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REVIEW ARTICLE

Integrin-Dependent Regulation of Small GTPases: Role in Cell Migration

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Abstract | Integrins play a vital role in regulating cell adhesion that drives cell attachment, spreading, and migration. They do so by recruiting and activating several downstream signaling pathways that control actin cytoskeleton remodelling, endocytic and exocytic trafficking, and membrane organization in cells. The spatial and temporal nature of this regulation supports the polarization, leading edge protrusion and trailing edge retraction vital for cell migration. By virtue of their dynamic but tightly controlled regulation, small GTPases activated by integrins constitute vital mediators in this pathway. Their activation in cells is driven by the differential recruitment of GEFs and GAPs. This review looks at the integrin-dependent activation, regulation, and role of the Rho family small GTPases Rac-1, RhoA, and Cdc42 along with the emerging contribution that Ral and Arf6 are making to this pathway. It also discusses the extensive crosstalk between these GTPases at the lamellipodial edge in a migrating cell.

1 Integrins, Cell Adhesion, and Migration

Integrins are major *trans*-membrane receptors that regulate the dynamic association between the extracellular matrix (ECM) and cells. In doing so, they drive changes at the plasma membrane and the actin cytoskeleton that help regulate many cellular processes, including cell migration.[1] Integrins are heterodimeric proteins consisting of an alpha and beta subunit that carry an extracellular domain that binds the extracellular matrix and triggers downstream signaling through a short cytoplasmic tail that also links it with the actin cytoskeleton.[2] In addition, integrin clustering can also trigger intracellular signaling that drives cell proliferation, survival, and migration in both normal and cancer cells.[3] The extracellular domain of integrins recognizes diverse matrix ligands, including fibronectin (e.g., α5β1, αvβ3, and α4β1), collagen (e.g., α1β1 and α2β1), and laminin (e.g., α2β1, α3β1, and α6β1). Integrins in general bind-specific motifs (like the RGD motif) within the matrix protein; thus, cells adherent to fibronectin could carry adhesions containing a diverse group of integrins. These can, in turn, differentially regulate adhesion dynamics, vital to processes like cell motility.[4, 5]

Cell migration is a fundamental physiological process involved in everything from embryogenesis to immune surveillance and wound healing.[6] It is also central to pathogenesis of diseases, contributing to tumor cell invasion and leukocyte transmigration.[7, 8] Movement of cells requires the precise coordination of four sequential cellular events.[9] (A) Recurring interaction of the cell with the extracellular matrix (ECM), through adhesion receptors, such as integrins, provides a link between the ECM and intracellular actin cytoskeleton. Integrin-ECM binding triggers the recruitment of cytoskeletal and signaling proteins creating dynamic adhesion complexes. These evolve in space and time, changing in size, shape, and force they experience.[10] (B) To initiate migration, these signals facilitate the development of a polarized cell morphology with a leading and a trailing edge. This supports the compartmentalization of membrane and signaling events needed for directed cell migration.[9] (C) These

ECM: Extra cellular matrix composed of proteins secreted by cells that are crosslinked to provide structural support and biochemical cues. These proteins include collagen, fibronectin, laminin, proteoglycans, and others. They bind cell surface receptors (like integrins) to relay information from the ECM to the cell. Their crosslinking and stiffness also provide mechanical cues to the cell.

RGD: The tripeptide Arg-Gly-Asp (RGD) was originally identified as the sequence within fibronectin that integrins recognize and bind.

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spatially restricted signals can now promote active actin remodelling and polymerization that pushes the cell membrane forward at the leading edge and helps retract it at the trailing edge.[11] (D) Migration finally also requires disassembly and recycling of adhesion sites. The balance between formation and disassembly of adhesion is tightly coordinated in space and time.[12] Along with adhesion disassembly,[13] integrin recycling also plays a role in adhesion turnover.[14–16]

Integrins lack an intrinsic catalytic activity and ligand binding induces their clustering that results in the formation of multiprotein complexes containing signaling and adaptor proteins that connect to the actin cytoskeleton. Proteins Talin, Kindlin, Vinculin, and α-actinin are known to mediate this crosstalk.[17, 18] The affinity of integrins for its ligands is also regulated by intracellular signaling (inside out signaling) that controls their activation (reviewed in [18]). The difference between the rate of actin polymerization and its retrograde flow defines the forward protrusion in a migrating cell. The resistance the plasma membrane offers to the polymerizing actin cytoskeleton at the leading edge drives the retrograde actin flow,[19] supported by contraction of actomyosin filaments.[20] Integrin-dependent adhesions by inhibiting retrograde flow help promote rapid protrusion. Integrins are also seen to regulate the organization of the plasma membrane by regulating the trafficking and spatial localization of raft microdomains at the leading edge of the migrating cell.[21, 22] This, in turn, regulates the membrane anchoring and activation of several adhesion-dependent signaling pathways.[23, 24] The maturation of integrin-mediated adhesions and the signals they generate are also regulated by the tension they experience which, in turn, also influences cell motility.[25, 26]

The rapid and dynamic nature of cell migration entails continuous remodelling of the cellular architecture. This requires rapidly activated and spatiotemporally regulated signaling networks that enable the cell to respond to external cues. Small GTPases (including those belonging to the Rho family) act as important molecular switches to drive such signaling processes.[27]

