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ScienceDirect
Cellular Signalling 19 (2007) 1621–1632
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Review

Scaffold mediated regulation of MAPK signaling and cytoskeletal dynamics: A perspective

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Received 5 April 2007; accepted 23 April 2007
Available online 5 May 2007

Abstract

Cell migration is critical for many physiological processes and is often misregulated in developmental disorders and pathological conditions including cancer and neurodegeneration. MAPK signaling and the Rho family of proteins are known regulators of cell migration that exert their influence on cellular cytoskeleton during cell adhesion and migration. Here we review data supporting the view that localized ERK signaling mediated through recently identified scaffold proteins may regulate cell migration.

Keywords: MP1; p14; Scaffold; Actin; Microtubule dynamics; Calpains; Rho; PAK; MEK; ERK; Ste5; Cell migration

Contents

1. Introduction

The mitogen-activated protein kinase (MAPK) pathways that activate ERK, JNK and p38 kinases play important roles in modifying the morphogenetic and motile responses of cells. These pathways receive inputs from both soluble growth factors and extracellular matrix proteins to regulate the localization, amplitude and duration of MAPK signaling, and hence the

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doi:10.1016/j.cellsig.2007.04.012

spectrum of targets phosphorylated. Emerging data implicate MAP kinase signaling in the control of F-actin and focal adhesion formation and turnover required for cell morphogenesis and migration. The goals of this brief review are to describe how ERK signaling is connected to these processes, and to use comparative interactome observations to speculate on possible novel connections where little or no direct experimental data exist. Where possible, we will suggest mechanisms by which ERK scaffolding proteins may be important in localizing signaling to sites of actin and adhesion turnover. We refer the reader to a recent review that summarized the influence of the p38 and JNK pathways on cell migration [1].

2. ERK activation in response to growth factors and adhesion signals

Binding of growth factors to cognate receptor tyrosine kinases at the plasma membrane promotes activation of Ras. Ras recruits Raf family kinases to the membrane where they are activated by incompletely understood mechanisms including phosphorylation/dephosphorylation and lipid binding. Active Raf phosphorylates and activates the dual specificity tyrosine and threonine kinases MEK1&2, which are upstream kinases for ERK1&2. Activated ERK can be translocated to the nucleus to regulate transcription or retained/routed within the cytoplasm to regulate diverse activities including cell motility, organelle structure, integrin signaling and cytoskeletal dynamics. Integrins are cell surface receptors composed of α and β chains that respond to extracellular matrix (ECM) components and signal to multiple intracellular pathways [2,3]. Integrin engagement following adhesion to ECM is also an activator of MAPK signaling [4,5]. Integrin activation of MAPK requires FAK autophosphorylation and c-Src activation [6], and has been proposed to involve SH2-mediated recruitment of the adapter protein Grb2 involved in growth factor activation of Ras to a site of c-Src phosphorylation (Y925) in FAK [7,8]. Alternatively, integrins may cooperate with growth factor receptors such as the PDGF, insulin and EGF receptors to activate their respective downstream signaling mechanisms [9–11]. However, while some laboratories find that Ras is required for ERK activation during adhesion [8,12,13], others find that inhibiting Ras is without effect on adhesion stimulated ERK activation [14,15]. Thus, alternative Ras-independent mechanisms may activate ERK during adhesion. In this regard, we and others have investigated the role of the small GTPases Rac and Cdc42 and their downstream effector PAK in activating ERK signaling during adhesion [16,17]. We and others have reported that c-Src and FAK dependent PAK phosphorylation of MEK1 may prime the ERK module for activation by low levels of Raf activity during adhesion [18], although it is possible that PAK may directly activate MEK1 in a Raf-independent fashion [19]. MEK1 phosphorylated by PAK1 transiently localizes to peripheral membrane structures and focal contacts during cellular adhesion to fibronectin, and exhibits enhanced ERK binding activity [18]. Note that these Ras-dependent and –independent pathways are not mutually exclusive: the population of MEK1 that is phosphorylated by PAK in response to adhesion may be sensitized to Raf activated downstream of integrin engagement, thereby coupling adhesion stimulated Rac-Cdc42 to MAPK signaling.

3. Rho family GTPases regulate actin dynamics

The Rho family of small GTPases including Rac, Cdc42 and Rho coordinate to control the formation, respectively, of lamellipodia, filipodia and stress fibers and focal adhesions [5,20–26]. The formation of these distinct structures is dependent on actin polymerization (Fig. 1A). A family of related WAVE and WASP/Scar proteins bridge Rac and Cdc42 to the Arp2/3 complex that functions to nucleate actin polymerization and facilitate dendritic branching of actin filaments [27,28]. Rac through its binding to IRSp53 regulates WAVE dependent activation of Arp2/3 [29–31] to stimulate accumulation of densely branched actin filaments in lamellae and membrane ruffles that drive membrane protrusion [32]. Similarly, Cdc42 directly interacts with WASP to stimulate actin polymerization in filipodia [33,34]. Formation of these dynamic actin structures at the cell periphery enables the cell to sample the surrounding substrate in preparation for cell movement.

In contrast, Rho can oppose Rac and Cdc42 function by limiting membrane protrusion [20]. Signaling through its effectors Rho kinase (ROCK) and mDia, Rho stimulates actin stress fiber formation by stimulating the monomeric actin binding protein profilin, and stabilizes stress fibers by inhibiting the activity of the actin depolymerizing protein, cofilin [35–40]. Bundling of actin filaments with myosin to form stress fibers, itself stimulated by ROCK and myosin light chain kinase, stimulates cell contraction and stabilization of focal adhesions [41–44], points of integrin-extracellular matrix contact between the ventral surface of the cell and the underlying substrate. Rho-mediated contractility is important for retraction of the cell rear and translocation of the cell body [26]. It follows that the coordinated assembly and disassembly of actin structures is subject to strict temporal and spatial control.

