independent, and at rest raft Lck is

maintained in an inactive (phosphorylated) state. Binding of ligand to T-cell receptor (TCR) leads to phosphorylation of its cytoplasmic tail by non-raft Lck, which in turn induces (1) binding of the TCR to the cortical actin cytoskeleton and (2) beginning of its association with rafts [Sedwick and Altman, 2002; Harder, 2004]. In contrast, and apparently due to ligand-mediated extracellular crosslinking of immunoglobulin Ig-molecules, raft recruitment in B-cell activation does not depend on the cortical actin cytoskeleton. In a similar way, inhibition of neurite outgrowth by MAG involves binding in trans to the gangliosides GT1b and GD1a, upstream of p75 and Rho activation [Vinson et al, 2001]. Raft recruitment in signaling may thus depend initially on a local partial immobilization process, which can be provided through loose anchorage to intra- or extracellular scaffolds.

The subsequent assembly of increasingly effective signaling platforms involves a sequential process of adapter and scaffolding protein recruitments to the original signaling complex. This is again illustrated best for T-cell activation, where receptor activation leads to recruitment of the kinase ZAP-70, which in turn recruits PLCγ1, PI3K and the adapter protein LAT. Subsequent phosphorylation of LAT at multiple sites creates docking sites for further signaling molecules. An additional checkpoint in T-cell activation involves persistent activation of raft-associated Lck through raft-recruited phosphatase CD45. Fully activated Lck then leads to raft-dependent recruitment of PKC-theta, and full T-cell activation [Sedwick and Altman, 2002; Harder, 2004].

Examples of signal-induced recruitment to rafts in the nervous system include GDNF-induced association of c-Ret [Paratcha et al, 2001], neurotrophin-induced association of p75- and Trk-receptors [Higuchi et al, 2003], Netrin-1-induced association of DCC and neuropilin, neuregulin-induced recruitment of ErbB4 through PSD-95 [Ma et al, 2003], and light-induced association of the phototransduction complex, upstream of further signaling [Nair et al, 2002].

While extracellular signals initiate raft-mediated signaling, the extent of this signaling is determined by the expression levels of cell-intrinsic factors. Thus, the controlled

assembly of raft-dependent platforms in signaling is influenced by the expression of raft-associated components such as flotillins, which affect both raft levels and processes of signal-induced raft clustering. In neurons, expression levels of the GAP43-like proteins GAP43, CAP23 and MARCKS influence the efficiency of raft-dependent signaling to the actin cytoskeleton, which regulates neurite outgrowth and synaptic plasticity [Laux et al, 2000]. GAP43-like proteins function by enhancing the accumulation and assembly of $PI(4,5)P_2$-rich plasmalemmal raft-based patches in a calcium/calmodulin- and PKC-regulated manner [Caroni, 2001].

2.1.2.2. Raft association modifies signaling

Raft association influences the strength and quality of signaling through at least two distinct types of mechanisms: 1) the activity of signaling molecules can be influenced by the local environment at rafts; 2) signaling molecules can interact with and modify distinct downstream components at and outside of rafts.

In two examples of how the local environment at rafts modifies signaling, PSD-95 recruits the voltage-gated channel Kv1.4 to rafts, where channel activity is modulated through raft-associated kinases [Wong and Schlichter, 2004], and the catalytic activities of neuronal-Src or Lyn are substantially higher when these cytosolic kinases are associated with a raft environment [Mukherjee et al, 2003]. Importantly, association with a raft environment does not simply activate signaling indiscriminately, but is often required for appropriate dosage of signaling. Thus, for example, NCAM-140 and NCAM-180 modulate surface and raft accumulation of Kir-3 channels, regulating in this way their functional impact [Delling et al, 2002].

In addition to influencing the signaling strength of individual components, raft association can profoundly influence signal quality through differential recruitment of signaling components at and outside of rafts. In one nice example involving GDNF signaling, c-Ret interacts with Shc outside rafts, but with the Src-type kinase Frs2 when associated with rafts. Only signaling through Frs2 and PI3K promotes neuronal survival

[Paratcha et al, 2001]. In a further dramatic example from the immune system, clonotypic elimination of activated T-cells requires association with rafts of Fas receptor in re-stimulated T-cells, before interaction with Fas-ligand [Muppidi and Siegel, 2004].

Importantly, the readout of signaling through cell adhesion molecules and ECM receptors depends on the extent and mechanisms through which these molecules associate with rafts. For example, a fraction of NCAM-140 can associate with lipid rafts upon palmitoylation at juxtamembrane intracellular residues. For NCAM-140 to promote neurite outgrowth in vitro, activation of two parallel pathways appears to be required: mediation of FGF signaling through non-raft NCAM, and activation of FAK and ERK by an NCAM-Fyn complex at rafts [Niethammer et al, 2002]. In addition, by associating to GPI-anchored receptor for GDNF (GFRα1), NCAM-140 can bind GDNF with high affinity, and mediate signaling to Fyn and FAK [Paratcha et al, 2003]. Furthermore, NCAM associates with and signals through GAP43 and Fyn at distinct subpopulations of growth cone raft complexes [He and Meiri, 2002].

2.1.2.3. Spatial control of signaling through rafts

Recent discoveries of signal-related trafficking, local targeting, and local accumulation of rafts have established lipid rafts as a major factor in spatial control of signaling.

Many of the molecular components regulating the actin cytoskeleton, cell motility and adhesion are associated with rafts. These include Rho-type GTPases, and the phosphoinositides PI(4,5)P_2 and PI(3,4,5)P_3 [Caroni, 2001]. This suggested that local accumulation of rafts may mediate some of the spatial specificity in Rho-GTPase signaling to the actin cytoskeleton. An important recent study now sheds light on how integrin signaling relates to raft distribution and Rac activity [del Pozo et al, 2004]. The study shows that integrin engagement leads to the accumulation of rafts at the cell surface. In contrast, when cells are detached from their substrate, rafts are endocytosed and accumulate intracellularly. Furthermore, the paper shows that in the absence of surface rafts, Rac-GTP fails to associate with the plasma membrane, and to activate the

downstream effector of integrins FAK. Together with those of previous studies [Grimmer et al, 2002], these results provide evidence that integrin signaling couples sensing of the local environment to the local accumulation of rafts required for Rac-GTP activity at the cell surface. A related study shows that activation of leading edge FAK downstream of integrin signaling leads to accumulation of GM1 to the leading edge, and to microtubule stabilization through Rho and mDia [Palazzo et al, 2004]. A cascade of signaling reactions downstream of integrin signaling may thus induce a sequential accumulation of distinct rafts at the site of activation, culminating in microtubule accumulation and cell polarization (Fig. 1).

In addition to sensing ECM molecules, certain integrins can also function as receptors for diffusible axon guidance molecules such as Netrin-1 [Yebra et al, 2003] or Sema7A [Pasterkamp et al, 2003], and inside-out activation of integrin receptors can be brought about by e.g. Sema3 or Eph/Ephrin signaling. Raft-dependent integrin signaling also mediates interactions between cells, such as axon-mediated survival of oligodendrocytes [Decker and ffrench-Constant, 2004]. Therefore, although the mechanisms through which integrin signaling mediates raft accumulation are not yet clear, this versatile receptor system can clearly play a major role in mediating local control of signaling through rafts.

Polarization of rafts, and the ways in which rafts are involved in setting up cell polarity and polarized signaling are nicely illustrated in chemotactic cell migration. In one of the first reports of raft polarization, Martinez-Arias and colleagues [Gomez-Mouton et al, 2001] showed that polarizing T-cells accumulate distinct raft components, including the gangliosides GM3 and GM1 at their leading and trailing edge, respectively. A subsequent study showed that while initial signaling is raft-independent, the presence of intact rafts is necessary for sustained Rac activation and actin polymerization [Pierini et al, 2003]. Significantly, in migrating leukocytes, CCR5-receptor and subsequently activated PI3K redistribute to leading edge rafts in a raft-dependent manner [Gomez-Mouton et al, 2004]. Taken together, these studies show that in polarized cell migration spatial signaling can be organized by concentrating gradient sensing machinery through rafts. As discussed below, this principle likely plays a major role in growth cone guidance.

Local delivery of rafts at defined sites on the cell surface can involve dedicated targeting mechanisms. The exocyst is an octameric complex involved in targeting proteins and vesicles to specified plasma membrane domains, thereby acting as a polarization cue. The raft-associated small G-protein TC10 is activated by a complex involving flotillin, and can then recruit the exocyst-component Exo70 to lipid rafts [Inoue et al, 2003]. In insulin signaling, activated TC10-exocyst complex then targets GLUT4-containing vesicles to appropriate plasma membrane fusion sites, thus acting as a landmark for polarized delivery of secretory vesicles [Inoue et al, 2003]. Together with a more universal PI3K signaling branch, this lipid raft-pathway provides spatiotemporal specificity to the insulin response. In a further important illustration of how rafts can influence membrane and cargo delivery at the cell surface, association of SNARE proteins (syntaxin, SNAP-25, VAMP) with plasmalemmal rafts can define docking and fusion sites for secretory vesicles [Lang et al, 2001], and regulated inclusion or exclusions from these microdomains can influence critical properties of exocytosis [Salaun et al, 2004]. In addition, the association of the VGCC Cav2.1 with such microdomains provides for a close spatial coupling of calcium influx sites and the exocytotic machinery [Taverna et al, 2004].

Recent studies have begun to illuminate the roles of lipid microdomains in growth cone guidance. An elegant study by Zheng and colleagues demonstrated that raft integrity is specifically required for growth cone turning responses induced by BDNF, Netrin-1, and Sema3A, but not glutamate [Guirland et al, 2004]. Treatments disrupting cholesterol-rich rafts specifically affected BDNF-induced attraction, whereas treatments affecting GM1-rich rafts interfered with BDNF-induced neurite extension. Importantly, signaling-competent rafts accumulated asymmetrically towards a BDNF-gradient, suggesting that the turning response may be brought about by a spatial rearrangement of lipid microdomains on the surface of growth cones. Like in integrin-mediated adhesion, raft-associated Rac1 plays a central and complex role in growth cone guidance, where it is implicated in both growth and collapse. In the absence of collapsing signals, Rac1 activity drives actin polymerization at the leading edge of growth cones, and raft-

associated proteins such as L1 can increase the levels of active Rac1 at the growth cone membrane. Upon collapsing signals (e.g. through ephrinA2), Rac1 activity is transiently lost, the growth cone switches to a "proof-reading" mode dominated by Rho-mediated contraction, and growth cone microdomains are redistributed.

2.1.2.4. Temporal control of signaling through rafts

Just like most lipid rafts are highly dynamic entities, signaling domains based on lipid rafts appear to consist of loosely interconnected and rapidly exchanging signaling modules. These dynamic properties are well suited to couple processes of domain assembly and disassembly to temporal control in signaling. While raft disruption often abolishes persistent signaling, stable anchorage to cortical cytoskeleton or extracellular components tends to prevent recruitment to raft-based signaling domains. As discussed in the next two sections, the mechanisms regulating the assembly and dynamics of raft-based signaling domains profoundly influence the duration and strength of signaling (Fig. 2).

The assembly of dynamic actin filaments at lipid rafts, and their loose association with cortical cytoskeleton are thought to stabilize signaling domains, whereas actin disassembly and endocytosis mediate their dispersal. Raft-associated proteins can mediate regulated anchorage to the actin cytoskeleton. For example, GAP43-like proteins anchor rafts to cortical actin cytoskeleton in a calcium/calmodulin- and PKC-regulated manner [Laux et al, 2000; Caroni, 2001; He and Meiri, 2002]. Likewise, loose interactions in trans with other cells or extracellular components can serve to prolong and enhance signaling through rafts. In contrast, a more stable association of individual components with the cytoskeleton can function to restrict their recruitment to signaling domains. Thus, upon activation by phosphorylation and $PI(4,5)P_2$, ERM proteins link specific membrane proteins at rafts to the cortical actin cytoskeleton. This linkage can for example be regulated by the assembly of a Cbp-EBP50-ERM complex, which restricts raft motility and prevents the formation of the immunological synapse [Itoh et al, 2002].

Raft components are selectively internalized through clathrin-independent/dynamin-dependent endocytosis under the control of Rho-GTPases [Lamaze et al, 2001]. A specialized path involves association with caveolae, a stable plasmalemmal compartment where phosphorylation of caveolin-1 is required to induce endocytosis [Thomsen et al, 2002; Lee et al, 2002]. For example, when raft-associated components such as integrin-α 2β1 are clustered, they can translocate along actin filaments to accumulate at caveolae [Upla et al, 2004]. In a nice illustration of differential regulation through raft-independent and -dependent endocytosis, TGFβ-receptor in clathrin-dependent endosomes associates with SARA (Smad anchor for receptor activation), leading to Smad phosphorylation and nuclear accumulation of Smads, wheras TGFβ-receptor in caveolae endosomes associates with Smad7-Smurf2 and is targeted for degradation [Di Guglielmo et al, 2003]. Interestingly, the ephrin signaling cascade appears to require a functional endocytic machinery for aspects of its function [Zimmer et al, 2003]. Thus, in ephrin-mediated repulsion, ephrin-induced transcytosis (a special form of endocytosis whereby ligand/receptor complex is internalized through a phagocytosis-like process) is required for interacting cells to detach. While it is not clear yet whether endocytosed ligand/receptor complexes continue to play an important role in ephrin signaling, NGF signaling from the periphery to the cell body does involve retrograde transport of endocytic vesicles containing activated receptors. Two distinct signaling pathways both involving rafts seem to exist: one via the TrkA receptor with fast internalization kinetics, and one via the p75 receptor with slower kinetics [Bronfman et al, 2003].

Residence of rafts at endosomes can also lead to selective rearrangements and mixing of raft-associated components important for signaling. Thus, secretion of Aβ is cholesterol-dependent, and is blocked by dominant-negative dynamin. APP and its key processing enzyme BACE-1 are thought to be associated with separate raft entities, which interact more effectively upon entering endosomes. This requirement for endocytosis can be overcome by antibody-induced co-patching of APP and BACE1 at the cell surface [Ehehalt et al, 2003].

2.1.2.5. Sustained signaling at synapses

Synapses are the major sites of information transfer, and hence of signaling in the nervous system. Not surprisingly, the assembly and dynamics of synapses involves raft assembly and trafficking. Synaptogenesis in vitro can be promoted by glia-derived cholesterol in association with ApoE4 lipoporoteins, suggesting that cholesterol-rich lipid rafts could be involved in synapse maturation and/or stability [Mauch et al, 2001]. However, while it is clear that raft accumulation and trafficking influence the dynamics of synaptic components in and out of the synapse, it is not yet clear whether and how they directly influence synapse formation, stability and turnover.

Synapses among cultured neurons accumulate raft markers, and raft disruption leads to loss of synapses in vitro [Bruses et al, 2001; Hering et al, 2003]. Synapse loss induced by raft disruption is partially prevented by treatments that stabilize the actin cytoskeleton, suggesting that rafts may stabilize synapses by promoting linkages to the cortical cytoskeleton, although alternative possibilities cannot be excluded. In addition, not all synapses in vitro are lost upon raft disruption, and resisting synapses exhibit larger sizes, suggesting that stronger synapses may be less dependent on stabilization through raft-dependent mechanisms.

Crucial components of synapses are clearly associated to rafts. Acetylcholine-receptor and the essential scaffold protein rapsyn are transported together from Golgi membranes to the postsynaptic complex at the neuromuscular junction in a raft compartment containing caveolin and flotillin [Marchand et al, 2002]. Likewise, most receptors at central synapses are partially associated to rafts, where ephrins and their receptors can affect raft association. Thus, ephrinB can recruit GRIP (glutamate receptor interacting protein), a scaffold protein interacting with AMPA receptors, to lipid rafts [Bruckner et al, 1999]. In addition, binding of ephrinB to EphB induces a direct interaction with NMDA receptors, and EphB kinase activity regulates synapse numbers in hippocampal neurons [Dalva et al, 2000]. Furthermore, EphB2 activation by ephrinB2 leads to NMDAR phosphorylation by a raft-associated src-like kinase, and can potentiate NMDA receptor-dependent calcium influx.

Recent evidence on how the exocyst is involved in synapse assembly and receptor trafficking points to extensive potential interactions between the exocyst complex and lipid rafts in neurons. Binding of a PDZ-binding domain of Sec8 to synapse-associated protein 102 (SAP102) is a prerequisite for normal delivery of NMDA receptors to the synapse [Sans et al, 2003]. If a PDZ binding domain on NMDA receptor is deleted, mutant NMDA receptor is trafficked to the cell surface, but not to the synapse. In addition, Sec8 binds to the scaffold and raft-associated protein PSD-95 [Riefler et al, 2003].

Raft-dependent mechanisms play major roles in regulating trafficking of receptors in and out of the synapse, and hence in synapse function. One powerful mechanism to rapidly and reversibly regulate association with rafts involves palmitoylation of cytosolic residues in transmembrane and cytosolic proteins. Among the proteins involved in glutamatergic transmission, those known to be palmitoylated include mGluR4, GluR6, GRIP, PSD93 and PSD-95. Studies on β-adrenergic receptors, Gα and NOS (nitrous oxide synthase) had suggested that agonist-induced depalmitoylation might be a general mechanism to attenuate receptor signaling through enhanced receptor endocytosis. Consistent with this notion, blocking palmitoylation of PSD-95 causes associated proteins like the Kv-channel 1.4 to be internalized [Wong and Schlichter, 2004]. In addition, interfering with palmitoylation of PSD-95 causes dispersal of PSD-95 and GluR1 clusters in a process requiring glutamate receptor activity and postsynaptic Ca^{2+} entry, and leading to increased GluR1 internalization [El-Husseini et al, 2002]. This important study provides evidence that one mechanism of activity-dependent synaptic plasticity involves reversible palmitoylation/depalmitoylation reactions on PSD-95, to regulate AMPA receptor levels at glutamatergic synapses [El-Husseini et al, 2002]. Reversible palmitoylation also influences the assembly of presynaptic components. Thus, distinct palmitoylation processes on GAD65 determine its trafficking to axon-specific endosomes, and its insertion and clustering at presynaptic sites [Kanaani et al, 2004].

2.1.3. Conclusions and future directions

It has become apparent that processes of raft-based domain regulation at the plasma membrane are directly coupled to spatial and temporal control of signaling. Thus, local raft recruitment and raft domain assembly have been shown to mediate spatial control of signaling in response to extracellular signals. Such raft-mediated polarization played a central role in directed motility processes such as cell migration, growth cone guidance and the local interactions of cells with their environment. Likewise, mechanisms controlling the dynamics of raft-associated components into and out of signaling domains have been shown to influence the duration, strength and quality of signaling. These principles of raft-mediated dynamics play major roles in synaptic plasticity. Future challenges mainly involve achieving a better understanding of raft-mediated regulation at the molecular level. This will include elucidating how specific lipid and protein components associated with lipid rafts interact in biological membranes in situ, identifying forms of subplasmalemmal cytoskeleton that regulate the assembly and trafficking of raft-based signaling domains, and elucidating the mechanisms that regulate trafficking of rafts to and from signaling domains. Because of the key roles played by lipid rafts in coupling local molecular organizations to signaling at membranes, the results of these studies will likely be of major importance to a mechanistic understanding of growth cone guidance, synapse assembly and circuit plasticity.

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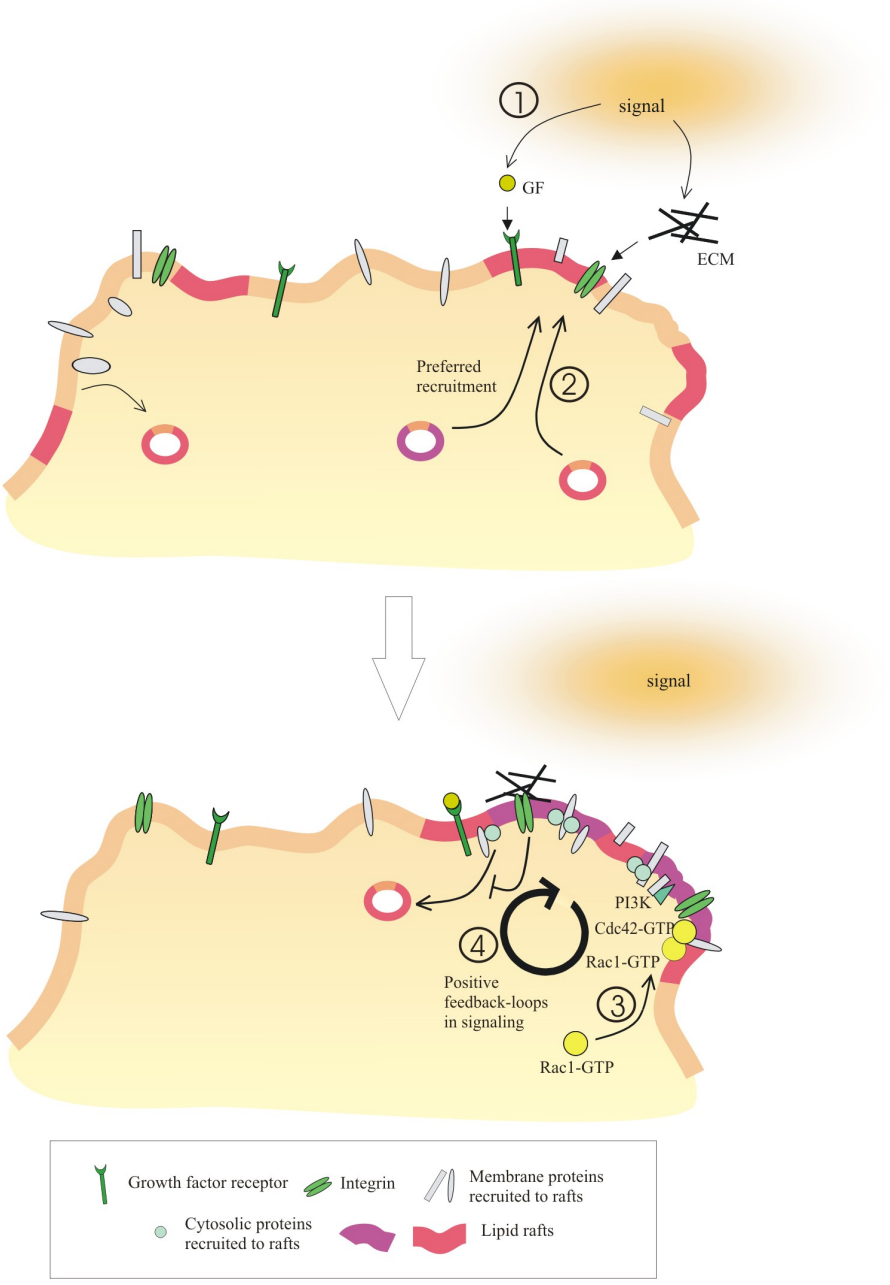
2.1.4. Figures and figure legends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Positive
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in signaling
3
Rac1-GTP
Growth factor receptor
Integrin
Membrane proteins
recruited to rafts
Cytosolic proteins
recruited to rafts
Lipid rafts

Figure 1: **Spatial control of signaling through rafts**.
Local extracellular signals (1) induce a preferential local recruitment and accumulation of lipid rafts to the cell surface (2) through integrin and/or growth factor receptors, specifying sites for local assembly of signaling components at raft-based domains. As one critical components for further downstream signaling, activated Rac1-GTP targets to the lipid raft domains (3), initiating local signaling to the actin cytoskeleton and to downstream effectors such as FAK. The sequential recruitment and assembly of signaling components to the raft-based domains promotes reverberating positive feedback loops (4), to amplify and sustain polarized signaling.

