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Membrane dynamics in cell migration

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Abstract

Migration of cells is required in multiple tissue-level processes, such as in inflammation or cancer metastasis. Endocytosis is an extremely regulated cellular process by which cells uptake extracellular molecules or internalise cell surface receptors. While the role of endocytosis of focal adhesions (FA) and plasma membrane (PM) turnover at the leading edge of migratory cells is wide known, the contribution of endocytic proteins *per se* in migration has been frequently disregarded. In this review, we describe the novel functions of the most well-known endocytic proteins in cancer cell migration, focusing on clathrin, caveolin, flotillins and GRAF1. In addition, we highlight the relevance of the macropinocytic pathway in amoeboid-like cell migration.

Abbreviations

AP-2, adaptor protein 2; Arp2/3, Actin related protein 2/3; Cav, caveolin; CHC, clathrin heavy chain; CLC, clathrin light chain; CLIC, clathrin-independent carrier pathway; DC, dendritic cells; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; ERK1/2, Extracellular signal Regulated Kinase ½; ERM, Ezrin, Radixin, Moesin; FA, Focal adhesions; FAK, focal adhesion kinase; FCHO2,F-BAR domain-containing Fer/Cip4 homology domain-only protein 2; Flot, flotillin; GRAF1, GTPase regulator associated with focal adhesion kinase-1; HR, hydraulic resistance; LPA, lipid lysophosphatidic acid; LPAR1, lipid lysophosphatidic acid receptor 1; MEK, mitogen activated protein kinase kinase; MMP, matrix metalloprotease; N-WASP, Wiskott-Aldrich Syndrome Protein; NMDAR, N-methyl-D-aspartate receptor; PAK1, P21-Activated Kinase 1; PDPN, podoplanin; PLA, podosome-like adhesion; PM, plasma membrane; RANK, receptor activator of nuclear factor-κB; ROCK, Rho-assocaited coiled coil-containing protein kinase; SCAR/WAVE, Suppressor of cyclic AMP receptor mutation/ WASP and Verprolin homologous protein; TRE, tubular recycling endosome

Introduction

Cell migration is an essential biological process involved in wound healing, embryonic development, immune responses and cancer metastasis (1,2). In brief, cellular migration is characterised by a cyclic process that consists of cell polarisation (3), protrusion extension at the leading edge, establishment of cell-substrate adhesions, forward movement of the cell body and detachment of the trailing edge of the cell (1). Importantly, the migratory cycle has been related to endocytosis and exocytosis (also known as recycling) of proteins contained in adhesive structures, which all together regulate the disassembly and further assembly of new adhesions (1). Cell protrusion is an actin-dependent process driven by promoters of actin nucleation and polymerization, such as Actin Related Protein 2/3 (Arp2/3), Wiskott-Aldrich Syndrome Protein (N-WASP) and Suppressor of cyclic AMP receptor mutation/ WASP and Verprolin homologous protein (SCAR/WAVE) (4,5). Of note, extracellular signals indirectly activate small GTPases, such as Rho, Rac and Cdc42, which are involved in the regulation of actin dynamics (5). Following cell protrusion, cell-substrate adhesions are formed and mediated by focal adhesions (FA) (1). FAs are integrin-based structures that mediate cell adherence to the substrate and regulate cell migration and spreading. Following integrin engagement with the extracellular matrix (ECM), multiple regulatory proteins are recruited to mediate FA connection to the actin cytoskeleton (1). This capability of responding to the mechanical properties of the ECM and transform the extracellular stimulus into intracellular signalling is defined as mechanotransduction (6). Outstandingly, the ECM goes beyond being a passive structure but it provides different types of mechanical signals and dimensionality. In three-dimensional (3D) environments, cells interact with and are embedded into an intricate network of fibres (6,7). Strikingly, cells exposed to 3D microenvironments present smaller FA than thier 2D counterparts (7). In 2D surfaces, ECM stiffness affects mechanosensing (7), which defines the act of sensing mechanical stimulus by cells (6). On the contrary, ECM stiffness varies in 3D environments depending on fibril alignment, intra- and extra-fibril crosslinking and ECM ligand density (7). Importantly, cancer cell migration contains elements of 2D and 3D environments. For instance, thick fibres could be potentially reminiscent of 2D surfaces (6), where cells show a

polarised, flat and spread morphology since they adhere to the substrate on one side (8). Cell spreading is defined as the process through which recently attached cells engage the ECM and is required prior to cell migration. Indeed, migrating cells share the same subcellular processes with spreading cells (1).

3D migration in invasive cancer cells comes in two different fashions: mesenchymal and amoeboid migration (9). The first one is characterised by an elongated morphology with membrane protrusions, while cells are rounded in amoeboid migration. Another significant difference is the presence of strong adhesive structures and ECM degradation in the mesenchymal state, characteristics that are absent in amoeboid migration (9). In particular, amoeboid migration in cancer utilises small and unsteady blebs, which may be on account of augmented intracellular pressure, which correlates with high membrane tension, or weak membrane-cortical cytoskeleton attachment (10). Interestingly, amoeboid-like cells present a flexible shape that allows them to squeeze into the ECM pores (8,10).

Endocytosis is defined as the process by which cells internalised a variety of molecules, including surface receptors, from the plasma membrane (PM) to cytoplasmic vesicles (11,12). Endocytic pathways are categorised depending on their morphology and mechanics into clathrin-mediated endocytosis, caveolin-dependent endocytosis, clathrin- and caveolin-independent pathways, such as clathrin independent carriers (CLICs) and macropinocytosis (12). Clathrin-dependent internalisation, characterised by the formation of polygonal clathrin-coated pits and vesicles, is the most widely-known form of endocytosis (13,14). These distinctive structures are possible because of the assembly of approximately hundred clathrin triskelia (15). The clathrin triskelion is a highly evolutionary conserved structure (16), composed of three clathrin heavy chains (CHCs) and three clathrin light chains (CLCs) that radiate form a centre (13,16). There are two CLC isoforms: CLCa and CLCb in vertebrates (16). CHCs are recruited together with CLCs to phosphatidylinositol 4,5-bisphosphate-enrich membranes, where they will contribute to membrane bending (14). The clathrin triskelion, however, cannot directly interact with the PM and adaptor proteins, such as adaptor protein 2 (AP-2) and epsin, are required (14). Other accessory adaptor proteins and curvature effectors are involved, such as Epidermal Growth Factor Receptor Pathway Substrate 15 (Eps15), which participates in AP2 clustering. Other relevant effectors

involved in nucleation of clathrin-coated pits are F-BAR domain-containing Fer/Cip4 homology domain-only proteins (FCHOs) (14,17). Complete invagination of the PM is followed by self-polymerisation of the GTPase dynamin around the neck of the pit, where it induces membrane scission (14). It is also worth noting that clathrin, together with other adaptor-coated vesicles, including AP-1, is implicated in the vesicle formation in the trans-Golgi network (TGN) (15,18). The TGN is the major secretory pathway of newly synthesised proteins to distribute them to different subcellular compartments (18).

Caveolins are the foremost components of caveolae, invaginations in cholesterol- and sphingolipid-rich membranes, also known as lipid rafts domains (19). These fatty acid-enriched membranes have been described to participate in endocytosis, exocytosis, transcytosis, as well as cholesterol, triacylglycerid and mitochondrial homeostasis (19).

Despite the fact that endocytosis at the leading edge of migrating cells has been reported to confine receptor-mediated signalling and promote integrin-mediated adhesion turnover (3), recent evidence shows that endocytic proteins regulate cell migration in a variety of ways. In this review, we will first discuss the newly described direct contribution of membrane trafficking-related proteins, such as clathrin, caveolin, flotillin and GRAF1 in cell migration. Secondly, the role of macropinocytosis – as an endocytic pathway – in overcoming the directional bias of cells for low hydraulic resistance environments will be considered.