2 Small GTPases

Small GTPases cycle between an active GTP-bound (Guanosine triphosphate) and inactive GDP-bound (Guanosine diphosphate) form to differentially bind downstream effectors when active and control downstream signaling.[28] Consensus amino-acid sequences mediate this nucleotide binding seen to be dependent on the presence of Mg^{2+} as a co-factor.[28] Small GTPases are inactivated by their intrinsic GTPase activity resulting in conversion of GTP to GDP turning off signaling.[29–31] In addition, small GTPases also undergo post-translational modifications, Ras and Rho family undergoing farnesylation, geranylgeranylation, and palmitoylation at their C-terminus,[32] while Arf family GTPases undergo myristoylation on their N-terminus.[33] These facilitate the targeting of GTPases to the cell and endo-membrane allowing for their spatial activation and binding of effectors.[34, 35] Alignment of the crystal structures of small GTPases in their active and/or inactive form revealed that they undergo very little structural rearrangement, except within the effector loop.[36] These changes are thought to mediate their association with upstream regulators and downstream effectors.[29, 37]

Activation state of small GTPases is regulated by three kinds of proteins. (A) Guanine nucleotide exchange factors (GEFs) that promote GTPase activation by displacing the Mg^{2+} from active site and triggering the switch from GDP to GTP.[38] (B) GTPase activating proteins (GAPs) inhibit GTPase activation by inserting a water molecule into the catalytic pocket of the GTPase and promoting their intrinsic GTPase activity (by a few 1000-fold).[39, 40] GEF and GAP interactions take place at the effector-binding interface in the GTPase and are also governed by their relative localization in cells.[32] (C) Finally, GDP dissociation inhibitors (GDI) bind GDP-associated GTPases and block the switch from GDP to GTP and help maintain GTPases in an inactive state.[29–31] GDI actively retrieve the GDP-bound form from the plasma membrane and facilitate their cytosolic or endo-membrane localization.[41, 42] Small GTPase activity can also be regulated by transient post-translational modifications, such as phosphorylation [43–46] or SUMOylation,[47] which regulate their localization and activation.[43, 44]

Determining the activation status of a small GTPase in a cell at any given time has been vital to studying their role in cellular processes like migration. The fact that GTPases undergo only a very small structural rearrangement on their activation means that antibodies fail to detect this change. Most of our early understanding of small GTPase function hence comes from studies of mutants that are locked in active or inactive GTPase conformations [48, 49] and from the

GEFs: Guanine nucleotide exchange factor; assists GDP–GTP exchange on a GTPase by displacing the magnesium ion from the active site favouring destabilization of GDP–GTPase interaction. This allows exclusion of the bound GDP from the active site. The substitution by a GTP is a result of relatively higher intracellular concentration of GTP than GDP (~10-fold).

GAPs: GTPase Activating Protein; enhances intrinsic GTPase activity of a GTPase by positioning water molecule for a nucleophilic attack on GTP in the GTP-binding pocket.

studies of their downstream effectors.[36, 50] Knowing that downstream effectors associate specifically with the active form of the GTPase, effector domains conjugated to beads were used to pull down and measure GTPase activity from cell lysates. Examples include GGA3 (Golgi-localized, gamma ear-containing, Arf-binding protein 3) for Arf6,[51] Sec5 Ral-binding domain for Ral,[52] and p21 activated kinase (PAK) for Rac1 and Cdc42.[53] This, however, does not provide spatiotemporal information about the active GTPase in cells. As an alternate approach, biosensors were designed to follow the activation state of GTPases in live cells or organisms using microscopy.[54] To date, several generations of probes have been developed to monitor binding of fluorescently tagged active GTPases to their downstream effectors by measuring Förster resonance energy transfer (FRET). Biosensors currently available include probes for Rac1,[55] RhoA,[56] Cdc42,[57] Arf6,[58] and Ral[59–61] These probes have provided much needed spatiotemporal information on the activation of these GTPases allowing for a better understanding of their role in processes like cell migration.

The spatial localization of small GTPases when considered with their regulation by GEFs, GAPs, GDIs,[62] and their ability to mediate actin polymerization and vesicular trafficking, makes them attractive candidates for integrin-dependent regulation of process like cell migration.[20] This review looks at five major small GTPases Rho GTPases (Rac-1, Rho, and Cdc42), Ral, and Arf6 focusing on their regulation by integrins and their spatiotemporal activation and function in migrating cells. It further goes on to look at their regulatory crosstalk and the implication it has in mediating their role in integrin-dependent cell motility.

3 Rac GTPase

The Rac subfamily comprises of four members Rac1, Rac2, Rac3, and RhoG;[63, 64] the ubiquitously expressed Rac1 is studied more extensively than others and has hence been discussed here. Rac GTPases regulate multiple molecular pathways, such as cell polarity,[65] cadherin-based cell–cell adhesions,[66] microtubule stabilization,[67] and actin polymerization,[68, 69] making it a major player in a process like cell migration. Active Rac1 is found to be localized to the plasma membrane,[70] endosomes,[71] cell–cell adhesions[72] and nucleus.[73] Activation of Rac1 is tightly coupled to its translocation to plasma membrane [74] mediated by its palmitoylation at Cysteine 178[75] and supported by its dissociation from RhoGDI.[76, 77] Integrin-mediated adhesion is known to regulate this plasma membrane targeting of Rac1 (supporting its interactions with effectors) by the regulation of the trafficking and plasma membrane delivery of raft microdomains that act as Rac1 binding sites.[23] These trafficking and targeting of rafts are, in turn, dependent on integrin-dependent activation of RalA and Arf6.[78, 79] In addition, PIPK-I α binds Rac1 and mediates its localization at the plasma membrane[80] as does the integrin-binding protein Tetraspanin (CD151).[81] Integrin-associated protein Talin binds the RacGEF Tiam1 to support localized activation of Rac at focal adhesions.[82] Focal adhesion kinase (FAK) phosphorylates RacGEF β-Pix to also recruit Rac at focal adhesions.[69, 74]