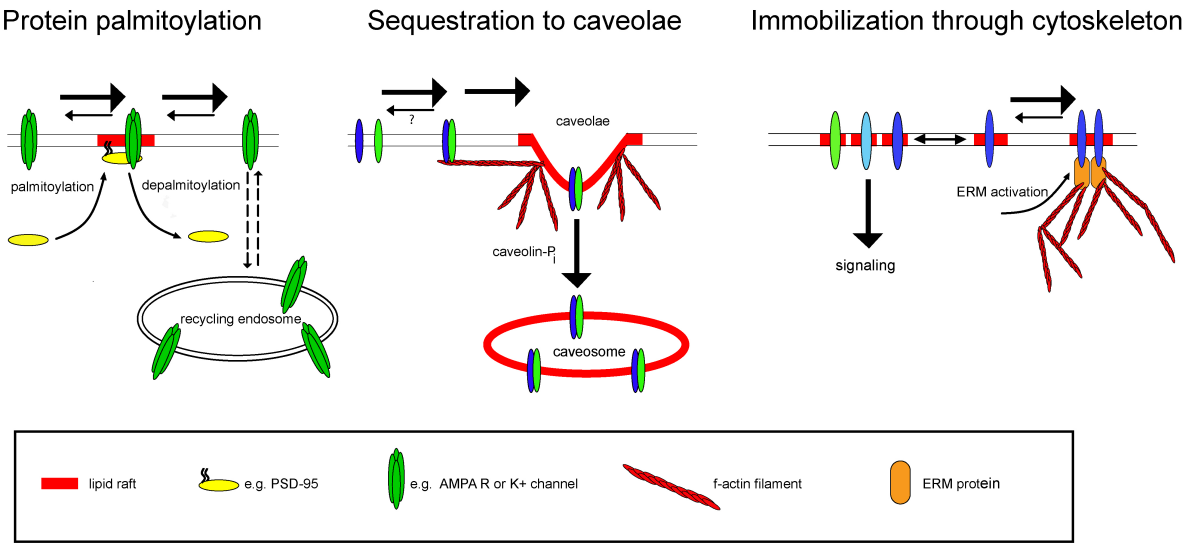

Figure 2: **Dynamics of signaling components in and out of raft domains**.

Left: In addition to direct regulation of raft association through palmitoylation, the residence time of raft-associated proteins such as AMPA receptors at rafts can be prolonged through binding to dually palmitoylated proteins such as PSD-95. Upon depalmitoylation, proteins dissociate from raft domains, and can be more readily internalized.
Center: Caveolae are specialized and comparatively stable lipid raft domains at the plasma membrane. Sequestration of proteins into caveolae (e.g. upon oligomerization and actin-dependent lateral translocation) removes them from active signaling pools. Signal-induced internalization to caveosomes persistently removes signaling components from the cell surface.
Right: Raft proteins can be prevented from interacting with raft domains by immobilizing them through cytoskeletal anchorage. For example, signaling can activate ERM proteins to bind to specific raft proteins and stably anchor them to the cortical actin cytoskeleton.

2.2. PIP-dependent microdomain assemblies capture microtubules to promote and control leading edge motility

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2.2.1. Introduction

Regulated motility at the cell surface mediates local interactions with the cell environment, cell polarization and oriented migration processes. Cell responses based on surface motility involve the regulation of actin dynamics (Pollard and Borisy, 2003). In addition, microtubules (MTs) play a decisive role in polarizing motility, and defining the specific positions along the cell surface where motility directs cell organization and behavior (Rodriguez et al., 2003; Gundersen et al., 2004). The sites and mechanisms through which MTs are captured at specific positions along the cell surface are thus of critical importance to organized motility and cell polarity. The lipid second messenger $PI(4,5)P_2$ is an attractive candidate to integrate signaling and coordinate actin and membrane dynamics in motility. Thus, $PI(4,5)P_2$ is concentrated at inner leaflet cholesterol-dependent lipid microdomains (rafts), which can accumulate locally to amplify signaling. Furthermore, $PI(4,5)P_2$ accumulates at sites of cell surface motility, and can modulate both actin dynamics and the assembly of membrane-associated protein coats mediating morphogenesis and membrane trafficking (Tall et al., 2000; Botelho et al., 2000; Rozelle et al., 2000; Martin, 2001; Yin and Janmey, 2003; Huang et al., 2004). These observations have raised the possibility that protrusive motility at the cell surface may be regulated through the local accumulation of raft domains enriched in $PI(4,5)P_2$ (Caroni, 2001; Yin and Janmey, 2003). However, whether and how rafts do accumulate locally has remained a controversial issue, and the role of $PI(4,5)_2$-rich rafts in regulating cell surface motility is not clear.

Plasmalemmal rafts are in principle well suited to play major roles in regulating motility at the cell surface (Golub et al., 2004). Thus, among the molecular components involved in actin cytoskeleton regulation, transmembrane proteins associated with rafts include receptor tyrosine kinases and activated integrins, and components associated with inner leaflet rafts include Rho-type GTPases, activated N-WASP, src-like kinases, ERM proteins, PI5-kinase, and $PI(4,5)P_2$ (e.g. Martin, 2001; del Pozo et al., 2004). MT-

dependent functions linked to cell surface motility also depend on raft integrity. Thus: 1) raft integrity is critically important to polarize cells (Pierini et al., 2003); 2) chemotacting cells accumulate and require distinct types of rafts and raft-associated signaling components at their leading and trailing edge (Gomez-Mouton et al., 2004); 3) neuronal growth cones polarize raft components during steering, and this polarization is essential for growth cone guidance (Guirland et al., 2004). Furthermore, two recent studies have provided evidence that sites of MT accumulation at the cell surface coincide with regions of the plasmamembrane enriched in raft markers (Pardo and Nurse, 2003; Palazzo et al., 2004). It thus seems that cell surface sites enriched in rafts might coincide with sites where MTs interact with the cell membrane, but the mechanisms linking cell surface rafts to MT capture and organized motility at the cell surface are not clear.

Members of the Rho-type family of small GTPases, key molecular switches linking cell surface signaling to the regulation of actin dynamics, play major roles in regulating cell motility (Etienne-Manneville and Hall, 2002). In addition, Rho-type GTPases regulate processes that shape cell dynamics, such as the assembly and dynamics of focal contact sites (Small and Kaverina, 2003), and the dynamics of microtubules (Rodriguez et al., 2003). Rho-type GTPases may thus promote the assembly of specific signaling complexes, possibly including PI(4,5)P_2-rich rafts, to link local signaling to actin-based motility and cell organization.

Here we investigated whether and how PI(4,5)P_2-rich rafts accumulate and organize to influence protrusive motility at the cell surface. We show that signals triggering lamellipodial motility at the cell edge, induce a rapid local accumulation of dynamic cholesterol- and PI(4,5)P_2-rich raft-based plasmalemmal domains with unique turnover properties for acylated raft components and PI(4,5)P_2. We further show that the patches capture and stabilize MT plus ends through patch-associated IQGAP1. MTs in turn promote the clustering of raft patches into spatially focused and temporally stable domains, restraining and polarizing motility. Taken together, our results suggest a two-step model for local control of motility and polarization in which: 1) local signaling at the cell surface induces raft patching through PI(4,5)P_2 and Cdc42 to promote motility; 2)

clustering of the patches into more stable platforms through MTs and PKA polarizes and organizes motility. As there are extensive similarities among the molecular requirements for processes involving sustained polarized signaling at the cell surface, these principles for local control of signaling and polarization through raft assembly and organization likely apply to further cellular processes.

2.2.2. Materials and methods

Reagents

Expression plasmids used in this study were kind gifts from the following sources: PLCδ 1-PH-GFP,

E. Tall, Stony Brook; cortactin-GFP, H.B. Peng, Hong Kong; synaptojanin, P. De Camilli, Yale; PI5-kinase, L. Machesky, Birmingham; dn-N-WASP, S. Lommel and T.E. Stradal, Braunschweig; dn-Cdc42 (HA-tag), dn-Rac (myc-tag), dn-Rho (myc-tag), A. Hall, London. The raft-targeted double-palmitoylation constructs (GFP or RFP (i.e. tetrameric dsRed) included the first 40 amino acids of GAP43, as described (Laux et al., 2000). DiD (DiD oil; $DiC_{18}(5)$ oil) was from Molecular Probes (working concentration: 10 μM). Inhibitor compounds and growth factors, with their final concentrations, were used as follows: cytochalasin D (2 μM, 30") (Sigma) , methyl-ß-cyclodextrin (5 mM, 10'-90') (Sigma), U73122 (1 μM, 20') (Sigma), nocodazole (10 μM, 5'), Rp-cAMP (100 μM, 30') (Biolog) , Sp-cAMP (50 μM, 30') (Biolog), Wortmannin (100 nM, 1hr) (Sigma), PDGF (100 ng/ml, 5') (Sigma), NGF (100ng/ml, 30" - 30') (Invitrogen), Bradykinin (20 mM, 15') (Sigma), Neomycin (10 mM) (Sigma). Signal PIP™ Kit was from Echelon Biosciences. Antibodies and fluorescent reagents were from the following sources: FLAG, anti-vinculin (Sigma); Arp3, IQGAP1 (Santa Cruz); cortactin (Upstate Biotech.); Dynamin II (BD transduction laboratories*)*; Tyr-tubulin, Ac-tubulin (Sigma); PKA RII (Upstate Biotech.); myc (Cell signaling); HA (Roche); alexa fluor 488 phalloidin (Molecular Probes); filipin (Fluka). The monoclonal antibody against PIP_2 was from K. Fukami, as described (Laux et al., 2000). Anti-p75 was a kind gift from Y. Barde, Basel;

the antibody against APC was a kind gift from I. Naethke, Dundee. All secondary antibodies were from Molecular Probes. All secondary antibodies were from Molecular Probes.

Cell culture and immunocytochemistry

Cell lines (COS-7, Swiss 3T3 and NIH 3T3, PC12B-GAP43 and PC12B-GAP43(ΔED) (Laux et al., 2000)) were cultured in DME, with 10% FCS and 10% HS (Gibco). Tissue culture dishes were coated with collagen (30μg/ml, Sigma). For replating experiments, cells were removed from semi-confluent dishes, and fixed 1hr after replating. For transient transfections, cells were treated with Lipofectamine 2000 reagent (Invitrogen), and analyzed 18-48 h later.

For carrier-mediated application of $PI(4,5)P_2$ to cultured cells, long chain synthetic phospholipids ($PI(4,5)P_2$ di C_{16}, final concentration 300 μM; Echelon) were resuspended in 4mM KCl, 150mM NaCl and 20mM Hepes, pH 7.2. Histone carrier H1 was resuspended in the same buffer, at a concentration of 100 μM. Carrier-phospholipid complex was formed by co-incubating phospholipid and carrier at room temperature for 10 minutes, followed by 10-fold dilution in DME with 10%HS and 5%FCS- DME. Cells were exposed to this $PI(4,5)P_2$-histone complex for 20 minutes, and then imaged. In control experiments, uptake and lamellipodial accumulation of carrier were verified with a fluorescent reagent ($PI(4,5)P_2$ C_6-NBD, C_{16}, Echelon).

To visualize cholesterol and raft markers, cells were fixed for 20 min at 37°C in 4% PFA, 0.4mM $CaCl_2$, 50mM sucrose, 100mM NaH_2PO_4, washed, and then incubated with antibody solution. For $PI(4,5)P_2$ stainings, cells were fixed in 4% PFA in DME with 2mM EGTA (30', 37°C), followed by 5 hr at 4°C. First antibody incubations were over-night at 4°C, in PBS, 0.2% saponin, 50mM glycine, 0.1% BSA, 1% FCS. In similar experiments, permeabilization of fixed cells at room temperature with 0.1% Triton-X100 instead of saponin led to a loss of PHδ1-GFP, raft markers and f-actin signal from most cell surface patches (not shown), suggesting that at room temperature these membrane domains are particularly sensitive to detergent extraction. Images were obtained and processed using a Nikon epifluorescence microscope (100x, oil immersion) and Act-1 software, or with an Olympus confocal microscope (63x, oil) and Fluoview software. All

experiments were carried out at least five times independently, and representative examples are shown in the figures.

For the analysis of Triton-insoluble fractions (Ledesma et al., 1998), cells were grown to 80% confluency, washed 3x with PBS, and scrapped from the culture dish in 1ml of 25mM TrisCl pH 7.5, 150mM NaCl, 5mM EDTA, protease inhibitor cocktail (Roche), and 1% Triton-X114. After homogenization (glass homogenizer, 50 strokes), cells were left for 1hr on ice, and centrifuged at 15'000g (15 min, 4°C). Pellet and supernatant were collected separately, equal amounts of protein were loaded on 10% SDS/PAGE gels, and protein contents visualized on immunoblots. In separate experiments, cells were homogenized as described above, homogenates made to 40% sucrose (with an 80% sucrose solution; 2ml total), and overlaid with 30% sucrose (3ml), 20% sucrose (3ml), 10% sucrose (3ml) and buffer (2ml). After centrifugation, (70'000g, 20h, 4°C), a raft band (R) was collected at the 10/20% sucrose interface, and the majority of membranes (M) was collected as a pellet.

Oligonucleotides for RNAi of rat IQGAP1 were 5'-AAGGGTGATAATGCTCACC-3', and 5'-AATGAGAGACTCACGGCAT-3' (synthesized by Ambion, UK). Cells were transfected with (or without; mock transfection) 20 nM of siRNA and 4 µl of Lipofectamine 2000 reagent in 35 mm dishes, with 0.9 ml of OptiMEM. After 4hr of incubation at 37°C, cells were switched back to growth medium (10%HS+5%FCS, DMEM), and analyzed 48hr later. Transfection efficiencies were 50-60%, and residual IQGAP1 signals were 0-20% of control.

Live imaging

Cells were imaged with a Zeiss Axioskop, a water immersion objective (Achroplan 100X/1.0W), filters to reduce fluorescent light, and a Hamamatsu digital CCD camera (C4742-95), controlled by QED Camera Plug-in for Power Mac G4 (QED Imaging Inc., Pittsburgh, PA). Images were acquired every 10 seconds, and imaging sessions ranged from 6-20 min. For live imaging, cells were kept at 37°C (heated microscope stage), in Tyroid's imaging buffer (2.68mM KCl, 0.5mM $MgCl_2$, 137mM NaCl, 0.36mM NaH_2PO_4, 5.5mM glucose, 1.8mM $CaCl_2$). For DiD imaging, cells were preincubated in growth medium with 10 µM DiD for 5 min, washed three times with PBS and imaged. Where

appropriate (e.g. non-fluorescent transgenes), the expression of transgenes in imaged cells was subsequently verified by immunocytochemistry. In control experiments, cells were reexamined 1-6 h after imaging, and exhibited no obvious signs of phototoxicity (e.g. blebbing).

A Zeiss confocal microscope (LM 510 meta, Axioplan2) was used to acquire live images (512x512 resolution; 8-bit images; 63x, N_A=0.95 objective) of double-labeled cells (DiI/PHδ1-GFP; ppRFP/ PHδ1-GFP) and for FRAP experiments. An Argon2 laser was used to excite GFP constructs (1% intensity); RFP and DiI constructs: HeNe1 laser (24% intensity). Two-colors acquisition was carried out using multi-tracking. Emission filters: BP505-530 (488nm excitation); LP560 (543nm excitation). During imaging, cells were kept in a microscope chamber at 37ºC. Step sizes for Z-sectioning were 0.5 μm (optical slices <2.3 μm).

Consistent with the notion that PHδ1-GFP predominantly visualizes raft-associated $PI(4,5)P_2$, PHδ1-GFP and ppgFP constructs yielded comparable results (not shown). In control experiments, DiD did not highlight comparable structures associated with ruffling lamellipods in NGF-treated PC12 cells (Suppl. Video 5), validating the use of PHδ1-GFP and ppGFP to visualize $PI(4,5)P_2$-rich raft patches associated with actin-based motility in living cells.

FRAP analysis

For FRAP measurements, the confocal pinhole was set at 1 Airy unit, and photobleaching was performed using 100 It (Argon2 laser; 100% intensity; rectangular regions of 20-30x20-30 pixels). Fluorescence recoveries and bulk photobleaching during the time series were analyzed using LSM software (version 2.5 SP2, Carl Zeiss Microimaging, Inc.). Images were acquired at intervals of 3.4-4.0 sec. All intensity values were corrected for bulk photobleaching caused by scanning. For FRAP of raft patches, structures with areas of 0.2-0.5 μm^2 (untreated cells), 1.5-2.5 μm^2 (+NGF), 0.5-1 μm^2 (nocodazole or Rp-cAMP, +NGF), or 3-4 μm^2 (Sp-cAMPS, +NGF) were analyzed.

The comparatively slow FRAP rates of ppGFP at $PI(4,5)P_2$-rich raft patches did not correlate with the relative intensities of the ppGFP signal at the patches (not shown).

Quantitative analysis

All data to be compared were acquired with identical camera settings, and images were analyzed quantitatively using Image-Pro 5 software (Media Cybernetics). All isolated cells from randomly selected fields (100x objectives) were analyzed. To compare intensities (arbitrary units) or define threshold values for patches, sets of data were calibrated by setting individual zero (no cells) and background fluorescence values inside cells (fixed cells: 50; PH-GFP: 80). Co-localization of antigen labeling with f-actin or raft markers (GAP43 unless stated otherwise) was assessed using the count/size function, and computing Pearson's correlation coefficients. Thresholds were set to include all clearly defined structures (values of 20-30 above background fluorescence). For Pearson's coefficients to f-actin patches, background values were defined at 120, to restrict the sample to lamellipodial f-actin. Where specified (edge), only structures within 2.5 μm of the cell edge were included. Images of double-labeled cells were then compared electronically. The Pearson's correlation coefficient (1=highest value, 0=lowest value) is a measure of the linear association between two variables.

For patch size analysis, all patches within 2.5x8 μm masks were analyzed. Where specified (edge), masks were placed with their long axis tangential to cell edge lamellipodia. Patches smaller than 0.2 μm^2 were considered as background, and excluded when deriving values for patch numbers and average patch size. Data from cells derived from 3-5 independent experiments were pooled and medial values per unit area were calculated. To derive labeling intensity ratios, intensities at the brightest patches or lamellipods of a given cell were related to average values at the corresponding structures of the same cell. Average MT to cell edge counts are given per cell, and only include MTs apparently ending at the cell edge (usually with bright tubulin heads).

For the analysis of fluorescent time-lapse recordings, individual cell edge patches were followed for at least 5 min, and data pooled for individual time points after the addition of NGF. Patch boundaries exhibited thresholds of at least 30 units over surrounding background values (a background value of 80 was subtracted from all patch intensity values). Both, edge patch intensity and area values reflect averages at individual patches.

For the analysis of phase-contrast time-lapse recordings, images from individual time frames were aligned, and entire cell edge regions not contacting neighboring cells were

analyzed. Occasional cells that did not respond to NGF with lamellipodial motility (less than 10-15% of total) were excluded from the analysis. Cell outlines from 20 sec time intervals were traced and overlaid using fiduciary marks, defining individual "difference cell edge areas" that had either extended or retracted (mean area values). For average motility values, the areas were all summed (irrespective of whether they represented advances or retractions), and normalized to 15 sec intervals, and total cell outline lengths (μm/15 sec). Average motility values were derived from sets of such comparisons within defined one-minute intervals, as indicated. Net/total motility ratios (100%) were derived by calculating individual net advance or retraction areas (the sum of all advances, minus the sum of all retractions) per unit time and particular cell edge region, and dividing these values by the total value of corresponding advance and retraction areas.

2.2.3. Results

PI(4,5)P_2-rich raft patches reflect distinct diffusion domains at the cell surface

To investigate how lipid rafts relate to protrusive motility at the cell surface, we stained quiescent, replated or PDGF-treated cells for the raft-associated components cholesterol (filipin), GAP43 and PI(4,5)P_2. While quiescent cells did not exhibit obvious sites of cell surface raft concentrations, raft components in replated cells were concentrated in patches at cell surface ruffling lamellae, where they co-distributed with intense labeling for f-actin (Suppl. Fig. 1). Significantly, while cells attached and spread to a comparable extent in the absence or presence of cyclodextrin, raft disruption led to a near to complete loss of lamellipods, and a dramatic reduction of ruffling motility (Suppl. Fig. 1; see also Grimmer et al, 2002).

To investigate the properties of raft accumulation sites associated with protrusive motility, we analyzed cells transfected with GFP (or RFP (tetrameric dsRed)) fusion proteins targeting to lipid rafts. For the purpose of this study, PC12 cells cultured on a

collagen substrate provided a particularly favorable experimental system, due to the comparatively large size of their raft patches. We found that a PLCδ1-PH-GFP construct (PHδ1-GFP) specifically targeting to plasmalemmal $PI(4,5)P_2$ (Tall et al., 2000), and an RFP construct targeted to the cytosolic face of cell surface rafts through a double-palmitoylation motif (ppRFP) co-localized at prominent patches near the cell edge of living PC12 cells (Fig. 1A). In contrast, when surface and intracellular membranes in the same living PC12 cells were labeled with the lipophilic dye DiD, which distributes homogeneously throughout the lipid phase of cellular membranes, no accentuation of the DiD signal was detected at cell surface sites highlighted by the raft markers (Fig. 1A). Furthermore, live imaging with PHδ1-GFP, followed by fixation, permeabilization with saponin, and staining for raft markers yielded closely comparable labeling patterns before and after fixation (Fig. 1B). We concluded that the patches reflect sites of $PI(4,5)P_2$ and raft marker accumulation at the surface of living cells.

