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Integrin trafficking in cells and tissues

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Abstract

Cell adhesion to the extracellular matrix (ECM) is fundamental to metazoan multicellularity and is accomplished primarily through the integrin family of cell-surface receptors. Integrins are internalised and enter the endo/exocytic pathway before being recycled back to the plasma membrane. The trafficking of this extensive protein family is regulated in multiple context-dependent ways to modulate integrin function in the cell. Here we discuss recent advances in understanding the mechanisms and cellular roles of integrin endocytic trafficking.

Introduction

In mammals, stable non-covalent interaction between 18 α- and 8 β-subunits generates 24 functionally distinct integrin heterodimers, the majority of which contain the β1-subunit (Fig. 1a). Whereas some integrins, such as α5β1, interact with a limited number of ECM ligands, others, such as αvβ3 and α4β1, have multiple ECM binding partners. In addition, the same ECM ligand can be engaged by different integrins[1] (Fig. 1a) and activate alternative signalling pathways. Therefore, the biological response to environmental cues is strongly influenced by which integrins are expressed and active on the plasma membrane. The extensive overlap in the signalling pathways of the mammalian integrin family, complicate the interpretation of loss-of-function experiments. Invertebrate model organisms have greatly broadened our understanding of integrin-driven processes *in vivo* as these express a smaller repertoire of integrins and integrin ligands[2] (Fig. 1a). Integrin function is regulated through multiple mechanisms including conformational changes, protein-protein interactions and trafficking[3–7]. In most cell types integrin function depends on a delicate balance between active and inactive receptors on the cell surface[8,9]and spatiotemporal control of integrin activation is key for efficient adhesion formation and cell motility[10]. Integrins are activated through “inside-out” signals: an intracellular signal promoting the binding of proteins such as talin and kindlin to the β-integrin tail switching the receptor into an extended conformation with high affinity for ECM ligands[11]. Integrin binding to ECM ligands, in turn, triggers “outside-in” signals that recruit protein complexes (Fig. 1b),

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consisting of scaffolding/adaptor molecules, kinases and phosphatases, to regulate cell behaviour. The network of proteins recruited to integrin adhesion sites[12,13], varies in composition and size giving rise to multiple classes of integrin-ECM adhesions (Fig. 1c).

Integrin adhesions are highly dynamic and undergo constant cycles of assembly and disassembly. The lifetime and composition of focal adhesions (FA) (described in Fig. 1c) strongly influences ECM-driven signalling and therefore cell behaviour. Adhesion turnover is regulated, in part, by integrin endocytosis and exocytosis back to the cell surface, typically referred to as recycling[4,14,15] (Fig. 2a). Endocytosed integrins are also able to transmit intracytoplasmic “inside-in” signals either through recruitment of focal adhesion kinase (FAK), and possibly other effectors[16,17], or by enhancing the signalling of co-trafficking growth factor receptors[18–20] (Fig. 1b).

Knowledge of the underlying mechanisms and the molecular specificity of integrin trafficking pathways is therefore fundamental in understanding integrin function. In this review, we will discuss recent insights into how integrins traffic and how integrin-trafficking pathways regulate cellular processes including animal development.

Integrin endocytic trafficking pathways

Unlike many other cell-surface receptors that undergo synchronized ligand-induced internalisation and degradation, integrins are constantly trafficked in cells. The Rab family of small GTPases are implicated in each step of this process[4,14,21] (Fig. 2a). Integrins are initially endocytosed by different mechanisms (Fig. 2b) and routed to Rab5-positive early endosomes (EE) where sorting decisions are made. A large proportion of integrins are recycled back to the plasma membrane through two distinct pathways (reviewed in[4,14]). As a result, integrins are remarkably stable with a half-life of 12–24 hours[22–24]. The constant flux of integrins between the plasma membrane and intracellular pools allows cells to probe the environment for adhesive and migratory cues and to adapt their mechanical and morphological properties to mount appropriate responses.

Clathrin-mediated and -independent endocytosis of integrins

Clathrin-mediated endocytosis (CME) is the best-studied integrin internalisation route[14,25]. Over 50 proteins identified as key components of the CME machinery coordinate membrane invagination, formation of clathrin-coated pits, cargo recruitment and vesicle scission[26] (Fig. 2b). In polarized cells, ECM-proximal integrins at the cell front are endocytosed via interaction with the clathrin adaptor Numb, the recruitment of which is inhibited by phosphorylation mediated by apical polarity determinants Par3-aPKC[27]. In mature disassembling FAs, kinesin KIF15, a microtubule motor, promotes the recruitment of clathrin adaptor Dab2 to integrins to trigger CME and FAK- and dynamin-dependent vesicle scission[28–30]. Following FA disassembly, endocytosed, ligand-unbound integrins can be maintained in an active state by talin and FAK within endosomes. This integrin pool transits from Rab5-positive EE to Rab11 recycling endosomes (RE) and is recycled to the cell front for polarised adhesion assembly and directional fibroblast cell migration[17]. Rab5- and FAK-dependent integrin endocytosis is also associated with ultrasound therapy-induced cell

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migration[31], suggesting that this pathway may be a more general mechanism for regulating cell migration.

FAK also plays a role in clathrin-independent endocytosis (CIE) of integrins. Galectin-3, an extracellular carbohydrate binding lectin, induces the formation of clathrin-independent carriers and internalization of receptors including β1-integrins by recruiting intracellular GRAF1 in complex with phosphorylated FAK to the receptors[32,33]. Other routes of integrin CIE include internalisation from caveolae[34–37], rapid macropinocytosis of unengaged integrins from the dorsal cell surface following PDGF stimulation[38] and Rab21-dependent internalisation via a yet undefined mechanism[39]. Recently, accumulation of an LL5 (pleckstrin homology like domain, family B)–liprin-α1–ERC1 (ELKS/Rab6-interacting/CAST family member 1) ternary complex at the cell periphery was associated with FA turnover through vinculin and paxillin displacement and active integrin internalisation into endosomes[40]. Whereas ERC-1 was shown to partially colocalise with caveolin, a definitive role for caveolae was not investigated here[41].