FRET sensors developed for detecting active Rac reveal a gradient of Rac1 activation in migrating cells, with higher activity at the front decreasing towards the cell interior.[70] High Rac activity is spatially, temporally, and in magnitude tightly correlated with lamellipodial protrusions at the leading edge of migrating cells. Studies in re-adherent neutrophils from mice expressing the Rac-FRET construct reveal maximum Rac activation in the first 8 min after readhesion. Localization of Rac activity in migrating neutrophils is also shown to be the highest at the leading edge with bursts of high activity lasting for 3–5 s. In addition, bursts of Rac activity were also detected at the periphery and trailing edges in these cells.[83] On integrin-dependent adhesion, Rac1 activation is the first step followed by activation of RhoA; this facilitates the early spreading of cells by extension of lamellipodia in all directions. However, at later stages, the Rac1-RhoA activation switch at leading edge of cell promotes directional migration. In addition, the decreasing spatial gradient of Rac1 activation from cell front till the trailing edge of cells ensures restricted lamellipodia formation—again favouring directed cell migration.[84, 85]

Rac activation drives cell migration by regulating actin polymerization at the leading lamellipodial edge that provides the driving force for membrane extension.[86, 87] Rac regulates the Arp2/3-WAVE complex to drive actin polymerization via multiple mechanisms, including its activation of PAK.[88] Maximal activity of the WAVE2 complex requires simultaneous interactions with prenylated membrane-bound Rac1 and acidic phospholipids.[89] RacGEF Tiam1 also directly binds to Arp2/3 to spatially facilitate Rac-1-dependent actin polymerization.[69] Rac activation suppresses local Rho activity supporting

Focal adhesions: They are large macromolecular assemblies of cell surface integrin receptors that cluster together when bound to the extracellular matrix (ECM) and recruit several adaptors and signaling proteins. These structures through actin-binding proteins induce actin stress fibre assembly. They hence act as important mechanical links and signaling hubs between the cell and the ECM.

FRET: Förster resonance energy transfer; Mechanism of energy transfer between two light-sensitive molecules (chromophores). A donor chromophore, in its electronic excited state, can transfer energy to excite an acceptor chromophore through non-radiative dipole–dipole coupling. The efficiency of this energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small changes in their relative distance.

Lamellipodial: Highly dynamic sheet like actin cytoskeletal projections in the front of a polarized migrating cell. These structures have ruf-fle-like plasma membrane organization and are rich in focal complexes and focal adhesions. They measure a few micrometers from the cell edge.

the formation of nascent adhesions essential for lamellipodia extensions.[28] Although mammalian Dia1—an actin nucleator protein—is activated only by the Rho family (i.e., RhoA, B, and C), the related Dia2 and Dia3 proteins can also be activated by Rac1 and Cdc42 to effect actin polymerization.[90] In the lamellipodium, cofilin inactivation that regulates actin depolymerisation is seen to be dependent on Rac1 and its effectors PAK1 and PAK2.[91]

Rac1 also regulates actomyosin contractility.[92] Rac activated by GEF Asef2 enhances phosphorylation of MyoII leading to greater cell contraction.[93] A ternary complex of RacGEF P-Rex1, Rac1, and actin remodelling protein Flightless 1 was recently shown to also influence cell contraction [94] and hence migration. Rac effector PAK1 antagonises MLCK function to also decrease actomyosin contractility.[95] Another important Rac effector IQGAP1 mediates active Rac1-dependent destabilization of cell–cell adhesions by directly binding α catenin and sequestering it from E-cadherin junctions.[96] This would favour cell–cell dissociation and cell migration. IQGAP1 also facilitates actin reorganization by binding N-WASP-Arp2/3.[97, 98]

Rac1 inhibition is pro-migratory owing to the fact that this Rac1-RhoA switch is essential for persistence of directional migration.[84, 85] This mechanism is also exploited in cancer cells where increased RacGAP1 expression favours Rac1 inactivation at cell front and promotes invasion through RhoA.[99] Another mediator of the Rac–Rho crosstalk is the Rac GAP, FilGAP, which is recruited to cell protrusions through its interaction with FilaminA[100] and Arf6.[101] This then functions to deactivate Rac1 at these sites upon phosphorylation by Rho effector ROCK.[100] Nuclear translocation of active Rac1 by Nucleophosmin-1 also allows for higher cytosolic RhoA activation promoting cell invasion.[102]

4 Rho GTPase

The Rho subfamily includes the isoforms RhoA, RhoB, and RhoC, which are 84% identical in sequence; differing largely in their C-terminus.[103] RhoA and RhoC localize to the plasma membrane or interact with RhoGDI in the cytoplasm, whereas RhoB localizes to endosomal membranes because of its unique C-terminal lipid modifications.[30, 103, 104] Studies by Ren et al. revealed that in the first 10–30 min of integrin-mediated adhesion, RhoA is rapidly and transiently inhibited followed by peak in activation between 60 and 90 min.[105] Integrins use p190RhoGAP to inhibit RhoA, which support the release of Rac-1 from RhoGDI and its activation. This, in turn, enhances cell spreading and migration in Rat1 fibroblasts.[106–109] RhoGEF p115 is also implicated in RhoA activation following its transient inhibition on adhesion of cells to fibronectin.[110] p190RhoGEF is also implicated in integrin-dependent RhoA activation downstream of FAK and Proline rich kinase2.[111] αv integrin-mediated adhesions recruit Rho GEF-H1 to activate RhoA and its effector mDia1 to support actin reorganization.[112] However, α5-β1 integrin localizing in smaller peripheral adhesions uses the Rho ROCK pathway to drive force generation.[112] β1 integrins mediate force-dependent RhoA activation specifically through GEFs GEF-H1 and LARG to cause cell stiffening in migrating cardiac fibroblasts. GEF-H1 further activates RhoA to support focal adhesion maturation essential for migration.[113]