4. Rho regulation of microtubules

In migrating cells, the nucleus is positioned behind the microtubule organizing center (MTOC), orienting the MTOC towards the leading edge [45,46], thereby creating a polarized microtubule array aligned along the direction of migration with their plus-ends oriented towards the leading edge [47]. Microtubules undergo net growth during migration that is thought to be essential for providing membrane and signaling molecules to membrane protrusions. The identification of proteins that accumulate at the growing tip of microtubules (so called +TIPs) supports the view that regional accumulation of proteins including APC, CLIP170 and CLASPs may stabilize plus ends and/or promote the assembly of microtubules with motors to transfer associated cargoes to the leading edge during membrane protrusion and migration. Considerable data now support the idea that Rho proteins regulate, and are regulated by, the microtubule cytoskeleton [47] in addition to regulating actin dynamics. Depolymerization of microtubules increases

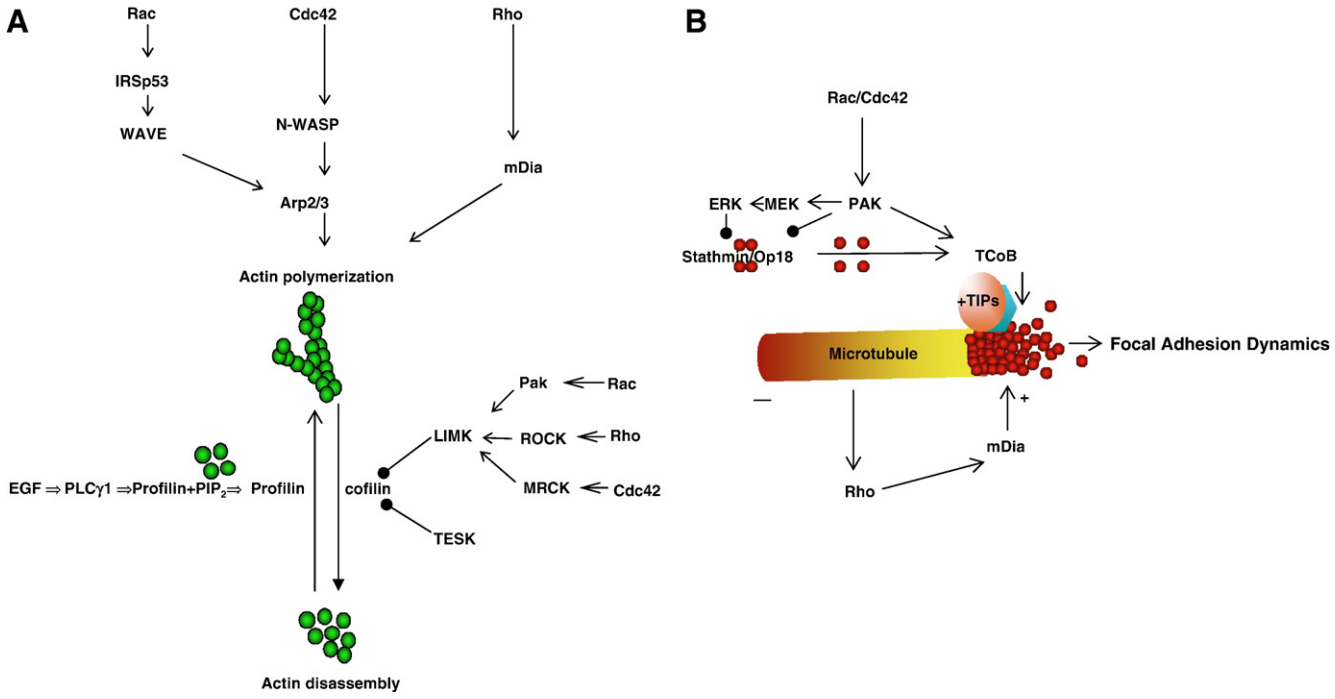

Fig. 1. Rho family of proteins regulate actin and microtubule dynamics. A) Membrane protrusion is governed by regulated assembly and disassembly of actin cytoskeleton. Activated Rac and Cdc42 respectively activate WAVE and WASP family of proteins to regulate actin polymerization through Arp2/3. Steady state actin bundles are maintained by the opposing effects of profilin-dependent assembly and cofilin-dependent disassembly. B) The Rho effector, mDia is required for stabilization of microtubules. Stable microtubules perhaps transport activated Rac to membrane protrusions. PAK, the downstream effector of Rac/Cdc42, and ERK downstream of activated PAK promote microtubule polymerization by inhibiting stathmin's tubulin sequestration activity thereby providing free tubulin for tubulin cofactor (TcoB) dependent α- and β- tubulin heterodimerization. PAK can directly activate TcoB and promote its tubulin polymerization activity. Microtubule dynamics may also facilitate transfer of calpain proteases in kinesin-dependent fashion to downregulate integrin signaling by proteolytic cleavage of FAK and dynamin-dependent disassembly of focal adhesions.

Rho GTP levels and results in cell contraction mediated by bundling of actin with myosin motors into stress fibers that exert tension on focal adhesions [48]. Conversely, polymerization of microtubules results in Rac activation and production of lamellipodia [49]. These opposing effects of depolymerization and polymerization are hypothesized to regulate the activities of Rho regulators including RhoGEFs. The RhoA exchange factors, p190RhoGEF [50] and GEF-H1 [51] bind microtubules and can result in activation of RhoA even though RhoA does not directly bind microtubules. A functional correlation between microtubules and Rho activity could also be drawn from studies on another exchange factor, TrioGEF that activates RhoG [52]. RhoG is an activator of Rac and Cdc42 that can promote membrane protrusion [53]. Neither RhoG nor TrioGEF binds directly to microtubules, but their activities on Rac- and Cdc42-dependent protrusion and localization to the cell periphery are dependent on an intact microtubule network [53]. This might imply that microtubule motors provide a link between Rac, Cdc42 and Rho and/or their activating GEFs and microtubules to transport activated Rho proteins to protrusions. Since kinesin (a predominantly plus end directed motor family) and its interactor, kinectin are required for RhoG mediated actin assembly [54], and RhoG binds kinectin [53], kinesin motors may transport activated RhoG to sites proximal to cell adhesions. Interestingly, the Rac GEF, ASEF is activated upon binding to APC, a plus end binding protein that surfs the growing tip of microtubules [55], perhaps providing a means to target active Rac to membranes during cell adhesion [56].

Microtubule dynamics is influenced by Rho proteins. Activation of Rho has a direct positive effect on microtubule stability at the leading edge through its downstream effector, mDia [57,58]. Additionally, Rac and Cdc42 can promote microtubule growth through PAK1 mediated phosphorylation and inhibition of the microtubule destabilizing protein stathmin/Op18 [59] and through PAK dependent activation of tubulin cofactor B that mediates tubulin heterodimeration [60]. Thus, microtubule dynamics may activate Rho proteins, which in turn feed back to control the net growth and stability of microtubules at the leading edge [47]. The combined effects of Rho proteins on actin and microtubule cytoskeleton and the dynamic interactions [61,62] between these two filament systems indicate that Rho proteins exert their influence on cell motility by coordinate control of cytoskeleton converging on focal adhesion dynamics (Fig. 1B).

5. Focal adhesion dynamics

Integrin ligation leads to the assembly of focal contact structures that mature to focal adhesions serving as both points of contact between the actin stress fiber network and the underlying substrate and as sites for signaling by numerous integrin stimulated pathways regulating focal adhesion dynamics and cell fate (e.g. apoptosis, proliferation, migration). The

formation of focal adhesions from nascent adhesions is determined by the rigidity of the matrix as sensed by integrins, which in turn modulate cell contractility primarily through signaling cascades impinging on the actomyosin system [63]. Activation of Rho stimulates contractility and focal adhesion formation by promoting bundling of actin filaments and associated myosin motors into stress fibers [44]. Rho-ROCK signaling influences contraction through effects on both the myosin and actin: ROCK elevates myosin light chain phosphorylation by inhibiting myosin phosphatase and/or by directly phosphorylating myosin light chain (MLC) [41–43]. ROCK also phosphorylates and activates LIM kinase [64], which in turn phosphorylates and inactivates the actin severing protein, cofilin [36,65].