Clathrin-coated plaques support cell spreading and migration

Clathrin triskelia, henceforth referred to as clathrin, is not only involved in the uptake of extracellular molecules but also controls cytokinesis (16,20), lymphocyte migration (16,21), cell protrusion and adhesion (22), as well as invasion (20). At the front of migrating lymphocytes, clathrin drives accumulation of actin and Arp2/3, which results in lymphocyte polarization during migration (21). In meningioma and cervical cancer HeLa cells, CHC is required to induce WAVE2-dependent lamellipodia formation and cell migration through Arp2/3 activation (22).

Moreover, clathrin has been described in other distinct pleiotropic structures, known as tubular clathrin/AP-2 lattices (TCAL), clathrin-coated plaques or flat clathrin-coated

structures (20,23). Although recent pieces of evidence show that this clathrin-coated plaques result from frustrated endocytosis (24), it is still not clear which extracellular or intracellular signals govern the induction of either clathrin-coated pits or this flat clathrin structures (23). Interestingly, clathrin coated structures resulting from frustrated endocytosis were reported to be mechanosensitive structures that responded to rigid substrates, i.e. stiff environments (24), as well as low-tension membrane conditions (25). Correspondingly, it has been reported that TCAL preferentially nucleate as semitubes around the long axis of collagen fibres, suggesting that the local curvature induced by collagen triggers clathrin assembly following the recruitment of clathrin adaptor proteins (*Figure 1*.) (20).

Strikingly, TCALs have been involved in contact-guided cell migration in response to extracellular cues, in an endocytosis-independent manner (23), but they have also been shown to inhibit cell migration under certain circumstances (26). Focal adhesions (FA) are considered mechanotransducers that signal mechanical and topographical remodelling of the ECM to intracellular structures (27), such as the actin cytoskeleton (1). ECM modification is induced by either exertion of cellular physical forces (28) or matrix metalloproteases (MMPs)-mediated digestion (29). Interestingly, a recent studydeposited to bioRxiv suggests that extracellular topographical cues, such as gelatin degradation, promote disassembly of FA at the leading edge of migrating U373 glioblastoma cells (23). According to this study, FA would be substituted by long-lasting TCALs, which would promote cell adhesion after proteolytic ECM remodelling (23), as well as cell spreading on collagen matrices (20). Similarly, TCALs surround collagen fibres to mediate protrusion stabilisation and anchoring to the matrix (*Figure 1*.) (20). Interestingly, it seems that αvβ5 and β1 integrins would be retained at the PM following FA disassembly and those integrins would be required for TCALs stabilization (23). Consistently with these findings, αvβ5 integrin engagement with the ECM protein vitronectin is required for clathrin-coated plaques formation (24,25). In particular, β1-integrin found at TCALs has been reported to recruit clathrin adaptor proteins, such as Dab2 and AP-2, which promote further integrin clustering at collagen fibres (*Figure 1*.) (20). Dab2 and AP-2 recruit EH domain scaffold proteins, such as Eps15, to regulate the size of clathrin-coated plaques and bring accessory proteins such as dynamin or FCHO2, protein that sense membrane curvature (30). In agreement with that, other

studies performed in immortalized keratinocytes established that other adaptor proteins, including Autosomal Recessive Hypercholesterolemia (ARH) protein and Numb, interact with the cytoplasmic region of β5 integrin in clathrin-coated plaques (25,31). Interestingly, Numb and Dab2 probably present interchangeable roles when mediating Eps15 recruitment to TCALs (25). Importantly, Eps15 seems to possibly mediate FA shift to clathrin-coated plaques and the localization of β5 integrin in those structures (23). In addition, Eps15 would be necessary for the stabilization of TCALs (23). More recently, CLCa isoform has been specifically identified as a key protein in clathrin-dependent cell spreading and successive migration in HEK293 (Human Embryonic Kidney), HeLa (Cervical cancer) and H1299 (Non-small cell lung carcinoma) cells (16). Cell spreading following integrin-dependent anchorage to the substrate requires WAVE2-mediated lamellipodia formation (16,22). Indeed, CLCa appears to promote the activation of the small Rho family GTPase Rac1 (*Figure 1*.) (16). WAVE-2 participates in rearrangements of the actin cytoskeleton via Arp2/3-dependent polymerisation of F-actin. Interestingly, CHC controls WAVE-2 recruitment to the PM, while Rac1 activity mediates its activation (16,22).

However, perdurable TCALs have been reported to oppose cell migration in HeLa cells (26). Intriguingly, flat clathrin lattices appear to perform as lipid lysophosphatidic acid receptor 1 (LPAR1) signalling nanodomains (26). Interestingly, lipid lysophosphatidic acid (LPA) induces migration via the activation of the Rho-associated coiled coil-containing protein kinase (ROCK) pathway, which then provokes actin cytoskeleton reorganization (32). Besides, LPA enhances the expression of MMPs, such as MMP2, which consequently results in increased cancer cell motility and invasion (*Figure 1*.) (32). Following LPA stimulation, LPAR1-containing TCALs are internalised in an N-WASP and Apr2/3 dependent way in HeLa cells (26), thus promoting cell migration. In fact, inhibition of TCAL internalisation has been shown to impair cell motility (26). More importantly, this may suggest that TCAL-endocytosis is required for maintaining the dynamics of clathrin-mediated adhesion and allow cell migration. Further research is needed to identify additional regulators of TCALs disassembly and turnover, as well as clarify the relation between the pro- and anti-migratory roles of clathrin. In addition, it is not clear whether clathrin-coated plaques are exclusively formed around

collagen fibres or are also involved in cell adhesion to other extracellular matrix components during cell migration.

Altogether, clathrin allows the adaptation of migrating cells to the surrounding environment by controlling WAVE2-mediated lamellipodia formation, actin cytoskeleton polymerization and, together with LPAR1, adhesion dynamics during cell migration.

Caveolin-1 signalling hub fosters cell migration

The caveolin family is constituted of three isoforms, Caveolin-1 (Cav-1), Caveolin-2 (Cav-2) and Caveolin-3 (Cav-3) (19,33,34). Of note, Cav-1 and Cav-2 have been associated with cell transformation (33,34). Cav-1 expression has been reported to be augmented in breast, colon, prostate, pancreas and oesophageal carcinomas, while Cav-2 is overexpressed in cervical cancer, lung adenocarcinoma, glioma and urothelial and oesophageal carcinomas (33). Cav-1 and Cav-2 have been described to regulate cell motility in androgen-insensitive prostate cancer cells (33).

Cav-1 expression is controlled by epigenetic mechanisms. At early stages of cancer or during cell transformation, Cav-1 is considered a tumour suppressor and its expression is diminished owing to methylation of the CpG islands present in CAV-1 promoter (35). As an example, Cav-1 levels are higher in non-transformed breast epithelial cells compared to ERα-positive breast tumorigenic cells, in which the CpG Island is hypermethylated (35,36). Interestingly, CAV1 promoter is demethylated as tumour progresses; in fact, re-expression of Cav-1 in lung adenocarcinoma fosters filopodia formation, cell migration and metastasis (35). Caveolin function in tumorigenesis remains ambiguous, as it has been shown to be both depleted and up-regulated in several cancers (33). On one hand, in peritoneal mesothelial cells, loss of Cav-1 leads to hyperactivation of mitogen activated protein kinase kinase (MEK)- Extracellular signal Regulated Kinase (ERK1/2)-Snail-1 pathway, which consequently enhances cell migration and invasion, suggesting that Cav-1 restrain cell migration in this context (37). Indeed, the peritoneum of Cav-1 deficient mice presents augmented fibrosis and epithelial to mesenchymal transition (EMT) (37).