These internalisation pathways underscore the importance of precise and spatially controlled integrin uptake. However, they fail to explain how cells choose a particular endocytic pathway and/or discriminate between different integrin heterodimers or integrin activation states.

Fine-tuning integrin endocytosis

The majority of integrin receptors share a common β1-subunit (Fig. 1a) with a conserved β-tail NPxY/NxxY motif that enables interaction with CME adaptors and endocytosis accessory proteins such as epidermal growth factor receptor substrate 8 (EPS8), Dab2 and Numb[42]. Hence, all β1-containing heterodimers can potentially be endocytosed via the same mechanism.

However, it is becoming apparent that different molecular pathways are employed to distinguish between integrin heterodimers, activation states, and trafficking routes to regulate specific biological processes (Table 1). For example, in endothelial cells, internalisation of active β1-integrin from nascent adhesions into Rab5 endosomes, requiring R-Ras and Rin2, leads to Rac activation, lamellipodia formation and directional cell motility[43]. Conversely, selective traffic of inactive β1-integrins, containing the α1, α2 and α3, but not αv or α5, chains, from the dorsal plasma membrane to the ventral side by a Dab2—EPS15 CME module may be important for maintaining the reservoir of intracellular integrins during HeLa cell migration[44,45]. How these two pro-migratory pathways are consolidated in cells remains unknown and, perhaps, each requires a distinct extracellular cue and/or is cell-type specific.

While many adaptors have been associated with β1-integrin endocytosis, a few have also been attributed to the trafficking of other β-subunits. In oral squamous carcinoma cells, the cytoskeletal regulator HAX-1 interacts with αvβ6-integrin and induces its internalization via clathrin to promote cancer cell migration and invasion[46]. Integrin-heterodimer-specific internalisation can also be regulated by integrin α-subunits that harbour a common endocytosis motif (Yxxϕ) recognised by clathrin adaptor protein 2 (AP2)[47]. This conserved

motif permits selective CME of integrins, containing the motif, over other receptors that lack it, but bind to the same ECM ligand. This could allow heterodimer-specific downstream signalling to initiate divergent responses to an ECM ligand.

Whereas the role for clathrin and/or its adaptors in integrin endocytosis is undisputed, there is some debate as to the precise conditions required for CME of integrins. A recent study highlighted a low-force microenvironment as a prerequisite for Dab2 recruitment to active β3 integrins[48]. Whether other clathrin adaptors are influenced by force is not known. Regardless, the cell clearly employs different mechanisms for selective CME of integrin heterodimers which may depend on extracellular cues, including ECM topology.

Integrin interaction with other cell-surface receptors, such as MET, Syndecan-4 and ADAM9, also influences endosomal traffic and cell adhesion and migration *in vitro and in vivo*[18,19,34,49–51] (Fig. 2c). In addition, PKCα activation, in response to an extracellular cue, leads to phosphorylation of FMNL2, an actin nucleator/assembly factor and FMNL2-dependent integrin endocytosis (Fig. 2c). Further, N-glycosylation of the α5-integrin β-propeller, favours integrin association with Syndecan-4 and subsequent receptor internalization[52]. Thus, interactions and signals at the cell surface can be vital for driving integrin endocytosis.

Rab GTPases also provide selectivity for integrin traffic. Rab21, Rab25 and Rab34 directly interact with integrins. The ubiquitously expressed Rab21 binds to α-subunit tails to induce integrin endocytosis, regulate FA turnover[39] and deliver active β1-integrin to FAK-positive signalling endosomes for suppression of anoikis and survival of disseminated cancer cells[16]. In contrast, epithelial-cell-specific Rab25 interacts with the β-subunit tail to recycle endocytosed α5β1-integrin locally within invasive protrusions in three-dimensional ECM[53]. In metastatic ovarian carcinoma cells, Rab25-dependent sorting of α5β1-integrin, endocytosed from the cell front, to lysosomes and exocytosis at the cell rear facilitates rear detachment by activating Src-mediated FA disassembly[24]. Rab34, a Golgi-associated protein, is upregulated in aggressive breast cancers and directly binds the cytoplasmic tail of β3-integrin. EGF stimulation recruits Rab34 to the plasma membrane and promotes Src-mediated tyrosine-phosphorylation of Rab34, which induces β3-integrin internalization and delivery to recycling compartments[54]. This protects β3-integrin from degradation and stimulates cancer cell migration and invasion[54]. In contrast, Rab35 appears to limit α5β1-integrin traffic as its inactivation by CLIC4 enhances α5β1-integrin endocytosis and Arf6-mediated recycling[55]. Concordantly, low Rab35 or elevated Arf6 expression enhances cell migration in some cancers[56], placing Rab35 as a potential obstacle in cancer progression.

Receptor internalisation and subsequent transit to the Golgi, a process known as retrograde traffic, also appears to impart selectivity to integrin-dependent processes, depending on the accessory proteins involved. For example, a Golgi-SNARE complex, consisting of syntaxin-6 and VAMP3, promotes integrin-heterodimer-specific recycling in different cell types. In HeLa and prostate cancer cells, this complex drives α3β1-integrin-mediated chemotactic cell migration[57], whereas in endothelial cells, syntaxin-6 is important for α5β1-integrin-mediated cell spreading and motility on fibronectin[58]. Interestingly, another SNARE complex consisting of syntaxin-16 is specific for the retrograde transport of inactive β1-

integrins[59]. Thus, retrograde transport may be a broad mechanism for the trafficking of β1-integrin-containing heterodimers, and the ECM landscape and the composition of the SNARE complex determine the heterodimer and/or the specific receptor activation state that is a cargo for retrograde transport.

Sorting of integrins between late and recycling endosomes

Endocytic cargo can be recycled or shuttled through late endosomes to lysosomes for degradation. Integrins, however have the potential to bypass this protein downregulation machinery, which explains their generally low degradation rate mentioned above. An important regulator of endocytosed β1-integrin fate is sorting nexin 17 (SNX17), which binds to the membrane distal β-integrin NxxY motif, and in cooperation with the retriever and WASH (WASP and SCAR homologue) complexes diverts the receptor from degradation to recycling[60–62] (Fig. 2d). This process depends on proper endosomal localization of SNX17[63] and is fundamental for efficient cell adhesion and migration[63].