Rac and Cdc42 also negatively regulate cofilin function through LIM kinases and thereby stabilize actin stress fibers. While Rac entrains PAK for this function [66,67], Cdc42 signals through MRCK [36,65]. Finally, the recently discovered TESKs can directly phosphorylate cofilin [68], perhaps in response to Rac signaling [69] (Fig. 1A). Regardless of the precise upstream components, cofilin phosphorylation results in reduced actin filament disassembly and thus stabilization of stress fibers. Stress fibers are therefore available to be anchored to mature focal adhesions [2,70] and can transmit contractile forces exerted by the actomyosin machinery to the extracellular matrix.

Increased cell contraction results in the recruitment of signaling proteins to the focal adhesions, and these regulate the activation state of integrins and focal adhesion stability [3,71–73]. Focal adhesions contain more than fifty proteins, many of which are tyrosine phosphorylated and/or themselves tyrosine kinases. Tyrosine phosphorylation at adhesion sites occurs after the initial recruitment of talin and paxillin apparently followed by FAK and vinculin to focal sites [74–77]. Tensin and zyxin are generally absent from nascent adhesions [74]. This ordered assembly promotes sequential tyrosine phosphorylation at nascent complexes and regulates the maturation and life-time of adhesions [74,77,78]. However, it should be noted that focal adhesions do not mature solely by accumulating signaling molecules; there is also an ordered exit of molecules during maturation, as evidenced by the decreased content of FAK and zyxin in mature adhesions [74,79]. RhoA might play a role in focal adhesion disassembly by recruiting c-Src to focal adhesions through activation of mDia [80]. Further, elevated RhoA activity in the leading edge [81] might determine mDia regulated stabilization of microtubules [57] that are required for targeted disassembly of focal adhesions [82] (see below) and actin-mediated recruitment of c-Src to membranes and focal adhesions [80].

The localization of tyrosine kinases to adhesion sites and increased phospho-tyrosine content of focal adhesions [83,84] led to the proposal that focal adhesions serve as signaling organelles where environmental cues are translated into cytoskeletal changes for the required cellular response [85]. Gene ablation studies confirm this view. For instance, while FAK is not needed for focal adhesion formation *per se*, the disassembly of focal adhesion components requires FAK dependent processes. Thus, FAK null cells contain enlarged focal adhesions and "thicker" actin stress fibres and are severely impaired in migration [86]. These defects are in part due to misregulation of Rho-ROCK signaling [87,88] but might also involve MEK-ERK signaling [79] and microtubules (see below).

6. ERK regulation of Rho-ROCK function

The presence of activated MEK and ERK on cytoskeletal structures and their association with focal adhesions and microtubules is consistent with the hypothesis that localized MAPK signaling modulates Rho signaling [89–95]. Indeed, considerable evidence indicates that the integration of Rho family GTPase and ERK signaling is important in the control of cell morphogenesis [96–103]. Integrin activation of MAPK requires c-Src and FAK, and ERK activity is required for FAK stimulated focal adhesion disassembly to promote cell migration [79,89]. Several studies have shown that FAK or v-Src can suppress the activities of Rho [48,87] and its effector, ROCK [88], and this pathway requires ERK signaling [79,104] to modulate actin and focal adhesion dynamics. Consistent with these observations, activated FAK and c-Src and activated ERK localize to focal adhesions [89]. It is presently not clear how MAPK regulates Rho and ROCK activities downstream of FAK, although logical targets would include the GEFs and GAPs that set GTP loading of Rho. Integrin engagement activates p190RhoGAP, a negative regulator of Rho [105], through the tyrosine kinases Src [106–110], FAK [111] and Arg [112,113]. p190RhoGAP localizes to structures relatively poor in stress fibers [108] and arc-like structures in EGF stimulated cells with concomitant reduction in actin stress fibers [114], suggesting a functional link between the RhoGAP and actin dynamics during cell spreading and growth factor signaling [114,115]. However, while activation of p190RhoGAP requires tyrosine phosphorylation of Y1105 [116–118], tyrosine phosphorylation alone appears insufficient to activate p190 RhoGAP activity, at least in response to growth factor stimulation [119,120]. It is currently not known whether p190RhoGAP is regulated by ERK signaling. In contrast, the FAK associated RhoGAP GRAF [121,122] is a substrate for ERK [123], but is not characterized with respect to regulation of Rho during acute adhesion and spreading. To our knowledge, the regulation of Rho GEFs by ERK signaling has not been characterized.

ERK is also implicated in the co-ordinate control of Rho and Rac activities in fibroblast models of transformation [101] and human tumor cells [103]. However, the precise mechanisms and targets through which ERK signals, and their spatiotemporal control, are not well understood. In these situations, ERK signaling does not acutely influence Rho function, but rather sustained ERK signaling selects for down-regulation of Rho or Rho effector function [101,103]. In colon carcinoma cells, loss of Fra1 results in an up-regulation of RhoGTP levels and ROCK activity as a result of activation of β_1 but not β_3 integrins [103]. Since Fra1 expression is controlled by ERK, these studies demonstrate that ERK activity promotes migration of colon carcinoma cells in part by Fra1-dependent suppression of Rho/ROCK activation. In contrast, Ras- and Raf-transformed fibroblasts exhibit increased motility in spite of high RhoGTP levels [101]. In these cases, sustained ERK activation uncouples

ROCK from Rho and thereby inhibits stress fiber formation [101]. These mechanisms evolve during cellular transformation and likely function to increase the motile and invasive behavior of tumor cells [101,103].

Distinct functional pools of ERK are hypothesized to be defined by outcome-specific scaffolds that couple ERK activation and/or function to select downstream targets in a spatially and temporally restricted manner [124,125]. Our recent observations have suggested a potential role for the MP1-p14 complex in coupling MAP kinase signaling to Rho [126]. MP1 is a small protein (14 kDa) identified as a MEK1 and ERK1 binding partner [127]; p14 is a protein tightly associated with late endodomes and lysosomes that was subsequently found to interact with high affinity with MP1 [128–130]. Specifically, we have found that MP1-p14 is important for PAK phosphorylation of MEK1 and ERK activation during adhesion to fibronectin, and that siRNA-mediated depletion of the complex causes a spreading defect in fibroblasts in concert with the formation of dense circumferential F-actin and large vinculin containing adhesions [126]. In contrast to the typical transient decrease in RhoGTP levels seen in control fibroblasts [48,109], cells depleted of MP1 exhibit increasing RhoGTP levels during replating on fibronectin. Furthermore, treatment of these cultures with either of two ROCK inhibitors causes dissolution of F-actin and adhesions and restores cell spreading [126]. These data suggest that MP1-p14 controls a population of ERK important for the remodeling of focal adhesion and actin structures during spreading. However, it should be noted that knock-down of MP1 and pharmacological inhibition of MEK signaling did not give identical results, indicating that MP1-p14 may signal through pathways in addition to ERK to regulate actin and focal adhesions. In this regard, it is perhaps relevant that MP1 also associates with active mutants of PAK1 [126]. The potential role of endosomal localization of MP1 in focal adhesion turnover is not yet known and will be discussed below. However, of particular interest is the recent finding that MP1, p14 and MEK control endosomal localization [131], perhaps consistent with a role for ERK regulation of endocytosis of adhesion components or trafficking of signaling proteins to and from dynamic adhesions (see below).