On the other hand, Cav-1 has been shown to localise at the trailing edge of fibroblasts, where it participates in rear polarization and promotes directional migration by regulating Rho GTPases and tyrosine protein kinase c-Src (*Figure 2B*.) (38,39). Moreover, Cav-1 interacts with Kinase Suppressor of Ras 1 (KSR1), a molecular scaffold that facilitates Ras-mediated activation of Raf/MEK/ERK signalling cascade, to induce H-Ras driven cell transformation in primary mouse embryo fibroblasts (40).

A recent paper deposited to bioRxiv reports that at the leading edge of invasive B16.F10 melanoma cells, Cav-1 appears to functionally cooperate with podoplanin (PDPN) to induce directional cancer cell migration in response to chemical or mechanical signals (e.g. tissue stiffening or ECM remodelling) (*Figure 2A*.) (41). PDPN (a glycosylated mucin-like protein) is highly expressed in several tumours, including melanoma, lung, colorectal, oral and breast cancer. Remarkably, PDPN expression correlates with higher metastatic potential and EMT (41). Under this circumstances, PDPN co-localising with Cav-1 probably triggers the activation of P21-Activated Kinase 1 (PAK1) (41), which ultimately activates ERK1/2 (41,42) and Ezrin, Radixin, Moesin (ERM) proteins to promote directional migration via regulation of the actin cytoskeleton (*Figure 2A*.) (41). Although Rho family small GTPases Cdc42 and Rac1 are major upstream regulators of PAK1 (42), endogenous expression of both Cav-1 and PDPN seems to regulate cell migration in a RhoA-, Rac1- and Cdc42- independent manner (41). Notwithstanding, PDPN has been reported to activate RhoA/C small GTPases, which activity is inhibited by C-type lectin receptor (CLEC-2) signalling to induce actomyosin cytoskeleton relaxation in stromal fibroblastic reticular cells (43). However, PDPN overexpression in the non-invasive breast cancer cells MCF7 have been shown to decrease active RhoA levels during collective migration, resulting in an increase of motility (44). Further research is required to clarify the role of Cav-1 and PDPN in controlling small GTPase signalling, since other yet to be described proteins could potentially explain those discrepancies; for example, unidentified regulators of this signalling pathway could participate by rapidly inhibiting RhoA GTPases after their activation in order to facilitate migration by relaxing the actomyosin cytoskeleton.

PDPN has also been shown to associate with CD44 (43,45), to promote the migration of stromal fibroblastic reticular cells in the lymph nodes (43). CD44 is a transmembrane glycoprotein that binds to ECM proteins, such as hyaluronan acid,

collagens and fibronectin (45). CD44 has been reported to participate in stiffness-dependent cell adhesion and motility (46). In addition, CD44 has recently been involved in sensing hyaluronic acid cleavage in hyperalgesia (47) and participate in collagenolysis during inflammation and fibrotic processes (48). CD44 specifically triggers the translocation and subsequent activation of c-Src, which triggers integrin β1 activation via a process known as inside-out signalling (45). Integrin activation results in its clustering and enhanced adherence to the matrix (*Figure 2A*.). This prevents the endocytosis of caveolin-containing lipid rafts (45) which could potentially prolong signalling in these domains and mediate cell migration together with CD44. Of note, caveolin has been shown to form a complex with integrins at focal adhesions (49). Integrins also regulate the activation of c-Src (50) and protein kinase C (PKC) (51), both of which phosphorylate caveolin (52), for the proper adhesion and spreading of cells (51,52).

In addition, Cav-1 seems to induce receptor activator of nuclear factor-κB ligand (RANKL)-dependent migration in gastric cancer (53). The receptor activator of nuclear factor-κB (RANK) is expressed in several malignancies, such as renal, breast and lung cancer. Infiltrating T cells secret RANKL to promote c-Src activity which successively triggers Cav-1 activation/phosphorylation (pCav-1) (53) and the signalling axis Phosphoinositide 3-kinase (PI3K)/Akt and ERK pathway (54) to support cell migration (*Figure 2A*.) (53). pCav-1 has been reported to sequester the regulatory subunit of PI3K, p85α, to prevent Rab5 inactivation. Rab5 activity results in recruitment of Rac1 GEF Tiam1 and subsequent Rac1 activation (*Figure 2A*.), which promotes actin cytoskeleton reorganization and enhances migration (55). Neddylation is a post-translational modification by the neural precursor cell expressed developmentally downregulated 8 (NEDD8) that resembles the process of ubiquitin conjugation and affects protein stability and function (56). Neddylation of Cav-1 importantly diminishes its phosphorylation by c-Src and prevents prostate cancer and glioblastoma cell migration (56). Overall, caveolins seem to form a signalling regulation platform where multiple proteins interact to modulate cell migration.

Flotillins promote cell migration by inducing FA turnover and EMT

Flotillins are lipid rafts proteins (57–60) that define specific microdomains in the plasma membrane, which act as signalling sites and, in addition, flotillins have been suggested to transduce regulatory signals to the actin cytoskeleton, as well as participate in cell-cell adhesion in zebrafish embryos (61). Flotillins have additionally been associated to membrane trafficking (57), including cholesterol-enriched exosome secretion in oligodendroglial cells (62), and participate in clathrin-independent endocytosis (57,58). The flotillin protein family comprises two well-preserved isoforms: flotillin-1 (Flot-1; reggie-2) and flotillin-2 (Flot-2; reggie-1) (57), which is indispensable for Flot-1 stabilization (58). Besides, Flot-1 and Flot-2 undergo posttranslational modifications by means of palmitoylation and myristoylation (57,58) in order to being recruited to the PM (57,58,60). The process of flotillin homo- and hetero-oligomerisation at the PM (58) additionally modulates its translocation to endosomal compartments (63) and function (57). Flotillins have been shown to be involved in actin cytoskeleton remodelling, cell adhesion and motility by modulating Rho GTPases activity (58).

In addition, Flot-1/2 has been implicated in promoting integrin-dependent cell-fibronectin adhesion, spreading, as well as directional migration (57). Consistently, Flot-1/2 downregulation correlates with increased non-directional migration in HeLa cells (57). Indeed, flot-2 knockout reduces metastasis formation in murine breast cancer models, while its overexpression enhances metastatic melanoma (57). Flot-1/2 controls E-cadherin and α5β1 integrin recycling, as well as focal adhesion turnover in a Rab11-dependent manner, which negatively regulates Rac1 activation (64). Flot-1/2 – depleted cells thus present high levels of active Rac1, which increases peripheral lamellae formation and random migration (65).

Flot-1 is highly expressed in lung adenocarcinomas, where its expression *in vitro* correlates with enhanced cell proliferation, migration and invasion (59). Recent evidence suggests that both Flot isoforms are located at FA, where they seem to be a required for the autophosphorylation of FAK (focal adhesion kinase), downstream of integrin engagement with the ECM (57). Phosphorylated FAK (pFAK) successively forms

a complex with c-Src, which further phosphorylates FAK (*Figure 3A.*), and promotes α-actinin phosphorylation (57). c-Src consequently binds to phosphorylated α-actinin, thereby disassembling the c-Src/FAK complex (*Figure 3A.*). pFAK can then be dephosphorylated by the phosphatase PTB-1B, accelerating FA turnover (57). Moreover, Flot localization to FAs correlates with myosin IIa activity, which is intriguingly arbitrated by Flot. Notwithstanding, it is not known whether myosin IIa activity is dependent on Flot localization to FA (57). In addition, FAK indirectly promotes ERK activation during cell spreading, resulting in Flot-1/2-mediated anchorage-independent proliferation (*Figure 3A.*) (57). Flot-1 has also been involved in the signal transduction of tyrosine kinase receptors, such as insulin-like growth factor receptor (IGF-1R). Palmitoylation of Flot-1 is indispensable for its translocation together with IGF-1R to the PM, where it promotes proliferation of prostate cancer cells (60) and actin remodelling by regulating RhoA, Rac1 and Cdc42 activity (*Figure 3A.*) (66).

