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Special Focus: Molecular Mechanism of Adhesion Complex Turnover

Bidirectional control of the inner dynamics of focal adhesions promotes cell migration

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Focal adhesions (FA) are bidirectional mechanical biosensors that allow cells to integrate intracellular and extracellular cues. Their function is tightly regulated by changes in molecular composition and also by variation in the spatio-temporal dynamics of FA components within this structure. A closely regulated turnover of FA proteins within FA sites allows cells to respond appropriately to their environment, thereby impacting on cell shape and function. FA protein dynamics are linked to FA maturation and rates of assembly and disassembly, and have a significant influence on tumor cell migration. Using the FRAP technique to investigate the hidden internal dynamics of FA, we identified two new regulators of FA dynamics and cell migration: the Mgat5/galectin-3 lattice and tyrosine phosphorylated caveolin-1 (pY14Cav1). In this short review we first introduce FA and their complex dynamic behavior. We then present the Mgat5/galectin-3 lattice and caveolin-1 and discuss their concerted role in FA dynamics, which defines previously unknown, interdependent roles in tumor cell migration. We conclude with a discussion of interesting unexplored avenues that might lead to a better understanding of the complex mechanism of FA dynamics.

Focal Adhesions: Apparent Stability Hides High Dynamism

Cell adhesion to the extracellular matrix (ECM) is a feature shared by nearly all cells in multi-cellular organisms, and recently cell-substrate adhesion has also been shown to power one of the modes of gliding motility in bacteria.[1] Cell adhesion to the ECM integrates intracellular and extracellular cues; it is essential for the preservation of tissue integrity and for organogenesis during embryonic development, and is also frequently associated with human pathologies. Cell-ECM adhesions are highly plastic and dynamic structures that enable cells to sense, integrate, adjust and react to a variety of intracellular and extracellular signals. Cell-ECM adhesions are bidirectional mechanical biosensors. In one direction they allow cells to translate extracellular signals (such as matrix composition or organization) into intracellular signals or mechanical cascades (such as cell proliferation or cellular contractility), while in the other direction they convert intracellular signals (such as RhoGTPase activity or integrin activation) into extracellular action (such as remodeling of ECM fibers).[2] The several types of cell-ECM adhesions differ from one another in morphology and molecular composition, though most are integrin-dependent, for example focal complexes, focal adhesions, fibrillar adhesions, podosomes and three-dimensional (3D) matrix adhesions.[3]

The first cell-ECM structures to be identified, and therefore the best-characterized, are the focal adhesions. Focal adhesions (FA) are highly dynamic structures and are the master control machinery of cell migration.[4] Optimal cell migration requires spatiotemporal feedback between actomyosin contraction, actin polymerization and continuous formation and disassembly of focal adhesions.[5] During cell migration, the assembly, maturation, translocation and disassembly of FA mediate, respectively, cell attachment, contraction, protrusion of leading edges and retraction of trailing edges.[6] Nevertheless, the first observations suggested that FA were mostly immobile molecular complexes in motile cells, whereas they performed centripetal translocation in stationary cells.[7] The advent of state-of-the-art microscopy techniques, such as FRAP (Fluorescence Recovery After Photobleaching), FCS (Fluctuation Correlation Spectroscopy), TIRF (Total Internal Reflection Fluorescence) and FSM (Fluorescence Speckle Microscopy), allowed closer examination of the internal dynamics of FA in living cells, and revealed the high dynamism of their molecular components.[8-11] Through this work it became clear that variation in the spatio-temporal dynamics both of FA structures and of their component molecules is a powerful mechanism used by cells to sense changes in their immediate environment. Changes in the molecular composition of FA have been extensively studied, and have a clear role in the regulation of FA function. Although less well studied, hidden variations in the internal dynamics of FA are at least as important, tightly controlling FA behavior and dynamics to regulate cell migration.[12,13]

FA structures are characterized by a continuous exchange of proteins (such as FAK, vinculin, paxillin) between the FA compartment at the plasma membrane and the cytosolic compartment, which participates in FA maturation rather than FA movement. Early, elegant work showed that increases in extracellular matrix