Studies done using a Rho sensor have revealed GEF-H1 to be required for localized RhoA activation at the leading edge of migrating HeLa cells.[114] Despite the fact that RhoA is activated at the back of the cell to control actomyosin contractility,[115] FRET probes reveal bulk of RhoA activation to occur directly at the leading edge of protruding lamellae in fibroblasts.[56, 116] In Rat mammary adenocarcinoma cells MtLN3, RhoA activation observed at the cell front showed a biphasic pattern with peaks of activity at 1 and 3 min post-EGF stimulation.[117] Transient bursts of RhoA activation were, however, simultaneously observed at the back of the cell during active retraction of the tail.[56] Further activation of RhoA was also observed in peripheral ruffles and on actively moving macropinosomes.[116, 118]

Active RhoA relieves autoinhibition of Rho-associated kinase/ROCK that phosphorylates MLC and inhibits the MLC phosphatase.[119] Phospho-MLC thus generated has greater actin-binding capacity and induces formation of stress fibres. This leads to the maturation of focal complexes to focal adhesions and greater contractility in cells to drive cell motility. ROCK also targets FAK and paxillin to control the formation and stability of focal adhesions.[50] Rho effector mDia1 regulates c-Src and Cdc42 localization thus affecting polarization and directed cell migration.[120] mDia1 also controls actin reorganization and microtubule alignment in these cells.[116, 121, 122]

The morphology of RhoA, RhoB, and RhoC lacking is very distinctive and so are their effects on localization of Rac1 activity and chemotactic

Focal complexes: Small/nascent focal adhesions are often referred to as focal complexes. They are present at the periphery of spreading or migrating cells are regulated by GTPases Rac and Cdc42.

cell migration.[123, 124] GEF-H1 activated RhoB affects focal adhesion dynamics by regulating active β1 integrin levels to control cell spreading and cell migration in cancer cells.[124] RhoB also regulates Akt activation to drive this pathway.[125–127] While a direct effector of RhoB remains to be identified, a role for mDia that binds RhoB is speculated.[128] Activation of RhoC when followed using a RhoC-FLARE sensor in conjunction with RhoA sensor showed their concomitant activation at the cell edge.[129] RhoC recruits its effector formin3 (FMNL3) to regulate migration in prostate cancer cells.[124] It is also over-expressed in metastatic pancreatic cancers, where its activation is essential for invasiveness.[130]

5 Cdc42 GTPase

Cell division control protein 42—Cdc42—was first identified in the budding yeast, *Saccharomyces cerevisiae*, as a small GTPase essential for regulating cell polarity.[131] Adhesion of mouse fibroblasts to fibronectin is seen to activate Cdc42/Rac1 effector PAK supporting the integrin-dependent activation of these GTPases. This was confirmed by inhibition of cell spreading by dominant negative mutants of Cdc42.[36] Active Cdc42 pulled down with its effector (GST-PAK) found that it is activated a minute after the astrocyte monolayer is wounded by a scratch.[132] This activation stays for most of the next 1 h.[132] The initial recruitment of Cdc42 to the leading edge of a migrating cell is integrin-dependent [132] which is supported by the integrin-dependent activation of Arf6 and resulting membrane trafficking.[78, 133] In neutrophils downstream of β_2 integrin, Rho GEF Vav1 and Vav3 activate Cdc42.[134] In 3D collagen gels, integrin α2-β1 uses the GEF βPix to regulate Cdc42 activation,[135] while in invasive glioblastoma cells, integrin αv-β8 was shown to bind directly to RhoGDI-1, releasing Cdc42 to support its activation.[136] Cdc42 is also found to transcriptionally upregulate the expression of β1 integrins through transcription factor SRF.[137] Integrin-dependent activation of Cdc42 was further shown to be negatively regulated by 14-3-3 ζ in CHO cells.[138] Recently, yeast 14-3-3 protein Rad24 was shown to competitively bind Cdc42 GEF Gef-1, thereby regulating the spatial activation of Cdc42.[139] A ternary complex of integrin α-4/paxillin/14-4-3-ζ was seen to be essential for activation of Cdc42 at the leading edge of migrating leukocytes and the Cdc42-dependent migration of these cells.[140]

Cdc42 inhibition leads to a loss of polarization in wound edge cells accompanied by a loss in Golgi realignment inhibiting overall migration.[141] Active Cdc42 fast cycling mutant (Cdc42-L28) in neuronal cells is shown to regulate neuronal polarity.[142] Spatial activation of Cdc42 using an optically activatable construct found this site to convert to a migrating front in the cell with detectable Rac1 activation. RhoA/ROCK activation was further triggered at the newly defined rear of the cell, demonstrating that Cdc42 activation alone is sufficient for cell polarization.[143] Detection of Cdc42 activation by the GDI-Cdc42 FLARE sensor[144] and Cdc42 Mero-CBD sensor[145] found most of Cdc42 to be active 2–3 μm behind the protruding cell edge, at sites of maturing focal adhesions.[144] This activation happened following the initiation of protrusion, suggesting that Cdc42 may function in reinforcement of protrusive forces rather than their initiation.[144] A role is also seen for Cdc42-binding GDI in creating the time lag preceding this activation.[146] Cdc42 activation was also decreased in the retraction phase of the membrane ruffle.[144] The spatial control of Cdc42 activity is not only achieved through recruitment of its regulators but also by recruitment of Cdc42 itself. In budding yeast, polarized recruitment of the GEF Cdc24p recruits and activates Cdc42p.[147, 148] Arf6-dependent vesicular trafficking promotes the accumulation of βPIX at the leading edge, which is also required for Cdc42 recruitment and activation.[133, 149, 150] It is also speculated that a Cdc42-driven feedback loop could enhance its accumulation at the leading edge.[151]