Furthermore, Flot-1 appears to be involved in EMT (60). In prostate cancer cells, Flot-1 is sumoylated with SUMO-2/3 by the E2 conjugating enzyme UBC9 in the endoplasmatic reticulum (ER). Sumoylation of Flot-1 triggers its relocation to the nucleus, where it interacts with, stabilises and hampers the proteosomal degradation of Snail – one of the foremost transcription factor involved in EMT, thereby promoting metastasis formation (*Figure 3B.*) (60). Taking everything into account, Flot-1/2 enhances directional cell migration by modulating Rho GTPases activity and promotes cell spreading via activation of FAK downstream of integrin engagement.

GRAF1 role in regulating cell migration

GTPase regulator associated with focal adhesion kinase-1 (GRAF1), also known as Oligophrenin-1-Like (67), is a Cdc42 and RhoA GTPase-activating protein (GAP) (68) that seems to play an essential role as a sculptor of the highly curved membranes that are characteristic of the clathrin-independent carrier (CLIC) pathway (69,70). In fibroblasts, CLICs constitute one of the foremost pinocytic routes (68), also categorised as caveolin- and clathrin-independent endocytic pathways (68,69). Cdc42 and RhoA activity is required and tightly circumscribed at the initial stages of CLIC (69); while at

the later stages, GRAF1-mediated Cdc42 and RhoA inactivation is necessary for the progression of CLIC-mediated endocytosis.

Outstandingly, GRAF1-positive CLICs have been reported to participate in cell surface dynamics (68), impacting actin remodelling (68,71), integrin trafficking (67,68,71), spreading, adhesion (67,68,70,71), polarization of motile cells (72) and 2D and 3D migration (71). GRAF1 role in cell migration seems contradictory; on the one hand, its expression is required for the turnover of the plasma membrane, which facilitates the pro-migratory activity of cells in 2D surfaces. On the other hand, GRAF1 represses amoeboid migration of cancerous cells in a 3D environment. Recent evidence shows CLICs to be directly recycled to the PM, resulting in an accelerated recycling during cell adhesion turnover and motility. CLICs, for instance, have been reported to internalise the ECM adhesion receptor CD44, which complexes with β1 integrin, at the front of migrating fibroblasts. In addition, CD44 can mediate directional persistence and actin cytoskeleton rearrangements through ERM proteins (72).

At the leading edge of migrating HeLa cells in a 2D surface, momentary interaction between GRAF1 and the Rho GTPase Cdc42 is required for modulating PM and actin dynamics during cell surface turnover (68). Cdc42 activity triggers GRAF1 recruitment to the PM, where it will promote membrane invagination. GRAF1 GAP activity concomitantly increases during membrane bending, which eventually causes Cdc42 inactivation and dissociation from the PM to possibly further permit scission of the plasma membrane and maturation of the recently formed endosomal compartment (*Figure 4A*.) (68). Similarly, Rac1 fosters the CLIC pathway by controlling GRAF1 membrane targeting (*Figure 4A*.) (70).

Moreover, downstream of CLIC generation, GRAF1 localises in phosphatidylinositol-(4,5)-bisphosphate-enriched (69,71) tubular recycling endosomes (TREs) where it has more recently been shown to indirectly interact with MICAL-L1 (Molecules Interacting with CAsL-Like1) and EHD1 (Eps15 Homology Domain containing protein 1) to induce TREs vesiculation (*Figure 4A*.) (67). GRAF1 similarly seems to be involved in the vesiculation of clathrin-independent incoming endosomes (67). Interestingly, this process is required for favouring β1 integrin recycling to the PM and, to a lesser extent, its lysosomal degradation (*Figure 4A*.) (67).

Podosome-like adhesions (PLA) are integrin-rich protrusive structures that resemble classical podosomes in macrophages (73). PLA are formed in the absence of large traction forces in non-transformed fibroblasts, (73) and during the first stages of spreading in platelets (74). Although those adhesions present classical podosome and invadopodia (also known as podosome-type adhesions (75)) markers, such as F-actin, N-WASP and Arp2/3, they lack the characteristic matrix metalloproteinases of Src-transformed fibroblasts (73). Despite this, the Virtanen group reported that podosome-like structures can degrade ECM to an extent (76). Despite the fact that PLA share many proteins with focal adhesions, N-WASP and the Arp2/3 complex are not found in the latter ones. Cdc42-recruited GRAF1 additionally modulates the dynamics of Src-induced PLA sites at the front of migrating cells (*Figure 4C*.) (70). GRAF1 deregulations consequently negatively impact on cell adhesion, spreading and migration by perturbing integrin recycling and PLA endocytosis.

Furthermore, besides it role in controlling integrin recycling and focal adhesion turnover, GRAF1 has recently been implicated in the maintenance of membrane tension homeostasis (71,77). GRAF1 appears to participate in constructing a repository of cytoplasmic membranes with which cells can respond to fluctuations regarding osmotic pressure and membrane tension (*Figure 4B*.) (71). In particular, GRAF1-induced endocytosis reduces membrane tension and cellular blebbing (*Figure 4B*) by downregulating RhoA activity. Of note, loss of GRAF1 in HeLa and SW480 colorectal cancer cells results in an increase in bleb formation, fostering amoeboid invasive 3D migration (71).

GRAF1 has further been reported to preserve the normal epithelial phenotype and, for this reason, it is contemplated as a tumour suppressor-type gene (77). Knockdown of GRAF1 in the non-malignant breast cells MCF10A apparently induces EMT (77), which may be mediated by the constitutive activation of RhoA/ROCK pathway, which expression is commonly correlated to metastases (77). Considerably, GRAF1 contribution to cell migration, either by promoting CLIC pro-mesenchymal migration or suppressing bleb-induced amoeboid migration, as well as EMT, has to be further studied since other yet unknown effectors may be involved in its role in tumorigenesis. Integrin-containing TRE, for instance, could potentially activate signalling pathways

that ultimately promote EMT. Indeed, it is well-known that integrin- and active FAK-positive endosomes signal cell survival and supress anoikis (78).

Thus far, this review has reported the migratory role of proteins involved in the regulation of endocytosis and membrane composition. In the next section, the role of the endocytic pathway macropinocytosis in overcoming the migration bias towards high hydraulic resistance pathways will be discussed.

Macropinocytosis in amoeboid migration

Barotaxis is commonly defined as the phenomenon by which migrating cells (e.g. neutrophils) follow tracks with minimum hydraulic resistance (HR) (79) or hydraulic pressure (80). The term HR is used to describe the resistance of the environment to fluid displacement with which can restrict amoeboid-like cell migration by limiting the interstitial liquid movement (79). HR consequently causes a small force imbalance at cellular level that biases cell migration to least hydraulic pressure paths (79). The Lennon-Duménil group has recently shown that highly barotactic dendritic cells (DCs) present an extremely polarised accumulation of actin and myosin II at the trailing edge, while barotactic-resistant DCs exhibited a symmetric accumulation of actomyosin at both the leading and trailing edge (79). *In vitro* experiments using low-friction Y-shaped bifurcations showed that barotactic DCs first extend two symmetric projections, one of which eventually retracts after accumulating actomyosin; for this reason, actomyosin regulators can impinge on barotaxis (79). Low-barotactic cells exhibit high macropinocytic activity at the leading edge (*Figure 5*.) (79). Macropinocytosis or internalisation of extracellular fluid is an actin-dependent process (81,82) induced by augmented intracellular calcium after activation of the Ras/Rac1 pathway (81). Outstandingly, the macropinosomes formed during barotactic-independent migration are retrogradely transported to the trailing edge where fluid is released and cell resistance to extracellular flow is therefore diminished. As a result, macropinocytosis thwarts the directional bias to migrate towards low HR environments (79).