Alternative integrin recycling routes are regulated by specific endocytic adaptor proteins such as APPL1, GGA2 and the CORVET complex[64–67] (Fig. 2d). APPL1 inhibits internalization of α5β1-integrin and simultaneously promotes its recycling back to adhesion sites, resulting in increased cell-surface α5β1 and reduced adhesion dynamics[64]. However, the role of APPL1 may be dependent on cell type as in lung cancer cells, APPL1 depletion has no effect on internalization or endosomal signalling of integrins[16]. Another possible link between APPL1 and integrin traffic is the CORVET complex, which mediates fusion of APPL1-containing EE[66]. Furthermore, CORVET subunits Vps3 and Vps8 localise to Rab4- and Rab11-positive RE in HeLa cells and participate in integrin recycling. Correspondingly, Vps3/Vps8 knockdown inhibits cell adhesion, spreading and migration on fibronectin and collagen[67] (Fig. 2d).

Similarly to internalisation, intracellular sorting of integrins is fine-tuned by other receptors and ECM ligands and this process impinges on efficient cell migration[14,20]. For example, the tyrosine phosphorylation status of Syndecan-4 acts as a switch controlling differential recycling of αvβ3- and α5β1-integrins, and the tightly balanced availability of these two receptors at the plasma membrane determines FA half-life and cell migration behaviour[50]. Association of inactive β1-integrins with ADAM9 metalloproteinase is required for optimal β1-integrin internalization, degradation and cell migration[49]. In fibroblasts, interaction between fibronectin and its receptor α5β1-integrin, promotes α5β1-integrin ubiquitination and routing towards lysosomal trafficking and degradation. This is suggested to limit intracellular accumulation of integrins destined for re-adhesion and thus allow more productive cell migration[22]. However, in cancer cells, it is the diversion of the α5β1-integrin receptor away from degradation towards recycling, in a process requiring WASH (Arp2/3 activator), that drives invasive cell migration[68]. Therefore, while it is clear that other cell-surface receptors and ECM cues actively participate in the regulation of integrin fate, the choice between integrin recycling and degradation, particularly during cell migration, appears to be context-dependent.

Altogether, these examples illustrate the complexity of integrin traffic and highlight the need to tightly control these events to modulate integrin-mediated functions in the organism.

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Integrin traffic in developing tissues

Expanding evidence links integrin trafficking to the normal execution of cellular processes such as cell adhesion[4,15], migration[14,20,69], matrix remodelling[70–72] and differentiation[73,74]. The concept that these pathways can be hijacked by cancer cells has been reviewed elsewhere[4,75–77]. Below we highlight the role of integrin trafficking pathways in regulating cellular processes fundamental for metazoan development and tissue homeostasis[2,78].

Integrin trafficking determines cell polarity and drives ECM remodelling

In polarised cell types, such as epithelial and endothelial cells, integrins control the orientation of the apico-basal axis by providing physical and mechanical cues (ECM engagement) and spatial positioning, and regulate molecular delivery to correct subcellular locations[79–81]. A striking example, is the peri-implantation stage of mouse embryos (E5.0), where the epiblast undergoes apico-basal polarisation and changes in cell morphology leading to formation of an epithelial rosette structure with a central cavity[82,83]. Interestingly, this polarization can be also recapitulated in stem cell colonies grown in Matrigel[82]. This process depends on ECM secreted by extraembryonic cell lineages and on selective, Rab6- and syntaxin-16-dependent, inactive β1-integrin retrograde traffic[59] (Fig. 3a). In fish retinal neuroepithelium, regulated integrin-ECM interaction at the basal side of cells is required for bending this flat cell layer into a hemispherical optic cup. Here, the amount of integrin at the basal endfeet is regulated by direct competition between β1-integrin and a membrane protein Opo for binding to CME adaptors[84]. Loss of Opo triggers excess integrin endocytosis and failure in optic cup bending[85] (Fig. 3b).

In endothelial cells, the removal of “old” fibronectin and the basolateral secretion of new fibronectin molecules are coupled to the cycle of Rab21-dependent α5β1-integrin recycling[70]. Fibronectin turnover is critical for fibronectin fibrillogenesis and vascular development[71,86]. Non-canonical interactions between angiopoietin-2 (Ang-2), a regulator of vascular homeostasis, and α5β1-integrin, leading to integrin activation, impacts fibronectin fibrillogenesis, in addition to destabilizing endothelial cell-cell junctions[87]. Ang-2 induces α5β1-integrin transition from peripheral FA to stable fibrillar adhesions (primary sites of fibronectin fibrillogenesis, Fig. 1b), positive for tensin1. In this study, the role of integrin traffic was not directly addressed; however, increased α5β1-integrin recycling into tensin1-positive adhesions has been linked to endothelial destabilization in sepsis[88]. Thus, dynamic intracellular movement of integrins and generation of fibrillar adhesions are important for both vascular development and maintenance of vascular homeostasis.

Links between metabolism and integrin trafficking

A cell’s metabolic state is intimately associated with integrin traffic and matrix remodelling[72]. In high-nutrient conditions, tensin expression is suppressed by the cellular metabolic sensor AMPK[89]. In contrast, nutrient depletion induces α5β1-integrin translocation to tensin-dependent fibrillar adhesions, from which ligand- and tensin-bound integrins and ECM components are endocytosed to lysosomes to provide cell nutrients[90]. In cancer-associated fibroblasts (CAFs), interactions between Src-phosphorylated Hic-5 (also

known as TGB1I1), β1-integrin and tensin1 stabilise β1-integrin on the cell surface, preventing receptor internalisation to lysosomes[91]. In starved human mammary epithelial cells, enhanced β4-integrin expression and endocytosis expedite laminin uptake and degradation in LAMP1-positive vesicles to generate the nutrients required for mammalian target of rapamycin complex 1 (mTORC1) activation and cell survival[92]. Notably, in the mammary glands of dietary restricted mice, resident mammary fibroblasts are the primary source of laminin production, and fibroblast-conditioned medium supports survival of starved mammary epithelial cells[92]. An intriguing question is how metabolic stress creates both matrix-devouring (epithelial cells) and matrix-producing (fibroblasts) entities within the same tissue[93].