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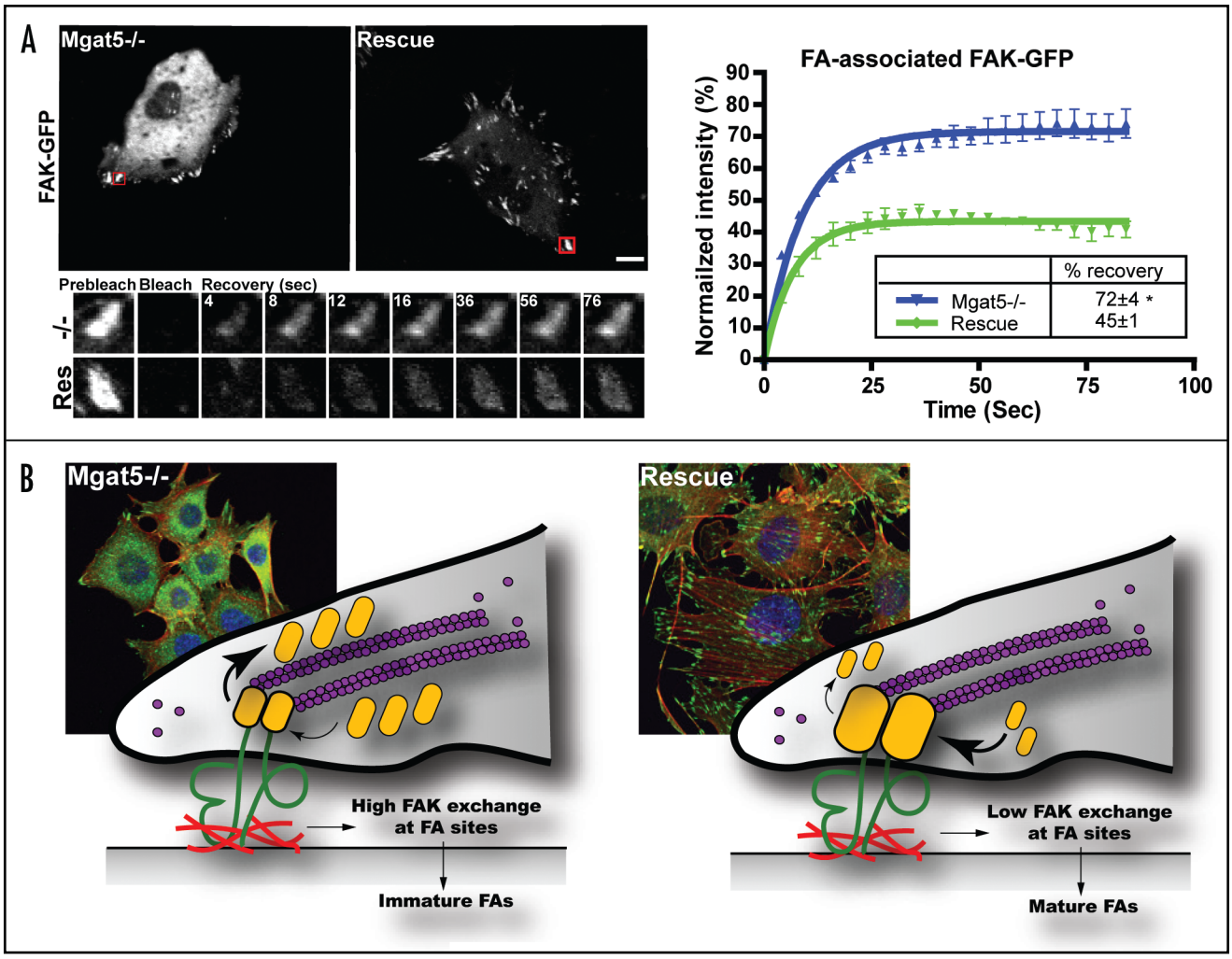