In polarized migrating cells, the positioning of the MTOC relative to the nucleus is thought to be an important early event. In astrocytes, fibroblasts, and epithelial sheets, the centrosome is relocated between the nucleus and the leading edge of the migration cell.[132, 152, 153] The Golgi complexcolocalizes with the MTOC[153] and their positioning contributes to the targeted delivery of processed proteins along microtubules to the cell front.[154] A integrin-Cdc42–Par6–PKCζ pathway is thought to recruit the microtubule motor protein Dynein to mediate this.[132] Apart from this, a PKCζ -GSK3β-APC-Dlg1 pathway has also been reported to cause MTOC reorientation downstream of the Cdc42/PAR6 complex.[155] The localization of protrusion at the leading edge was, however, mediated by Cdc42 effector PAK recruiting βPix and Rac1 which, thereby, regulate actin reorganization. The PAR6/aPKC pathway functioned only for Golgi and MTOC reorientation.[156] In 2005, Gomes et al. observed that the MTOC reorientation is accomplished by retrograde movement of nucleus behind the MTOC

Fast cycling mutant: A small GTPase mutant that does not stably bind either GTP or GDP effectively allowing the nucleotide exchange to be independent of GEF and GAPs. This mutant hence has a higher cycling rate of nucleotides. In a GTP-rich cellular milieu, these mutants better represent the active state of a GTPase.

and maintenance of MTOC at its location. The PAR6/aPKC/Dynein pathway was involved in keeping the MTOC immobile at the cell centroid, while a Cdc42–MRCK-myosinII pathway channelizes actin retrograde flow to move the nucleus.[157]

6 Ral GTPase

Ral GTPases are key regulators of multiple cellular functions, including cytokinesis, mitochondrial fission, cell survival, and cell migration.[158–161] Ral isoforms, RalA and RalB, share 82% sequence identity but regulate distinct cellular functions [162] mediated by their differential activation and/or localization in cells.[163, 164] Integrin-mediated adhesion differentially regulates the activation of RalA (but not RalB), in mouse fibroblasts.[79] This further supports a RalA-Arf6-exocyst-dependent membrane trafficking pathway to drive adhesion-dependent signaling and cell spreading.[79, 164] The kinetics of integrin-dependent RalA activation suggests that it is transiently activated at the early adhesion time points (15 min). Ral is further seen to localize to sites of active integrin signaling, membrane ruffles[79] and focal adhesions,[165] and specialized structures like invadopodia.[166] Growth factors, such as EGF, seen to work with integrins to drive common pathways, also activate Ral at sites of active integrin function like membrane ruffles.[60, 167] The regulation of Ral activation is mediated by the spatiotemporal localization of RalGEFs and/or GAPs.[158, 168] It remains unclear which GEF/GAP directly helps mediate integrin-dependent Ral activation. The cell-type-specific nature of integrin-dependent Ral isoform activation and function also remains untested.

Invadopodia are membrane protrusions and sites of active ECM degradation by the trans-membrane type 1 matrix metalloproteinase (MT1-MMP, also known as MMP14) typically seen in invasive cells.

Active Ral talks to a series of downstream effectors to help regulate cell migration. RalA effector FilaminA, known to be required for induction of actin stress fibres and filopodia,[169] is recruited by active RalA.[170] Active Ral-mediated binding of exocyst components Sec5[171] and Exo84[172] regulates exocyst function.[173] This, in turn, mediates the delivery of raft microdomains at the plasma membrane which act as anchoring sites for small GTPases like Rac to regulate their activation and function.[23, 79] Interestingly, in NRK epithelial cells, RalB similarly engages the exocyst complex to guide polarized delivery of secretory vesicles and drive directional cell movement.[174] Exocyst-mediated delivery of PIPK-I-gamma-i2 promotes PIP2 synthesis at the cell front to support activation of multiple GEFs at these sites.[175] The Ral-exocyst complex is also seen to mediate trafficking of α-5 integrins[44] and β1 integrin.[175] Active Ral is further seen to activate Arf6[164] that, in turn, supports multiple pathways (discussed under Arf6 subheading) that drive cell migration.[78, 101, 176] Active RalB-Sec5 also binds Rho GEF (GEFH1) to regulate RhoA activation.[177] While FRET sensors detecting active RalA and RalB are available and have shown their activation to be spatially restricted,[59–61] the localization of active Ral in migrating cells remains to be tested.

Exocyst complex: An octameric protein complex evolutionarily conserved from yeast to mammals that binds cargo loaded vesicles ensuring their delivery at specific sites on plasma membrane.

In renal carcinoma cells, Prostagandin E2 (PGE2)-mediated activation of RalA through the RalGAP protein RGC2 promotes invasiveness.[163] RalA-mediated regulation of the exocyst complex in invasive prostate cancer cell line PC3 is seen to be essential for single cell as well as collective cell migration.[178] The Ral-exocyst complex also interacts with aPKC to mediate JNK activation, phosphorylate paxillin at the leading edge, and regulate focal adhesion stability.[174] Proteome analysis of focal adhesions has reported the presence of RalA.[179] Exocyst component Exo70 also directly interacts with the Arp2/3 complex facilitating its interaction with WAVE2, promoting actin branching at the lamellipodia edge in migrating cells.[180] RalB, more than RalA, was also found essential for formation invadopodia in PDAC cell lines trough regulation of its effector RalBP1.[166] In bladder cancer cells, indeed, active RalB is seen to promote cell migration.[161] In breast cancer cell line, MDA-MB-231, however, loss of either Ral isoforms, was seen to inhibit their invasive phenotype.[181] In cancers, RalGEFs and RalGAPs are, however, seen to regulate Ral activation and drive tumor cell migration. RalGDS in breast cancer cells,[182] RalGAP-β and RalGAP-α2 in pancreatic cancer cell lines,[61] and RalGAP-α2 in bladder cancer cells[183] all help mediate cell migration. This suggests differential regulation and role for RalA and RalB along cell migratory pathways in cancer cells.