7. Microtubule and endocytic regulation of focal adhesions

One might envision focal adhesions to be destabilized in at least three ways. First, stress fibers might be targeted for disassembly, thus reducing contractile forces on focal adhesions. Second, the linkages between stress fibers and focal adhesions may be severed. Third, structural components of focal adhesions themselves may be degraded or recycled. Available experimental data implicating ERK and ERK scaffolds in these mechanisms will be discussed below.

Focal adhesion disassembly is not simply the reversal of assembly. While active Rho is required for focal adhesion assembly and stress fiber formation, RhoGTP alone is insufficient to promote the formation of focal adhesions and stress fibers in all cases. In mitotic cells and in Ras transformed cells, elevated Rho activation does not stimulate focal adhesion and F-actin formation [101,132]. In these situations, Rho activity appears to be uncoupled from its known effector functions(s). Biochemical and cell imaging data have illuminated an intimate connection between microtubule dynamics and focal adhesion disassembly. Depolymerizing microtubules with nocodazole treatment results in accumulation of enlarged focal adhesions with increased phosphotyrosine content, which has led to the proposal that Rho mediated cell contraction might be countered by the microtubule architecture [73,133–135]. Time lapse imaging studies indicated that growing microtubules target focal adhesions for subsequent disassembly [133,136]. A single focal adhesion may be targeted multiple times and a single microtubule tip may target multiple focal adhesions suggesting dynamic interaction of microtubule and actin filament systems and their associated molecular motors [136,137]. Consistent with this view, inhibiting the kinesin motors (predominantly plus-end directed motors of the microtubule system) can interfere with focal adhesion disassembly without affecting microtubule dynamics or targeting to focal adhesions [137,138]. Thus, microtubules may direct kinesin motors bound to disassembly factors to focal sites to promote adhesion turn over. The molecular nature of factors that are delivered to focal adhesions by microtubules have remained elusive [134,137,139] but might include the class of calcium activated proteases, calpains [140,141] (see below). Interestingly, Ezratty et. al., recently provided evidence that microtubule stimulated disassembly of focal adhesions can be inhibited by a dominant negative form of dynamin, a master regulator of endocytosis [82]. Dynamin, a large GTPase, forms a collar around membrane invaginations to cause scission of budding vesicles that are targetted to the endocytic membrane system. Dynamin self assembly and GTPase activity are significantly enhanced by microtubules [142–144]. Further dynamin has been identified in complex with actin regulators, including profilin and ROCK [145]. These studies raise the possibility that microtubules targeting focal adhesions may stimulate dynamin-dependent formation of endocytic vesicles that can internalize structural or signaling components stabilizing focal adhesions. As integrin signaling suppresses internalization of membrane domains [146], one attractive possibility is that kinesin mediated transfer of calpains to focal adhesions could relieve integrin inhibition of endocytic domains and thus promote endocytic clearance of integrins and signaling molecules from adhesions. Endocytosis may also allow initiation of integrin signaling at newly protruding membranes by recycling receptors and signaling modules to the leading edge [147,148].

8. ERK and microtubule and motor dynamics

While mechanistic connections between ERK signaling, microtubules and endocytosis of focal adhesion components remain to be determined, we will speculate on possible novel links. First, dominant-negative dynamin inhibits ERK activation in response to many receptor tyrosine kinase and G-protein coupled ligands [149], and much evidence indicates that ERK signaling continues on endosomes internalized following receptor stimulation [124]. Interestingly however, Moolenaar

and colleagues [149] determined that internalization of a plasma membrane receptor *per se* was not required for dynamin's effect on ERK activation, since phorbol ester stimulated activation of ERK was also blocked by dominant-negative dynamin. Furthermore, activation of Ras, Raf and MEK proceeded normally in the presence of the inhibitory dynamin mutant. These data suggest that internalization of active MEK is the dynamin-dependent step in ERK activation by these soluble ligands. Consistent with this view, active MEK has been localized at the plasma membrane and in intracellular structures believed to be vesicles [149] and on early endosomes [150].

Less is known about dynamin's role in ERK activation downstream of adhesion receptors. Maness and colleagues have determined that dynamin-mediated endocytosis of the neural cell adhesion molecule L1 following homotypic interaction or antibody-induced clustering is essential for ERK activation and neurite outgrowth [151]. L1 dependent activation of ERK can be blocked by preventing L1 endocytosis and internalized L1 colocalizes with ERK [152]. While L1 is not an integrin, it functionally interacts with β_1 integrins to potentiate neuronal migration toward ECM through endocytosis and MAP kinase signaling [153]. Though definitive data are lacking, the finding that L1 associates with β_1 integrins and signals through some of the same intermediates that β_1 integrins use (e.g. Src, Rac, PI3K, ERK) suggest that L1 may stimulate ERK activation through β_1 integrins. Interestingly, while L1 signaling to ERK is independent of FAK and Ras, it does require Rac and PI3 kinase [151] consistent with the possibility that L1 activates ERK primarily through PAK-dependent phosphorylation of Raf and/or MEK1.

A review of the available literature suggests interesting potential connections between ERK scaffolding proteins and microtubule/endosome-mediated turnover of focal adhesions. For instance, MP1 associates tightly with p14, a protein tightly associated with late endosomes and lysosomes [129,130], and this localization is important for ERK activation in response to EGF [128]. MP1-p14 appears important for focal adhesion remodeling during cell spreading on fibronectin [126]. These data might suggest that endosomes containing the p14-MP1 complex traffic active MEK1 downstream of dynamin-mediated scission at the plasma membrane to enable ERK activation and subsequent focal adhesion disassembly in response to growth factor stimulation. However, the finding that MP1 does not associate with early endosomes, but rather with late endosomes and lysosomes [128] is not obviously consistent with this hypothesis. EGF stimulated ERK does not colocalize with MP1/p14 on late endosomes at early time points suggesting sustained activation of ERK might require endosomal maturation or transfer of MEK and ERK from an early endosomal compartment to the late endosomal population containing MP1/p14. Alternatively, MP1 and/or p14 might directly regulate the localization of active ERK since recent evidence has shown that MP1/p14 is required for the correct localization of late endosome/lysosome compartment to the perinuclear regions in the cell [131] and might have a role in endosomal biogenesis [154].