Figure 1. Increased FAK exchange at FA sites correlates with FA maturation and directional cell migration. Adapted from ref 8. (A) Mgat5$^{-/-}$ and Mgat5-Rescue cells transfected with FAK-GFP were imaged before laser bleaching of one FA-containing region of interest (ROI; red square). A time-lapse sequence (in seconds) shows the corresponding ROI before photobleaching (prebleach), immediately after photobleaching (bleach) and during recovery (recovery). Quantification of FAK-GFP fluorescence over time in FA region of interest (ROI) is presented for Mgat5$^{-/-}$ and Rescue cells. Percentage of recovery (boxes) shows the extent of FAK-GFP mobile fraction. *$p < 0.05$. Bar, 20 μm. (B) Mgat5$^{-/-}$ and Rescue cells were immunofluorescently labeled with Hoechst (blue) and with Alexa568-phalloidin (red) and anti-vinculin (green) antibodies. The diagram depicts the differential dynamics of FA components, and their impact on FA maturation, highlighted by the FRAP technique.

rigidity strengthen integrin-cytoskeleton linkages while increased cytoskeletal contractility increases FA area.[14,15] This biomechanical response suggests that physical distortion of FA induces variations in the density and recruitment of both structural and signaling molecules within FA complexes. FA thus appear to act as signaling platforms, such that increased expression and membrane localization of components increases the number (or average lifetime) of FA complexes able to interact with signal transduction proteins, thus increasing the extent of activation of downstream processes.[16] One of the best techniques for measuring the internal dynamics of complex structures is FRAP (Fig. 1A). The time and extent of fluorescence recovery provide valuable information about the reorganization and renewal of the analyzed structure. The FRAP behavior of FA proteins is tightly regulated by intracellular concentration as well as their strength of binding to other FA proteins.[12] For example, the turnover of FA-targeted integrins is relatively slow (5–10 min)[8,11] compared with that of proteins such as FAK or paxillin (<2 min)[8,9] (Fig. 1A). This continual turnover of FA proteins allows cells to use changes in adhesion structures to adjust cell shape and function. Consistent with this, FA protein dynamics are tightly linked to FA maturation and rates of assembly and disassembly, and have a significant influence on tumor cell migration (Fig. 1A). Our recent studies have identified two regulators of FA dynamics and cell migration: the galectin lattice and tyrosine phosphorylated caveolin-1 (pY14Cav1). Our work has defined previously unknown, interdependent roles for galectin-3 and pY14Cav1 in tumor cell migration, whereby transmembrane crosstalk between these factors promotes FA turnover and tumor cell migration by stabilizing components within FA.[8] In the following sections, we present each of these partners in turn and then discuss their concerted influence on FA dynamics.

The Galectin Lattice: Master Control Machinery of Plasma Membrane Organization

Galectins constitute a family of proteins defined by a conserved carbohydrate recognition domain (CRD) that specifically binds β-galactoside-containing N-glycans. This interaction produces a

thermodynamically stable array of lectins and glycoproteins at the cell surface, called the galectin lattice.[17] The mammalian galectin family contains 15 members and these proteins regulate a variety of important cellular phenomena involved in cell activation, cell adhesion, cytokine secretion and cell death.[18] High affinity N-glycan ligands for galectins are produced by a Golgi processing enzyme, β1,6N-acetylglucosaminyltransferase V (GlcNAc-TV), encoded by the mammalian *Mgat5* gene. The N-glycan branching pathway and the number of N-glycans present on glycoproteins both influence the ability of glycosylated receptors to bind galectins.[19] Expression of Mgat5 and branched N-glycans is increased in various carcinomas,[20] while galectin-3 is associated with tumor progression and cancer metastasis in several tumor types.[21] The concept of a galectin lattice was introduced by studies by the group of Jim Dennis, our collaborator. These studies showed that the binding of galectins to Mgat5-modified N-glycans in the T cell receptor restrict T cell activation and autoimmunity by hampering T cell recruitment to antigen presentation sites.[17] We subsequently conducted extensive FRAP analysis to examine protein dynamics at the cell surface. These studies showed that Mgat5-dependent recruitment of cytokine receptors (EGFR) to the galectin lattice enhances signaling potential by downregulating receptor endocytosis.[22,23] Further work, including the study described here, demonstrated that Mgat5-dependent recruitment of glycosylated integrins promotes their clustering, decreases local exchange of FA proteins, and favors subsequent FA turnover[8,24] (Fig. 1A). These studies thus provide definitive evidence for the existence of a galectin lattice that restricts cell surface diffusion of glycosylated receptors at the plasma membrane. Our work further demonstrates that recruitment to the lattice promotes not only homotypic but also heterotypic interactions among glycosylated receptors at the cell surface, allowing cross-stimulation of associated downstream effect (reviewed in ref. 25).