7 Arf6 GTPase

Arf6 is the sole member of Class III Arf GTPases that is primarily localized at plasma membrane and recycling endosomal compartments[184–186] and to special structures like invadopodia in cancer cells.[187, 188] Arf6 is seen to be essential for migration of variety of cell types, including Schwann cells,[189] Smooth muscle cells,[190] and several epithelial cell lines.[188, 191] Arf6 regulates cell migration, though several mechanisms include regulation of cortical actin reorganization,[192] activation of Rac1,[186] adherence junction turnover,[191]

membrane recycling,[78] integrin recycling, and focal adhesion turnover.[193] Arf6 activation itself is interestingly regulated by integrin-ECM engagement downstream of RalA GTPase in mouse embryonic fibroblasts. The kinetics of adhesion-dependent Arf6 activation suggests its regulation to also be rapid (like RalA).[78, 79] This is mediated by the recruitment of the Arf6 GEF ARNO by the Ral effector RalBP1.[78, 164] IQGAP1 is also seen to act downstream of β1 integrins to regulate Arf6 activation possibly through the Arf6 GEF HERC1.[194] Arf6 is also a critical mediator of β1-integrin recycling detected in endothelial cells derived from Arf6 −/− mice[195] and several other cell types, including mouse fibroblasts, human fibroblasts, ovarian carcinoma cells, and melanoma cells.[78, 196–198] The β1 integrin recycling in these cells is tightly coupled to Arf6-mediated endocytosis of β3 integrins which regulates their surface levels and focal adhesion stability.[197] The relative levels of β1 and β3 integrins, in turn, determine the speed and persistence of migrating cells.[4] Arf6 controlled endocytosis of integrins is regulated by Dynamin,[199] whereas its recycling is mediated by effectors PIP5K, exocyst complex, and Rab-11 interacting FIP3/4.[200–202] Another Arf6 effector—GGA3—in association with sorting nexin SNX17 ensures proper localization of integrins α2, α5, and β1, and prevents their lysosomal degradation.[203]

Apart from regulation of integrin trafficking, another major contribution of Arf6 to cell migration is via its regulation of Rac1 activation.[186, 204] Arf6 determines plasma membrane localization of RacGEF Kalirin[176] and RacGAP FilGAP[101] through direct GTP-dependent association. Another RacGEF DOCK180/ELMO complex mediates Arf6-dependent Rac1 activation by mechanism not yet known.[149] Arf6 effector Nm23H1 is also reported to compete for RacGEF Tiam1 and regulates its activity at cell–cell junctions.[199] IQGAP1 is also seen to act as important mediator of Arf6-dependent Rac-1 activation.[194] The integrin-RalA-Arf6 pathway is also seen to mediate the spatial delivery of raft microdomains that act as plasma membrane anchoring sites that mediate Rac activation.[23, 79, 164] Arf6 promotes migration of epithelial cells by mediating E-cadherin trafficking and adherence junction turnover.[191, 199, 205] ArfGEF BRAG2 (GEP100) controls Arf6 activation in this pathway. BRAG2-activated Arf6 also promotes β1 integrin internalization through the microtubule motor protein MAP4K4 leading to the disassembly of focal adhesions and endothelial cell migration.[206–208] Arf GEF Cytohesin2 (ARNO) (seen to work downstream of Ral) also directly interacts with paxillin to activate Arf6 and guide pre-adipocyte migration.[209, 210] Cytohesin2 associates with PIP2 to regulate β1 integrin recycling as well.[211]

Like their GEFs, ArfGAP ACAP4, via interaction with Grb2, also mediates β1 integrin recycling and cell migration.[212] Arf6 GAP Centaurin α2 further regulates Arf6 inactivation at the PM to effect cortical actin reorganization.[213] ArfGAP1 interacts with non-phosphorylated α4 integrin and paxillin at the sides and rear of migrating cells to reduce Arf6 and Rac activation at these sites. This helps to facilitate the generation of the Rac activation gradient from the front of a migrating cell.[214, 215] ArfGAP proteins ACAP1 and ARAP2 localize in distinct endosomal compartments and have opposing effects on Arf6-dependent β1 integrin internalization and thereby focal adhesion stability.[216, 217] Such a distinct localization of active Arf6 in two distinct vesicular compartments was also reported earlier.[199] This suggests that two subcellular pools of active Arf6 may be helping influence directional cell migration.[216] A FRET-based Arf6 activity sensor is available for Arf6 but has not been used to evaluate Arf6 activation in migrating cells.[58]

8 Crosstalk Between Small GTPases at the Lamellipodial Edge

Since small GTPases are known to activate overlapping pathways, their crosstalk provides the much needed regulatory control processes like cell migration need. The ability of RhoA and Rac to inhibit each other,[84, 85] Cdc42 to activate Rac,[218] Arf6 to activate Cdc42,[219] Ral to activate Arf6,[164] and the Ral-Arf6 crosstalk to regulate Rac activation at the plasma membrane [23, 79] all reveal the extent of their regulatory overlap (Fig. 1). Functionally, this overlap controls two major pathways in cells that actively affect cell migration, actin cytoskeletal reorganization, and vesicular trafficking.