The association of cell surface proteins and lipids with lipid microdomains does not by itself affect their diffusion rates in the membrane as determined in FRAP experiments (Kenworthy et al., 2004). To determine whether $PI(4,5)P_2$-rich raft patches might reflect plasmalemmal domains distinct from surrounding membranes at the cell surface, we carried out a FRAP analysis in PC12 cells expressing ppGFP. FRAP of ppGFP at homogeneously labeled ventral plasma membrane facing the substrate yielded rapid recovery rates comparable to published values, and edge-to-center gradients of fluorescence recovery consistent with lateral diffusion (Fig. 1C). Similar rapid recovery rates were detected for dorsal surfaces outside patch areas (not shown). In marked contrast, FRAP at dorsal (brightly labeled) patches was consistently slower (about 3-fold), and recovery patterns exhibited no evidence for edge-to-center gradients (Fig. 1C). Similar slow FRAP values were obtained for raft-accumulating substrate facing podosomes, i.e. well defined structures at the plasma membrane (Fig. 1C). We concluded that $PI(4,5)P_2$-rich raft patches associated with protrusive motility reflect membrane compartments with distinct dynamic properties at the cell surface.

A rapid local accumulation of PI(4,5)P_2-rich raft patches at the cell edge anticipates protrusive motility

To investigate how the redistribution of cell surface raft patches relates to protrusive motility, we analyzed the short-term responses of PC12 cells to NGF. In the absence of NGF, PC12 cells exhibited a near to complete absence of protrusive motility at the cell edge (Suppl. Video 1). Upon the addition of NGF, phase-contrast time-lapse recordings revealed substantial protrusive motility, which started 45-60 sec upon the addition of the growth factor, peaked at 4-8 min, and subsided after 15-20 min in the presence of NGF (Fig. 2A; Suppl. Video 2). Pretreatment of the cultures with 5 mM cyclodextrin, suppressed most NGF-induced protrusive motility, except for a brief phase of thin lamellae and spike extension at the cell edge (Fig. 2A; Suppl. Video 3).

Consistent with a critical role of rafts in promoting ruffling motility, NGF induced a rapid redistribution and gradual accumulation of PI(4,5)P_2-rich raft patches along the edge of PC12 cells (Figs. 2B-D). The appearance of numerous, initially small raft patches at the cell edge was detectable from 20 sec on, and preceded NGF-induced loss of stress fibers and focal adhesions (i.e. a process preceding protrusive motility; Fig. 2D). Further raft patch accumulation accompanied the appearance (from 45-60 sec on) of intensely labeled f-actin structures (Fig. 2D) rich in Dynamin2, cortactin and Arp3 at these same sites (i.e. a process coinciding with ruffling motility; see Fig. 4B).

In the absence of NGF, cell surface PI(4,5)P_2-rich patches exhibited little dynamics and/or motility (PHδ1-GFP ; not shown). Within 20-40 sec upon the addition of NGF, new distinct PI(4,5)P_2-rich patches appeared on the surface of PC12 cells adjacent to the cell edge (Fig. 2E; Suppl. Video 4). Individual patches behaved as coherent and highly dynamic surfaces for periods of up to 5 minutes and more (Fig. 2E), and their positions correlated with those of protruding motility (> 90% of new patch sites exhibited lamellipodial motility within the next 3 min). Although the patterns of PHδ1-GFP patch motility did in part overlap with ruffling lamellipods, they did not directly reflect the distribution of motile lamellae. Thus, thin substrate-associated protruding

lamellae usually exhibited inverted (lowest at front) gradients of PHδ1-GFP signal, and raft marker signals were consistently low at the leading edge of thin protruding lamellae. We concluded that lamellipodial motility is preceded by a redistribution of cell surface rafts, and coincides with the accumulation of distinct $PI(4,5)P_2$-rich raft patch assemblies at sites exhibiting motility.

NGF enhances $PI(4,5)P_2$ turnover and reduces raft component exchange rates at cell surface raft patches

To explore the possibility that NGF might influence actin-based motility through the accumulation of $PI(4,5)P_2$ at leading edge raft patches, we carried out FRAP experiments in cells expressing PHδ1-GFP, in the absence and presence of NGF. In the absence of NGF, FRAP values for $PI(4,5)P_2$ at patch, non-patch and podosome regions of the plasmamembrane resembled those for the raft marker ppGFP (Fig. 2F). In contrast, NGF substantially accelerated $PI(4,5)P_2$ FRAP rates at raft patches, whereas it slightly slowed down corresponding FRAP values for ppGFP (Fig. 2F). Neither ppGFP nor PHδ 1-GFP FRAP rates outside raft patches or at podosomes were affected by NGF (Fig. 2F). The differences in PHδ1-GFP recovery rates at and outside patches, as well as inside patches in the absence or presence of NGF argued against the possibility that exchange rates with unbound cytosolic pools of PHδ1-GFP were the rate limiting factor in these experiments. Instead, our findings suggest that $PI(4,5)P_2$ levels at raft patches are primarily controlled through hydrolysis and synthesis rates of $PI(4,5)P_2$ at the patches, which are accelerated by NGF.

Cell surface raft patching depends on $PI(4,5)P_2$, Cdc42, and N-WASP

We next addressed the mechanisms underlying the accumulation of $PI(4,5)P_2$-rich raft patches associated with cell surface motility. To discriminate between mechanisms inducing motility itself, and specific requirements to induce raft patching, we first analyzed PC12 cells cultured in the absence of NGF, which exhibited well-defined raft patches on their dorsal surface. We found that overexpression of the $PI(4,5)P_2$

phosphatase synaptojanin (Cremona and De Camilli, 2001) or of dominant-negative (dn) Cdc42 abolished raft patching in quiescent cells, whereas dn-Rac and the PI3-kinase inhibitor Wortmannin (and dn-Rho, not shown) did not (Fig. 3A,B). Furthermore, a dn-N-WASP construct (Lommel et al., 2001) effectively suppressed cell surface raft patches in PC12 cells (Fig. 3A,B), and so did disruption of the actin cytoskeleton with cytochalasin (not shown).

Dn-Cdc42 or dn-N-WASP did not prevent the initial accumulation of PI(4,5)P_2 at the edge of NGF-treated cells, but PI(4,5)P_2-rich patches were abnormally small, weakly labeled and short-lived (Fig. 3B,C; Suppl. Video 6). In contrast, dn-Rac suppressed the appearance of new PI(4,5)P_2 signal, raft patches and motility in NGF-treated cells (Figs. 3B,C). A specific inhibitor of PI(4,5)P_2-hydrolizing PLC enzymes (U73122) failed to restore any PI(4,5)P_2 accumulation in the presence of dn-Rac, and inhibiting PI3-kinase did not suppress NGF-induced PI(4,5)P_2 accumulation and patching in the absence of dn-Rac (not shown), suggesting that it might be the failure to locally generate PI(4,5)P_2 in response to NGF that prevented raft patching in the presence of dn-Rac. In support of this interpretation, carrier-mediated delivery of PI(4,5)P_2 partially rescued NGF-induced raft patch accumulation in the presence of dn-Rac (not shown, but see Fig. 4E), and overexpressing synaptojanin suppressed NGF-induced actin remodeling and lamellipod formation in PC12 cells (not shown). We concluded that the sustained accumulation of PI(4,5)P_2-rich rafts into distinct patches depends on a specific mechanism involving PI(4,5)P_2, Cdc42, N-WASP and actin cytoskeleton integrity (Fig. 3D).

Raft patch accumulation is required to promote sustained protrusive motility at the leading edge

We next investigated how the accumulation of PI(4,5)P_2-rich rafts into patches might influence cell surface motility. As expected, NGF induced a redistribution of the actin cytoskeleton in PC12 cells, from a stress fiber pattern characteristic of quiescent cells to a prominent ruffling lamellipodial pattern, and this process depended on raft integrity (Fig. 4A). Furthermore, we detected a pronounced accumulation of Dynamin2,

cortactin, Arp3 and f-actin at raft patches along the cell edge in the presence of NGF (Fig. 4B,C). This accumulation was greatly reduced in cells overexpressing dn-Cdc42 or dn-N-WASP, and abolished in cells overexpressing Synaptojanin (Fig. 4C). Accordingly, phase-contrast time-lapse recordings revealed that in the presence of dn-Cdc42 or dn-N-WASP, both intensity and duration of NGF-induced cell surface motility were greatly reduced (Fig. 4D). Furthermore, carrier-mediated delivery of $PI(4,5)P_2$ induced spontaneous cell edge motility in the absence of NGF, and rescued some NGF-induced motility in the presence of dn-Rac (Fig. 4E). Taken together (see also Suppl. Material), these results suggest that the local accumulation of raft patches in a process requiring $PI(4,5)P_2$, Cdc42 and N-WASP enhances and sustains signaling for actin assembly and protrusive motility in NGF-treated cells.

Additional evidence that raft patch accumulation promotes protrusive motility

To provide further evidence relating the accumulation of cell surface raft patches to motility, we compared PC12 clones expressing wild-type GAP43, or a GAP43(ΔED) mutant lacking the basic activation domain (Laux et al., 2000). In a previous study, we had reported that GAP43(ΔED) also accumulates at rafts, but that cells expressing GAP43(ΔED) exhibit reduced spreading, and impaired neurite outgrowth in vitro and in vivo (Laux et al. 2000). We now find that GAP43(ΔED) interferes with both, raft patch accumulation at the surface, and cell surface motility in PC12 cells (Fig. 4F). We conclude that a local accumulation process of $PI(4,5)P_2$-rich raft patches plays a critical role in promoting and sustaining signaling for protrusive motility at the cell surface.

Capture and stabilization of microtubules at cell edge raft patches through IQGAP1

Although patch accumulation was required to promote sustained protrusive motility, and although sites where raft patches accumulated were predictive of where motility would develop along the cell edge, their actual positions did not directly coincide with those of motile lamellipods. This raised the issue of how patch and lamellipod

distributions might be related causally. To explore the possibility that this relation might involve MTs, we compared the distributions of raft patches and MTs near the cell edge. We found a striking co-distribution of MT ends with raft patches at the edge of quiescent and NGF-treated PC12 cells (Fig. 5A,B), where MTs were stabilized (Suppl. material, and Suppl. Fig. 2). Disruption of lipid rafts with cyclodextrin led to a near to complete loss of MTs targeting the cell edge (Fig. 5C), and overexpression of dn-Cdc42 (or dn-N-WASP, not shown), greatly reduced MT capture at the edge of these cells (Fig. 5D), suggesting that capture specifically depended on the presence of raft patches.

To investigate the mechanism through which MTs are captured at raft patches we first analyzed the distribution of proteins implicated in MT capture or targeting at the cell edge. We found that the MT-plus-end associated protein APC, which has been implicated in targeting of MTs to leading edges (Näthke et al., 1996), also co-distributed with cell edge raft patches (Suppl. Fig. 3). However, this association depended on both, raft and MT integrity, suggesting that APC is brought to the patches by MTs. We further found that IQGAP1 accumulated at the cell edge, where it co-distributed with raft patches (Fig. 6A). While the association of IQGAP1 with the cell edge depended on raft integrity, it was only partially affected by a disruption of MTs with nocodazole (Fig. 6A). To investigate whether the presence of IQGAP1 is required for MTs to be captured at cell edge patches, we carried out knockdown (RNAi) experiments for IQGAP1. We found that while mock-transfected cells continued to capture MTs at leading edge raft patches, down-regulation of IQGAP1 led to a near to complete loss of MTs targeting the leading edge in these cells (Fig. 6B, C). In addition, leading edge raft patches in the absence of IQGAP1 were strikingly small, and were not concentrated into a few major patches in NGF treated cells (Fig. 6D). We concluded that cell edge raft patches capture MT plus ends through IQGAP1 (Fig. 6E).

Stabilization of MTs captured at cell edge raft patches

It is well established that MTs are particularly unstable near cell edge regions exhibiting motility, where the activity of MT binding proteins such as CLASPs is

required for their stabilization (Rodriguez et al., 2003). The vast majority of MTs in PC12 cells were labeled with an antibody against tyrosinated tubulin (Tyr-tubulin), suggesting that they are comparatively dynamic (not shown). In support of this conclusion, nocodazole induced a rapid loss of cell edge MTs (see 30 sec nocodazole data, Suppl. Fig. 2C) in PC12 cells. However, even 5 min after the addition of nocodazole, we continued to detect some β-tubulin signal at cell edge raft patches (see Suppl. Fig. 4, and Fig. 6A), suggesting that MT plus-ends may be stabilized at these sites. Consistent with this interpretation, labeling with the Tyr-tubulin antibody did not highlight the distal heads of MTs near the cell edge, in the absence or presence of NGF (Suppl. Fig. 2A). In contrast, an acetylated tubulin antibody (Ac-tubulin) that binds to stabilized MTs, selectively decorated distal end stretches of MTs contacting cell edge raft patches, in the absence or presence of NGF (Suppl. Fig. 2A). In a second set of experiments aimed at providing evidence that MTs captured at $PI(4,5)P_2$- and raft-rich patches are relatively stable, we separated PC12 proteins into TritonX100-soluble (S) and -insoluble (P) fractions, and analyzed these fractions for raft, IQGAP1 and tubulin contents on immunoblots. We found that a majority of the raft-associated protein GAP43, and most IQGAP1 were recovered in the Triton-insoluble fraction (Suppl. Fig. 2B), and that acute treatments reducing the levels of surface $PI(4,5)P_2$ (Bradykinin or Neomycin; see Laux et al., 2000), and thus of $PI(4,5)P_2$-rich patches in PC12 cells, led to a redistribution of GAP43 and IQGAP1 to Triton-soluble fractions (Suppl. Fig. 2B). Significantly, a large fraction of Tyr-tubulin was soluble, whereas Ac-tubulin was strongly concentrated in the insoluble fraction, from which it was dislocated by Bradykinin or Neomycin to an extent comparable to that of the raft patch-associated proteins (Suppl. Fig. 2B). In a third type of experiments we analyzed the distribution of MTs in PC12 cells treated for brief periods with nocodazole. As shown in Suppl. Fig. 2C, while nocodazole induced a rapid and dramatic loss of MTs from the cell edge in these cells (30 sec nocodazole data), many MTs targeting raft patches were partially retained when nocodazole treatments were very brief (20 sec; arrows). We concluded that MTs captured at $PI(4,5)P_2$-rich raft patches near the cell edge are partially protected against depolymerization.

Raft patch clustering through microtubules is required to organize protrusive motility at the cell surface

To investigate how MTs might influence cell surface raft patches and NGF-induced motility, we analyzed PC12 cells treated with nocodazole. Treatments sufficient to disrupt most MTs in PC12 cells (3 min nocodazole), led to a marked fragmentation of raft patches in the absence of NGF (Fig. 7A). Furthermore, while some raft patches did accumulate at the cell edge in response to NGF, they failed to assemble in larger clusters and to coincide with intense phalloidin signals in the presence of nocodazole (Figs. 7A, B). Analysis of PI(4,5)P_2-rich patch dynamics in living NGF-treated cells revealed two consistent alterations in the absence of intact MTs: 1) a major impairment in PI(4,5)P_2-rich patch condensation and persistence; 2) rapid forward dissipation of PI(4,5)P_2-rich raft patches, coinciding with the emergence of rapidly advancing motile lamellae at the cell edge (Fig. 7C; Suppl. Video 7).

Phase-contrast time-lapse imaging revealed that NGF-treated cells with disrupted MTs consistently lacked coherent, spatially defined regions of sustained lamellipodial motility (i.e. regions exhibiting rapidly alternating forward and backward protrusive motility). This was reflected in the initial appearance of multiple small motile lamellae, followed by apparently unrestrained large-scale motility, leading to the formation of highly heterogeneous protrusions in these cells (Fig. 7D,E; Suppl. Video 8). Significantly, and in contrast to treatments interfering with raft patching, nocodazole did not affect the duration of NGF-induced motility, and only partially affected its intensity (Fig. 7D). These observations provide evidence that MT integrity is specifically required to promote the local concentration of PI(4,5)P_2-rich patches into few stable clusters, and to promote spatially focused and temporally stable ruffling motility at the cell edge.

Microtubules target PKA to cell edge raft patches, to promote clustering and organize motility in response to cAMP

In a search for mechanisms that might mediate the effects of MTs on leading edge raft clustering and the organization of cell surface motility, we focused on PKA, a protein kinase that has been implicated in regulating motility at the leading edge (e.g. O'Connor et al., 2001). We found that the regulatory subunit of PKA (RII) accumulated at leading edge PI(4,5)P_2-rich raft patches, where it co-distributed with MTs (Fig. 8A). Disruption of cell surface rafts with cyclodextrin induced a loss of RII from the cell edge (not shown). In addition, disruption of MTs led to a near to complete loss of RII from cell edge raft patches (Fig. 8A), suggesting that RII is delivered to these patches through MTs.

To investigate a possible involvement of PKA-mediated signaling in the local regulation of raft patch clustering and cell surface protrusive motility by MTs, we used membrane-permeable agonists (Sp-cAMPS), or antagonists (Rp-cAMPS) of PKA. Sp-cAMPS accelerated the appearance of PI(4,5)P_2 clusters at the cell edge in response to NGF, enhanced their compaction, and prolonged their half-lives (Figs. 8B,C). This was reflected in a sustained focusing and restriction of small-scale protrusive motility to a few sites along the cell edge (Suppl. Video 9; see also Fig. 8B,D). In contrast, Rp-cAMPS interfered with raft clustering, and reduced patch PI(4,5)P_2 labeling intensity and actin accumulation at lamellipods (Figs. 8B,C; Suppl. Video 10). This was reflected in a marked enhancement of unfocussed and uncontained large-scale motility (Fig. 8D). While the effects of Rp-cAMPS were clearly reminiscent of those induced by nocodazole, the cAMP antagonist did not affect the capture of MTs at cell edge raft patches (not shown). Consistent with the notion that Sp-cAMPS and Rp-cAMPS acted through PKA delivered to the leading edge by MTs, neither the agonist nor the antagonist affected raft assembly and leading edge motility in the presence of nocodazole (Fig. 8C,D). We conclude that one mechanism through which MTs organize motility at the leading edge involves the local delivery of PKA at raft patches, to promote their clustering.

<u>Microtubules and cAMP enhance the turnover of PI(4,5)P_2 and reduce that of a membrane-associated raft component at raft patches</u>

To investigate the mechanisms through which MTs and cAMP influence $PI(4,5)P_2$-rich raft clustering, we carried out FRAP experiments with PHδ1-GFP and ppGFP. Disruption of MTs led to a 2-fold reduction in the turnover rate of $PI(4,5)P_2$, and a more than 3-fold reduction in the turnover rate of ppGFP, specifically at patches (Fig. 9A-C). Inhibition of PLC enzymes (U73122, 1 μm) did not noticeably enhance $PI(4,5)P_2$ levels at patches in the presence of nocodazole (not shown), suggesting that the reduced $PI(4,5)P_2$ levels more likely reflect reduced local synthesis in the absence of intact MTs. As predicted, Rp-cAMPS mimicked the inhibitory effect of nocodazole on $PI(4,5)P_2$ FRAP, whereas Sp-cAMPS further accelerated the turnover rate of $PI(4,5)P_2$ at patches (Fig. 9A-C). Significantly, Rp-cAMPS did not mimic the effect of nocodazole on ppGFP FRAP rates at patches (Fig. 9C). Thus, while nocodazole greatly slowed down the turnover of ppGFP at patches, Rp-cAMPS accelerated it more than 2-fold (Fig. 9C). This result suggests that the turnover of ppGFP at patches involves MT-dependent trafficking of vesicles, and that the exchange of ppGFP through trafficking is inversely correlated to the degree of clustering of the patches. In support of this interpretation, Sp-cAMPS, which enhanced $PI(4,5)P_2$ patch clustering, led to a reduction in the turnover rate of ppGFP at patches (Fig. 9C). Taken together, these results suggest that MTs, PKA and ultimately cAMP levels, influence $PI(4,5)P_2$-rich patch clustering through their effects on patch $PI(4,5)P_2$ metabolism and levels, and that increases in patch clustering lead to corresponding reductions in the rates of raft trafficking to and from the clusters.

2.2.4. Discussion

We have investigated mechanisms of cell surface $PI(4,5)P_2$-rich raft accumulation associated with motility, and their role in regulating protrusive motility at the cell edge. We show that signals initiating protrusive lamellipodial activity trigger local raft patching processes depending on $PI(4,5)P_2$, Cdc42, and N-WASP, which lead to the accumulation

of distinct plasmalemmal domains rich in PI(4,5)P_2 and raft markers. We further show that these domains capture and stabilize MT plus ends through patch-associated IQGAP1. MTs in turn promote the clustering of raft patches into spatially focused and temporally stable domains, restraining and polarizing motility. In the following sections we discuss properties of these novel mechanisms, their proposed roles in organizing motility, and how these findings provide a framework to integrate related observations on how cell surface dynamics interfaces with cell polarity and organization.

PI(4,5)P_2-rich raft patches: distinct plasmalemmal domains associated with protrusive motility

We have provided evidence for the local accumulation of distinct PI(4,5)P_2- and raft-rich assemblies (patches) specifically associated with lamellipodial protrusive motility at the cell surface. These results provide an experimental paradigm to investigate how lipid rafts can contribute to local control of signaling at the cell surface. Thus, while the existence of cholesterol- and sphingolipid-enriched lipid microdomains, and their importance in cell trafficking and motility are well established (e.g. Wilson et al., 2004; Golub and Caroni, 2004), whether and in what ways lipid rafts can organize into higher order domains at defined sites on the cell surface had remained controversial. Our finding that FRAP values for ppGFP at patches were substantially slower than those detected outside the patches provides evidence that lateral diffusion into and out of the patches is restricted. That FRAP for ppGFP at patches was greatly reduced in the absence of intact microtubules, suggests that it might involve directed vesicle trafficking to and from the patches. Accordingly, in addition to accumulating raft-associated components promoting actin dynamics and cell signaling, PI(4,5)P_2-rich raft patches may provide distinct domains for raft-dependent trafficking at the cell surface. Such domains may, for example, be involved in the recycling and re-sensitization of cell surface receptors promoting signaling for motility.

PI(4,5)P_2- and Cdc42-dependent raft patching promotes sustained protrusive motility

Our studies provide evidence that the accumulation of rafts in patches at the cell surface depends on signals by Cdc42, $PI(4,5)P_2$, and N-WASP. This signaling mechanism ties in well with recent studies addressing mechanisms of cell motility regulation. Thus, integrin-dependent raft recruitment (del Pozo et al., 2004) may operate upstream of raft patching, and patching regulation could ensure tight spatial regulation of motility through Cdc42-recruiting complexes at the cell membrane, and tight dynamic regulation of motility through $PI(4,5)P_2$ synthesis and hydrolysis (Fig. 10A). Anchorage of activated N-WASP to raft-associated components may direct specific patterns of Arp2/3-mediated actin polymerization, to assemble membrane-associated actin scaffolds restricting the diffusion of signaling components. Although our experiments do not address directly the mechanism through which N-WASP promotes raft patching, we speculate that this might involve spatial containment through actin filament bundles, and adapter proteins such as LAT (in T-cells) and GAP43-like proteins (Golub and Caroni, 2004).