Caveolin-1: A Role Beyond Caveolae Formation

Caveolins are integral membrane proteins involved in the biogenesis of caveolae, which are flask-shaped invaginations involved in signal transduction and vesicular transport.[26] More precisely, caveolae are smooth, cholesterol-sensitive invaginations of the plasma membrane that constitute a stable, morphologically defined lipid raft domain.[27] Identification of caveolin-1 (Cav1), also known as VIP-21, as the major constituent of caveolae provided the first marker of these structures.[28,29] However, it was recently shown in PC3 cells lacking PTRF-Cavin that Cav1 expression does not induce caveolae formation.[30,31] The existence of PTRF-Cavin and potentially of other regulators indicates that Cav1 is not sufficient for caveolae formation, and also that Cav1 can be stably and functionally expressed outside caveolae. Our studies highlight a novel membrane domain, the "Cav1 scaffold", and explain how this non-caveolar Cav1 domain regulates receptor signaling and dynamics.[23]

Although the functions of Cav1 outside caveolae remain poorly understood, they are the subject of intensive study.[26,32] For example, Cav1 molecules have been detected in cells lacking caveolae and in non-caveolar regions of cells such as cytoplasm, focal adhesions, the Golgi apparatus, the extracellular milieu, and the nucleus, suggesting potential functions for Cav1 in a variety of cell signaling and mechanotransduction phenomena.[26,32,33] Recent work from our lab indicates that Cav1 negatively regulates EGFR signaling even when expressed at levels below the threshold for caveolae formation.[23] Caveolin-1 has been proposed to interact with integrins, most probably outside of caveolae.[34,35] Cav1 is also a substrate of Src kinase[36] and our studies show an association of Cav1 with cell transformation and tumor progression, although its exact role here remains unclear.[37] Downregulation of Cav1 results in decreases of focal adhesion number, cell polarization and directional cell movement.[38,39]

Phosphorylation of Cav1 on tyrosine 14 regulates domain recruitment of various signaling adapters, such as kinases and phosphatases, suggesting that Cav1 signaling activity might be equally as important as its structural role in caveolae formation. Indeed, Cav1, in particular its tyrosine phosphorylated form, regulates and stabilizes FAK exchange in focal adhesions, enabling FA-mediated signaling and cell migration in tumor cell lines.[8] Analysis of lipid order in focal adhesions showed that Cav1, and specifically tyrosine phosphorylatable Cav1, organizes ordered membrane domains within focal adhesions that are even more highly ordered than lipid rafts.[40] Interestingly, Cav1 mutants in which Y14 is replaced with either a negatively charged glutamate or a positively charged arginine both functioned as phosphomimetics, suggesting that conformational changes in Cav1 upon tyrosine phosphorylation enable the formation of ordered membrane domains within focal adhesions, impacting on their structure and dynamics.[8] Cav1 Y14 has also been shown to promote cell polarization and directional migration in mouse embryonic fibroblasts by enabling Src-mediated RhoGTPase activity.[39] More recently, we showed that tyrosine-phosphorylated Cav1 functions as an effector of Rho/ROCK signaling in the regulation of FA turnover, defining a feedback loop between Rho/ROCK, Src and phosphorylated Cav1 in tumor cell migration.[41]

Concerted Regulation of Focal Adhesion Dynamics by Tyrosine Phosphorylated Caveolin-1 and the Galectin Lattice