Integrin traffic regulates cell adhesion in development

Drosophila models have provided important insights into the developmental roles of integrin traffic *in vivo*. Surprisingly, in apparently very stable structures, like the myotendinous junctions (MTJs) (Fig. 3c), integrins are dynamically turned over by clathrin- and Rab5-mediated endocytosis[94]. Although the dynamics of this process somewhat decrease as development proceeds, it is comparable to the reported values for integrin turnover in fibrillar adhesions *in vitro*[94]. Integrin dynamics in MTJs depends on muscle tension and on integrin activation state. Increased tension reduces integrin endocytosis, whereas loss in tension promotes both endocytosis and exocytosis[95]. In addition, whereas integrin inactivating mutations or reduced ECM levels trigger increased integrin turnover, integrin activation decreases integrin turnover[96]. It remains unknown whether tension increases integrin activity and whether muscle tension and integrin activation converge on the same pathway to regulate integrin dynamics.

Similar to other trafficking events[97], integrin recycling in MTJs depends on phosphoinositide turnover in vesicle membranes. In *Drosophila*, loss of a lipid phosphatase Myotubularin, which cleaves the EE lipid PI(3)P, causes the accumulation of the β1-integrin ortholog, βPS integrin, (Fig. 1a) inside cells in large abnormal PI(3)P-positive endosomal compartments and leads to myofiber detachment from the cuticle[98]. Symptoms of Myotubularin deficiency can be alleviated by simultaneous depletion of the class II PI3-kinase Pi3K68D[98]. These observations can be explained by the need for PI(3)P cleavage before an endosomal cargo can undergo exocyst-mediated exocytosis[99] (Fig. 3c). Myotubularin loss in *Drosophil*a manifests only in late pupal stages and resembles the condition of Myotubularin mutation in humans, X-linked myotubular myopathy, where β1-integrin is mislocalized to the perinuclear compartment[98]. Myotubularin loss also causes wing blistering, in line with the requirement for integrin–ECM adhesion and integrin trafficking for *Drosophila* wing morphogenesis (Fig. 3d)[100,101]. In summary, integrin turnover occurs even in stable adhesive structures and allows for flexible response to changing conditions such as fluctuating muscle tension. The dynamic nature of adhesions is not surprising given that based on single molecule tracking, integrin-ligand binding events do not exceed ~80 s in human epithelial cells *in vitro*[102]. This flexible system might constitute a conserved homeostatic mechanism, and the routes taken by integrins at different developmental stages might be tightly regulated to exert specific functions as required.

Integrin trafficking impinges on cell migration in development

During Metazoan development cells actively migrate either individually or collectively. Integrin recycling is necessary for the migration of many cell types including distal tip cells (Fig. 3a), cranial neural crest cells (NCC) (Fig. 3e), neuronal growth cones (Fig. 3f), and immune cells (the latter reviewed in[14,20,103]). In NCC, integrin receptors for fibronectin and laminin are recycled through Rab4 and Rab11 pathways (Fig. 3e) and inhibition of integrin recycling impedes NCC migration *in vitro*[104]. Integrin trafficking is crucial also for collective cell migration or morphogenetic movements of cell sheets. During Xenopus gastrulation, the protein XGIPC1 (also known as kermit2) promotes Rab21-mediated α5β1-integrin endocytosis and fibronectin ECM assembly by directly binding to the cytoplasmic domain of α5-integrin[105]. Loss of XGIPC1 function disrupts fibronectin ECM in embryos and causes defects indicative of slower mesoderm cell migration[105].

Whereas integrins are required for normal morphogenesis of the central nervous system[106,107], integrin trafficking is dispensable for the glial-guided radial migration of neurons, a dominant migratory mode in the cerebral cortex and cerebellum. Instead, the tissue-layering defects caused by integrin perturbation, reflect the requirement for integrin-mediated adhesion and signalling earlier in development and for radial glia attachment to the ECM[108,109]. However, active integrin recycling plays a major role later in neuronal development[110]. Axonal growth cones use the Rab-coupling protein(RCP)-dependent integrin recycling pathway, also employed by invasive cancer cells for effective migration[14,111]. In NGF-differentiated PC12 cells and in dorsal root ganglion neurons, α9β1-integrin is trafficked through RCP, Rab11 and Arf6 RE (Fig. 3f)[112,113] and perturbing this pathway reduces integrin cell-surface levels, resulting in inhibited axonal growth[113]. The use of particular recycling pathways seems to be neuron type-specific. Retinal ganglion cell axonal growth cones in *Xenopus* embryos require Rab4 and Rab5, but not Rab11, presumably for local integrin recycling within the growth cone to support migration[114]. Development of the axon initial segment in mature neurons precludes integrins and Rabs from entering the axon and axon regeneration[115,116]. Integrin traffic also influences dendrite morphogenesis. In hippocampal neurons, Ndr2 kinase phosphorylates the β1-integrin cytoplasmic domain and stimulates Rab5 and Rab11-dependent trafficking to increase active integrin at the cell surface, triggering premature dendritic branching[117]. Thus, whereas adhesion and ligand availability are important regulators of cell morphology and positioning within a developing tissue, integrin trafficking coordinates an integral mechanism for delivering integrins and effectors to drive the formation of highly organised tissues and may be an important factor in the regulation of stem cell niches.