We find that raft patching is required to promote and sustain motility at the leading edge. This is consistent with reports that the disruption of rafts diminished responses such as membrane ruffling and pinocytosis, and prevented sustained activation of actin dynamics in neutrophils (Grimmer et al., 2002; Pierini et al., 2003). As $PI(4,5)P_2$-rich platforms assembled at regions of prospective motility, these results suggest that assembled rafts provide spatial domains of enhanced signaling for motility. The patches may also contribute to sustain signals for protrusive motility through recycling and trafficking of signaling components at patches (Huang et al., 2004). In addition, locally regulated dynamics of the patches might contribute to distinct forms of actin-based protrusive motility such as protrusive lamellae (Mouneimne et al., 2004) and ruffling lamellipods (Fig. 10A).

Mechanism of microtubule-dependent clustering of $PI(4,5)P_2$-dependent raft patches

Our results provide evidence that there is a specific association between MT plus ends and plasmalemmal $PI(4,5)P_2$-dependent raft patches. In addition, we find a requirement for patch IQGAP1 in the capture of MTs at raft patches. IQGAP1 might

specifically couple motility receptor activation and the recruitment of Rac-GTP and Cdc42-GTP to raft patches, to the capture of MTs at those raft patches (Fukata et al., 2002; Yamaoka-Tojo et al., 2004). The mechanisms through which MTs target to rafts at the cell edge could involve extension along actin filament bundles (e.g. Palazzo et al., 2004), and the selective regulation of MT dynamics near the cell edge (Rodriguez et al., 2003). The arrangement of MTs with respect to rafts would be consistent with a "pause" mode of interaction, in which MTs deliver components to defined sites at the cell edge, including PKA. Our results are reminiscent of findings that Cdc42 signaling defines sites of MT capture at the cell surface in yeast and in polarized astrocytes (Gundersen, 2002; Etienne-Manneville and Hall, 2003), and it will be interesting to determine whether cell surface raft assemblies are also involved in those experimental settings.

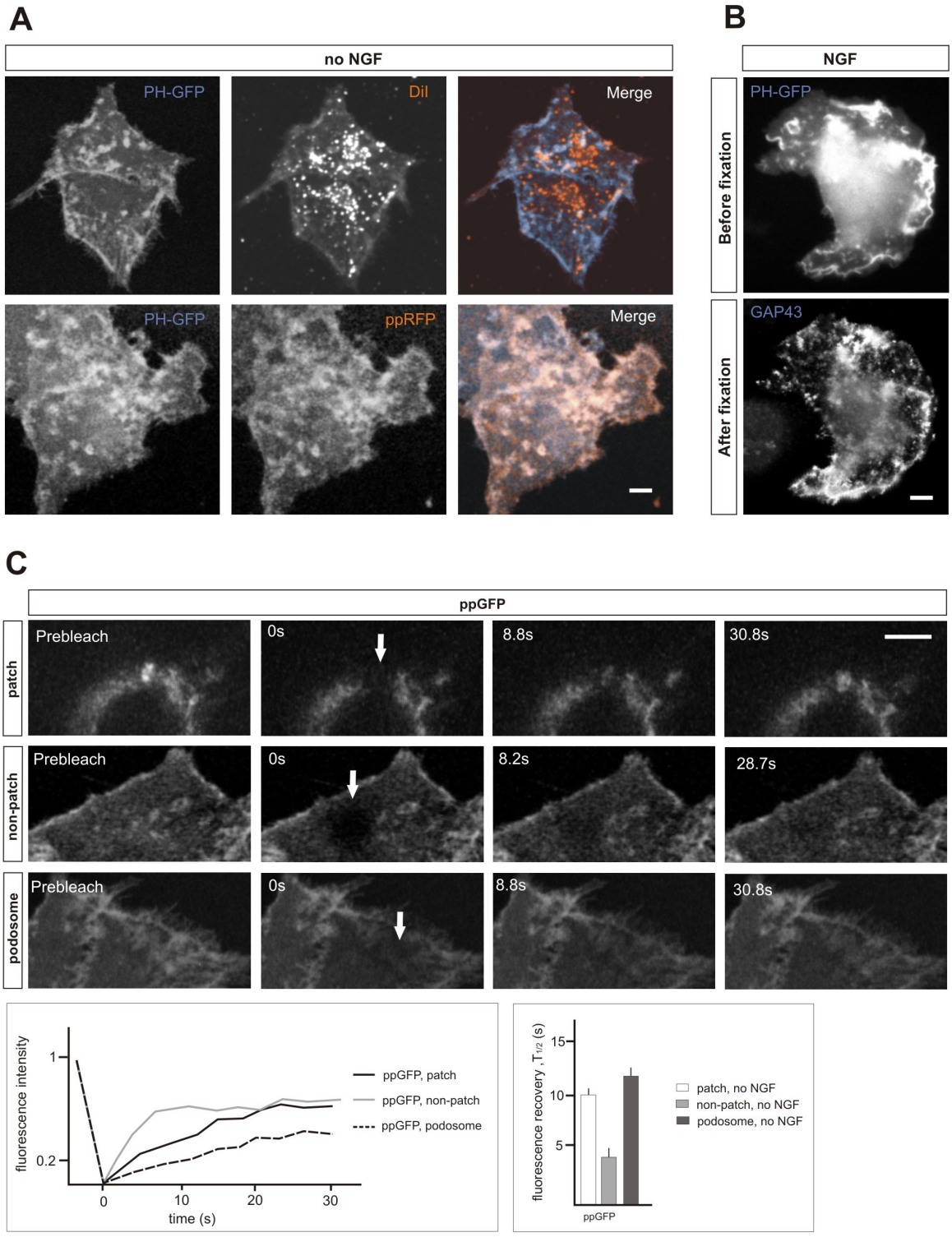
We show that the mechanisms through which MTs promote patch clustering at the leading edge include delivery of PKA, whose activity promoted clustering and focused motility. This provides a potential mechanism to couple local signaling to adenylate cyclases and phosphodiesterases to the organization and steering of motility at the leading edge. We further show that MTs and cAMP enhance the accumulation and turnover rates of $PI(4,5)P_2$ at patches, a process well correlated with the extent of patch clustering. Patch clustering through MTs and cAMP may thus involve enhanced $PI(4,5)P_2$ accumulation, to augment the anchorage of raft patches to the cortical cytoskeleton, reducing their lateral spread and dynamics (Raucher et al., 2000). Since PKA can activate Rac and inhibit Rho, we speculate that one possible mechanism may involve the activation of PI5-kinase downstream of MTs, PKA and Rac. Such a mechanism would be consistent with the observation that overexpressing PI5-kinase led to a dramatic condensation of cell edge raft patches, and a reduction of NGF-induced motility in these cells (not shown).

Organization of protrusive motility by microtubules through clustering of raft patches

This study provides novel evidence about the role of MTs in cell surface motility. Our results are in good agreement with previous reports that MT integrity is needed to

contain and steer motility (e.g. Gordon-Weeks, 2004; Guirland et al., 2004), and provide a candidate mechanism involving the clustering and spatial confinement of raft patches that promote motility. Taken together, our results suggest a model whereby moderate cell surface local $PI(4,5)P_2$ levels, together with Cdc42, drive the assembly of $PI(4,5)P_2$-dependent raft-based plasmalemmal domains promoting motility, whereas higher $PI(4,5)P_2$ levels at these domains, induced through MTs and PKA, reversibly enhance their compaction and anchorage to the cell cortex, restraining motility (Fig. 10B). This tight spatial and temporal regulation of motility through cell surface $PI(4,5)P_2$-rich raft patches and MTs could provide an exquisitely sensitive mechanism for directed cell migration and neuronal growth cone navigation. There are striking similarities between processes of cell surface domain patching and polarized clustering in cell motility, cytokinesis, and synapse formation. These include the spatio-temporal sequences of signaling platform assembly, and their molecular requirements (e.g. Weston et al., 2000; Wu et al., 2003). The mechanisms of raft-based patching and clustering reported in this study may therefore reflect general principles for local activation, polarization and organization at the cell surface.

2.2.5. Figures



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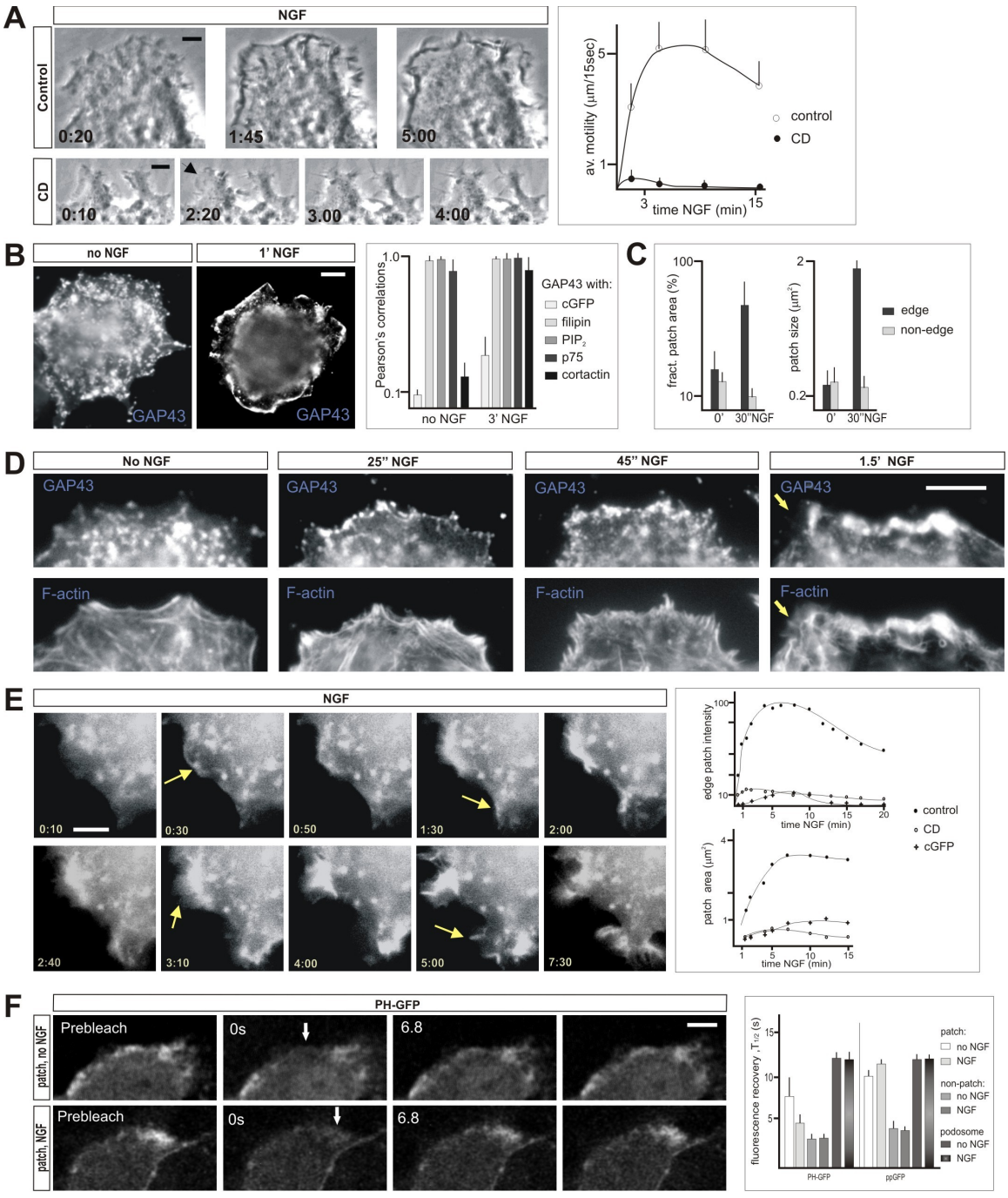

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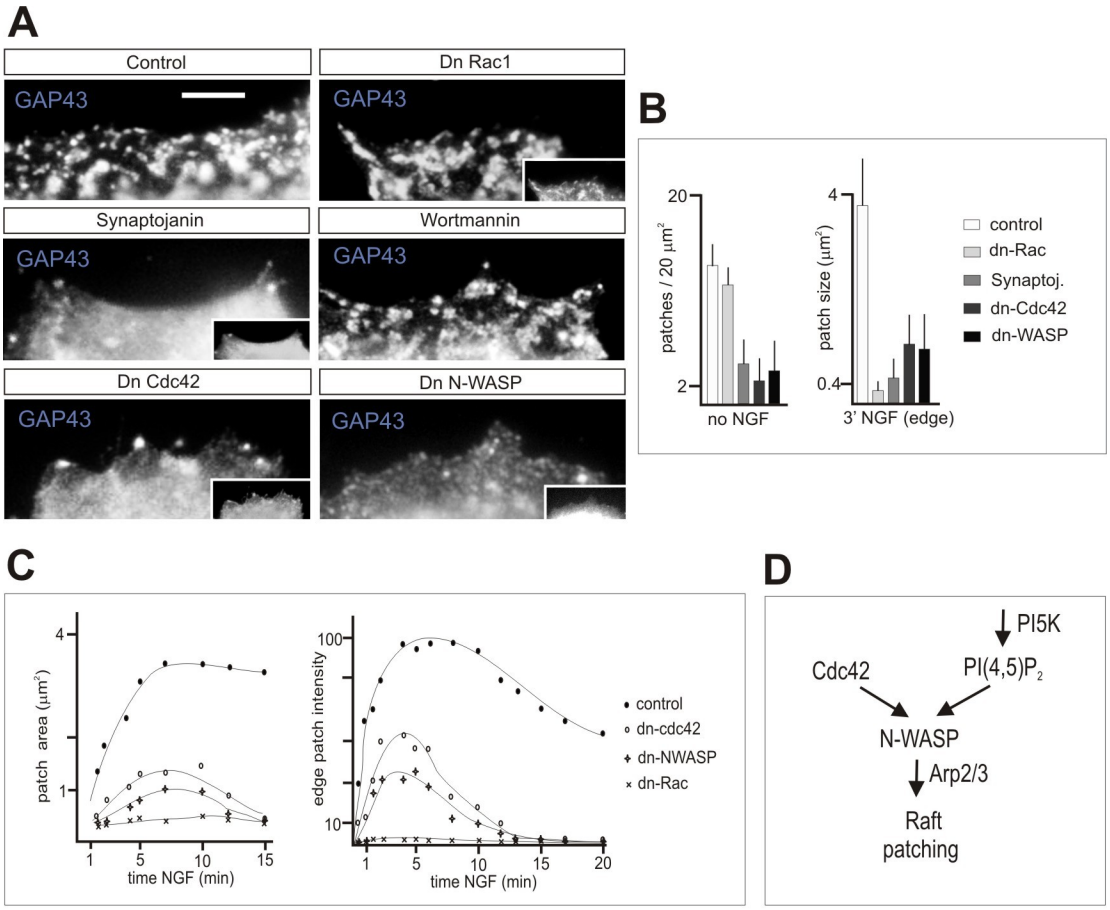

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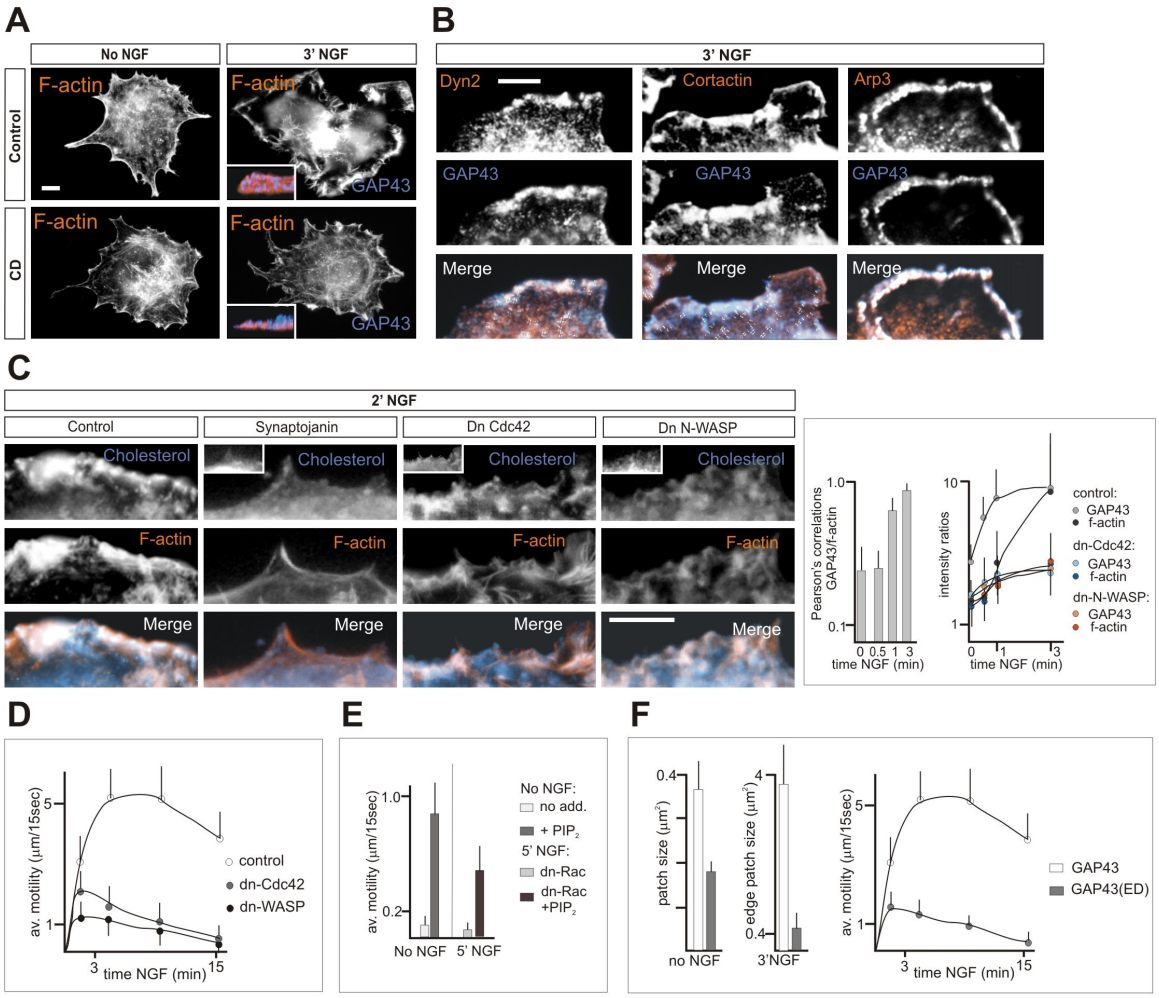

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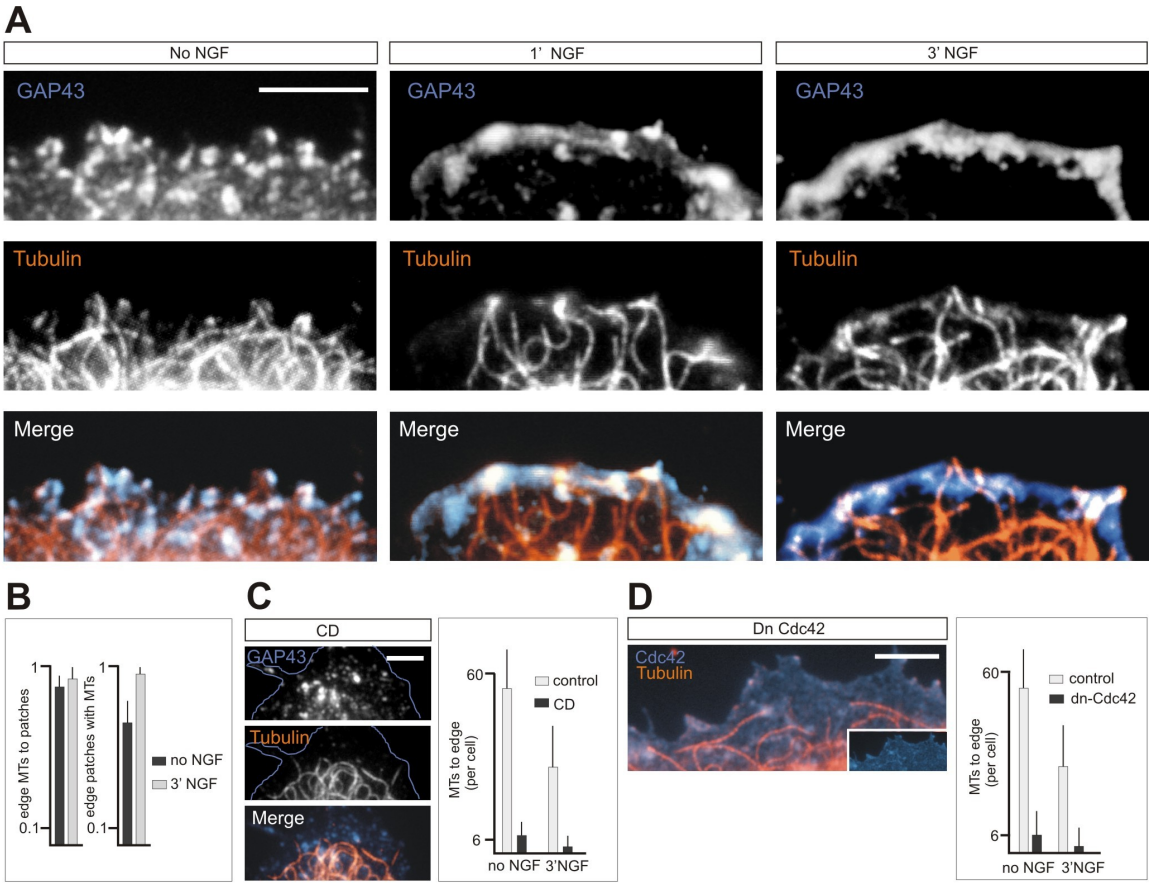