In 2006, we showed that binding of extracellular Gal-3 to Mgat5-modified N-glycans induced FN fibrillogenesis and cell motility via activation of α5β1-integrin.[24] Our more recent work shows that the Mgat5/galectin lattice regulates FA dynamics and that this activity is dependent on the expression of tyrosine-phosphorylated Cav1.[8] Using FRAP, we showed that Gal-3 and pY14Cav1 act in concert to increase the immobile fraction of focal adhesion components such as FAK, paxillin and α5-integrin in FA,[8] consistent with the formation of stable membrane domains within FA.[40] Gal-3-mediated integrin activation therefore acts through pY14Cav1 to induce a membrane organization within FA that restricts exchange of FAK and other FA components and enables FA turnover. In two additional studies, pY14Cav1 has been shown to recruit Csk, thereby inhibiting Src activity and leading to a p190RhoGAP-dependent increase in RhoGTP levels;[39] this regulates the localization of pY14Cav1 to cell protrusions as well as the dynamics of FAK at focal adhesion sites, and thus influences tumor cell migration.[41] Thus, pY14Cav1 sufficiently stabilizes FA components at FA sites to allow FA maturation, substrate adherence at cellular protrusions, and directional motility. Indeed, Cav1, but not Cav1Y14F, promotes both FA turnover and protrusive activity in MDA-435 tumor cells, implicating pY14Cav1 in tumor cell migration.[8]

The high signaling potential of FA has been confirmed by the use of the fluorescent probe Laurdan, which labels ordered membrane domains. Studies with this probe have demonstrated

that the FA membrane is more highly ordered than caveolae and other cholesterol-dependent raft domains.[40] Membrane order in FA is reduced in Cav1$^{-/-}$ MEFs and is more efficiently restored in cells transfected with wild-type Cav1 than in cells transfected with the non-phosphorylable mutant form. Our finding that pY14Cav1 reduces the availability of FA-localized FAK for dynamic exchange is consistent with its ability to promote membrane order within FA,[40] and suggests that regulation of membrane order within FA by pY14Cav1 is a determinant of the dynamics and stabilization of FA components.

A key discovery of our studies is that the effects of pY14Cav1 on FA dynamics require the expression of the Mgat5/Gal-3 lattice. Gal-3 forms pentamers, shows close molecular interaction on the cell surface,[42,43] and exhibits affinity for N-glycans in proportion to GlcNAc branching.[44] Recruitment of receptors to the galectin lattice regulates signaling.[17,22] Gal-3-mediated clustering of Mgat5-modified N-glycans stimulates tyrosine phosphorylation of FAK, favors FA organization and maturation (Fig. 1 and reviewed in ref. 8), slows α5-integrin exchange[8] and promotes the organization of integrins into focal and fibrillar adhesions.[24] Lateral association of integrins into clusters generates FA precursors and results from a combination of various extracellular and intracellular stimuli, including ligand binding, integrin activation, and actin polymerization.[45] Introduction of an N-glycosylation site at the interface of the I-like and hybrid domains of β3 integrin forces the integrin to adopt an extended conformation that results in integrin activation and clustering and slows integrin exchange.[11] Gal-3-dependent integrin activation is enhanced by addition of soluble RGD ligand, suggesting that, although it may not be sufficient, gal-3-integrin clustering promotes activation.[24] Consistent with a role for Gal-3 in integrin clustering, we found that Mgat5 rescue of Mgat5$^{-/-}$ ESC cells induces FA formation and cell spreading;[8] however, Mgat5 rescue did not induce FAK stabilization in FA, suggesting that the Mgat5/Gal-3 lattice is not the only actor in FA maturation. Indeed, clustering is not sufficient for integrin αIIbβ3-mediated signaling, which also requires integrin conformational changes that involve separation of integrin transmembrane domains.[46]

Regulation of FA dynamics by the galectin lattice requires the expression of pY14Cav1. Mgat5 rescue of Mgat5$^{-/-}$ ESC cells does not rescue Cav1 expression, FAK tyrosine phosphorylation, or the efficiency of FAK exchange in these cells. In contrast, rescue of Cav1 expression with wild-type Cav1, but not Cav1Y14F, enables proper FAK exchange at FA sites and subsequent FA disassembly.[8] Therefore, intracellular expression of pY14Cav1 and the extracellular presence of the Mgat5/Gal-3 lattice are both key determinants of proper FAK exchange at FA sites. Cav1 binds directly to integrins, recruits Src-kinase, regulates FAK phosphorylation, and couples integrin activation to Ras-ERK signaling.[35,47,48] Cav1 phosphorylation has also been shown to be a consequence of integrin-mediated mechanotransduction after shear stress is applied to endothelial cells,[49] and is transiently lost upon integrin dissociation from ligand.[50] Both Gal-3 and pY14Cav1 are therefore required, but not necessarily sufficient, for activation of Src kinases, which results in the stabilization of FA components and promotes FA signaling, disassembly and translocation.[8] The interdependence of FA turnover on both extracellular Gal-3 and intracellular pY14Cav1 is an elegant example of the outside-in signaling that regulates integrin activation and cell adhesion.[51]