Future Perspectives

Our knowledge of integrin trafficking pathways is rapidly expanding. However, how cells choose a specific internalization route or routes for different integrins is unclear. An important aspect is the composition of the plasma membrane surrounding integrins and the impact of the physical properties derived from lipid composition, membrane tension and curvature. The formation of nanoclusters of active and inactive integrins in the same FA and the different kinetics at which specific heterodimers are internalized may also be key in

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understanding pathway specificity[118,119], but more work is needed to clarify these aspects of integrin behaviour. Whereas the precise spatial details of integrin redelivery to the cell surface remain to be elucidated, exocytosis of integrins [23] and other cargo seems to preferentially occur at focal adhesions[120]. Whether these hotspots of integrin exocytosis depend on integrin activation state, cellular signalling or ECM substrate are the next exciting questions. Furthermore, understanding why certain Rabs mediate the transport of specific integrins requires further investigation. Many of these questions may be answered by new imaging probes for integrins and super-resolution microscopy within more relevant *in vivo* models (BOX 1). Furthermore, integrin reconstitution on artificial liposomes[121,122] may help in understanding the role of membrane composition on the recruitment of specific endocytic adaptors and to identify core complexes needed to direct heterodimer-specific traffic. Understanding integrin endocytosis is also of clinical importance. This route is being exploited as a mechanism for cytotoxic drug uptake in the development of anticancer therapies[123]. Finally, it is critical to not only understand how integrin traffic occurs at the cellular level but also how this complex process gives rise to distinct biological functions in tissues.

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Box 1

New tools to study integrin trafficking

Lattice light sheet microscopy (LLSM)

Integrin trafficking is a dynamic process and therefore best studied by live fluorescence microscopy techniques. Fast moving endocytic vesicles need to be captured at high spatio-temporal resolution while avoiding illumination-induced phototoxicity on the living sample[135,136]. LLSM fulfils these criteria as the new iteration of the method uses adaptive optics to correct for sample-induced image distortions and allows capture of intracellular events in a cell within intact tissues[137,138]. Measurements of the lifetime of endocytic events and vesicle trajectories within human stem cell-derived organoids and zebrafish embryos have demonstrated the power of this technique[137]. LLSM also enables imaging of fluorescently tagged endogenous proteins without the need for overexpression.

3D models

Studying integrins in physiologically relevant 3D model systems may reveal biological events not observed on stiff 2D substrates. For example, growing cells in soft 3D collagen matrices has unravelled clathrin tubular lattices as mediators of integrin-based cell adhesion during cell migration[139].

Integrin tags

Ectodomain labelling of β1, β3 and αL-integrins, which preserves receptor functionality has been achieved[23,102,140]. Besides creating fusions with fluorescent proteins, ectodomain tagging also enables other applications. For example, the cell-surface population of integrin β1 has been selectively tagged by a cell non-permeable Halo tag ligand, which allowed the kinetics of β1 endocytosis to be measured[23]. Additionally, pH probes based on red fluorescent SNAP tag ligands[141] could be used to capture endo and exocytosis, taking advantage of endosome acidification in comparison to the extracellular space.

Synchronised endocytosis

Unlike other membrane receptors, integrin endocytosis cannot be easily synchronized by addition of ligand, because ECM ligands are constantly present in cell culture conditions. Synchronized CME of integrins could be achieved by adapting the inducible system of recruiting clathrin-binding protein fragments to specific membrane proteins[142], for instance to allow local optogenetic activation of integrin endocytosis with subcellular precision to steer cellular behaviour.

In summary, by combining these methods it is possible to capture integrin behaviour at the cytoplasmic membrane and in endosomes with improved spatiotemporal precision and to probe them in diverse experimental contexts.