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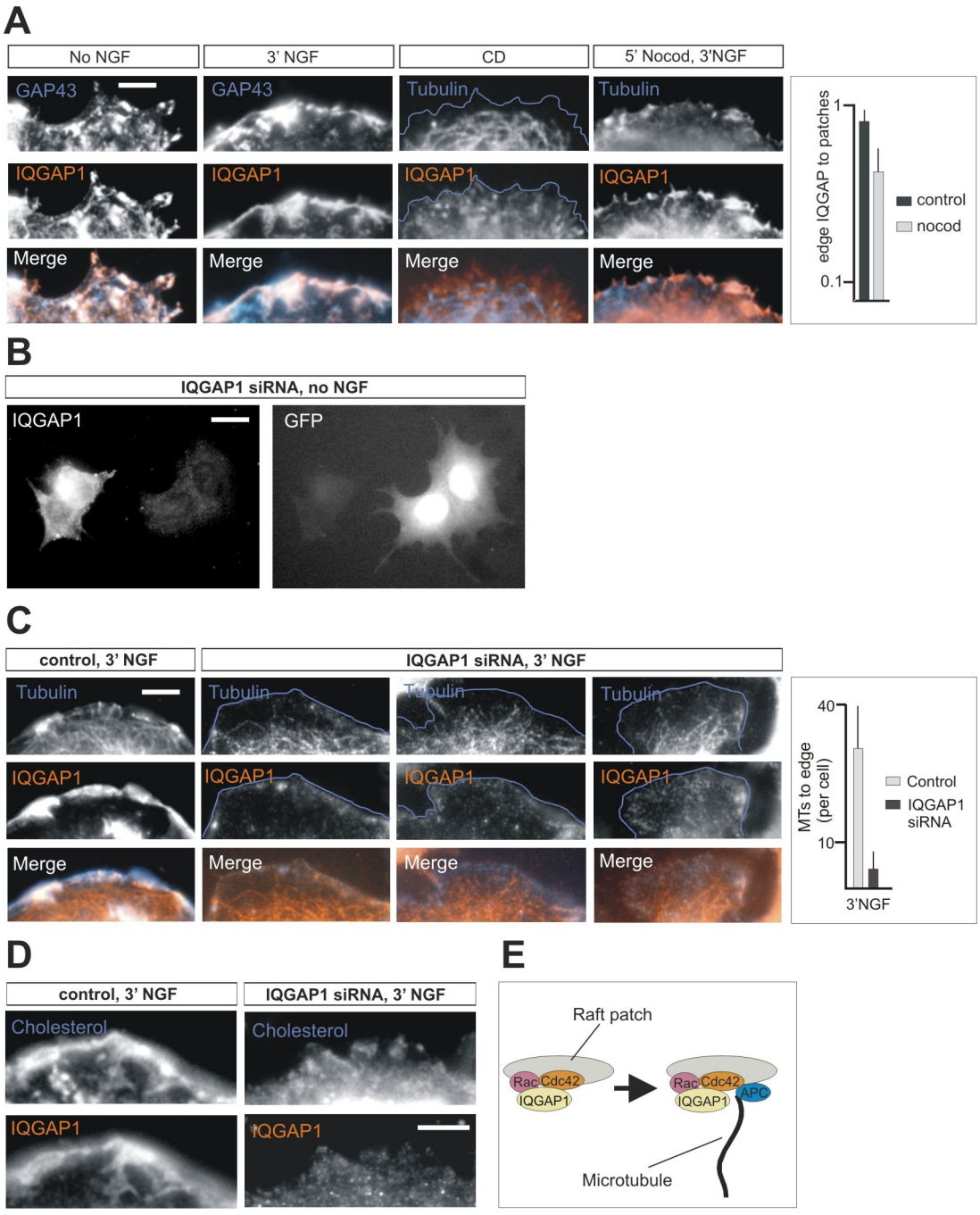
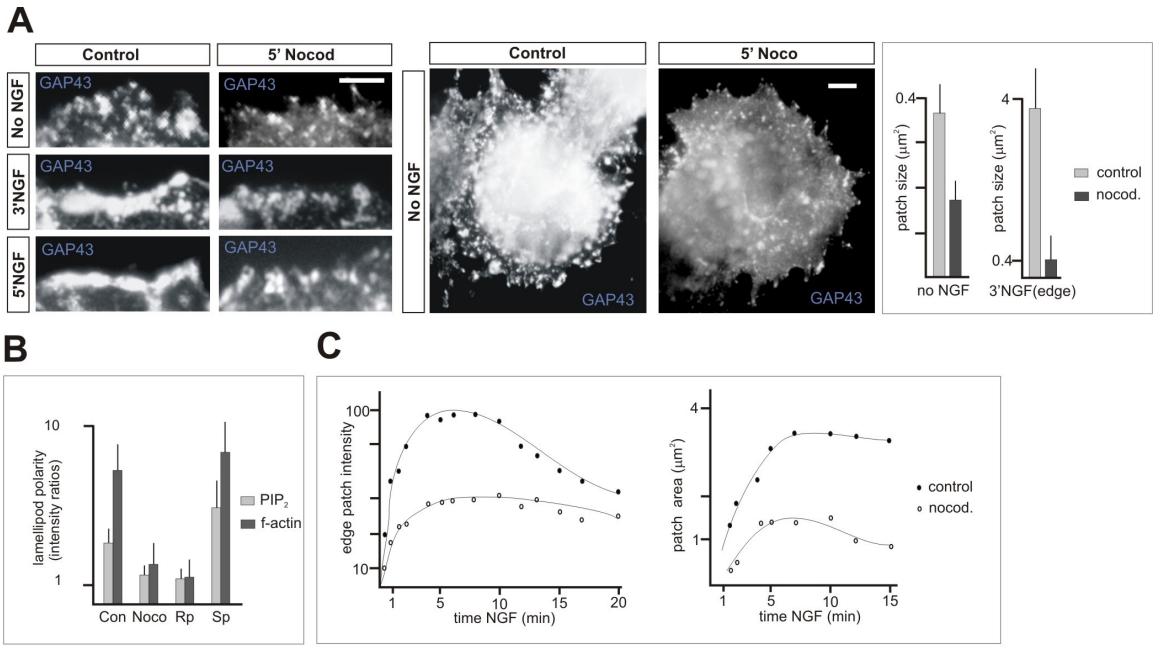
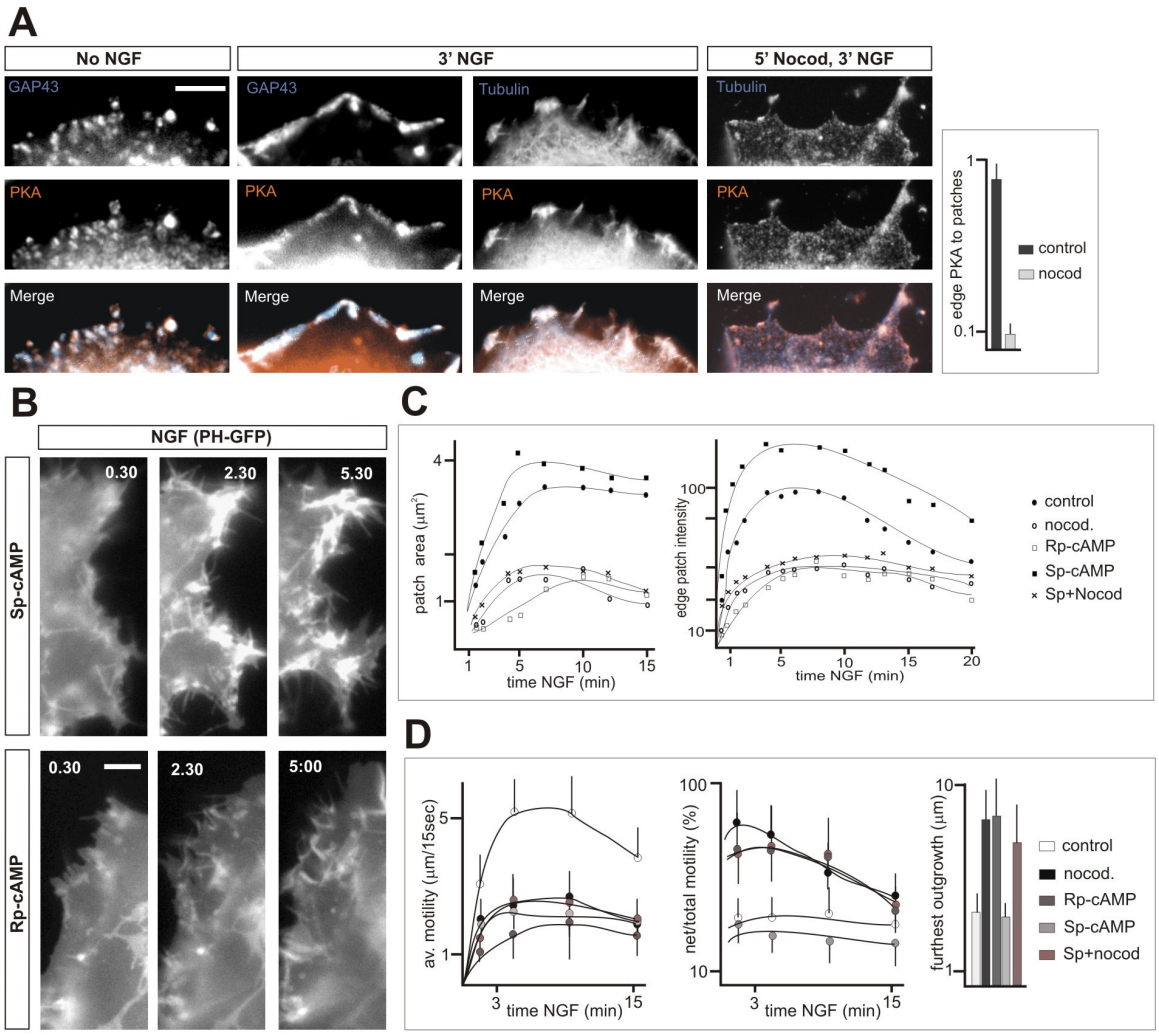


Golub and Caroni, Fig.6



Golub and Caroni, Fig7



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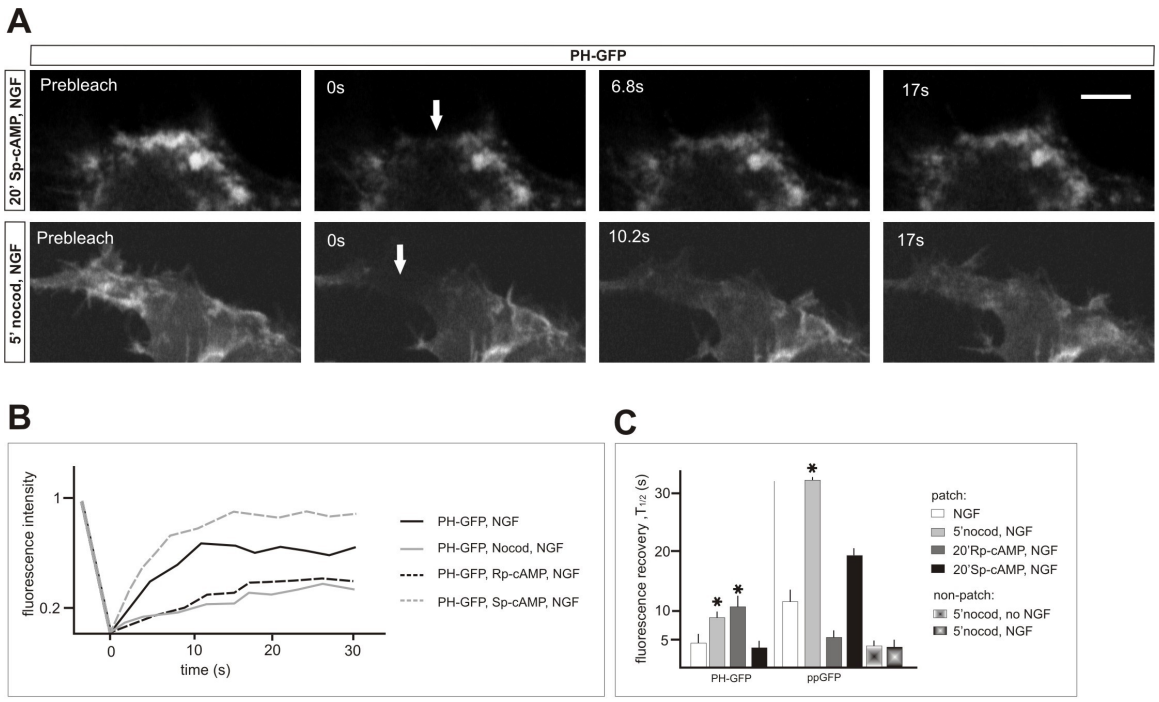
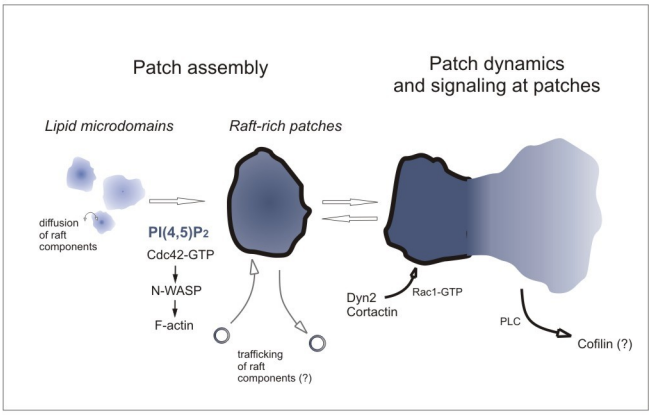


Golub and Caroni, Fig.9

A



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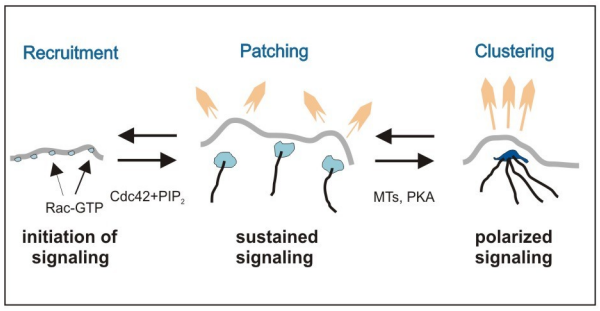
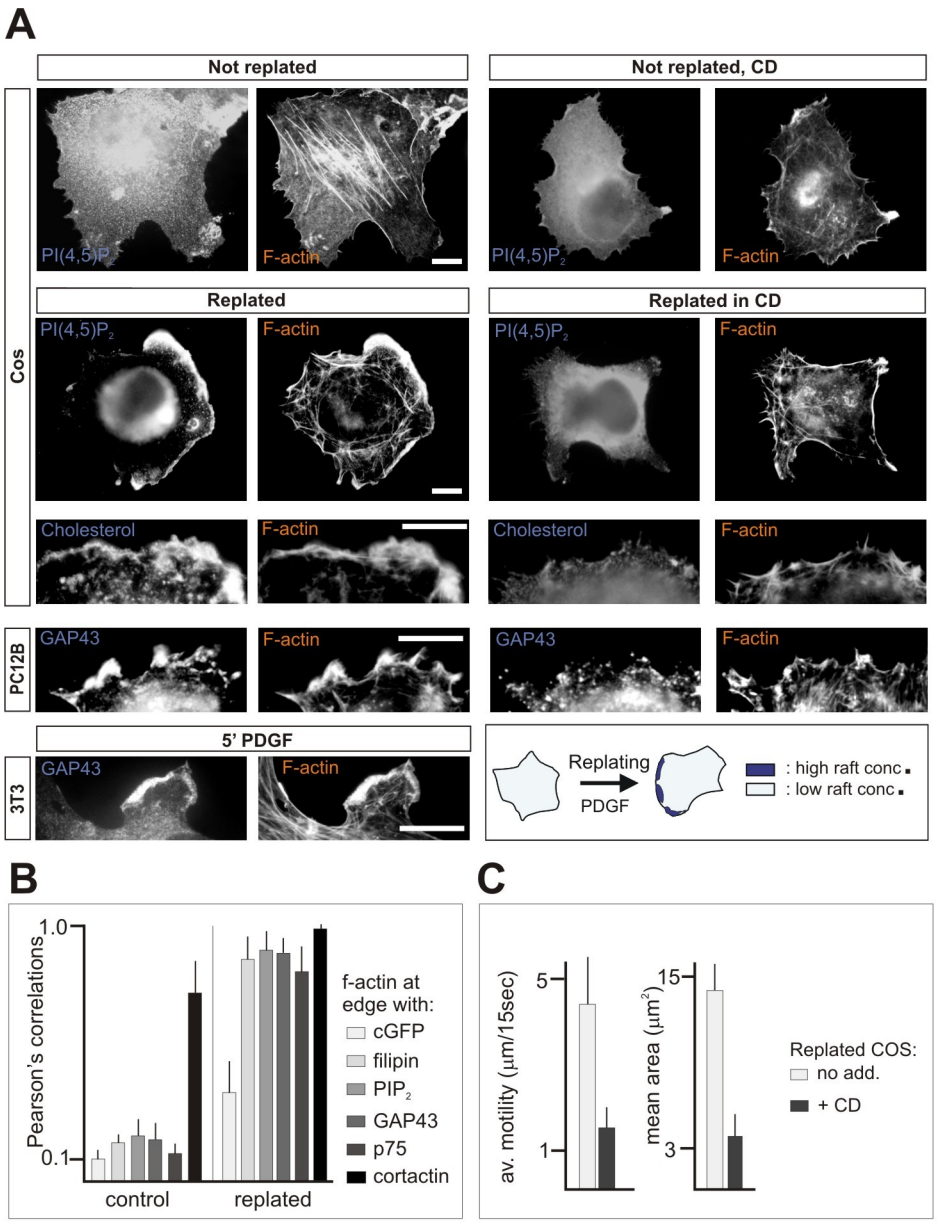
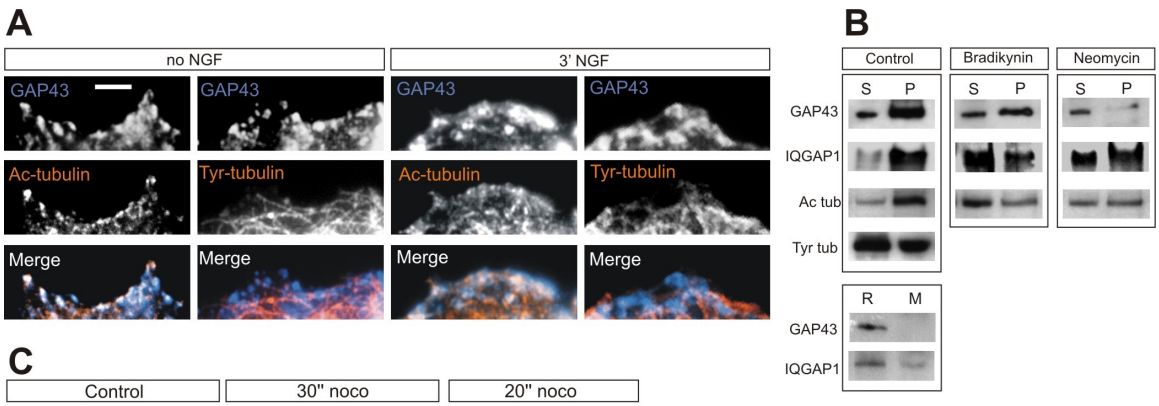


Golub and Caroni, Fig.10



Golub and Caroni, Suppl. Fig.1



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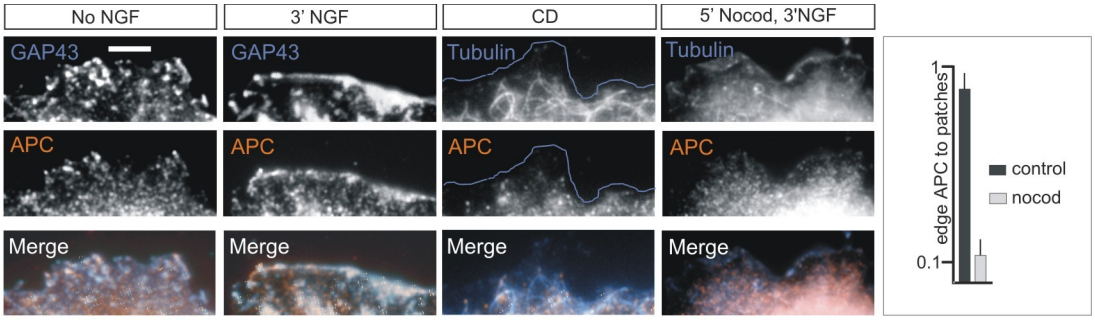

Golub and Caroni, Suppl. Fig. 3

2.2.6. Figure legends

Figure 1

Visualization and FRAP-based validation of PI(4,5)P_2-rich raft assemblies in living PC12 cells.

(A) Visualization of PI(4,5)P_2-rich raft assemblies in living cells. Top row: PHδ1-GFP highlights PI(4,5)P_2-rich patches that are not emphasized by the lipophilic dye DiD. Lower row: Co-distribution of PI(4,5)P_2-rich raft patches visualized with PHδ1-GFP and ppRFP. The images are z-stacks including all planes of these double-labeled living cells.

(B) Comparable labeling patterns for PI(4,5)P_2-rich raft complexes in living and fixed PC12 cells. The live-cell image (PH-GFP) was acquired 5' after the addition of NGF. Fixative was added within 15-20 sec after image acquisition, fixed cells were labeled for GAP43, and the PH-GFP-positive cell was retraced.

(C) FRAP for ppGFP reveals specific immobilization of raft markers at PI(4,5)P_2-rich raft patches. PC12 cells in the absence of NGF. Images are single confocal sections (patch: confocal plane slightly above substrate; non-patch and podosome: bottom plane of cells). Arrows: bleached area at end of photobleaching time. Representative FRAP curves (individual experiments) and average FRAP half-lives (N=15) are also shown in the figure.

Bars: 3 μm.

Figure 2

Rapid redistribution and accumulation of PI(4,5)P_2-rich raft patches at the cell edge upon induction of protrusive motility.

(A) NGF-induced protrusive motility at the cell edge depends on raft integrity. Left: Phase-contrast time-lapse recordings of PC12 cells treated with NGF in the absence or presence of (CD). Arrow: growth of a thin lamellipod in the presence of cyclodextrin (CD). Right: Quantitative analysis of NGF-induced protrusive motility (forward and

backward displacements of cell edge per unit time), without (control) and with cyclodextrin. N=15 cells.

(B) NGF induces a rapid redistribution of cell surface raft patches. Left: redistribution of raft patches (GAP43) from the dorsal surface (no NGF) to the edge (1 min NGF) of NGF-treated cells. Right: Co-distribution of raft-associated components at cell surface patches in PC12 cells in the absence and presence of NGF. Pearson's values of 1.0 reflect a complete overlap of compared signals.

(C) Rapid redistribution of raft patches in NGF-treated cells. Fractional patch area: fraction of surface area labeled with raft marker (GAP43). Times: no NGF, 30'' NGF. N=15.