Our data support a model in which Gal-3-bound Mgat5-modified integrins and Cav1 work in concert to generate intra-FA domains (Fig. 2). In the absence of the Mgat5-Gal-3 lattice, the high cytosolic concentration and exchange rate of FA components such as FAK keeps FA in an immature state. Expression of the Mgat5/Gal-3 lattice induces α5β1-integrin clustering and activation, enabling FA formation and cell spreading; however, this is not sufficient to stabilize FAK in FA domains, and therefore cannot produce mature FA. FA maturation also requires the co-expression of pY14Cav1, which stabilizes recruited constituents in FA. This favors FA turnover by enabling the formation of an organized and stabilized membrane domain within the FA[40] and by promoting RhoGTPase activity and subsequent FA maturation.[39,41] Together with the established interconnection between pY14Cav1 and integrins,[35,47,49,50,52] these results conclusively demonstrate a role for pY14Cav1 in FA domain organization and function. However, it is important to note that the evidence for stable association of pY14Cav1 within FA is based mainly on a single monoclonal antibody that crossreacts with phospho-paxillin.[30] While this does not exclude localization of pY14Cav1 to FA, it is important to consider that the contribution of pY14Cav1 to FA maturation and cell migration might be mediated by signals resulting from increased RhoGTPase activity. Stabilization of FA components by the concerted action of Mgat5/Gal-3 lattice and pY14Cav1 favors proper exchange of FA components at FA sites. Ultimately, pY14Cav1 promotes FA disassembly, which is intimately linked to the coordinated phases of cell retraction and protrusion that underlie directed cell migration.

Concluding Remarks and Unexplored Avenues

Our studies clearly demonstrate that coexpression of the Mgat5/galectin lattice and Cav1 in tumor cells results in their concerted action via pY14Cav1 to promote FA turnover and tumor cell migration. These observations are consistent with the poor prognosis associated with elevated Cav1 expression in a number of tumor types (reviewed in ref. 37) and with the increased expression of Mgat5-modified N-glycans in various carcinomas. Our results raise many questions and open up avenues of research that are likely to be very productive over the coming years. For example, it will be interesting to discover whether recruitment to the galectin lattice promotes interaction between growth factor receptors and integrins, which is known to regulate epithelial-mesenchymal transition, angiogenesis, survival and tumor cell invasion.[53] Another issue is whether expression of the Mgat5/galectin lattice increases plasma membrane order or increases RhoGTPase activity via integrin activation. Our studies and work by other groups suggest that the tyrosine 14 residue of Cav-1, through modulation of FA dynamics and tumor cell migration, might be a key factor in metastasis formation during cancer progression.[8,39,41,54] However, the study of metastasis behavior in vitro requires appropriate cell migration models that provide a good approximation to the in vivo situation. For example, the recent availability of engineered 3D microenvironments for modeling cancer progression has revealed that the engagement of both the ventral and the dorsal cell surfaces produces drastically different phenotypes compared with traditional 2D cultures.[55] Whereas cell-matrix adhesion structures and cell migration in 3D have already been extensively characterized, it is interesting that the dynamics of cell-matrix adhesions in 3D