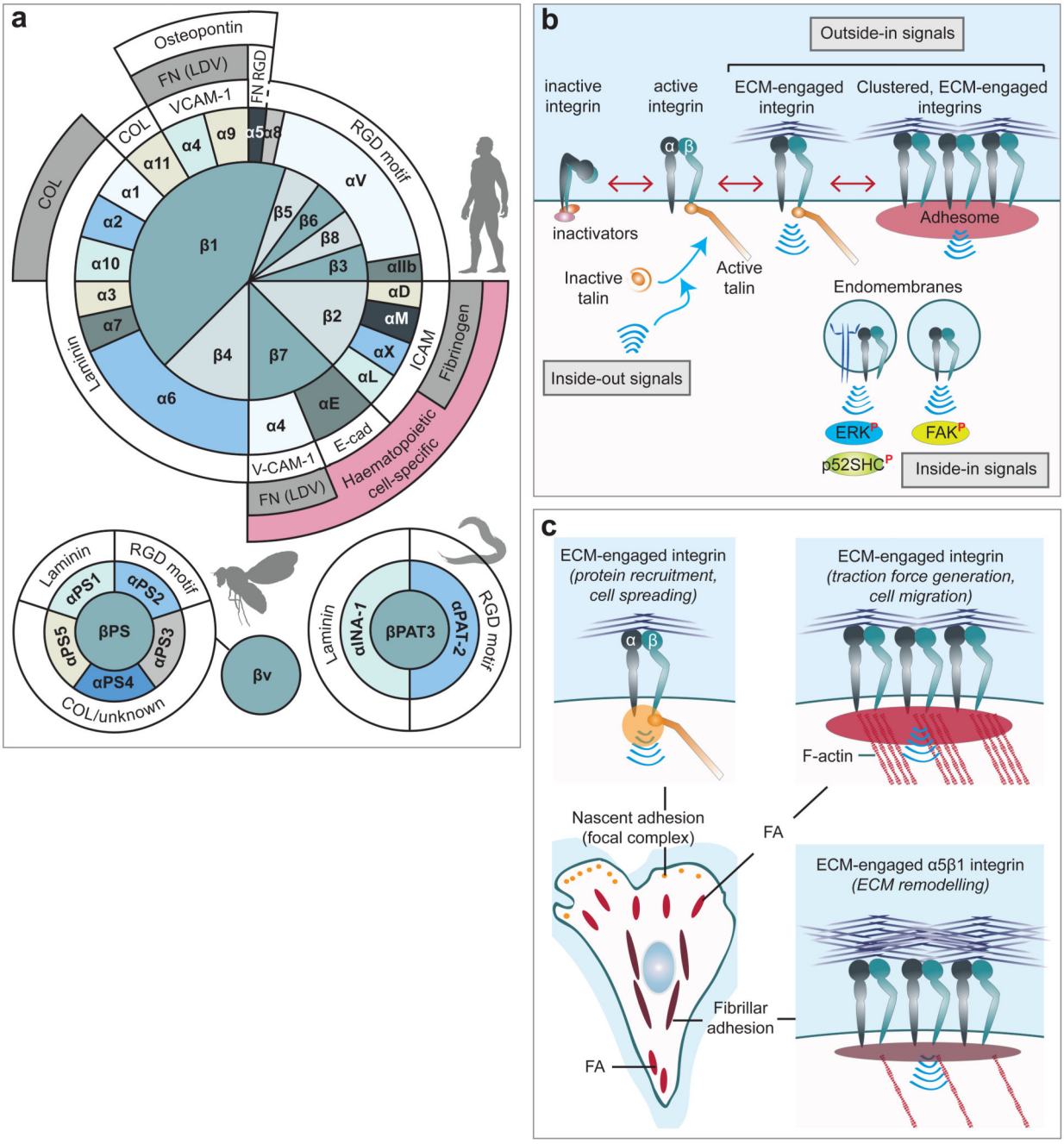

Figure 1. Composition and function of the integrin family.

a, The reported pairing between integrin α and β subunits and the ECM ligand/s for each heterodimer are illustrated for mammals, *Drosophila* and *C. elegans*. In mammals, α5β1-integrin binds to the fibronectin RGD motif, whereas α4β1 binds to the fibronectin LDV motif. COL: collagen; E-cad: E-cadherin; FN: fibronectin; ICAM: intercellular cell adhesion molecule; VCAM-1: vascular cell adhesion molecule 1. For a more comprehensive/exhaustive list of integrin ligands, please refer to[1].

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b, Integrins are bidirectional signalling molecules. Inside-out signals regulate talin binding to integrin β-tails and thus tightly control integrin affinity for ECM ligands. Subsequent ECM binding triggers recruitment of protein complexes (scaffolding and adaptor proteins, kinases and phosphatases, etc.) to the integrin cytoplasmic tails to promote integrin downstream signalling (outside-in signalling). Integrins can also signal from within endosomes (inside-in signalling) to support FAK activity and suppress anoikis[16] or to promote signalling downstream of co-trafficking MET to support anoikis resistance, tumour growth and cancer cell dissemination to lungs[19]. The superscripted P in red indicates phosphorylation. ERK, extracellular signal-regulated kinase; p52SHC, p52 isoform of SHC-transforming protein 1.

c, Integrin-ECM adhesions, *in vitro*, are defined based on localisation, components and maturation stage. Nascent adhesions (focal complexes) represent initial integrin receptor clustering in response to ECM engagement and recruitment of adaptor and signalling proteins to the integrin tails. These small protein assemblies mature into focal adhesions (FA), which serve to anchor actin stress fibres and are vital for generation of contractile force. Fibrillar adhesions are mature α5β1-integrin adhesions, and prominent sites of fibronectin fibrillogenesis, that result from the centripetal translocation of this specific integrin heterodimer towards the cell body.

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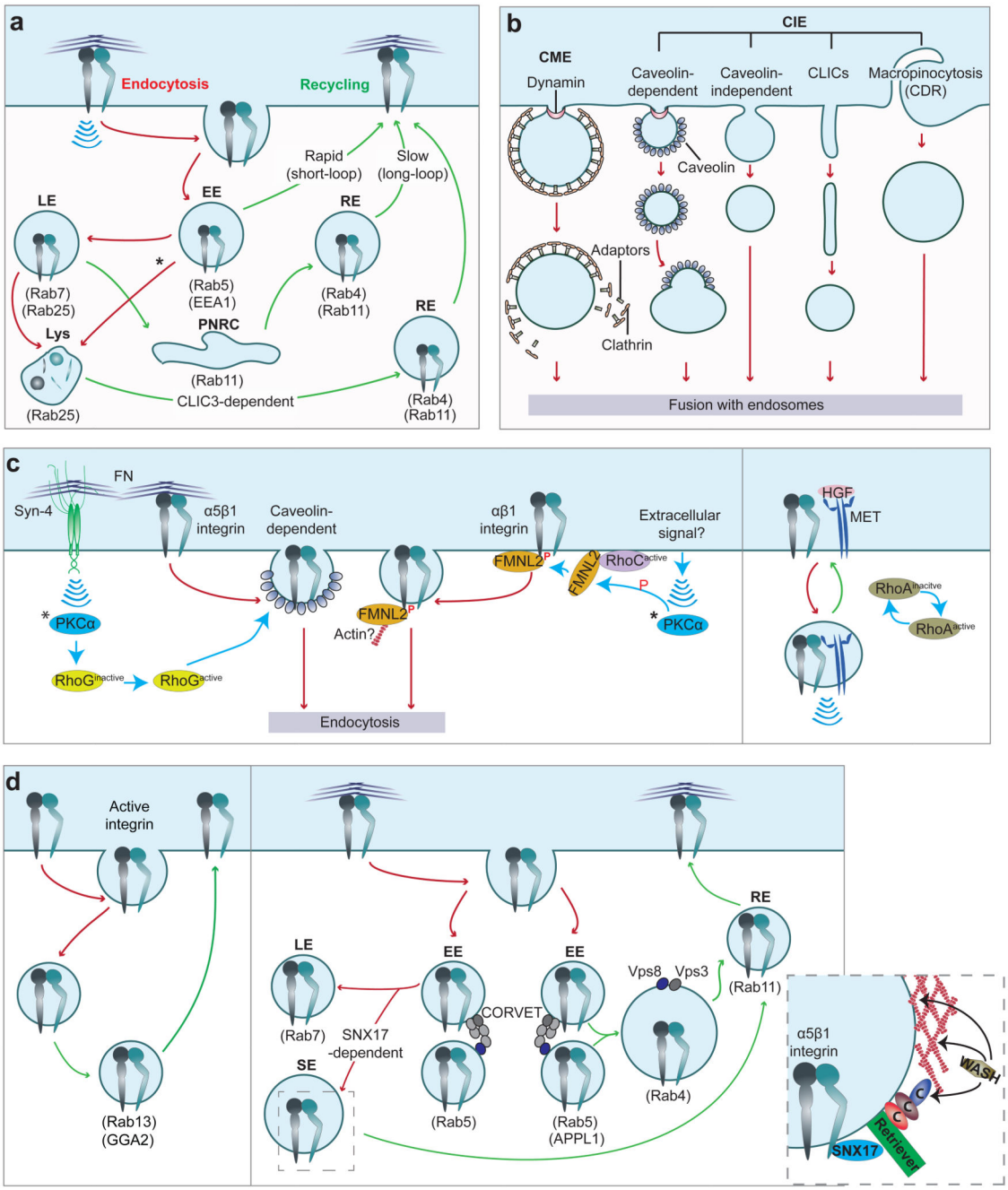