(D) Redistribution and accumulation of raft patches at the cell edge in response to NGF precedes actin rearrangements associated with lamellar and lamellipod motility.

(E) Dynamics of NGF-induced cell surface $PI(4,5)P_2$ patches visualized with PHδ1-GFP. Left: Live imaging of PH-GFP. Arrows: at 0:30 and 1:30: new cell edge $PI(4,5)P_2$-rich patches predicting lamellipodial motility; at 3:10: $PI(4,5)P_2$-rich domain extending with a lamellipod; at 5:00: appearance of a new distal domain. Right: Quantitative analysis of cell edge PH-GFP patches in NGF-treated PC12 cells. Average values; control: N=15; cyclodextrin and cGFP: N=4.

(E) Rapid, NGF-stimulated turnover of $PI(4,5)P_2$ at raft patches. Representative examples (A) and quantitative analysis of FRAP half-lives (B) for PHδ1-GFP and ppGFP at and outside $PI(4,5)P_2$-rich raft patches. Images are single confocal sections. N=15.

Bars: 3 (A-E) and 2 (E) μm.

Figure 3

Raft patch accumulation at the cell surface depends on $PI(4,5)P_2$, Cdc42 and N-WASP.

(A) Requirement for $PI(4,5)P_2$, Cdc42 and N-WASP to accumulate raft patches at the surface of naïve PC12 cells (no NGF).

(B) Quantitative analysis of experiments as shown in (a). Raft marker: GAP43. N=30 cells.

(C) Impaired assembly and persistence of NGF-induced cell surface PI(4,5)P_2-rich patches in the presence of dn-Cdc42 or dn-N-WASP. Analysis of PH-GFP lives imaging recordings. Dn-Cdc42, dn-N-WASP: N=10; dn-Rac: N=4.
(D) Proposed mechanism to induce cell surface raft patching. PI5K: PI-5-kinase.
Bar: 2 μm.

Figure 4

Requirement for PI(4,5)P_2-rich raft patches to promote actin cytoskeleton accumulation and sustained protrusive motility at the leading edge.
(A) NGF-induced assembly of f-actin-rich lamellipods depends on raft integrity. The insets show representative x-z profiles of leading edges double-labeled for GAP43 (blue, cell surface) and f-actin (orange).
(B) Accumulation of proteins involved in actin-based membrane motility (Dynamin2, cortactin and Arp3) at raft patches in NGF-treated PC12 cells.
(C) Actin cytoskeleton accumulation at raft patches in NGF-treated cells. Left: Prominent accumulation of f-actin at cell edge raft patches 2 min after the addition of NGF. Right: Co-distribution of raft marker and f-actin signal, and relative raft and f-actin labeling intensities at cell edge patches. N=10 cells.
(D) Reduction in the extent and persistence of NGF-induced protrusive motility in cells expressing dn-Cdc42 or dn-N-WASP. Analysis of phase-contrast time-lapse recordings. Average values; N= 10 cells.
(E) Exogenously added PI(4,5)P_2 promotes lamellipod motility in the absence of NGF, and some NGF-induced motility in the presence of dn-Rac. N=8 cells.
(F) PC12 cells stably expressing a GAP43(ΔED) construct interfering with the accumulation of raft patches at the cell surface exhibit reduced NGF-induced lamellipodial motility. Raft patches: N=30 cells; motility: N=10 cells.
Bars: 3 μm.

Figure 5

Capture of microtubules at cell edge raft patches.
(A,B) MT ends associate with cell edge raft patches in naïve and NGF-treated PC12 cells.

(B) MT capture at the cell edge depends on raft integrity. Left and middle panel: Raft disruption (CD, 10') leads to loss of MTs associated with the cell edge. Right panel: Analysis of data as shown in (a). The extents to which MTs specifically associate with raft patches at the cell edge are given in fractional values (value of 1=100%). N=30 cells.
(C) MT capture at the cell edge depends on raft patching. N=30 cells.
Bars: 2 μm.

Figure 6

Raft patches capture MTs through IQGAP1.
(A) Association of IQGAP1 with cell edge raft patches. The accumulation of IQGAP1 at the cell edge depended on raft integrity, but not on MT integrity. Blue outline (CD): cell edge. Quantitative analysis (fractional values): no NGF, N=30 cells.
(B) Knockdown of IQGAP1 in PC12 cells. Transfected cells co-expressed GFP.
(C) Capture of MTs at raft patches depends on IQGAP1. Quantitative analysis of MTs to edge: N=30 cells.
(D) Fragmentation of raft patches (cholesterol) in the absence of IQGAP1.
(E) Model of how IQGAP1 may provide a physical link, from cell edge raft patches (Rac-GTP and Cdc42-GTP) to MT plus-ends.
Bars: 2 (A, C, D) and 5 (B) μm.

Figure 7

Raft patch clustering and organized protrusive motility at the leading edge depend on intact MTs.
(A) Fragmentation of cell surface raft patch assemblies in the absence of intact MTs. Quantitative analysis: Influence of MT integrity on raft complex size; N=30 cells. Bar: 2 μm.
(B) Influence of MT integrity on the polarization of lamellipodial $PI(4,5)P_2$ and f-actin accumulation in NGF-treated cells (5' NGF; N=30 cells). The intensity ratios are a measure for the extent to which lamellipodia with the highest labeling values differ from average lamellipodial labeling values for any given cell. Rp: Rp-cAMPS; Sp: Sp-cAMPS.

(C) Influence of MT integrity on the dynamics of $PI(4,5)P_2$ patches. Live imaging of PH-GFP expressing cells; average values, N=10 cells.
(D) Influence of MT integrity on the patterns of NGF-induced motility in PC12 cells. Net/total motility is a measure for locally organized motility (see methods). N=10 cells.
(E) Schematic of NGF-induced motility with and without intact MTs. Colors indicate areas exhibiting motility during the time intervals indicated at the bottom; the two colors represent lamellipodial configurations at two consecutive time points (violet before blue).

Figure 8

MTs target PKA to cell edge raft patches, to promote patch clustering and spatially constrain protrusive motility.
(A) Targeting of PKA to cell edge raft patches through MTs. Quantitative analysis: no NGF, N=30 cells.
(B) The activity of PKA promotes $PI(4,5)P_2$ signal (PHδ1-GFP) accumulation and patch compaction at the cell edge. Bars: 2 μm.
(C) Rp-cAMPS mimics the effects of nocodazole on PH-GFP patch dynamics, whereas Sp-cAMPS enhances patch clustering in a MT-dependent manner. Average values; N=10 cells.
(D) Rp-cAMPS mimics the effects of nocodazole on NGF-induced motility, and MT disruption suppresses any effect of Sp-cAMPS on leading edge motility. N=10 cells.

Figure 9

Microtubules and cAMP augment FRAP rates for $PI(4,5)P_2$ at raft patches. All experiments: PC12 cells treated with NGF.
(A) Representative examples of PHδ1-GFP FRAP at patches in the presence of the cAMP analogue Sp-cAMPS or nocodazole. Single confocal sections; arrows point to bleached area at end of photobleaching time (0 sec after photobleaching). Bar: 2 μm.
(B) Representative FRAP curves (normalized) for PHδ1-GFP at raft patches.
(C) Quantitative analysis of FRAP experiments for PHδ1-GFP and ppGFP at raft patches, with or without intact microtubules, Sp-cAMPS or Rp-cAMPS. The values are FRAP

half-lives; N=15. Asterisks: in these experiments fluorescence did not recover to original values (see curves in (B)), and half-lives are given for plateau values.

Figure 10

(A) Proposed model of how local PI(4,5)P_2 metabolism, together with Cdc42, drives the accumulation and dissipation of dynamic PI(4,5)P2-dependent raft-rich patches, which provide signaling platforms for protrusive motility at the cell surface.
(B) Proposed model of leading edge motility control through PI(4,5)P_2-rich raft assemblies and MTs. MTs captured at raft patches through IQGAP1 target PKA to the leading edge, promoting clustering of raft patches by enhancing PI(4,5)P_2 accumulation at patches. This leads to a focusing and polarization of signaling and motility.

Supplementary material

Suppl. Figure 1

Cell surface rafts accumulate at motile lamellipods.
(A) COS, 3T3 and PC12 cells were analyzed 1h after replating on a collagen substrate, or 5' after the addition of PDGF (3T3). Note accumulation of cell surface rafts (cholesterol, GAP43, PI(4,5)P_2) in replated cells at sites of f-actin assembly at lamellipods, and absence of f-actin-rich lamellipods in cells spreading in the presence of cyclodextrin. Bars: 4 μm.
(B) Co-distribution of raft markers (filipin, PI(4,5)P2, GAP43, p75) with intense lamellipod f-actin in replated cells. In control experiments, cytosolic GFP (cGFP) did not exhibit a comparable co-distribution with f-actin at lamellipods. Pearson's values were derived from 2.5x8 μm masks (Materials and Methods). Values of 1.0 reflect a complete overlap of compared signals. Control: non-replated cells. N=45 (3 independent experiments).
(C) Inhibition of lamellipodial motility in COS cells replated (45') in the presence of cyclodextrin (CD). Average motility (μm/15sec) is a measure for the medial extent of

forward or backward extension per unit time at the cell edge; mean area (μm^2) is a measure for the average size of surface extension or retraction per 10 sec interval at the leading edge (see Experimental Procedures). N=9 cells (3 independent experiments).

Suppl. Figure 2

Stabilization of microtubules at PI(4,5)P_2-rich raft patches.
(A) MT ends at cell edge raft patches are selectively labeled with an Ac-tubulin antibody. Tyr-tubulin antibody (dynamic MTs) decorates most MTs, but not their ends at raft patches; Ac-tubulin antibody (stable MTs) decorates MT ends at cell edge rafts patches.
(B) Selective association of stable MTs with a Triton-insoluble fraction enriched in raft markers and IQGAP1. PC12 cell proteins (no NGF) were fractionated in a Triton-soluble (S) and -insoluble (P) fraction, and analyzed by immunoblots. GAP43 and IQGAP1 were also enriched in a low-density floating raft (R) fraction; M: membrane fraction sedimenting in sucrose gradient. Bradykinin and Neomycin reduce accessible plasmalemmal PI(4,5)P_2, interfering with raft patching in PC12 cells (see Laux et al., 2000); this led to a partial redistribution of raft markers, IQGAP1 and Ac-tubulin to the Triton-soluble fraction. Equal amounts of protein were loaded on each gel slot.
(C) Partial protection of patch-associated MTs against depolymerization by nocodazole. Most MTs towards the edge of undifferentiated PC12 cells are highly dynamic, and are depolymerized upon a 30'' treatment with nocodazole. Upon briefer treatments with nocodazole (20''), more stable MTs in these cells are frequently associated with raft patches.
Bars: 2 μm.

Suppl. Figure 3

Association of APC with cell edge raft patches. The accumulation of APC at the cell edge depended on raft and MT integrity. Quantitative analysis: no NGF, N=30 cells.

Supplementary videos

Supplementary Video 1

Phase-contrast time-lapse recording of PC12 cells in the absence of NGF. Total recording time 05:16 min. Frames were acquired at the rate of 1/10 sec, and the video run at 15 frames/sec.

Supplementary Video 2

Phase-contrast time-lapse recording of NGF-treated PC12 cells. This recording and all following ones begin 10-15'' after the addition of NGF. Total recording time 13:00 min. Frames were acquired at the rate of 1/10 sec, and the video run at 15 frames/sec. See also Fig. 2A.

Supplementary Video 3

Phase-contrast time-lapse recording of PC12 cell pretreated with cyclodextrin, and then with NGF. Total recording time 05:16 min. Frames were acquired at the rate of 1/10 sec, and the video run at 15 frames/sec.

Supplementary Video 4

GFP-fluorescence time-lapse recording of NGF-treated PC12 cell expressing PHδ1-GFP. Total recording time 05:00 min. Frames were acquired at the rate of 1/10 sec, and the video run at 15 frames/sec. See also Fig. 2D.

Supplementary Video 5

Time-lapse recording of NGF-treated PC12 cells labeled with DiD. Total recording time 06:24 min. Frames were acquired at the rate of 1/10 sec, and the video run at 15 frames/sec

Supplementary Video 6

GFP-fluorescence time-lapse recording of NGF-treated PC12 cells co-expressing PHδ1-GFP and Dn-Cdc42. Total recording time 07:16 min. Frames were acquired at the rate of 1/10 sec, and the video run at 15 frames/sec.

Supplementary Video 7

Phase-contrast time-lapse recording of NGF-treated PC12 cells in the presence of nocodazole (added 5 min before NGF). Total recording time 14:00 min. Frames were acquired at the rate of 1/10 sec, and videos run at 15 frames/sec. See also Suppl. Fig. 3.

Supplementary Video 8

GFP-fluorescence time-lapse recording of NGF-treated PC12 cells expressing PHδ1-GFP in presence of nocodazole (added 5 min before NGF). Total recording time 07:06 min. Frames were acquired at the rate of 1/10 sec, and videos run at 15 frames/sec. Note long $PI(4,5)P_2$-rich ridges at the edge of lamellipods lifting from the substrate (see Results section).

Supplementary Video 9

GFP-fluorescence time-lapse recording of NGF-treated PC12 cells expressing PHδ1-GFP in the presence of Sp-cAMP (added 30 min before NGF). Frames were acquired at the rate of 1/10 sec, and videos run at 15-frames/sec. Total recording time 06:24 min.

Supplementary Video 10

GFP-fluorescence time-lapse recording of NGF-treated PC12 cells expressing PHδ1-GFP in presence of Rp-cAMP (added 30 min before NGF). Total recording time 06:24 min. Frames were acquired at the rate of 1/10 sec, and

3. Discussion

This study has provided answers concerning several unresolved issues on lipid rafts, and its results point to new issues and hypotheses for experimental investigation.

One of the issues addressed involves clustering of lipid rafts. Thus, while the existence of lipid rafts has not been contested recently, clustering of rafts, its molecular mechanisms and its functional implications, are under hot debate. While the notion of lipid rafts as signaling platforms had appeared attractive, serious doubts and criticisms were raised following the application of recent technical advances in cell biology to questions of lipid raft clustering. For example, once lipid rafts could be visualized in living cells using GFP fusion constructs, clustered patterns previously detected in fixed and permeabilized cells could not be detected anymore. Biophysical methods led to raft size estimates in the range of 50 nm, i.e. well below the resolution limit of light microscopy, raising further doubts about the reality of raft patches detected in previous studies. Furthermore, experimental settings thought to involve raft clustering such as T-cell activation yielded unconvincing evidence when re-examined with FRET techniques, raising doubts about the general notion of signal-induced raft clustering (Glebov and Nichols, 2004). Against this background, this study now provides clear evidence for the existence of raft patches in living cells, and shows how these are induced to form, how they relate to trafficking, and how they promote actin-based motility.

A second important issue addressed in this study involves the capture of MTs at the cell surface, a process thought to play an important role in cell polarity and directed cell motility. This study provides evidence that raft patches capture MTs through patch IQGAP1, and that this capture in turn mediates targeting of signaling components involved in promoting, sustaining and focusing actin-based motility. In doing so, this

study identifies for the first time a plasmalemmal structure involved in MT capturing, and provides evidence relating this capture to the local regulation of actin dynamics.

3.1. Raft clustering

We have used the Fluorescence Recovery After Photobleaching (FRAP) technique on PH-PLCδ1-GFP (PH-GFP) or palmitoylated-RFP (i.e. a raft-targeted construct) transfected PC12B-GAP43 cells to monitor the dynamic properties of raft patches and their components. The analysis demonstrated that the exchange of raft components at patches exhibits properties clearly distinct from those of the same components outside the patches. While values measured outside patches were closely comparable to those reported in recent studies by others (Kenworthy et al, 2004), values at patches were substantially slower. This suggested that while outside patches exchange involves diffusion in the plane of the membrane, diffusion is strongly restricted at patches. Instead, exchange at patches depended on the integrity of MTs which target patches, suggesting that it involves vesicle trafficking.

This study also provided insights about the mechanisms promoting raft patching at the cell surface. We discovered that raft patching involves dual signaling through the small RhoGTPase Cdc42 and $PI(4,5)P_2$. This dual signaling was necessary for the activation of N-WASP, and actin assembly, which were in turn required for raft patching. Our results thus suggest that a pathway involving local signaling to PI5-kinase ($PI(4,5)P_2$ synthesis) and Cdc42 promotes raft patching through N-WASP and local actin assembly. The latter may promote patching by providing a linkage from the membrane patch to the cortical cytoskeleton. Such a model highlights the importance of regulated membrane-cortex interactions in promoting and sustaining spatial control of signaling at the cell surface.

Our results have provided evidence that rather than lateral diffusion, local $PI(4,5)P_2$ synthesis is the predominant mechanism to promote patch assembly. The results also show that enhanced raft patching at the leading edge in the presence of motogenic signals

such as NGF involves enhanced local $PI(4,5)P_2$ synthesis, and that this is regulated through a cAMP-dependent pathway involving PKA delivered to raft patches through MTs. Accordingly, raft patching could be regulated in an exquisitely dynamic manner through mechanisms converging on the regulation of local $PI(4,5)P_2$ levels at plasmalemmal rafts. The patches in turn appear to be required for sustained local signaling promoting motility. This may involve in part the accumulation of components involved in signaling to the actin cytoskeleton at the raft platforms. In addition, this may also involve mechanisms coupling signaling to trafficking, e.g. mechanisms targeting activated receptors to specific sites at the cell surface, and recycling desensitized receptors from and to the cell surface.

The versatility of surface signaling through rafts appears to be further enhanced through the apparent existence of cell- and state-specific factors affecting raft patching. Thus, we found that raft clustering is not exhibited by all cell types and under all circumstances. Cos-1 cell cultures, for example, exhibit homogenous distributions of raft components under resting conditions, and only exhibit raft clustering when stimulated to spread, i.e. upon integrin activation. Furthermore, quiescent PC12 cells exhibit prominent raft patches on their dorsal surface when cultured on a collagen substrate, but nearly no dorsal patches when cultured on laminin. It is therefore possible, that raft patching and motility at the cell surface might be controlled through the expression and activation state of integrin signaling components. This mode of regulation would be attractive in that it would allow for multiple levels of control of cell motility and polarization through receptor expression, ligand-mediated activation, and inside-out mechanisms linking intracellular signaling states to the accumulation of signaling competent integrins at the cell surface.

3.2. N-WASP function and rafts

Our results provide novel information with respect to the possible functions of N-WASP (and WASP). Thus, recent studies have suggested that contrary to previous expectations,

WASP proteins may not play a major role in initiating actin polymerization for lamellipodial motility. Instead, WAVE proteins seem to be the dominant mediators of signal-induced actin polymerization downstream of Rho-type GTPases (Bompard and Caron, 2004). These recent findings suggested that the role of Arp2/3 and actin dynamics activation through WASP/N-WASP had to be reinvestigated. On the other hand, WASP was found to be required for the assembly of the immunological synapse. Thus, T cells from Wiskott-Aldrich syndrome (WAS) patients were impaired in their ability to cluster GM1 glycosphingolipid during T cell activation, suggesting a critical role for WASP in the movement and subsequent aggregation and clustering of lipid rafts during this process (Dupre et al, 2002). As T-cell activation also involves the activity of Cdc42, it is conceivable that the mechanism of TCR clustering resembles that of raft patching. Interestingly, signaling through N-WASP might be particularly versatile, and thus mediate several distinct cellular responses. Thus, the recent discovery of a novel N-WASP regulator (Toca-1) downstream of Cdc42 and PI(4,5)P_2, suggests the possibility that modulation of N-WASP activity by co-factors might mediate its specific effects on raft clustering and surface motility (Ho et al, 2004).

3.3. Sites of MT capture at the cell surface

The existence of distinct cortical patches capturing MTs had been deduced from the results of several recent studies, e.g. those monitoring the capture of MTs at the cell cortex during cytokinesis (Kodama et al, 2004; Nelson, 2003; Pardo and Nurse, 2003). The need for the identification of such patches had been recognized, and authoritative comments have emphasized how this would contribute to the elucidation of mechanisms directing cell polarity (Kodama et al, 2004; Horton and Ehlers, 2003).Our study now identifies one such MT capture site as PI(4,5)P_2-rich and Cdc42-dependent plasmalemmal raft patches. The involvement of patch-associated IQGAP1 provides a potential mechanism linking raft Cdc42-GTP and Rac-GTP to MT capture. These findings thus provide a direct link between the local regulation of actin dynamics and the capture of MTs at the cell surface. These mechanisms likely play an important role in

directed cell motility processes such as cell migration and growth cone navigation. Furthermore, our results raise the testable possibility that further sites of MT capture at the cell surface involved in cell polarization might involve similar mechanisms of cell surface raft patching.

3.4. Cdc42 and rafts in polarization

The identification of Cdc42 as one of the factors involved in raft patching, and thus MT capture at the plasmalemma ties in particularly well with previous findings about the roles of Cdc42 in cell polarity. Thus, several studies have established that Cdc42 plays a crucial role in organizing polarity, e.g. through its downstream effectors Par and aPKC (Kay and Hunter, 2001; Johnson, 1999; Gotta et al, 2001).

A recent review had pointed out that although much is known about Cdc42 downstream targets that affect polarity, "how exactly Cdc42 does accomplish that polarity is not yet known" (Raftopoulou and Hall, 2004). Referring to the role of Cdc42 in yeast schmoo formation, the same review elaborated that "generating cellular asymmetry is not necessarily the same as generating asymmetry in the correct orientation. Thus, in yeast lacking Cdc42p, pheromone induces an asymmetrical, stochastic accumulation of schmoo components on the membrane surface, but these are transient. The role of Cdc42 is to stabilize this activity in the correct location with respect to the external cue". While emphasizing that mechanisms are presently not known, the review speculated that local positive feedback loops involving Cdc42 may take a prominent role. Clearly, a role for Cdc42 in directing patching of $PI(4,5)P_2$-rich rafts to capture MTs and promote sustained signaling might be consistent with such requirements.

3.5. Outlook

One of the most intriguing issues raised by this study involves the possible existence of a direct link between raft patching, regulated endocytosis and cell regulation. How and

where does a cell "decide" what to do with the signal it receives, and how and where does it mount a response to that signal? An attractive answer could be – by signaling through endosomes. Thus, subsequent to internalization of activated receptors, cells have the option to terminate a signal (e.g. by tagging and targeting receptors for degradation), to prolong it (e.g. by reactivating and recycling receptors to the cell surface), to modify it (through cross-interactions at the level of signaling endosomes), and to localize it (e.g through trafficking endosomes and secretory vesicles to specific sites on the cell membrane). It is known that endosomes can and do signal, and that clathrin-dependent and clathrin-independent endocytosis generate endosomes with distinct signaling properties (Di Guglielmo et al, 2003). Trafficking from and to raft patches might thus influence quality and specify location in cell signaling.