It is interesting to note that the crystal structure of MP1/p14 bears a striking similarity to the Roadblock/LC7 dynein light chain homodimer [130,155,156]. Roadblock/LC7 is assembled with members of two other distinct light chain families, namely LC8 (DLC1), and Tctex1, into a 1.2 MDa cytoplasmic dynein complex that functions as a minus end-directed molecular motor to traffic cargo from the plus end of microtubules to the MTOC in the perinuclear region. The function of DLC is regulated by PAK phosphorylation on DLC-Ser88 [157] that enhances endosomal trafficking and macropinocytosis [158]. Apart from its well known role as a component of dynein motors, DLC is also present as a stoichiometric subunit of the myosin V motors that transport short-range vesicles in the cell cortex [159,160] raising the possibility that PAK regulation of DLC function may affect traffic through both filament systems. Furthermore, like MP1/p14, LC8 (DLC1) also interacts with PAK1, a kinase that phosphorylates both MEK1 and LC8 [157]. One intriguing possibility is that MP1/p14 may incorporate into the dynein motor complex through the LC7-like motif in p14 and thus tether MEK1, ERK1 and PAK1 to the dynein motor complex on microtubules. Since MP1 binds preferentially to inactive MEK1 [127] and active PAK1 [126], one might speculate that MP1/p14 could serve to integrate MEK and PAK signals into the microtubule and motor machinery. Alternatively, PAK1 bound to DLC1 through PAK1 N-terminus, might bridge the MP1/p14 dimer bound to its C-terminus to molecular motors on microtubules facilitating specific targeting to cellular sites of action (Fig. 2).

Together, this model suggests that MEK and ERK found on early endosomes may be transferred or matured to late endosome/lysosomes through their interaction with MP1/p14 tethered to dynein and associated dynactin complex. Implicit in this model is the requirement of microtubule motor dependent movement of MEK and ERK to the late endosomal compartment (Fig. 2). It is conceivable that signaling components localized to the perinuclear endosomal population could be transferred by kinesin dependent recycling traffic to effect anterograde movement of MEK and ERK to membrane proximal sites in a similar manner suggested for Rab-dependent recycling of integrins [147,148].

IQGAP may also serve to localize MEK and ERK [161,162] to dynamic microtubules. IQGAP is a calmodulin regulated actin binding protein with a GAP-like domain that while lacking GAP activity toward Rho facilitates retention of Cdc42 and Rac in their GTP bound states [163]. IQGAP binds CLIP170 a protein that associates with the growing tips of microtubules and may regulate microtubule dynamics through its interaction with other +TIPs [164,165]. IQGAP has the potential to crosslink the actin filament system to growing tips of microtubules, and hence may help localize microtubule tips to focal sites. It is not known if IQGAP and CLIP170 bind directly to dynein motors, however, it is possible that regional accumulation of MEK, ERK bound to IQGAP at growing tips of microtubules can carry the signaling complex to membrane protrusions and adhesions coupled to microtubule dynamics.

Disassembly and recycling of adhesion constituents occurs either by the disruption of linkages anchoring cytoskeleton to focal adhesions [166] or by endocytosis of integrins themselves and of focal adhesion components [167]. Recent data supports the view that dynamic traffic at the leading edge and from recycling compartments regulates integrin signaling and cell

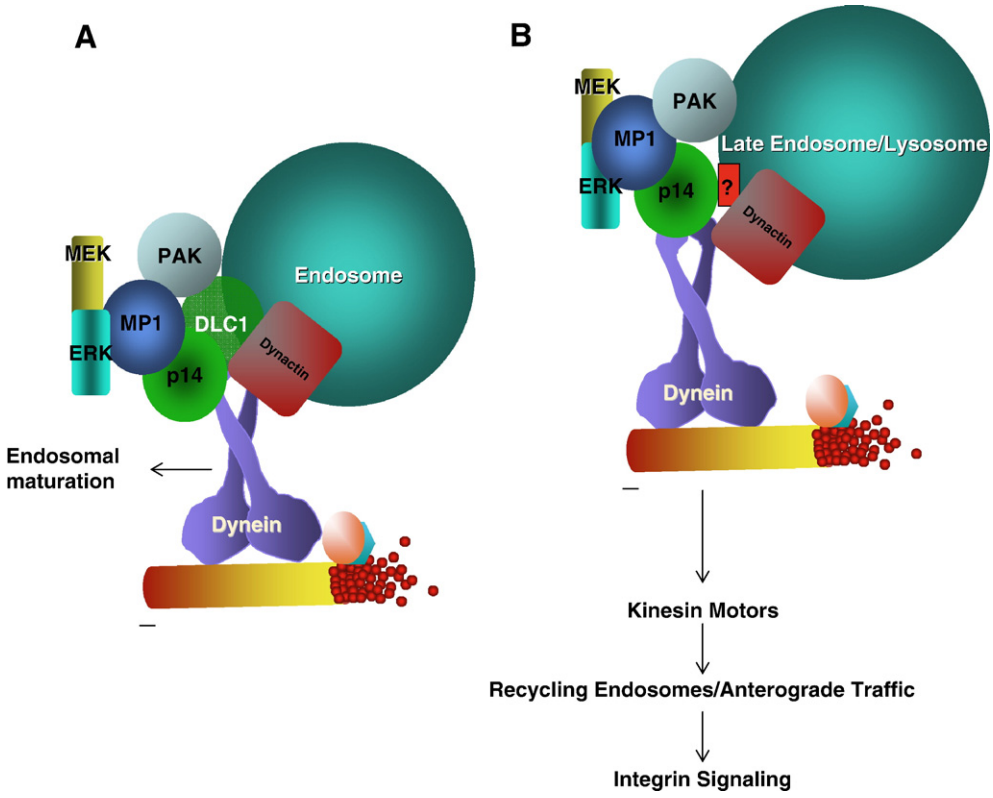

Fig. 2. MP1/p14 may integrate Rho and PAK signaling on endosomes. MP1/p14 regulate integrin activation of MAPK and focal adhesion disassembly. MP1/p14 bind active forms of PAK and regulate PAK phosphorylation of MEK during acute adhesion. PAK also binds and activates the dynein light chain (DLC1). We speculate that the LC7 dynein light chain motif of p14 may tether PAK-MEK-ERK-MP1 complex to dynein motors facilitating their transfer through endocytic system (A). Alternatively, MEK-ERK-MP1 complex could be activated by PAK at late endosome by virtue of p14 interaction with MP1 (B). Further transfer of this activated complex to kinesin motors could potentially traffic the complex through recycling endosomes or anterograde traffic to focal sites.