Figure 2. Fine-tuning integrin endocytosis and recycling.

a, Summary of integrin trafficking pathways. Integrins are internalised into Rab5-positive early endosomes (EE). EE mature into late endosomes (LE), which fuse with lysosomes (Lys) for integrin degradation. Under certain conditions (Rab25 and chloride intracellular channel protein 3 (CLIC3) expression), integrins exit LE/Lys compartments and are recycled back to the plasma membrane[24]. Integrins also traffic to multivesicular bodies (highlighted by *) and to the perinuclear recycling compartment (PNRC). This process occurs within 20 minutes, while degradation takes several hours[133] resulting in the majority of integrins being

recycled back to the cell surface. Each step requires a spatiotemporal hierarchy of interactions between integrins, endocytic adaptors, Ras and Arf GTPase family members and other molecules. The Rab GTPases involved are indicated.

b, Integrin receptor internalisation mechanisms, broadly classified as clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE, including caveolin-dependent pathways, micropinocytosis and clathrin-independent carriers (CLICs)). CDR, circular dorsal ruffles.

c, Integrin endocytosis can be fine-tuned by extracellular-initiated signals. Left-hand panel: Syndecan-4(Syn-4)—fibronectin interaction activates PKCα and RhoG and promotes caveolin-dependent α5β1-integrin endocytosis, attenuated adhesion and increased cell migration[34]. PKCα also phosphorylate FMNL2 (recruited by RhoC). Phospho-FMNL2 (superscripted P in red indicates phosphorylation) interaction with the α-integrin GFFKR motif drives αβ1-integrin internalisation[124]. The role of actin nucleation and the extracellular signal regulating this pathway remain undefined. *PKCα activation occurs at the plasma membrane. Right-hand panel: MET receptor—β1-integrin interaction induces integrin endocytosis, collective mesenchymal cell migration, in a HIP1- (clathrin adaptor) and RhoA-dependent manner[51], and “inside-in” signalling[19] (see Fig. 1b).

d, Recruitment of specific endosomal adaptors imposes selectivity to integrin recycling pathways. Left-hand panel: GGA2, an Arf effector has been implicated in Rab13-mediated recycling of β1-integrin[65] (a non-peer-reviewed study). Right-hand panel: Different populations of Rab5-positive EE, defined by the presence of Rab5 effectors EEA1 (not shown), APPL1 and the CORVET complex promote different recycling routes[64,66,67]. CORVET mediates fission and fusion of EE that mature into LE. Here, Rab5 is replaced by Rab7 and integrins enter LE. Integrins escape degradation by interaction with SNX17 (components in inset)[60–62]. CORVET components Vps3/Vps8 localise to Rab4-positive endosomes (fusion of APPL1-positive EE). Vps3/Vps8 vesicles deliver integrins to the plasma membrane through Rab11-positive recycling endosomes. SE, sorting endosomes.