Observations made during the course of this study have provided possible entry points to investigate relations between raft patching and endocytosis in signaling for cell motility. Thus, by comparing PC12 clones expressing raft-associated proteins influencing patch accumulation (GAP43, CAP23), we have observed the existence of a very good correlation between the extent and size of cell surface raft patches and that of signal-induced endocytosis in cells treated with motogenic growth factors (not shown). If signaling and/or trafficking of such endosomes would differ depending on the state of assembly of raft patches, then this might provide a possible mechanism relating raft patches to ruffling motility. Thus, the enhanced endocytosis triggered as a consequence of NGF receptor activation, coupled to trafficking of raft-enriched vesicles to raft patches at the leading edge would ensure sustained motility near sites of raft patch assembly. As a consequence cells with more and larger raft patches would exhibit larger lamellipodia, longer neurites, stronger focal adhesions and more pronounced stress fiber systems, which is exactly what we observe.

A further line of evidence suggesting that endosomes might provide decision centers for signaling, comes from immunology, where it was shown that antigen presenting dendritic cells use macropinocytosis to capture antigens into macropinosomes, where they process them for subsequent delivery to the surface and presentation to T-cells (Symons and Rusk, 2003). Macropinocytosis happens beneath the ruffling dorsal membrane of cells

treated with growth factors; subsequent to uptake, macropinosomes could accumulate signaling components such as activated receptors, and recycle these components to sites of cell-cell signaling.

A further line for future investigations involves the role of cell surface rafts in growth control and cancer. Thus, as signaling through lipid rafts provides specificity and inside-out control in cell regulation, it seems likely that the loss of control over signaling characteristic of cancer cells will turn out to involve dysregulation of trafficking and polarization pathways involving lipid rafts and their assemblies.

4. References

Ahn, S., J. Kim, C.L. Lucaveche, M.C. Reedy, L.M. Luttrell, R.J. Lefkowitz, and Y. Daaka. 2002. Src-dependent tyrosine phosphorylation regulates dynamin self-assembly and ligand-induced endocytosis of the epidermal growth factor receptor. J Biol Chem. 277:26642-51.

Amann, K.J., and T.D. Pollard. 2001. The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. Nat Cell Biol. 3:306-10.

Arber, S., F.A. Barbayannis, H. Hanser, C. Schneider, C.A. Stanyon, O. Bernard, and P. Caroni. 1998. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature. 393:805-9.

Askham, J.M., P. Moncur, A.F. Markham, and E.E. Morrison. 2000. Regulation and function of the interaction between the APC tumour suppressor protein and EB1. Oncogene. 19:1950-8.

Bajno, L., X.R. Peng, A.D. Schreiber, H.P. Moore, W.S. Trimble, and S. Grinstein. 2000. Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation. J Cell Biol. 149:697-706.

Ballestrem, C., B. Wehrle-Haller, B. Hinz, and B.A. Imhof. 2000. Actin-dependent lamellipodia formation and microtubule-dependent tail retraction control-directed cell migration. Mol Biol Cell. 11:2999-3012.

Benting, J.H., A.G. Rietveld, and K. Simons. 1999. N-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells. J Cell Biol. 146:313-20.

Blanchoin, L., K.J. Amann, H.N. Higgs, J.B. Marchand, D.A. Kaiser, and T.D. Pollard. 2000. Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar proteins. Nature. 404:1007-11.

Blanchoin, L., and T.D. Pollard. 2002. Hydrolysis of ATP by polymerized actin depends on the bound divalent cation but not profilin. Biochemistry. 41:597-602.

Bompard, G., and E. Caron. 2004. Regulation of WASP/WAVE proteins: making a long story short. J Cell Biol. 166:957-62.

Botelho, R.J., M. Teruel, R. Dierckman, R. Anderson, A. Wells, J.D. York, T. Meyer, and S. Grinstein. 2000. Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. J Cell Biol. 151:1353-68.

Bronfman, F.C., M. Tcherpakov, T.M. Jovin, and M. Fainzilber. 2003. Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. J Neurosci. 23:3209-20.

Brown, R.E. 1998. Sphingolipid organization in biomembranes: what physical studies of model membranes reveal. J Cell Sci. 111 (Pt 1):1-9.

Bruckner, K., J. Pablo Labrador, P. Scheiffele, A. Herb, P.H. Seeburg, and R. Klein. 1999. EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. Neuron. 22:511-24.

Bruses, J.L., N. Chauvet, and U. Rutishauser. 2001. Membrane lipid rafts are necessary for the maintenance of the (alpha)7 nicotinic acetylcholine receptor in somatic spines of ciliary neurons. J Neurosci. 21:504-12.

Carlier, M.F., and D. Pantaloni. 1986. Direct evidence for ADP-Pi-F-actin as the major intermediate in ATP-actin polymerization. Rate of dissociation of Pi from actin filaments. Biochemistry. 25:7789-92.

Carlier, M.F., and D. Pantaloni. 1997. Control of actin dynamics in cell motility. J Mol Biol. 269:459-67.

Caroni, P. 2001. New EMBO members' review: actin cytoskeleton regulation through modulation of PI(4,5)P(2) rafts. Embo J. 20:4332-6.

Carvalho, P., J.S. Tirnauer, and D. Pellman. 2003. Surfing on microtubule ends. Trends Cell Biol. 13:229-37.

Clague, M.J. 1999. Membrane transport: Take your fusion partners. Curr Biol. 9:R258-60.

Clarke, J.H. 2003. Lipid signalling: picking out the PIPs. Curr Biol. 13:R815-7.

Cook, T.A., T. Nagasaki, and G.G. Gundersen. 1998. Rho guanosine triphosphatase mediates the selective stabilization of microtubules induced by lysophosphatidic acid. J Cell Biol. 141:175-85.

Cooper, J.A., and D.A. Schafer. 2000. Control of actin assembly and disassembly at filament ends. Curr Opin Cell Biol. 12:97-103.

Coue, M., V.A. Lombillo, and J.R. McIntosh. 1991. Microtubule depolymerization promotes particle and chromosome movement in vitro. J Cell Biol. 112:1165-75.

Cremona, O., and P. De Camilli. 2001. Phosphoinositides in membrane traffic at the synapse. J Cell Sci. 114:1041-52.

Dalva, M.B., M.A. Takasu, M.Z. Lin, S.M. Shamah, L. Hu, N.W. Gale, and M.E. Greenberg. 2000. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell. 103:945-56.

Daub, H., K. Gevaert, J. Vandekerckhove, A. Sobel, and A. Hall. 2001. Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16. J Biol Chem. 276:1677-80.

De Matteis, M.A., and A. Godi. 2004. PI-loting membrane traffic. Nat Cell Biol. 6:487-92.

Decker, L., and C. ffrench-Constant. 2004. Lipid rafts and integrin activation regulate oligodendrocyte survival. J Neurosci. 24:3816-25.

del Pozo, M.A., N.B. Alderson, W.B. Kiosses, H.H. Chiang, R.G. Anderson, and M.A. Schwartz. 2004. Integrins regulate Rac targeting by internalization of membrane domains. Science. 303:839-42.

Delling, M., E. Wischmeyer, A. Dityatev, V. Sytnyk, R.W. Veh, A. Karschin, and M. Schachner. 2002. The neural cell adhesion molecule regulates cell-surface delivery of G-protein-activated inwardly rectifying potassium channels via lipid rafts. J Neurosci. 22:7154-64.

Desai, A., S. Verma, T.J. Mitchison, and C.E. Walczak. 1999. Kin I kinesins are microtubule-destabilizing enzymes. Cell. 96:69-78.

Di Guglielmo, G.M., C. Le Roy, A.F. Goodfellow, and J.L. Wrana. 2003. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol. 5:410-21.

Doughman, R.L., A.J. Firestone, M.L. Wojtasiak, M.W. Bunce, and R.A. Anderson. 2003. Membrane ruffling requires coordination between type Ialpha phosphatidylinositol phosphate kinase and Rac signaling. J Biol Chem. 278:23036-45.

Dupre, L., A. Aiuti, S. Trifari, S. Martino, P. Saracco, C. Bordignon, and M.G. Roncarolo. 2002. Wiskott-Aldrich syndrome protein regulates lipid raft dynamics during immunological synapse formation. Immunity. 17:157-66.

Eden, S., R. Rohatgi, A.V. Podtelejnikov, M. Mann, and M.W. Kirschner. 2002. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature. 418:790-3.

Ehehalt, R., P. Keller, C. Haass, C. Thiele, and K. Simons. 2003. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol. 160:113-23.

El-Husseini Ael, D., E. Schnell, S. Dakoji, N. Sweeney, Q. Zhou, O. Prange, C. Gauthier-Campbell, A. Aguilera-Moreno, R.A. Nicoll, and D.S. Bredt. 2002. Synaptic strength regulated by palmitate cycling on PSD-95. Cell. 108:849-63.

Etienne-Manneville, S., and A. Hall. 2001. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell. 106:489-98.

Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. Nature. 420:629-35.

Etienne-Manneville, S., and A. Hall. 2003. Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature. 421:753-6.

Evangelista, M., D. Pruyne, D.C. Amberg, C. Boone, and A. Bretscher. 2002. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nat Cell Biol. 4:32-41.

Fivaz, M., L. Abrami, and F.G. van der Goot. 1999. Landing on lipid rafts. Trends Cell Biol. 9:212-3.

Friedrichson, T., and T.V. Kurzchalia. 1998. Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. Nature. 394:802-5.

Fukami, K., K. Matsuoka, O. Nakanishi, A. Yamakawa, S. Kawai, and T. Takenawa. 1988. Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. Proc Natl Acad Sci U S A. 85:9057-61.

Fukata, M., T. Watanabe, J. Noritake, M. Nakagawa, M. Yamaga, S. Kuroda, Y. Matsuura, A. Iwamatsu, F. Perez, and K. Kaibuchi. 2002. Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. Cell. 109:873-85.

Galjart, N., and F. Perez. 2003. A plus-end raft to control microtubule dynamics and function. Curr Opin Cell Biol. 15:48-53.

Glebov, O.O., and B.J. Nichols. 2004. Lipid raft proteins have a random distribution during localized activation of the T-cell receptor. Nat Cell Biol. 6:238-43.

Godi, A., P. Pertile, R. Meyers, P. Marra, G. Di Tullio, C. Iurisci, A. Luini, D. Corda, and M.A. De Matteis. 1999. ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. Nat Cell Biol. 1:280-7.

Golub, T., S. Wacha, and P. Caroni. 2004. Spatial and temporal control of signaling through lipid rafts. Curr Opin Neurobiol. 14:542-50.

Gomez-Mouton, C., J.L. Abad, E. Mira, R.A. Lacalle, E. Gallardo, S. Jimenez-Baranda, I. Illa, A. Bernad, S. Manes, and A.C. Martinez. 2001. Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci U S A. 98:9642-7.

Gomez-Mouton, C., R.A. Lacalle, E. Mira, S. Jimenez-Baranda, D.F. Barber, A.C. Carrera, A.C. Martinez, and S. Manes. 2004. Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. J Cell Biol. 164:759-68.

Gordon-Weeks, P.R. 2004. Microtubules and growth cone function. J Neurobiol. 58:70-83.

Gotta, M., M.C. Abraham, and J. Ahringer. 2001. CDC-42 controls early cell polarity and spindle orientation in C. elegans. Curr Biol. 11:482-8.

Green, J.M., A. Zhelesnyak, J. Chung, F.P. Lindberg, M. Sarfati, W.A. Frazier, and E.J. Brown. 1999. Role of cholesterol in formation and function of a signaling complex involving alphavbeta3, integrin-associated protein (CD47), and heterotrimeric G proteins. J Cell Biol. 146:673-82.

Grimmer, S., B. van Deurs, and K. Sandvig. 2002. Membrane ruffling and macropinocytosis in A431 cells require cholesterol. J Cell Sci. 115:2953-62.

Gruenberg, J. 2001. The endocytic pathway: a mosaic of domains. Nat Rev Mol Cell Biol. 2:721-30.

Gruenberg, J., G. Griffiths, and K.E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. J Cell Biol. 108:1301-16.

Guirland, C., S. Suzuki, M. Kojima, B. Lu, and J.Q. Zheng. 2004. Lipid rafts mediate chemotropic guidance of nerve growth cones. Neuron. 42:51-62.

Gundersen, G.G., and J.C. Bulinski. 1988. Selective stabilization of microtubules oriented toward the direction of cell migration. Proc Natl Acad Sci U S A. 85:5946-50.

Gundersen, G.G., E.R. Gomes, and Y. Wen. 2004. Cortical control of microtubule stability and polarization. Curr Opin Cell Biol. 16:106-12.

Gundersen, G.G. 2002. Evolutionary conservation of microtubule-capture mechanisms. Nat Rev Mol Cell Biol. 3:296-304.

Hagen, S.J., D.P. Kiehart, D.A. Kaiser, and T.D. Pollard. 1986. Characterization of monoclonal antibodies to Acanthamoeba myosin-I that cross-react with both myosin-II and low molecular mass nuclear proteins. J Cell Biol. 103:2121-8.

Harder, T. 2004. Lipid raft domains and protein networks in T-cell receptor signal transduction. Curr Opin Immunol. 16:353-9.

Harder, T., R. Kellner, R.G. Parton, and J. Gruenberg. 1997. Specific release of membrane-bound annexin II and cortical cytoskeletal elements by sequestration of membrane cholesterol. Mol Biol Cell. 8:533-45.

Harder, T., P. Scheiffele, P. Verkade, and K. Simons. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol. 141:929-42.

Harder, T., and K. Simons. 1999. Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. Eur J Immunol. 29:556-62.

Hayden, J.H., S.S. Bowser, and C.L. Rieder. 1990. Kinetochores capture astral microtubules during chromosome attachment to the mitotic spindle: direct visualization in live newt lung cells. J Cell Biol. 111:1039-45.

He, Q., and K.F. Meiri. 2002. Isolation and characterization of detergent-resistant microdomains responsive to NCAM-mediated signaling from growth cones. Mol Cell Neurosci. 19:18-31.

Henley, J.R., E.W. Krueger, B.J. Oswald, and M.A. McNiven. 1998. Dynamin-mediated internalization of caveolae. J Cell Biol. 141:85-99.

Hering, H., C.C. Lin, and M. Sheng. 2003. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. J Neurosci. 23:3262-71.

Higuchi, H., T. Yamashita, H. Yoshikawa, and M. Tohyama. 2003. PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts. Embo J. 22:1790-800.

Hiltbold, E.M., N.J. Poloso, and P.A. Roche. 2003. MHC class II-peptide complexes and APC lipid rafts accumulate at the immunological synapse. J Immunol. 170:1329-38.

Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science. 279:519-26.

Ho, H.Y., R. Rohatgi, A.M. Lebensohn, M. Le, J. Li, S.P. Gygi, and M.W. Kirschner. 2004. Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. Cell. 118:203-16.

Honda, A., M. Nogami, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A.J. Morris, M.A. Frohman, and Y. Kanaho. 1999. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell. 99:521-32.

Hopkins, C.R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. Cell. 35:321-30.

Horton, A.C., and M.D. Ehlers. 2003. Neuronal polarity and trafficking. Neuron. 40:277-95.

Howard, J., and A.A. Hyman. 2003. Dynamics and mechanics of the microtubule plus end. Nature. 422:753-8.

Huang, S., L. Lifshitz, V. Patki-Kamath, R. Tuft, K. Fogarty, and M.P. Czech. 2004. Phosphatidylinositol-4,5-bisphosphate-rich plasma membrane patches organize active zones of endocytosis and ruffling in cultured adipocytes. Mol Cell Biol. 24:9102-23.

Hunter, A.W., M. Caplow, D.L. Coy, W.O. Hancock, S. Diez, L. Wordeman, and J. Howard. 2003. The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. Mol Cell. 11:445-57.

Hyman, A.A., D. Chretien, I. Arnal, and R.H. Wade. 1995. Structural changes accompanying GTP hydrolysis in microtubules: information from a slowly hydrolyzable analogue guanylyl-(alpha,beta)-methylene-diphosphonate. J Cell Biol. 128:117-25.

Ikonen, E. 2001. Roles of lipid rafts in membrane transport. Curr Opin Cell Biol. 13:470-7.

Infante, A.S., M.S. Stein, Y. Zhai, G.G. Borisy, and G.G. Gundersen. 2000. Detyrosinated (Glu) microtubules are stabilized by an ATP-sensitive plus-end cap. J Cell Sci. 113 (Pt 22):3907-19.

Inoue, M., L. Chang, J. Hwang, S.H. Chiang, and A.R. Saltiel. 2003. The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature. 422:629-33.

Itoh, K., M. Sakakibara, S. Yamasaki, A. Takeuchi, H. Arase, M. Miyazaki, N. Nakajima, M. Okada, and T. Saito. 2002. Cutting edge: negative regulation of immune synapse formation by anchoring lipid raft to cytoskeleton through Cbp-EBP50-ERM assembly. J Immunol. 168:541-4.

Janes, P.W., S.C. Ley, and A.I. Magee. 1999. Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J Cell Biol. 147:447-61.

Janmey, P.A., and U. Lindberg. 2004. Cytoskeletal regulation: rich in lipids. Nat Rev Mol Cell Biol. 5:658-66.

Janmey, P.A., and T.P. Stossel. 1987. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. Nature. 325:362-4.

Jimbo, T., Y. Kawasaki, R. Koyama, R. Sato, S. Takada, K. Haraguchi, and T. Akiyama. 2002. Identification of a link between the tumour suppressor APC and the kinesin superfamily. Nat Cell Biol. 4:323-7.

Johannes, L., and C. Lamaze. 2002. Clathrin-dependent or not: is it still the question? Traffic. 3:443-51.

Johnson, D.I. 1999. Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiol Mol Biol Rev. 63:54-105.

Jones, D.H., J.B. Morris, C.P. Morgan, H. Kondo, R.F. Irvine, and S. Cockcroft. 2000. Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment. J Biol Chem. 275:13962-6.

Kanaani, J., M.J. Diacovo, D. El-Husseini Ael, D.S. Bredt, and S. Baekkeskov. 2004. Palmitoylation controls trafficking of GAD65 from Golgi membranes to axon-specific endosomes and a Rab5a-dependent pathway to presynaptic clusters. J Cell Sci. 117:2001-13.

Kaverina, I., O. Krylyshkina, M. Gimona, K. Beningo, Y.L. Wang, and J.V. Small. 2000. Enforced polarisation and locomotion of fibroblasts lacking microtubules. Curr Biol. 10:739-42.

Kaverina, I., O. Krylyshkina, and J.V. Small. 1999. Microtubule targeting of substrate contacts promotes their relaxation and dissociation. J Cell Biol. 146:1033-44.

Kaverina, I., K. Rottner, and J.V. Small. 1998. Targeting, capture, and stabilization of microtubules at early focal adhesions. J Cell Biol. 142:181-90.

Kay, A.J., and C.P. Hunter. 2001. CDC-42 regulates PAR protein localization and function to control cellular and embryonic polarity in C. elegans. Curr Biol. 11:474-81.

Kenworthy, A.K., B.J. Nichols, C.L. Remmert, G.M. Hendrix, M. Kumar, J. Zimmerberg, and J. Lippincott-Schwartz. 2004. Dynamics of putative raft-associated proteins at the cell surface. J Cell Biol. 165:735-46.

Kenworthy, A.K., N. Petranova, and M. Edidin. 2000. High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. Mol Biol Cell. 11:1645-55.

Kirschner, M., and T. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. Cell. 45:329-42.

Kodama, A., T. Lechler, and E. Fuchs. 2004. Coordinating cytoskeletal tracks to polarize cellular movements. J Cell Biol. 167:203-7.

Komarova, Y.A., A.S. Akhmanova, S. Kojima, N. Galjart, and G.G. Borisy. 2002. Cytoplasmic linker proteins promote microtubule rescue in vivo. J Cell Biol. 159:589-99.

Komarova, Y.A., I.A. Vorobjev, and G.G. Borisy. 2002. Life cycle of MTs: persistent growth in the cell interior, asymmetric transition frequencies and effects of the cell boundary. J Cell Sci. 115:3527-39.

Krauss, K., and P. Altevogt. 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. J Biol Chem. 274:36921-7.

Krylyshkina, O., K.I. Anderson, I. Kaverina, I. Upmann, D.J. Manstein, J.V. Small, and D.K. Toomre. 2003. Nanometer targeting of microtubules to focal adhesions. J Cell Biol. 161:853-9.

Kurzchalia, T.V., and R.G. Parton. 1999. Membrane microdomains and caveolae. Curr Opin Cell Biol. 11:424-31.

Kusch, J., A. Meyer, M.P. Snyder, and Y. Barral. 2002. Microtubule capture by the cleavage apparatus is required for proper spindle positioning in yeast. Genes Dev. 16:1627-39.

Lai, E.C. 2003. Lipid rafts make for slippery platforms. J Cell Biol. 162:365-70.

Lamaze, C., A. Dujeancourt, T. Baba, C.G. Lo, A. Benmerah, and A. Dautry-Varsat. 2001. Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. Mol Cell. 7:661-71.

Lang, T., D. Bruns, D. Wenzel, D. Riedel, P. Holroyd, C. Thiele, and R. Jahn. 2001. SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. Embo J. 20:2202-13.

Lassing, I., and U. Lindberg. 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature. 314:472-4.

Laux, T., K. Fukami, M. Thelen, T. Golub, D. Frey, and P. Caroni. 2000. GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J Cell Biol. 149:1455-72.

Le, P.U., G. Guay, Y. Altschuler, and I.R. Nabi. 2002. Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. J Biol Chem. 277:3371-9.

Ledesma, M.D., J. Abad-Rodriguez, C. Galvan, E. Biondi, P. Navarro, A. Delacourte, C. Dingwall, and C.G. Dotti. 2003. Raft disorganization leads to reduced plasmin activity in Alzheimer's disease brains. EMBO Rep. 4:1190-6.

Lee, H., D.S. Park, X.B. Wang, P.E. Scherer, P.E. Schwartz, and M.P. Lisanti. 2002. Src-induced phosphorylation of caveolin-2 on tyrosine 19. Phospho-caveolin-2 (Tyr(P)19) is localized near focal adhesions, remains associated with lipid rafts/caveolae, but no longer forms a high molecular mass hetero-oligomer with caveolin-1. J Biol Chem. 277:34556-67.

Li, F., and H.N. Higgs. 2003. The mouse Formin mDia1 is a potent actin nucleation factor regulated by autoinhibition. Curr Biol. 13:1335-40.