Such a crosstalk among GTPases is best characterized between RhoA and Rac1 (Fig. 2) at the lamellipodial edge. The Rac–Rho crosstalk at the lamellipodial edge involves active Rac1-mediated binding of p190RhoGAP allowing it to directly suppress RhoA activity.[220] Rac1-dependent generation of reactive oxygen species (ROS) inhibits the low molecular weight protein tyrosine phosphatase (LMW PTP) promoting p190RhoGAP phosphorylation and inhibition of Rho.[221] The Rac effector p21 associated kinase (PAK) also contributes to its suppression of RhoA signaling by regulating RhoA-specific GEFs p115,[222]

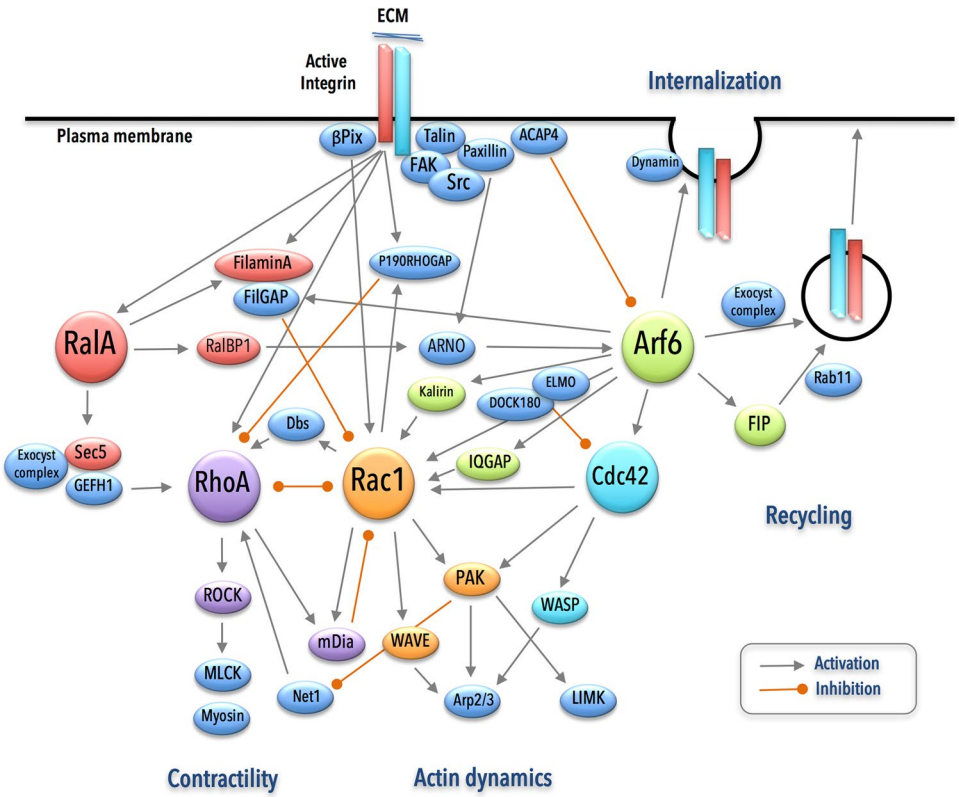

Figure 1: This schematic lists proteins (GEFs, GAPs, and adaptor proteins) that positively and negatively regulate the crosstalk between Rac1, RhoA, Cdc42, Ral, and Arf6 downstream of integrins to support cellular contractility and actin dynamics vital for cell migration. Direct effectors for each GTPase in this schematic are represented in the *same color*

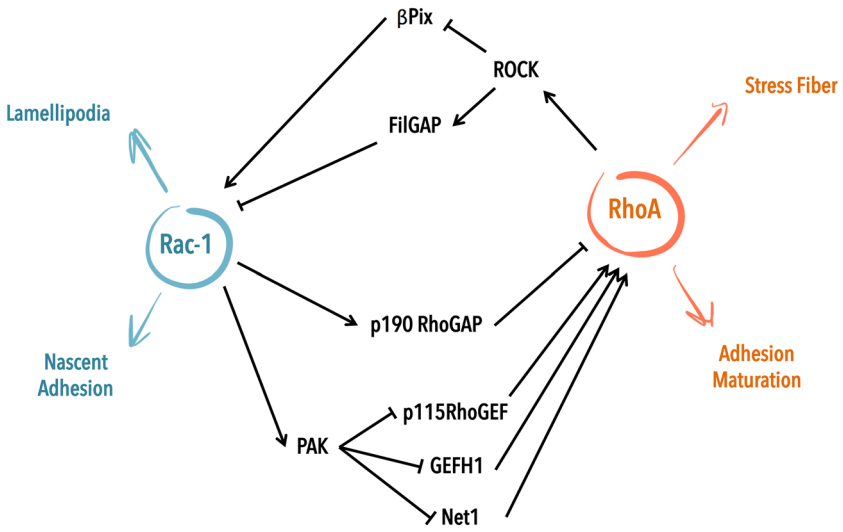

Figure 2: Crosstalk between Rac-1 and RhoA. This schematic lists proteins that positively and negatively regulate the crosstalk between Rac-1 and RhoA. ***Arrows*** indicate positive regulation (activation), while ***closed lines*** indicate negative regulation (inhibition). Together, this crosstalk allows for the differential activation of Rac and Rho to support lamellipodial protrusion and tail retraction in migrating cells (adapted from Guilluy et al. [227])

Net1,[223] and GEFH1.[224] Rac-1 also undergoes nucleocytoplasmic shuttling, mediated by Nucleophosmin-1, which, in turn, regulates cytosolic RhoA activation.[102] Arf6 also regulates Rac1 activation by engaging multiple RacGEFs[149, 176, 199] as well as RacGAP FilGAP.[225] Arf6 is also seen to work with RalA to mediate the exocytic delivery of raft microdomains to the plasma membrane, which, in turn, could support Rac-1 targeting to raft/non-raft boundaries promoting its activation.[226] The spatial regulation of membrane trafficking and targeting could help restrict Rac activation and its crosstalk with Rho at the lamellipodial edge.