migration [147,148] that might involve distinct endosomal populations [148,168,169]. This view is consistent with early work that demonstrated a dynamic endomembrane system in migratory cells, whereas stationary cells exhibited greatly reduced endocytic mobility [170]. Since endocytic motility is directly influenced by microtubule and actin architecture, perhaps the most direct way MAPK signaling may regulate microtubule dynamics is through its phosphorylation and inactivation of tubulin sequestering protein, stathmin/Op18. ERK phosphorylation of stathmin/Op18 at serines 25 and 38 [171] inhibits its tubulin sequesteration activity thereby providing free tubulin for microtubule polymerization (Fig. 1B). Stathmin is also a substrate for PAK and cdk5 [59,172]. Stathmin is phosphorylated by PAK at serine 16 and probably at serine 63 [59,172], and by cdk5 at serines 25 and 38 which are also sites for MAPK [173]. The net outcome of these inactivating phosphorylations on stathmin is net growth of microtubules. Further, microtubule polymerization can act as a positive feed back to hyperphosphorylate stathmin through an associated kinase(s) to inhibit its depolymerizing effect [174].

ERK activity may also impact microtubule dependent trafficking independent of its effects on microtubule stability. This is best understood for melanosomes, a specialized pigment organelle of melanocytes related to lysosomes. Microtubule dependent bidirectional movement of melanosomes requires a functional MEK-ERK complex on melanosomes bound by an unknown scaffold molecule [175,176]. The small GTPases, Rab7, Rab8 and Rab27, through their interaction with motors, have been implicated in melanosome movement [177–182] and a few Rabs are targets for ERK phosphorylation [183], raising the possibility that ERK signaling may influence Rab functions necessary for melanosome traffic. ERK dependent traffic of melanosomes is regulated by minus end directed dynein motors, that raised the speculation that ERK may directly regulate dynein activity [184] and thereby influence intracellular mobility of signaling complexes and organelles. Extending these to endosomal traffic, one can hypothesize that ERK signaling regulates cell migration through its influence on Rabs and molecular motors that control dynamic partitioning of signaling modules to specific locales in the cell.

9. Regulation of focal adhesions and actin by proteolysis

The calcium-activated protease calpain 2 serves at least three roles in regulated cell movement. First, calpain activity may stimulate integrin activation and clustering that leads to the formation of stress fibers and focal adhesions [140,166,185,186]. This is thought to be achieved in part by calpain cleavage of talin, a cytoskeletal anchor that links integrins to actin filaments. Calpain cleavage of talin exposes the N-terminal FERM domain that is then free to bind directly to integrin β subunits [187–189] and forge linkages with actin filaments.

Second, and paradoxically, calpain also stimulates actin and focal adhesion disassembly through proteolytic cleavage of proteins important for focal adhesion and F-actin stability. Calpain cleavage of talin here is a rate limiting step in disassembly of other focal adhesion components including zyxin, paxillin and vinculin [190]. Recent studies have demonstrated that growth factor stimulation of calpain activity and cellular deadhesion is dependent upon ERK [191,192]. Calpain is directly phosphorylated by ERK in response to EGF stimulation and this increases calpain's proteolytic activity [96]. MEKK1 and FAK, both upstream regulators of ERK in some circumstances, are required for calpain activity, since cells deficient in these proteins exhibit lower calpain activity and reduced migration [193]. Further, calpain proteolysis of Rho removes the lipid-bearing membrane targeting sequence from active Rho thereby disrupting localized Rho signaling [194], and calpain-mediated proteolytic inactivation of WASP, cortactin and spectrin may result in F-actin turnover [195,196].

FAK is also a target for calpain proteolysis in v-src transformed cells [197]. The relationship between FAK and calpain is complex: FAK is required to recruit calpain to focal adhesions and for calpain activation, and is subsequently a target for proteolysis during focal adhesion turnover [197]. Expression of FAK deficient for calpain binding prevents focal adhesion turnover. Indeed, FAK can serve as an adapter to assemble calpain together with its activator ERK, and this assembly is necessary for calpain proteolysis of FAK [197]. While proteolysis of FAK at first glance appear at odds with FAK's role in stimulating adhesion turnover [79,86,198], proteolysis probably occurs subsequent to, or in parallel with, activation of effectors executing adhesion turnover. One might speculate that proteolysis of FAK is a negative feedback event that prevents liberated FAK from destabilizing adjacent adhesions. However, it should be noted that calpain cleavage of FAK may be restricted to v-src-transformed cells [141,196].

Third, calpain 2 is required for efficient lamellipodial extension at the leading edge of motile fibroblasts, but it suppresses protrusions elsewhere [140,166,190,199]. How these opposing functions are controlled is unclear at present. Down-regulation of signaling from focal adhesions may require the concerted action of microtubules and calpain. Indeed, calpain may be one of the "relaxing" factors targeted to focal adhesions by microtubules for focal adhesion disassembly [140]. Src [200], FAK and MEKK1 [193] appear to regulate calpain activity in part through ERK phosphorylation of the protease [96,192]. FAK null cells exhibit less stable microtubules [201], lower calpain activity [193], decreased ERK signaling [198,202,203] and reduced migratory potential due to defects in focal adhesion disassembly [86], consistent with the view that signaling from FAK to ERK may alter migratory responses through ERK stimulated calpain proteolysis of focal adhesion components and actin remodeling, and microtubule dynamics.

10. Multiple ERK scaffolds regulate the diverse functions of the ERK cascade

Cells have evolved numerous mechanisms to selectively activate generic kinases such as MEK and ERK in a context specific manner to regulate a select set of downstream targets. This specificity is likely important in maximizing the activity of a selected module and in preventing spurious cross-talk by insulating an activated module [125]. The kinetics, strength, and duration of MAPK activities differ between growth factors. Cell fates determined by these MAPK components also differ depending on the strength and duration of signaling. For example, in some systems the platelet-derived growth factor (PDGF) stimulates sustained ERK activity and results in S-phase entry or cell differentiation whereas epidermal growth factor (EGF) stimulates transient ERK activity that does not drive cells in to S-phase and does not promote cell differentiation [204–207]. Duration and strength dependent modulation of cell fate appear to operate in neuronal PC12 cells, NIH3T3 fibroblasts [208], macrophages [209] and lymphocytes [210,211]. It is becoming apparent that cell fates determined by different growth factors that activate the same MAPK components requires a spatio-temporal control of MAPK targeting, sequestration and activation. In recent years, several proteins with presumptive scaffold functions toward ERK cascade components have been identified that fulfill some of these requirements. For example, the kinase suppressor of Ras (KSR) acts as a scaffold to assemble and activate MAPK pathway at the plasma membrane in response to pathway stimulation. ERK activation can alter the intracellular location of KSR so as to promote signaling from the plasma membrane [212]. The MP1/p14 scaffold as mentioned above regulates EGF stimulated MAPK signaling on late endosomes/lysosomes and is required for MAPK activation during acute adhesion events [126,128]. It appears that sustained pathway activation may require coordinated control by KSR and MP1/p14 complex to continue signaling from the plasma membrane to late endosomes. Sef1 assembles MEK on the Golgi membrane potentially regulating a subset of activities attributed to the generic cascade including Golgi structure and inheritance [213]. The GRK interacting GIT1 might link GPCR signaling to MAPK and through its interaction with MEK and ERK to membrane trafficking steps through its GAP function towards ARFs [214], and was recently shown to regulate ERK activation in focal adhesions following growth factor stimulation [214–216]. Additionally, several other cytosolic binding partners of MEK-ERK including Sprouty and Spred [217,218], and PEA15 [219] have been identified recently that may serve to anchor MAPKs to distinct intracellular compartments.