Of note, interstitial fluid pressure is extremely high at the tumour boundaries and it has been reported to be concomitant with invasiveness and lymphatic metastasis (83).

In murine pancreatic neuroendocrine tumours as well as in human and mouse breast malignancies, the interstitial flow can additionally support cancer invasion by regulating the localization of the neuronal receptor and calcium channel N-methyl-D-aspartate receptor (NMDAR) to the plasma membrane (PM) and the secretion of its major agonist: glutamate (83,84) (*Figure 5*.). In the hippocampus, NMDAR mediates the translocation and further activation of Ras (85) and, in addition, glutamate-induced activation of NMDAR increases intracellular calcium levels (*Figure 5*.) (84). Furthermore, certain malignant cells (e.g. ERα-negative breast cancer cells) occasionally migrate in an amoeboid-like fashion in three-dimensional cultures and *in vivo* (86). The latter raises the intriguing hypothesis that NMDAR activation could induce macropinocytosis in amoeboid-like migrating cancer cells in order to allow their dissemination to high HR regions.

Summary points

- Clathin promotes cell migration on ECM through the generation of TCALs. Following FA dissociation, TCALs assemble in response to extracellular cues, such as fibrillar collagen or ECM degradation. Under these conditions, flat clathrin-coated lattices act as adhesive structures that seem to be necessary for cell migration. CLCa is particularly important in promoting actin reorganization prior to migration. Finally, LPAR1 is recruited to TCALs and its downstream activity triggers ROCK activation, as well as endocytosis of these clathrin-coated plaques, allowing the cell to move forward.
- Cav-1 has been reported to act as a tumour suppressor-like gene, but also promote cell migration in cancer. Cav-1 is present at the front and rear of migrating cells. At the leading edge, Cav-1 forms a signalling platform where it interacts with several proteins, such as small GTPases and c-Src, to foster cell migration.
- In the endoplasmic reticulum, Flot-1/2 is sumoylated by UBC9, which triggers its translocation to the nucleus where it inhibits the degradation of the pro-metastatic transcription factor Snail. Flot-1/2 additionally promotes FA turnover and actin remodelling following the activation c-Src and small GTPases, respectively.

- GRAF1 presents a controversial role in migration. On the one hand, GRAF1 participates in FA turnover and integrin recycling, which are required in 2D cell migration. On the other hand, GRAF1 suppresses bleb-induced 3D amoeboid migration in cancer. GRAF1 has also been involved in preserving the epithelial phenotype and supressing EMT.
- Macropinosomes formed at the front of amoeboid-migrating cells are retrogradely transported and released so that resistance to the extracellular flow decreases, which allows cell migration towards high HR environments.

Conclusions

Cell migration is required in various physiological and pathological processes, such as tumour invasion and subsequent metastasis(1,2), which is responsible of the vast majority of cancer deaths. Therefore, having a deeper understanding of the molecular pathways that govern cell migration, especially in 3D environments, is essential to prevent cancer dissemination. The role of endocytic proteins in cell migration goes beyond promoting FA turnover; in fact, these proteins seem to be active participants of cell migration by activating downstream regulators of migration, such as small GTPases or actin-remodelling proteins.

Clathrin has been shown to have a role in promoting cell migration on ECM through the generation of TCALs. During cell migration cycle, cells adhere to the substrate via FAs (1). Interestingly, following FA dissociation, TCALs would assemble in response to extracellular cues, such as fibrillar collagen, ECM degradation or stiff substrates. Under these conditions, flat clathrin-coated lattices would act as reinforcing adhesive structures that would contribute to cell spreading. Of note, cell adhesion to the ECM is necessary to generate traction forces to pull cells forward during migration (87). Migration of cells is a dynamic actin-dependent process (4), in this context CLCa is particularly important in promoting actin reorganization prior to migration. Finally, adhesion structures disassemble at the end of the migratory cycle. On the one hand, LPAR1 is probably recruited to TCALs where its downstream activity triggers ROCK activation, which is involved in actin-cytoskeleton reorganisation and its activity

correlates to metastasis (32,77). On the other hand, LPAR1 provokes endocytosis of these clathrin-coated plaques, allowing cells to move forward.

Furthermore, Cav-1, another endocytic-related protein, has been reported to act as a tumour suppressor-like gene, but also promote cell migration in cancer (35). Cav-1 has been described to be at the front and rear of migrating cells. At the leading edge, Cav-1 apparently forms a signalling platform where it interacts with several proteins, such as small GTPases and c-Src, to foster cell migration by facilitating cell adhesion and spreading (51,52). In addition, Cav-1 containing signalling hubs further support cell migration by activating pro-migratory and pro-invasive signalling pathways.

Moreover, Flot-1/2 promotes FA turnover (64), being implicated in the endocytosis and recycling to the plasma membrane of adhesion structures involved in cell migration. Flot-1/2 is further involved in actin remodelling following c-Src and small GTPase activation (57,66). In the endoplasmic reticulum, Flot-1/2 is sumoylated by UBC9, which triggers its translocation to the nucleus where it inhibits the degradation of the pro-metastatic and EMT transcription factor Snail (60). Another endocytic-related protein is GRAF1, which have been reported to present a contradictory role in migration. On the one hand, GRAF1 promotes 2D migration by participating in FA turnover and integrin recycling (71) while, on the other hand, GRAF1 suppresses bleb-induced 3D amoeboid migration in cancer (71). Interestingly, GRAF1 has also been implicated in preserving the epithelial phenotype and supressing EMT (77).

Finally, macropinocytosis, an endocytic pathway, has been shown to contribute to 3D amoeboid migration. In summary, macropinosomes formed at the front of amoeboid-migrating cells are retrogradly transported and released (79). Consequently, the resistance to the extracellular flow decreases, which allows cell migration towards high HR environments. Further research is however needed to fully decipher the novel role of the reviewed endocytic proteins and macropinocytosis in cell migration, in 3D in vivo-like conditions, and unravel possible cancer-specific targets to supress metastasis.

Figure legends

Figure 1. Schematic representation of TCALs at the leading edge of migrating cells. Following FA disassembly, integrins are retained in the PM where they promote the stabilisation of TCALs. TCALs recruit clathrin adaptor proteins, such as Dab2, to promote further integrin clustering at collagen fibres. Dab2 brings to the membrane accessory proteins to sense membrane curvature and further stabilise clathrin-coated plaques. CLCa promotes Rac1 activity to subsequently mediate WAVE-2 activation leading to actin remodelling. Finally, LPAR1-resident clathin-coated plaques, besides triggering MMPs secretion and ROCK activity, are required to terminate cell adhesion and allow migration.

Figure 2. Scheme of Cav-induced migratory pathways in the leading and trailing edge. At the front of migrating cells, Cav-1 forms a signalling platform where it interacts with multiple signalling proteins, including c-Src, Rac1, Cdc42, PKC and PAK1, to eventually promote cell adhesion, spreading and directional migration (A). Cav-1 has been described to localise at the rear of cells to promote cell polarisation and directional migration (B).