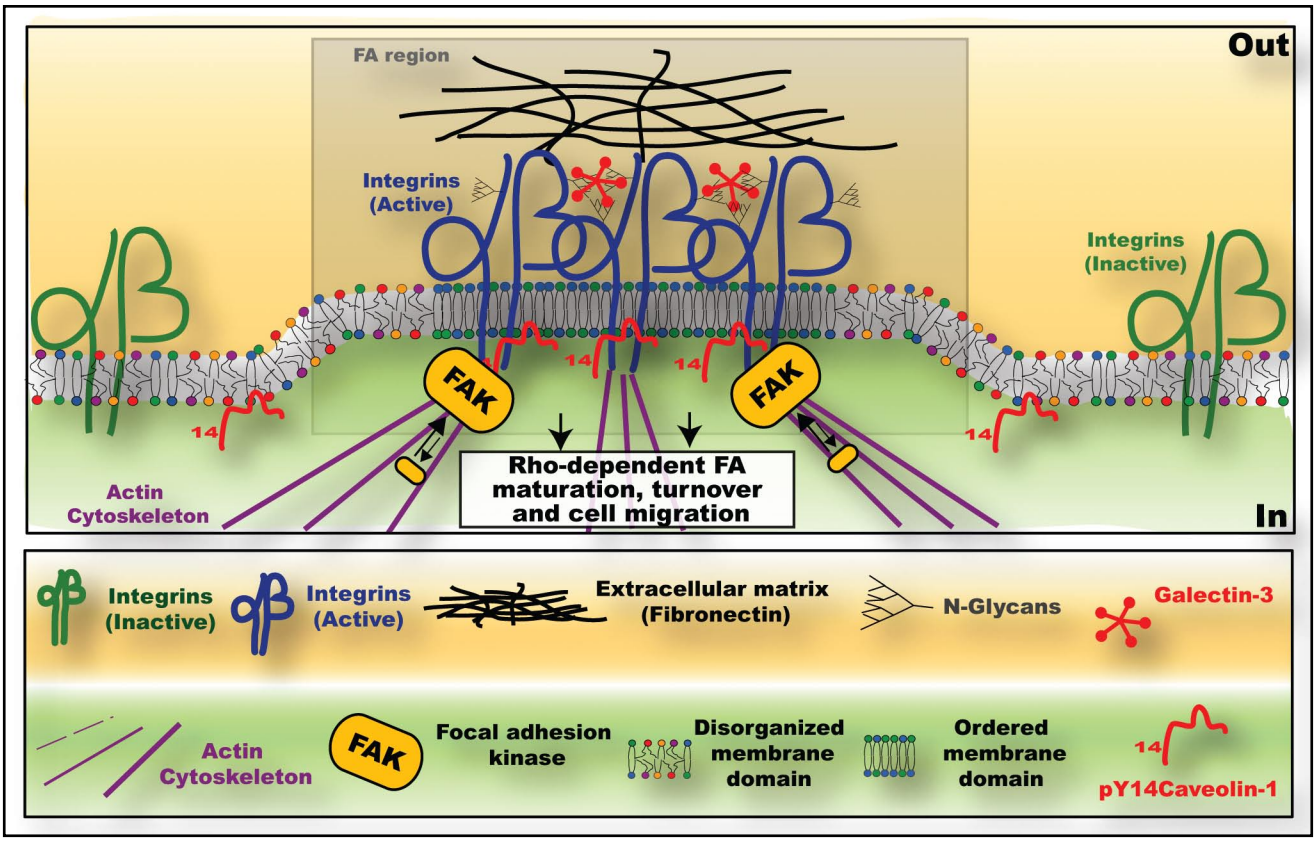

Figure 2. The Mgat5/Galectin-3 lattice and pY14Cav1 work in concert to stabilize FAK exchange at FA sites. Adapted from.[8] The Mgat5/galectin lattice mediates integrin clustering and activation and can induce the formation of FA and cell spreading, but does not favor FAK stabilization in FA domains. Expression of pY14Cav1 results in the formation of an ordered membrane domain and activation of RhoGTPase activity that results in the stabilization within the FA of integrin, FAK and paxillin as well as, potentially, other FA components. Stable association within FA of FAK, its Src-dependent phosphorylation and the associated recruitment of signaling partners within FA lead to disassembly and turnover of FA and directed cell migration.

have never been investigated. This is even more surprising since FA appear to be lost in a 3D environment and replaced by a so-called "cell-matrix adhesion."[56] Given the importance of FA dynamics in 2D cell migration and the relevance of cell-matrix adhesion in a tissue-like environment, tackling adhesion dynamics in 3D deserves intense and careful investigation.

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