Recent large scale interaction studies in *Drosophila* [220] supports the view that scaffolds can link signaling molecules to active transport mechanisms which might be an inherent property of dynamic signaling. We have briefly outlined some of these interactions in Fig. 3 with a focus on MP1/p14. *Drosophila* MP1 interacts with Sufu (suppressor of fused) that negatively regulates transcription in the Hedeghog pathway by binding and retaining the transcription activators *Cubutis interruptus* (Ci) in *Drosophila* and Glis in mammals to a cytosolic compartment [221]. MP1 also binds PIAS1 (protein inhibitor of STAT1) and the transcriptional activator, p300/CBP-co-integrator protein that are themselves binding partners for Sufu. MP1 in this context may serve to retain many regulators

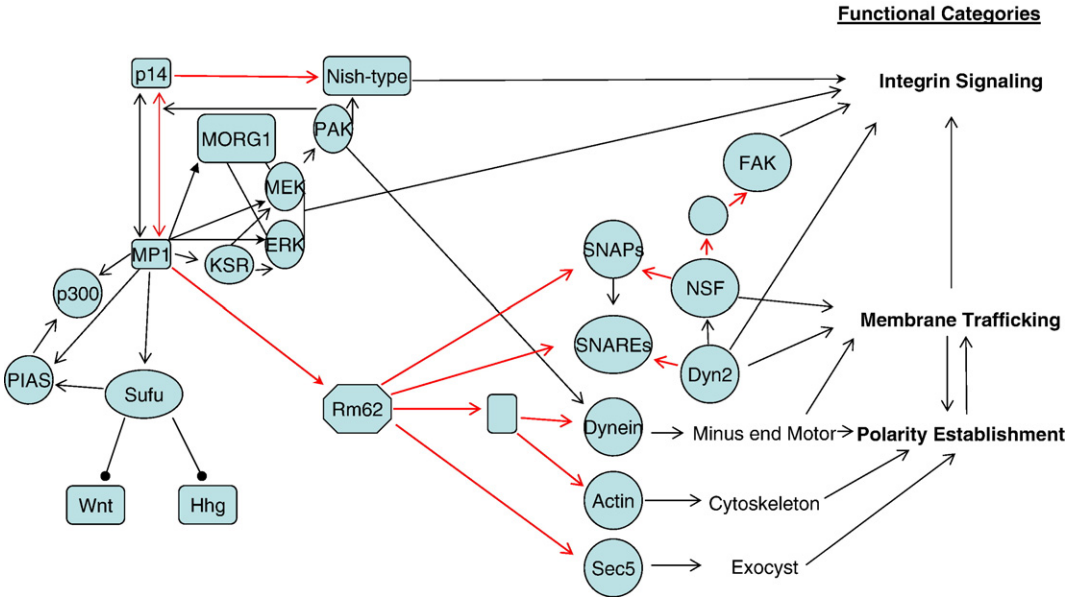

Fig. 3. An interactome of MP1/p14. Figure is a composite of *in vitro* and yeast two-hybrid data from mammalian cells and *Drosophila*. Data from whole genome interaction map from *Drosophila* (red arrows) (www.biogrid.com) was culled to show potential connection of MP1/p14 and ERK signaling to intracellular trafficking machinery that might be important for integrin signaling and cell migration. Novel uncharacterized interactions of MP1 suggest that it might also regulate suppressor of fused (Sufu) implicated in hedgehog (Hhg) and Wnt signaling through its interactions with Sufu-binding partners PIAS (protein inhibitor of STAT), and p300 (transcriptional coactivator). MP1 interacts with a RNA-helicase-like protein Rm62 which is a major interacting hub with 25 associations in the *Drosophila* interactome. Rm62 interacts either directly or indirectly to the actin cytoskeleton, dynein motors, and components of exocyst complex that regulates polarity in yeast and animals. Further, Rm62 interactions with SNAPs and SNARES might incorporate MP1 to membrane trafficking steps. Interaction of MP1 with WD-containing MORG might regulate LPA induced ERK activation whereas KSR-MP1 interaction might be required for sustaining growth factor signaling.

and effectors of Sufu in the cytoplasm and inhibit hedgehog and Wnt signaling [221]. In spite of its small size (14 kDa), MP1 has the potential to engage many systems through its interaction with Rm62 [220]. Rm62 is a putative RNA-helicase that is required for dsRNA-mediated silencing, transposon silencing and heterochromatin structure in *Drosophila* [222]. A mammalian equivalent of Rm62 is as yet unidentified, however, high-confidence interaction data support the possibility that MP1 through its association with Rm62 (or analogous protein in mammalian cells) can interact with dynein motors and actin cytoskeleton. Further, critical membrane fusion determinants including SNAPs, SNAREs, NSF and dynamin2 interact with Rm62 either directly or indirectly prompting the speculation that MP1 and its interacting partners may be functionally coupled to trafficking steps. Further experimental evidence is needed to verify these interaction studies.

11. Yeast Ste5p: a paradigm for mammalian MAPK scaffold

In *Saccharomyces cerevisiae*, the MAPKKK homolog Ste11 and its upstream activator Ste20 (a PAK homologue) are used in most MAPK pathways including the pheromone response, starvation/invasive growth, vegetative growth/cell wall synthesis and osmolarity response pathways, and the MEK homologue Ste7 is used in all but the latter pathway [223,224]. Specificity within each of these pathways is achieved in part by distinct scaffold proteins that assemble specific sets of kinases to effect distinct endpoints. Ste5 has emerged as a critical regulator of the mating response by virtue of its ability to assemble Ste11 and Ste7 to activate the MAPK, Fus3. Ste5-Ste11-Ste7 is recruited to membrane sites in response to pheromone where it can bind the PAK homolog, Ste20. Ste20 is activated by Cdc42, a major regulator of polarity establishment. Membrane recruitment of Ste11-Ste7 bound to Ste5 is required for Ste20 activation of Ste11 and propagation of pheromone signal down the cascade. Several detailed interaction studies have demonstrated that Ste5 regulates pheromone response in yeast by actively mobilizing kinases to sites of action to regulate cytoskeletal dynamics and polarized growth during *schmoo*ing [223,224]. Ste5 regulates the actin polymerization regulator Bem1, which in turn is required for specific localization of Cdc42 to membrane where it is activated by Cdc24. These interactions and activations in response to positional cues and cell cycle stages are crucial for establishment of cellular polarity.