Figure 3. Diagram of the molecular pathways involved in Flot-mediated migration. Downstream integrin engagement with ECM proteins, Flot isoforms are required for FAK autophosphorylation. Following pFAK dissociation from c-Src, α-actinin is recruited and pFAK is dephosphorylated, which promotes FA turnover. Besides, FAK-mediates indirect ERK1/2 activation and promotes anchorage-independent proliferation (A). At the PM, palmitoylated Flot-1/2 regulates actin remodelling through Rac1, RhoA and Cdc42. In the endoplasmic reticulum, Flot-1/2 can be sumoylated by UBC9, which triggers its translocation to the nucleus where it inhibits Snail degradation, thereby promoting EMT (B).

Figure 4. Schematic representations of GRAF1 roles in cell migration. GRAF1 is recruited to the membrane by small GTPases that regulate early stages of CLIC formation. Following GRAF1 recruitment, GRAF1 inhibts RhoA and Cdc42 to allow CLIC progression. Of note, GRAF1-mediated inhibition of RhoA eventually supresses EMT. GRAF1-induced vesiculation is required for integrin recycling to the plasma membrane (A). GRAF1-negative cells cannot reduce membrane tension, thereby leading to cell

blebbing and increase invasion in 3D environments (B). GRAF1 interacting proteins in PLA (C).

Figure 5. Representation of barotactic and barotactic-resistant cells. Barotactic cells follow low HR paths, while barotactic-resistant cells present high levels of macropinocytosis at the leading edge. Exocytosis of macropinosomes at the trailing edge diminishes cell resistance to the extracellular flow, which allow them to follow high HR paths. Interstitial pressure increases the expression of NMDAR and the exocytosis of its ligand, glutamate. Downstream activation of NMDAR triggers Rac activity and an increase of intracellular calcium levels, which could potentially induce macropinocytosis to induce amoeboid-like migration.

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Figure 1.

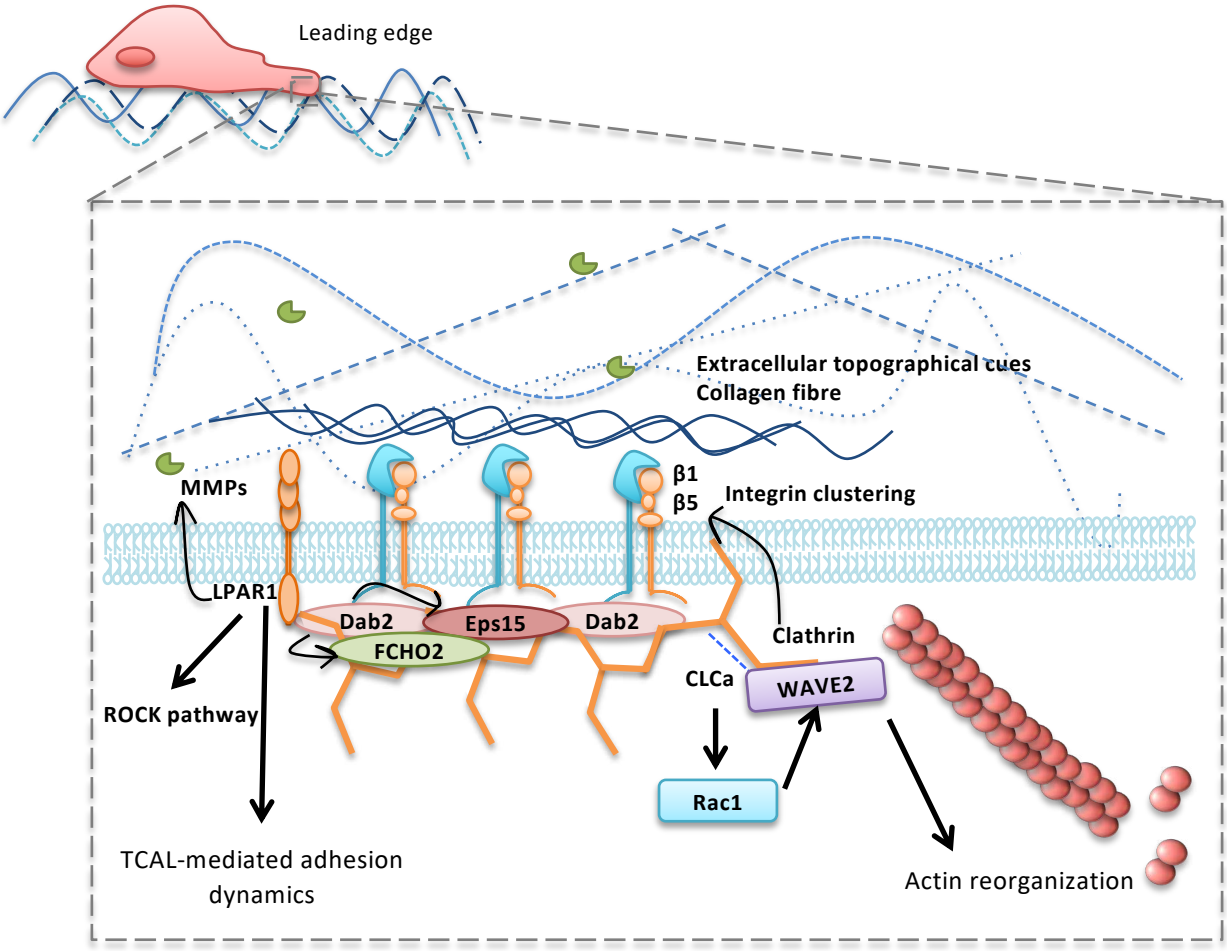

Leading edge
Extracellular topographical cues
Collagen fibre
MMPs
β1
β5
Integrin clustering
LPAR1
Dab2
Eps15
Dab2
FCHO2
Clathrin
CLCa
WAVE2
ROCK pathway
Rac1
TCAL-mediated adhesion dynamics
Actin reorganization

Figure 2.