Ling, K., R.L. Doughman, A.J. Firestone, M.W. Bunce, and R.A. Anderson. 2002. Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. Nature. 420:89-93.

Lommel, S., S. Benesch, K. Rottner, T. Franz, J. Wehland, and R. Kuhn. 2001. Actin pedestal formation by enteropathogenic Escherichia coli and intracellular motility of Shigella flexneri are abolished in N-WASP-defective cells. EMBO Rep. 2:850-7.

Ma, L., L.C. Cantley, P.A. Janmey, and M.W. Kirschner. 1998. Corequirement of specific phosphoinositides and small GTP-binding protein Cdc42 in inducing actin assembly in Xenopus egg extracts. J Cell Biol. 140:1125-36.

Ma, L., Y.Z. Huang, G.M. Pitcher, J.G. Valtschanoff, Y.H. Ma, L.Y. Feng, B. Lu, W.C. Xiong, M.W. Salter, R.J. Weinberg, and L. Mei. 2003. Ligand-dependent recruitment of the ErbB4 signaling complex into neuronal lipid rafts. J Neurosci. 23:3164-75.

Machesky, L.M., S.J. Atkinson, C. Ampe, J. Vandekerckhove, and T.D. Pollard. 1994. Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. J Cell Biol. 127:107-15.

Malecz, N., P.C. McCabe, C. Spaargaren, R. Qiu, Y. Chuang, and M. Symons. 2000. Synaptojanin 2, a novel Rac1 effector that regulates clathrin-mediated endocytosis. Curr Biol. 10:1383-6.

Mallet, W.G., and F.R. Maxfield. 1999. Chimeric forms of furin and TGN38 are transported with the plasma membrane in the trans-Golgi network via distinct endosomal pathways. J Cell Biol. 146:345-59.

Manes, S., and A.C. Martinez. 2004. Cholesterol domains regulate the actin cytoskeleton at the leading edge of moving cells. Trends Cell Biol. 14:275-8.

Manes, S., E. Mira, C. Gomez-Mouton, R.A. Lacalle, P. Keller, J.P. Labrador, and A.C. Martinez. 1999. Membrane raft microdomains mediate front-rear polarity in migrating cells. Embo J. 18:6211-20.

Marchand, J.B., P. Moreau, A. Paoletti, P. Cossart, M.F. Carlier, and D. Pantaloni. 1995. Actin-based movement of Listeria monocytogenes: actin assembly results from the local maintenance of uncapped filament barbed ends at the bacterium surface. J Cell Biol. 130:331-43.

Marchand, S., A. Devillers-Thiery, S. Pons, J.P. Changeux, and J. Cartaud. 2002. Rapsyn escorts the nicotinic acetylcholine receptor along the exocytic pathway via association with lipid rafts. J Neurosci. 22:8891-901.

Martin, T.F. 2001. PI(4,5)P(2) regulation of surface membrane traffic. Curr Opin Cell Biol. 13:493-9.

Mauch, D.H., K. Nagler, S. Schumacher, C. Goritz, E.C. Muller, A. Otto, and F.W. Pfrieger. 2001. CNS synaptogenesis promoted by glia-derived cholesterol. Science. 294:1354-7.

Maxfield, F.R., and T.E. McGraw. 2004. Endocytic recycling. Nat Rev Mol Cell Biol. 5:121-32.

Mayor, S., S. Sabharanjak, and F.R. Maxfield. 1998. Cholesterol-dependent retention of GPI-anchored proteins in endosomes. Embo J. 17:4626-38.

Mejillano, M.R., S. Kojima, D.A. Applewhite, F.B. Gertler, T.M. Svitkina, and G.G. Borisy. 2004. Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. Cell. 118:363-73.

Mimori-Kiyosue, Y., N. Shiina, and S. Tsukita. 2000. Adenomatous polyposis coli (APC) protein moves along microtubules and concentrates at their growing ends in epithelial cells. J Cell Biol. 148:505-18.

Mitchison, T.J. 1993. Localization of an exchangeable GTP binding site at the plus end of microtubules. Science. 261:1044-7.

Mouneimne, G., L. Soon, V. DesMarais, M. Sidani, X. Song, S.C. Yip, M. Ghosh, R. Eddy, J.M. Backer, and J. Condeelis. 2004. Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. J Cell Biol. 166:697-708.

Mukherjee, A., L. Arnaud, and J.A. Cooper. 2003. Lipid-dependent recruitment of neuronal Src to lipid rafts in the brain. J Biol Chem. 278:40806-14.

Muller-Reichert, T., D. Chretien, F. Severin, and A.A. Hyman. 1998. Structural changes at microtubule ends accompanying GTP hydrolysis: information from a slowly hydrolyzable analogue of GTP, guanylyl (alpha,beta)methylenediphosphonate. Proc Natl Acad Sci U S A. 95:3661-6.

Munro, S. 2003. Lipid rafts: elusive or illusive? Cell. 115:377-88.

Muppidi, J.R., and R.M. Siegel. 2004. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. Nat Immunol. 5:182-9.

Nair, K.S., N. Balasubramanian, and V.Z. Slepak. 2002. Signal-dependent translocation of transducin, RGS9-1-Gbeta5L complex, and arrestin to detergent-resistant membrane rafts in photoreceptors. Curr Biol. 12:421-5.

Nathke, I.S., C.L. Adams, P. Polakis, J.H. Sellin, and W.J. Nelson. 1996. The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. J Cell Biol. 134:165-79.

Nelson, W.J. 2003. Adaptation of core mechanisms to generate cell polarity. Nature. 422:766-74.

Niethammer, P., M. Delling, V. Sytnyk, A. Dityatev, K. Fukami, and M. Schachner. 2002. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. J Cell Biol. 157:521-32.

Niwa, R., K. Nagata-Ohashi, M. Takeichi, K. Mizuno, and T. Uemura. 2002. Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell. 108:233-46.

Nogales, E., M. Whittaker, R.A. Milligan, and K.H. Downing. 1999. High-resolution model of the microtubule. Cell. 96:79-88.

O'Connor, K.L., and A.M. Mercurio. 2001. Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells. J Biol Chem. 276:47895-900.

Oh, P., D.P. McIntosh, and J.E. Schnitzer. 1998. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J Cell Biol. 141:101-14.

Ojala, P.J., V. Paavilainen, and P. Lappalainen. 2001. Identification of yeast cofilin residues specific for actin monomer and PIP2 binding. Biochemistry. 40:15562-9.

Palazzo, A.F., T.A. Cook, A.S. Alberts, and G.G. Gundersen. 2001. mDia mediates Rho-regulated formation and orientation of stable microtubules. Nat Cell Biol. 3:723-9.

Palazzo, A.F., C.H. Eng, D.D. Schlaepfer, E.E. Marcantonio, and G.G. Gundersen. 2004. Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. Science. 303:836-9.

Palazzo, A.F., H.L. Joseph, Y.J. Chen, D.L. Dujardin, A.S. Alberts, K.K. Pfister, R.B. Vallee, and G.G. Gundersen. 2001. Cdc42, dynein, and dynactin regulate MTOC reorientation independent of Rho-regulated microtubule stabilization. Curr Biol. 11:1536-41.

Pantaloni, D., R. Boujemaa, D. Didry, P. Gounon, and M.F. Carlier. 2000. The Arp2/3 complex branches filament barbed ends: functional antagonism with capping proteins. Nat Cell Biol. 2:385-91.

Paratcha, G., F. Ledda, L. Baars, M. Coulpier, V. Besset, J. Anders, R. Scott, and C.F. Ibanez. 2001. Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron. 29:171-84.

Paratcha, G., F. Ledda, and C.F. Ibanez. 2003. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell. 113:867-79.

Pardo, M., and P. Nurse. 2003. Equatorial retention of the contractile actin ring by microtubules during cytokinesis. Science. 300:1569-74.

Parton, R.G., and A.A. Richards. 2003. Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic. 4:724-38.

Pasterkamp, R.J., J.J. Peschon, M.K. Spriggs, and A.L. Kolodkin. 2003. Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. Nature. 424:398-405.

Pelham, R.J., and F. Chang. 2002. Actin dynamics in the contractile ring during cytokinesis in fission yeast. Nature. 419:82-6.

Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol. 3:473-83.

Perez, F., G.S. Diamantopoulos, R. Stalder, and T.E. Kreis. 1999. CLIP-170 highlights growing microtubule ends in vivo. Cell. 96:517-27.

Pierini, L.M., R.J. Eddy, M. Fuortes, S. Seveau, C. Casulo, and F.R. Maxfield. 2003. Membrane lipid organization is critical for human neutrophil polarization. J Biol Chem. 278:10831-41.

Pollard, T.D., and C.C. Beltzner. 2002. Structure and function of the Arp2/3 complex. Curr Opin Struct Biol. 12:768-74.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. Cell. 112:453-65.

Pralle, A., P. Keller, E.L. Florin, K. Simons, and J.K. Horber. 2000. Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J Cell Biol. 148:997-1008.

Prior, I.A., C. Muncke, R.G. Parton, and J.F. Hancock. 2003. Direct visualization of Ras proteins in spatially distinct cell surface microdomains. J Cell Biol. 160:165-70.

Pruyne, D., M. Evangelista, C. Yang, E. Bi, S. Zigmond, A. Bretscher, and C. Boone. 2002. Role of formins in actin assembly: nucleation and barbed-end association. Science. 297:612-5.

Raftopoulou, M., and A. Hall. 2004. Cell migration: Rho GTPases lead the way. Dev Biol. 265:23-32.

Raucher, D., T. Stauffer, W. Chen, K. Shen, S. Guo, J.D. York, M.P. Sheetz, and T. Meyer. 2000. Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. Cell. 100:221-8.

Rieder, C.L., and E.D. Salmon. 1998. The vertebrate cell kinetochore and its roles during mitosis. Trends Cell Biol. 8:310-8.

Riefler, G.M., G. Balasingam, K.G. Lucas, S. Wang, S.C. Hsu, and B.L. Firestein. 2003. Exocyst complex subunit sec8 binds to postsynaptic density protein-95 (PSD-95): a novel interaction regulated by cypin (cytosolic PSD-95 interactor). Biochem J. 373:49-55.

Rietveld, A., and K. Simons. 1998. The differential miscibility of lipids as the basis for the formation of functional membrane rafts. Biochim Biophys Acta. 1376:467-79.

Robinson, R.C., K. Turbedsky, D.A. Kaiser, J.B. Marchand, H.N. Higgs, S. Choe, and T.D. Pollard. 2001. Crystal structure of Arp2/3 complex. Science. 294:1679-84.

Rodriguez, O.C., A.W. Schaefer, C.A. Mandato, P. Forscher, W.M. Bement, and C.M. Waterman-Storer. 2003. Conserved microtubule-actin interactions in cell movement and morphogenesis. Nat Cell Biol. 5:599-609.

Roper, K., D. Corbeil, and W.B. Huttner. 2000. Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. Nat Cell Biol. 2:582-92.

Rozelle, A.L., L.M. Machesky, M. Yamamoto, M.H. Driessens, R.H. Insall, M.G. Roth, K. Luby-Phelps, G. Marriott, A. Hall, and H.L. Yin. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. Curr Biol. 10:311-20.

Salaun, C., D.J. James, and L.H. Chamberlain. 2004. Lipid rafts and the regulation of exocytosis. Traffic. 5:255-64.

Sankaram, M.B., and T.E. Thompson. 1990. Interaction of cholesterol with various glycerophospholipids and sphingomyelin. Biochemistry. 29:10670-5.

Sans, N., K. Prybylowski, R.S. Petralia, K. Chang, Y.X. Wang, C. Racca, S. Vicini, and R.J. Wenthold. 2003. NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. Nat Cell Biol. 5:520-30.

Schafer, D.A., P.B. Jennings, and J.A. Cooper. 1996. Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. J Cell Biol. 135:169-79.

Schroeder, R., E. London, and D. Brown. 1994. Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci U S A. 91:12130-4.

Schutz, G.J., G. Kada, V.P. Pastushenko, and H. Schindler. 2000. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. Embo J. 19:892-901.

Sedwick, C.E., and A. Altman. 2002. Ordered just so: lipid rafts and lymphocyte function. Sci STKE. 2002:RE2.

Sharma, D.K., A. Choudhury, R.D. Singh, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 2003. Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. J Biol Chem. 278:7564-72.

Sharma, P., R. Varma, R.C. Sarasij, Ira, K. Gousset, G. Krishnamoorthy, M. Rao, and S. Mayor. 2004. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. Cell. 116:577-89.

Sheff, D.R., E.A. Daro, M. Hull, and I. Mellman. 1999. The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. J Cell Biol. 145:123-39.

Simons, K., and R. Ehehalt. 2002. Cholesterol, lipid rafts, and disease. J Clin Invest. 110:597-603.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. Nature. 387:569-72.

Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 1:31-9.

Small, J.V., G. Isenberg, and J.E. Celis. 1978. Polarity of actin at the leading edge of cultured cells. Nature. 272:638-9.

Small, J.V., and I. Kaverina. 2003. Microtubules meet substrate adhesions to arrange cell polarity. Curr Opin Cell Biol. 15:40-7.

Stowers, L., D. Yelon, L.J. Berg, and J. Chant. 1995. Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42. Proc Natl Acad Sci U S A. 92:5027-31.

Sun, H.Q., M. Yamamoto, M. Mejillano, and H.L. Yin. 1999. Gelsolin, a multifunctional actin regulatory protein. J Biol Chem. 274:33179-82.

Svitkina, T.M., and G.G. Borisy. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. J Cell Biol. 145:1009-26.

Symons, M., and N. Rusk. 2003. Control of vesicular trafficking by Rho GTPases. Curr Biol. 13:R409-18.

Tall, E.G., I. Spector, S.N. Pentyala, I. Bitter, and M.J. Rebecchi. 2000. Dynamics of phosphatidylinositol 4,5-bisphosphate in actin-rich structures. Curr Biol. 10:743-6.

Taverna, E., E. Saba, J. Rowe, M. Francolini, F. Clementi, and P. Rosa. 2004. Role of lipid microdomains in P/Q-type calcium channel (Cav2.1) clustering and function in presynaptic membranes. J Biol Chem. 279:5127-34.

Thomsen, P., K. Roepstorff, M. Stahlhut, and B. van Deurs. 2002. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. Mol Biol Cell. 13:238-50.

Tzima, E., W.B. Kiosses, M.A. del Pozo, and M.A. Schwartz. 2003. Localized cdc42 activation, detected using a novel assay, mediates microtubule organizing center positioning in endothelial cells in response to fluid shear stress. J Biol Chem. 278:31020-3.

Upla, P., V. Marjomaki, P. Kankaanpaa, J. Ivaska, T. Hyypia, F.G. Van Der Goot, and J. Heino. 2004. Clustering induces a lateral redistribution of alpha 2 beta 1 integrin from membrane rafts to caveolae and subsequent protein kinase C-dependent internalization. Mol Biol Cell. 15:625-36.

van Meer, G. 2002. Cell biology. The different hues of lipid rafts. Science. 296:855-7.

Varma, R., and S. Mayor. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature. 394:798-801.

Vieira, O.V., R.J. Botelho, L. Rameh, S.M. Brachmann, T. Matsuo, H.W. Davidson, A. Schreiber, J.M. Backer, L.C. Cantley, and S. Grinstein. 2001. Distinct roles of class I and class III phosphatidylinositol 3-kinases in phagosome formation and maturation. J Cell Biol. 155:19-25.

Vinson, M., P.J. Strijbos, A. Rowles, L. Facci, S.E. Moore, D.L. Simmons, and F.S. Walsh. 2001. Myelin-associated glycoprotein interacts with ganglioside GT1b. A mechanism for neurite outgrowth inhibition. J Biol Chem. 276:20280-5.

Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science. 283:680-2.

Wang, E., P.S. Brown, B. Aroeti, S.J. Chapin, K.E. Mostov, and K.W. Dunn. 2000. Apical and basolateral endocytic pathways of MDCK cells meet in acidic common endosomes distinct from a nearly-neutral apical recycling endosome. Traffic. 1:480-93.

Watanabe, N., T. Kato, A. Fujita, T. Ishizaki, and S. Narumiya. 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat Cell Biol. 1:136-43.

Watanabe, T., S. Wang, J. Noritake, K. Sato, M. Fukata, M. Takefuji, M. Nakagawa, N. Izumi, T. Akiyama, and K. Kaibuchi. 2004. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. Dev Cell. 7:871-83.

Waterman-Storer, C.M., W.C. Salmon, and E.D. Salmon. 2000. Feedback interactions between cell-cell adherens junctions and cytoskeletal dynamics in newt lung epithelial cells. Mol Biol Cell. 11:2471-83.

Weaver, A.M., M.E. Young, W.L. Lee, and J.A. Cooper. 2003. Integration of signals to the Arp2/3 complex. Curr Opin Cell Biol. 15:23-30.

Weston, C., B. Yee, E. Hod, and J. Prives. 2000. Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. J Cell Biol. 150:205-12.

Wilson, B.S., S.L. Steinberg, K. Liederman, J.R. Pfeiffer, Z. Surviladze, J. Zhang, L.E. Samelson, L.H. Yang, P.G. Kotula, and J.M. Oliver. 2004. Markers for detergent-resistant lipid rafts occupy distinct and dynamic domains in native membranes. Mol Biol Cell. 15:2580-92.

Wong, W., and L.C. Schlichter. 2004. Differential recruitment of Kv1.4 and Kv4.2 to lipid rafts by PSD-95. J Biol Chem. 279:444-52.

Woodman, P.G. 2000. Biogenesis of the sorting endosome: the role of Rab5. Traffic. 1:695-701.

Wu, J.Q., J.R. Kuhn, D.R. Kovar, and T.D. Pollard. 2003. Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. Dev Cell. 5:723-34.

Yamaoka-Tojo, M., M. Ushio-Fukai, L. Hilenski, S.I. Dikalov, Y.E. Chen, T. Tojo, T. Fukai, M. Fujimoto, N.A. Patrushev, N. Wang, C.D. Kontos, G.S. Bloom, and R.W. Alexander. 2004. IQGAP1, a novel vascular endothelial growth factor receptor binding protein, is involved in reactive oxygen species--dependent endothelial migration and proliferation. Circ Res. 95:276-83.

Yebra, M., A.M. Montgomery, G.R. Diaferia, T. Kaido, S. Silletti, B. Perez, M.L. Just, S. Hildbrand, R. Hurford, E. Florkiewicz, M. Tessier-Lavigne, and V. Cirulli. 2003. Recognition of the neural chemoattractant Netrin-1 by integrins alpha6beta4 and alpha3beta1 regulates epithelial cell adhesion and migration. Dev Cell. 5:695-707.

Yin, H.L., and P.A. Janmey. 2003. Phosphoinositide regulation of the actin cytoskeleton. Annu Rev Physiol. 65:761-89.

Zacharias, D.A., J.D. Violin, A.C. Newton, and R.Y. Tsien. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. Science. 296:913-6.

Zebda, N., O. Bernard, M. Bailly, S. Welti, D.S. Lawrence, and J.S. Condeelis. 2000. Phosphorylation of ADF/cofilin abolishes EGF-induced actin nucleation at the leading edge and subsequent lamellipod extension. J Cell Biol. 151:1119-28.

Zimmer, M., A. Palmer, J. Kohler, and R. Klein. 2003. EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. Nat Cell Biol. 5:869-78.

CURRICULUM VITAE

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Date of birth:	September 2nd , 1976
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Education

Mar 2000 – present	Postgraduate study in Cell Biology at the University of Basel, Phil II
Sep 1995 – Mar 2000	University of Zagreb, Faculty of Natural Sciences, Biology Department: Graduated March 2000: MSc. Molecular Biology (grade: 98%)
Sep 1991 – Jun 1995	Gymnasium, Zagreb, Croatia

Scholarships and awards

1994	Third prize at Regional Physics Competition, Zagreb, Croatia
1995	First prize at Regional Biology Competition, Zagreb, Croatia
1995	First prize at the National Competition of young biologists, Murter, Croatia
1996	"Scholarship of the Croatian Ministry of Science and Technology"
1998	"Scholarship of the University of Zagreb" (awarded to top 75 students)
1999	"Scholarship of the City of Zagreb"

Professional experience

Mar 2000 – present

Friedrich Miescher Institute for Biomedical Research (Novartis Research Foundation)

Ph.D. student in the Laboratory of Dr. Pico Caroni, Dept. of Neurosciences,

In the lab, we are interested in synaptic plasticity and what cellular and molecular mechanisms underlie these changes. My project involves illuminating the mechanisms promoting and controlling cell surface motility involved in a variety of cellular functions starting from migration during development to plastic changes neurons undergo during development and regeneration.

We have found that local assembly of dynamic PIP2-rich raft platforms anticipate, promote and sustain lamellipodial and filopodial motility at the leading edge. Rac-dependent accumulation of PIP2 at the cell edge, together with Cdc42, induces raft assembly through N-WASP.

We've also found that PIP2-rich platforms enhance local signaling for motility, capture and stabilize microtubules that target PKA at the cell edge, coupling local regulation of motility to cell organization.

Jun 1999 – Feb 2000

Dept. of Molecular Genetics of Eucaryotes, Ruđer Bošković Institute, Zagreb, Croatia

MSc. student, Laboratory of Dr. Mary Sopta for Gene Regulation

I worked on the regulation of transcription in the model organism *Saccharomyces cerevisiae* on the novel protein XTC1 that has been found to co-purify with yeast RNA polymerase II.

In the lab, we showed that Δxtc1 mutants show galactose auxotrophy, arresting in the Start phase of the cell cycle, as well as a more efficient transcription of the reporter gene then the wt cells.

These results, together with previously published results, indicated that Xtc1p could be a component of RNA polymerase II holoenzyme with a repressing function.

Publications:

Laux T, Fukami K, Thelen M, **Golub T**, Frey D, Caroni P
"GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism"
J Cell Biol. 2000, 149:1455-72.

Golub T, Wacha S and Caroni P
"Spatial and temporal control of signaling through lipid rafts" *Curr Opin Neurobiol* 2004, 14(5): 542-550, review.

Golub T and Caroni P
"Spatial control of actin-based motility through plasmalemmal PI(4,5)P_2-rich raft assemblies", *Biochem.Soc.Symp.* 2005, 72:119-27.

Golub T and Caroni P
"Regulation of the leading edge motility by PI(4,5)P2-dependent lipid microdomains"
J Cell Biol., 2005, 169(1):151-165.

Languages

English – excellent
German – good
Croatian – native speaker

Computer literacy

Microsoft Word, Microsoft Excel, Microsoft Power Point, Adobe Photoshop, Corel Draw/Photo Paint, Image-Pro5, Web page design

Hobbies

Swimming, dancing, aerobics, painting, choir singing, b/w photography