Even though no mammalian protein shares significant sequence similarity with Ste5, the disparate binding functions of Ste5 may be distributed among several scaffolding proteins in mammals [127]. For example, MORG binds MP1, MEK and ERK and might regulate ERK activation in response to GPCR signaling [225], whereas MP1/p14 might be involved in select set of growth factor signaling and integrin signaling responses [126,225]. Further, KSR regulates early growth factor activation of MAPK signaling at the plasma membrane by membrane recruitment of MEK and ERK to Raf [226]. Although KSR can also bind MP1 the functional relevance of this interaction is not known [227] (Fig. 4).

Where studied, scaffolds appear to possess a common function in regulating cytoskeletal and membrane dynamics that

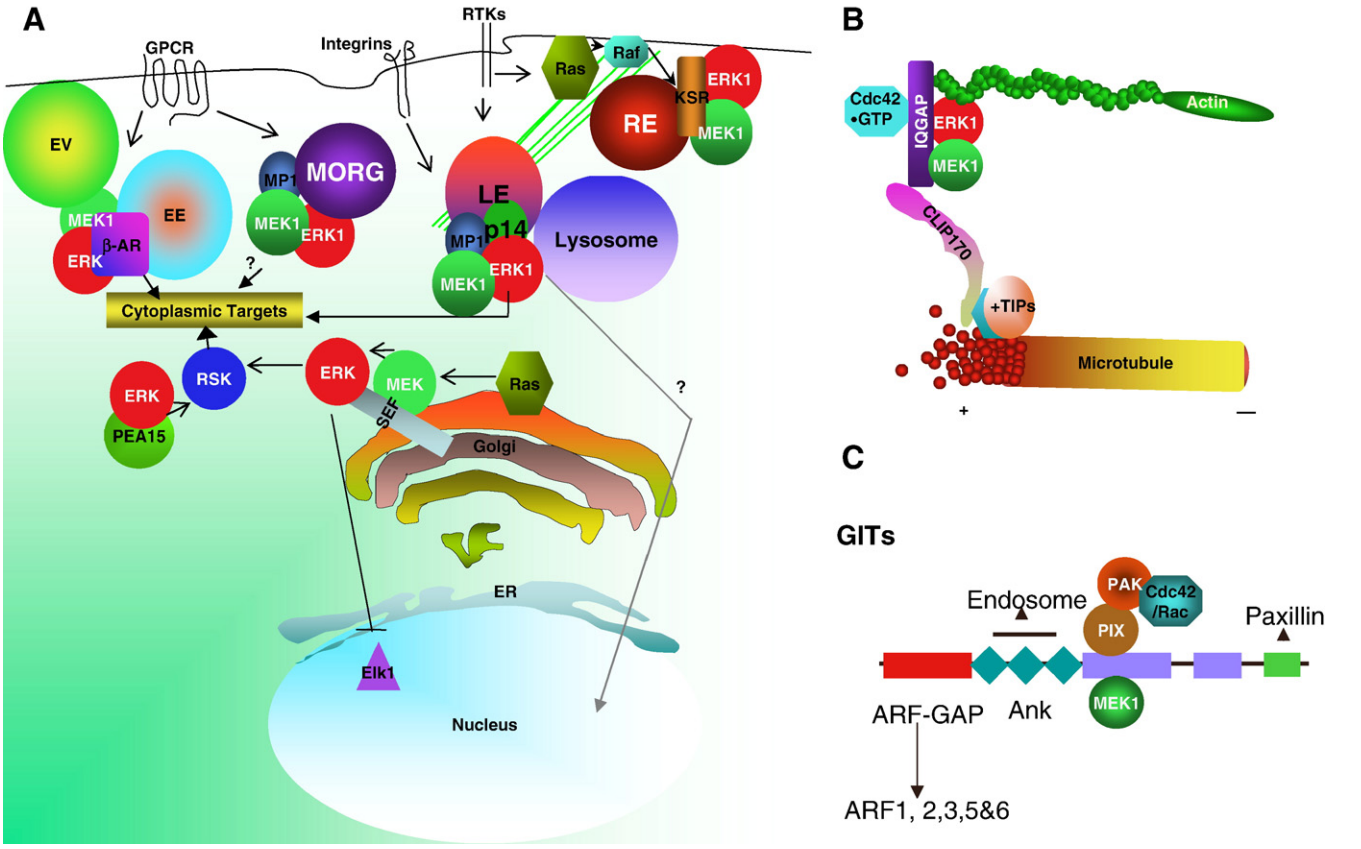

Fig. 4. Multiple ERK scaffold regulate growth factor and adhesion signaling. A). GPCR mediated MAPK activation is regulated by arrestin and MORG. KSR is constitutively localized to the cytoplasm and to a tubulo-vesicular recycling compartment. Pathway activation promotes membrane recruitment of KSR-MEK-ERK where the module is activated by membrane bound Raf. MP1/p14 is required for the late phase of growth factor signaling on late endosomes. B). IQGAP, an actin binding protein retains Cdc42 and Rac in their GTP bound states. MEK and ERK bound to IQGAP may be targeted to growing tips of microtubules through IQGAP interaction with CLIP170 (a +TIP). **C)**. GRK interacting GITs regulate signaling from GPCRs and integrins. GITs possess GAP activity towards membrane traffic regulators, ARFs. The multimeric interaction of GITs couple MEK and ERK to membrane trafficking steps through the GAP functions of GIT. EV, endocytic vesicles, EE, RE and LE refer to early, recycling and late endosomes, respectively.

determines cell morphogenesis [126,228]. By analogy to the various roles ascribed to the yeast Ste5, it is tempting to speculate that mammalian scaffolds may serve as shuttles to translocate signaling molecules to specific locations in response to a stimulus to regulate cytoskeletal architecture underlying cell morphogenesis. Extending the emerging view in yeast indicating that Ste5 and Bem1 establish polarity landmarks [229] where membrane addition and protrusive growth can occur, mammalian scaffold proteins may coordinate with post-Golgi and recycling membrane compartments to create signaling hubs proximal to adhesion sites to modulate membrane protrusion and migration. It would not be surprising to find that mammalian homologues of yeast polarity establishment proteins including exocyst components and Rabs in addition to Cdc42 would be critically involved in integrin signaling and may have an even greater role in directed migration.

Acknowledgment

Research in our laboratory is funded by National Institutes of Health grants, RO1 GM068111 to A.D.C. and P20 RR18766 (project principal investigator, A.D.C.; overall principal investigator, Stephen M. Lanier) and start-up funds provided by the Department of Pharmacology and Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center. We thank Electa Park for comments on the manuscript.

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