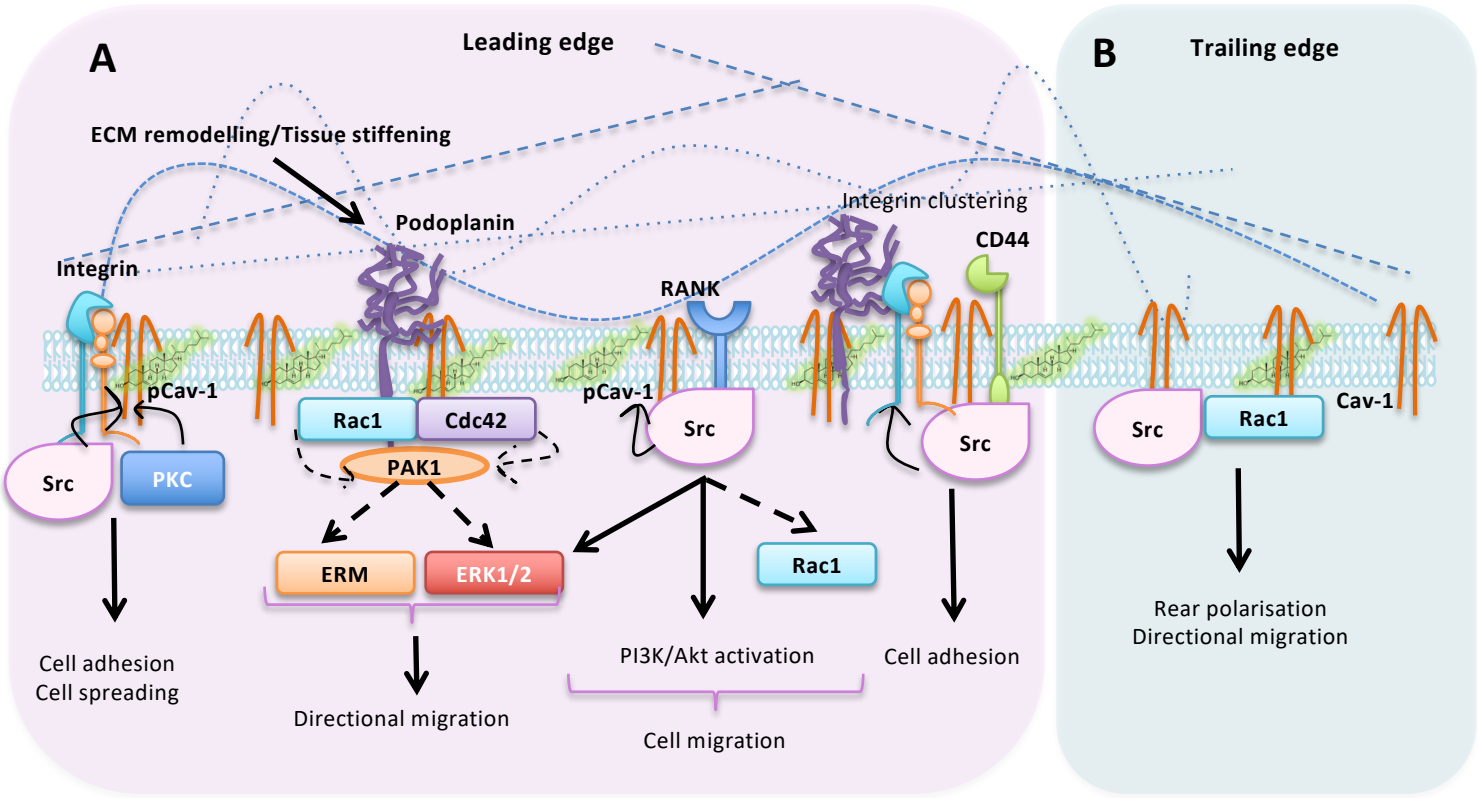

A
Leading edge
B
Trailing edge
ECM remodelling/Tissue stiffening
Podoplanin
Integrin
pCav-1
Src
PKC
Cell adhesion
Cell spreading
Rac1
Cdc42
PAK1
ERM
ERK1/2
Directional migration
RANK
pCav-1
Src
Rac1
PI3K/Akt activation
Cell migration
Integrin clustering
CD44
Src
Cell adhesion
Src
Rac1
Cav-1
Rear polarisation
Directional migration

Figure 3.

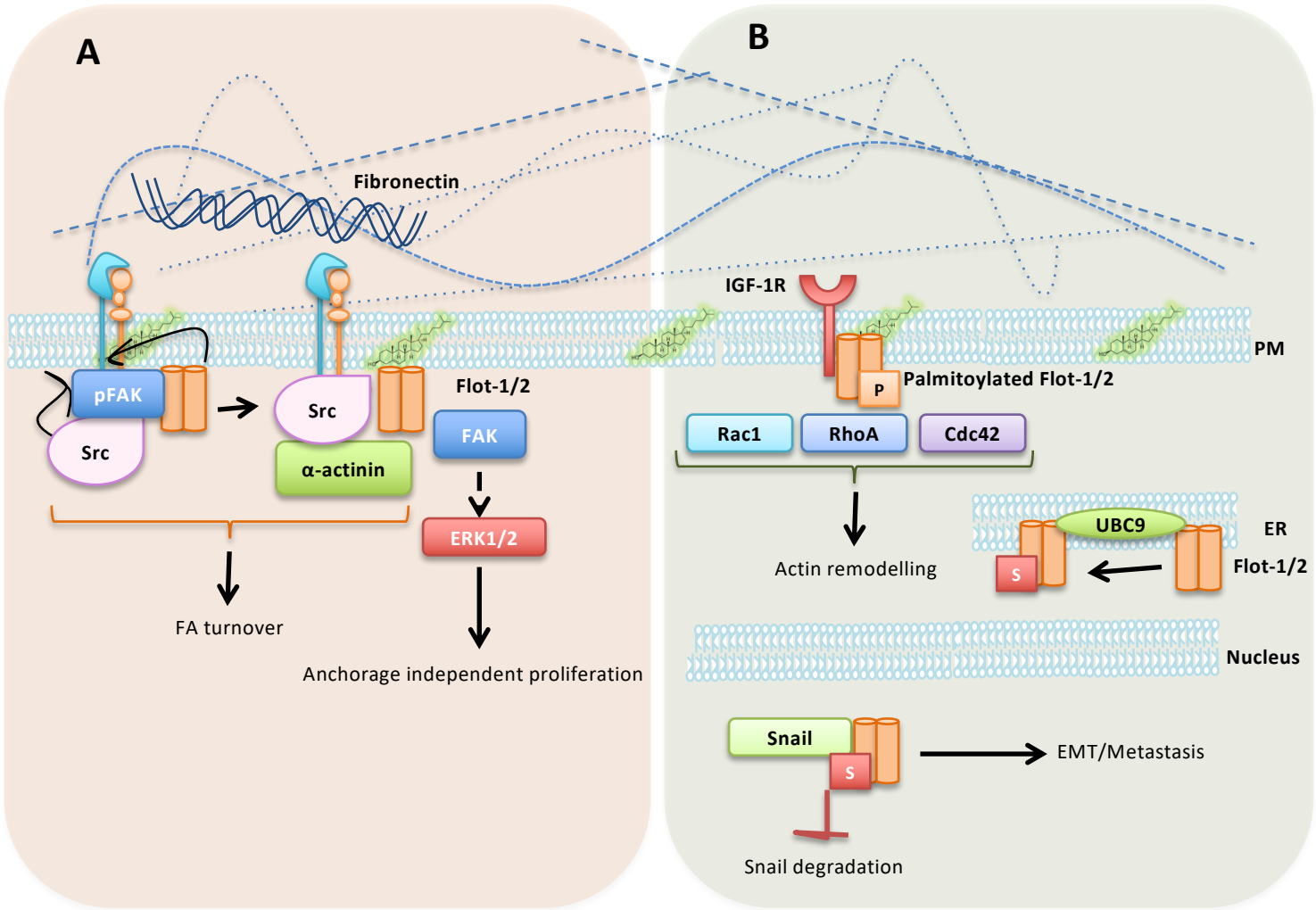

A
Fibronectin
pFAK
Src
Src
α-actinin
Flot-1/2
FAK
ERK1/2
FA turnover
Anchorage independent proliferation
B
IGF-1R
P
PM
Palmitoylated Flot-1/2
Rac1
RhoA
Cdc42
Actin remodelling
UBC9
S
ER
Flot-1/2
Nucleus
Snail
S
EMT/Metastasis
Snail degradation

Figure 4.