The Rho–Rac crosstalk (Fig. 2) is, however, mediated by the Rho effector ROCK that phosphorylates FilGAP to inhibit Rac-1 activation.[100] FilGAP is, in turn, recruited through its interaction with FilaminA[100] and Arf6.[225] Active Ral also binds FilaminA mediating its localization, which could further affect Rho mediated Rac-1 inhibition.[170] Competition between Rho proteins for binding RhoGDI could also allow for one Rho protein to regulate the stability and/or activation of the other.[227] In invasive rat adenocarcinoma cells, RhoA activation suppresses Rac1 activity to allow for Cdc42 to act as the predominant regulator of cell protrusion. Inhibition of RhoA in these cells causes Rac1 (instead of Cdc42) to mediate this protrusion. Hence, RhoA could also act as a mediator of Rac1 vs Cdc42 activity and downstream signaling.[117] Spatial accumulation at the lamellipodial edge of Cdc42 and its GEF βPix is mediated by Arf6 dependent membrane exocytosis.[133] Ral GTPase as an upstream regulator of both Arf6[164] and exocyst[228, 229] could further support Cdc42 localization. A distinct pool of Cdc42 is also seen to exist at the Golgi which acts as a reservoir for its plasma membrane pool and could be trafficked by the Ral-Arf6 pathway.[230] Cdc42 effectors by binding Rac1 GEF Tiam1[231] and ArfGEF17 could also drive Rac1 activation during lamellipodium formation.[218]

Computational multiplexing correlates the spatiotemporal activation profiles of Rho GTPases with respect to membrane protrusion and retraction cycle in fibroblasts (Fig. 3). These results were also validated by simultaneous use of biosensors for up to two GTPases in a cell. They reveal that RhoA was activated at the protruding cell edge and its temporal activity correlated with the protrusion retraction cycle. Rac1 and Cdc42 on the other hand were activated after a lag of about 40 s after initiation of protrusion and at a distance of approximately 1.8 μM from the cell edge (Fig. 3).[144] These studies indicated that, indeed, RhoA would function to initiate protrusion via actin reorganization simultaneously suppressing Rac1 activation at cell edge. Inhibition of Rho accompanied by the activation of Rac1 and Cdc42 could allow for the reinforcement of protrusive forces by acting on stable focal adhesions that are located about 2 μM from cell edge.[144] Regression analysis of Cdc42 and Rac1 activities has also demonstrated that their responses induce membrane protrusion but also cause membrane retraction in the perimeters. This analysis hence predicts a role for Rac1 in persistent migration and Cdc42 in random migration.[232]

9 Open Questions in the Field

Much of our understanding of the spatial and temporal regulation of small GTPases during cell migration is restricted to the leading edge of migrating cells. There again, this information is limited to small GTPases of the Rho family. With the emerging role for GTPases, like Ral and Arf6,

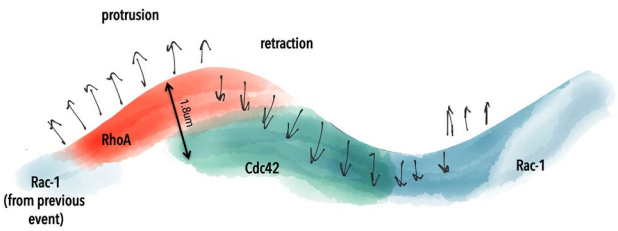

Figure 3: This schematic illustrates the spatial and temporal activation of Rho GTPases (RhoA—*red*, Cdc42—*green*, and Rac-1—*blue*) at the lamellipodial edge of a migrating cell during protrusion (*arrows pointing up*) and retraction or stalling (*arrows pointing down*). The *coloring* and *shading* indicate how activation of RhoA, Rac, and Cdc42 changes over space and time after the initial induction of a protrusive edge. Rac-1 activation (*blue*) is initiated as RhoA activation drops (*red*). This activation of Rac-1 initially overlaps with Cdc42 activation (*green*), though Rac-1 activation is sustained longer, fading into the next protrusion event (adapted from Machacek et al. and Welch et al.[144, 239]).

in regulating Rho GTPases[100, 101, 149, 170, 176, 177] and membrane organization,[78, 79, 233] understanding their spatiotemporal regulation in a migrating cells remains of much interest. The dynamic nature of membrane organization and trafficking at the leading edge and the role of this could have also remained unexplored. As has been discussed in this review, the localization and role for small GTPases are also reported at the nucleus and at focal adhesions.[73, 165, 217, 234] The role of the cross-talk among these GTPase, seen at the lamellipodia edge, which could have at these sites, is only beginning to emerge and needs further testing. The differential localization and role of GEFs and GAPs at sites of GTPase regulation and function are also an open question [135, 158, 235] which, however, is limited by the availability of reagents to detect and study many of these molecules. It is known that small GTPases have distinct roles in the regulation of random and directed migration in 2D matrices.[232, 236] With cellular morphology, integrin-dependent focal adhesion organization and cellular migration being all distinctly different in cells growing in 3D,[237, 238] the localization, regulation, and role of small GTPases in this microenvironment could also be different and are hence of direct interest to the field.

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