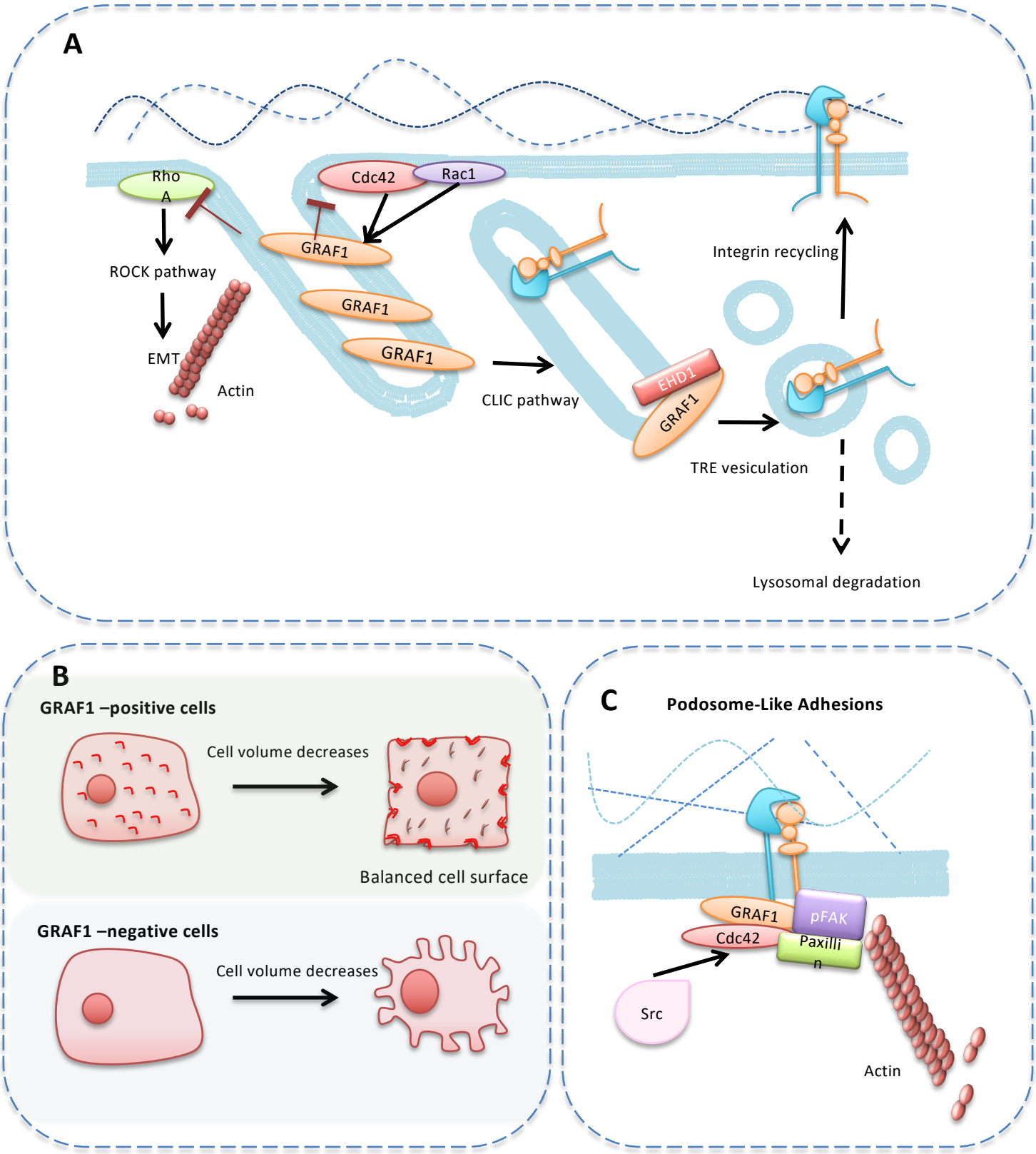
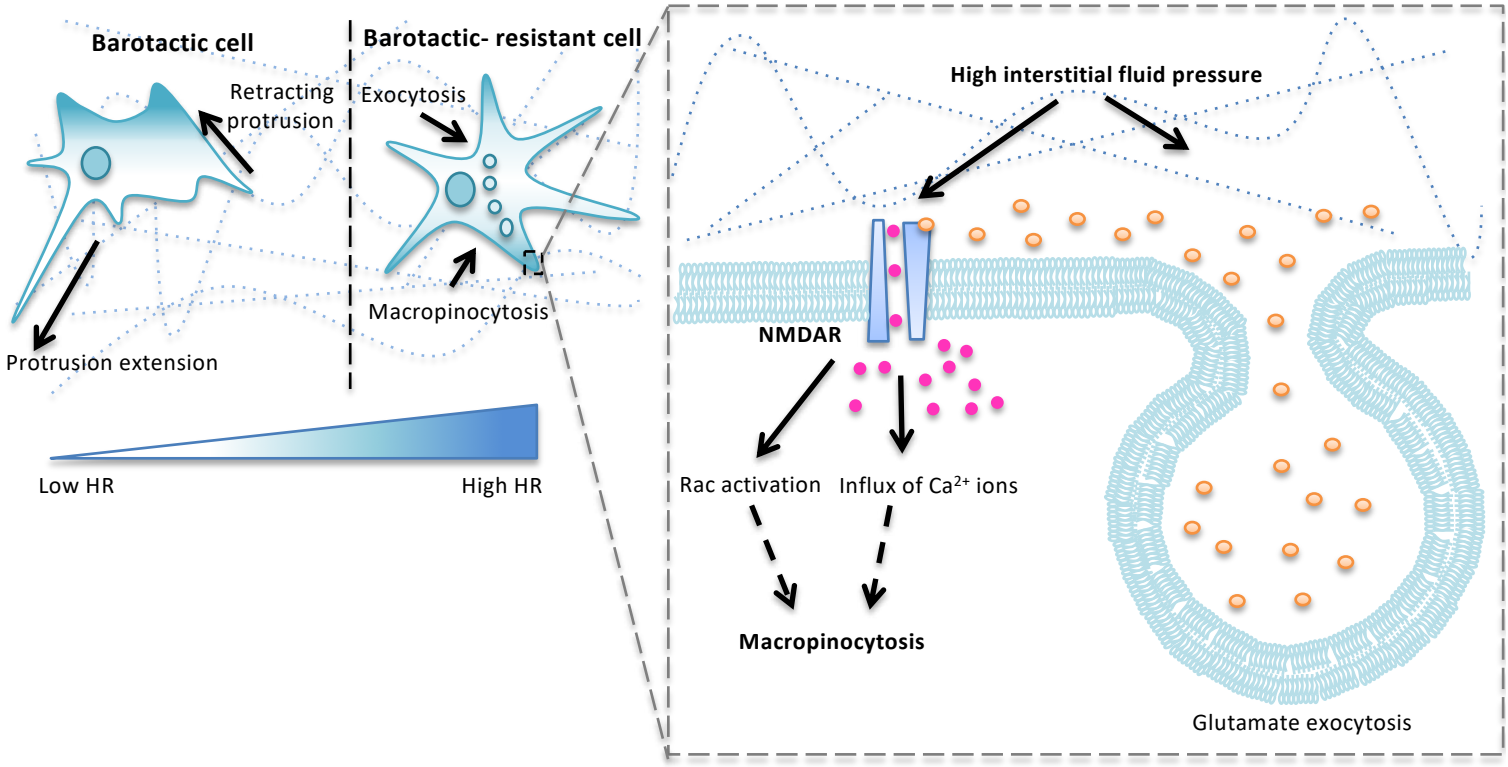

A
Rho A
ROCK pathway
EMT
Actin
Cdc42
Rac1
GRAF1
GRAF1
GRAF1
CLIC pathway
EHD1
GRAF1
TRE vesiculation
Integrin recycling
Lysosomal degradation
B
GRAF1 –positive cells
Cell volume decreases
Balanced cell surface
GRAF1 –negative cells
Cell volume decreases
C
Podosome-Like Adhesions
GRAF1
pFAK
Cdc42
Paxillin
Src
Actin

Figure 5.


Barotactic cell
Retracting protrusion
Protrusion extension
Barotactic- resistant cell
Exocytosis
Macropinocytosis
Low HR
High HR
High interstitial fluid pressure
NMDAR
Rac activation
Influx of Ca2+ ions
Macropinocytosis
Glutamate exocytosis