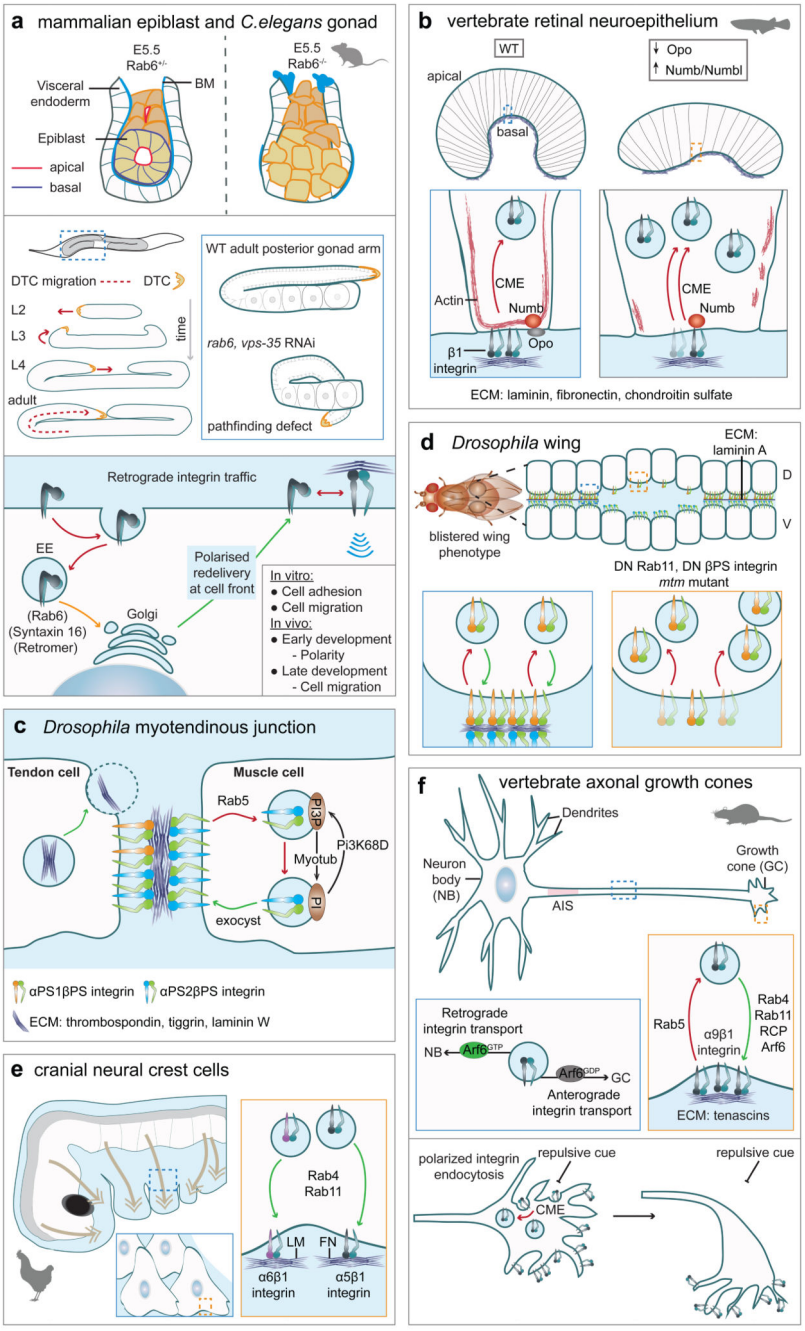

Figure 3. Integrin trafficking in development.

a, Retrograde transport of inactive β1-integrin (bottom) is required for polarized cell behaviours during development. Epiblast cell rosette structure fails to form in Rab6 mutant embryos[59] (top). Integrin retrograde traffic is also required for persistent migration of a distal tip cell (DTC) in *C. elegans* gonad (middle). Knockdown of Rab6 or the retromer component Vps35 causes a DTC pathfinding error and morphogenetic defects[59].

b, Integrin CME during optic cup formation is facilitated by binding of Numb and Numb-like to the β1-integrin NPxY motif and is inhibited by competitive binding of these adaptors

to the NPxF motif of the membrane protein Ojoplano (Opo). Loss of *Opo* or overexpression of adaptors result in excess integrin endocytosis, decreased cortical actin in basal endfeet, failure in basal constriction and a flat retina[84,85].

c, In MTJ, the muscle cell (αPS2βPS-integrins) is attached to the tendon cell (αPS1βPS- and αPS2βPS-integrins) indirectly through ECM. Integrin turnover in MTJ is increased by reduced availability of ECM and decreased by raised muscle tension, elevated integrin outside-in activation, or expression of Rab5 mutants[95,96]. Cleavage of PI(3)P by myotubularin (MTM1) prevents receptor accumulation in endosomal-related inclusions[98].

d, Cellular layers forming *Drosophila* wing are held together by adhesion of αPS1βPS-integrin (dorsal, D) and αPS2βPS-integrin (ventral layer, V) to the ECM secreted in between. In wing imaginal disc, βPS-integrin trafficking is mediated by Rab11[101], which when disrupted leads to increased apical cell area, intracellular βPS-integrin accumulation and disorganised actin cytoskeleton. The ensuing change in cell shape from columnar to cuboidal (not shown) leads to separation of cell layers and blisters. DN, dominant negative; mtm mutant, myotubularin mutant.

e, Recycling of α5β1- and α6β1-integrins is required for migration of cranial NCC[104]. Integrins are recycled through Rab4 and Rab11 pathways in a laminin substrate-dependent manner.

f, α9β1-integrin recycling within the growth cone and long-range axonal integrin traffic are required for efficient axon growth. Rab5-regulated endocytosis is followed by recycling through Rab4, Rab11 or Arf6 pathways. Activation of Arf6 promotes retrograde transport of integrin-containing vesicles towards the neuron body[112]. Chemorepellent cues trigger β1-integrin endocytosis on one side of the growth cone, which changes direction of growth cone migration[134].

Table 1

Recent examples of molecular pathways regulating specific integrin trafficking routes and/or biological processes.

Recent players in integrin trafficking from the last three years are highlighted in the table. FIP5, family-interacting protein 5; LRP1, prolow-density lipoprotein receptor-related protein 1; RN-tre, related to the N terminus of tre oncogene.; PAK, p21-activated kinase 1; ANKFY1, ankyrin repeat and FYVE domain containing 1; HUVEC, human umbilical vein endothelial cell; RPE-1, retinal pigmented epithelial cell.

Molecule/modification	Mechanism and/or phenotype	Model system	Reference
Plasma membrane / endocytic pathways			
ADAM9	Binds to inactive β1-integrins and induces their endocytosis and intracellular degradation. This allows adhesion turnover and cancer cell migration.	Prostate cancer and fibrosarcoma cells	[49]
FMNL2	Upon phosphorylation by PKCα, FMNL2 mediates actin assembly-dependent endocytosis of αβ1-integrins. Phospho-FMNL2 promotes invasion of melanoma cells.	Melanoma and HeLa cells	[124]
Hic-5	HIc-5 interaction with tensin1 prevents β1-integrin internalization and lysosomal degradation and instead promotes fibrillar adhesion formation.	Fibroblasts	[91]
Liprin-α1, LL5 & ERC1	Complex aids focal adhesion turnover by displacing adhesion components and inducing internalization of active β1-integrins into Rab7 endosomes. Drives protrusion formation at the leading edge during migration and invasion of breast cancer cells.	Breast cancer cells and fibroblasts	[40, 41]
N-glycosylation of α5-integrin	Glycosylation of the α5-integrin β-propeller (extracellular domain) promotes interactions with Syndecan-4, endocytosis of active α5β1-integrin and cell migration in cancer cells. No effect on cell spreading.	HeLa, breast cancer and glioblastoma cells	[52]
RN-TRE	RN-TRE, a Rab5 GTPase inactivating protein (GAP), is recruited by EPS8 to β1-integrin and inhibits receptor endocytosis	Fibroblasts	[125]
Tensin1-3	Tensins bring ligand-occupied integrins from peripheral adhesions to subnuclear adhesions; promoting their endocytosis via Arf4 and subsequent degradation in lysosomes.	Ovarian cancer cells	[90]
Recycling pathways			
Ankyrin B	Recruits RabGAP1L to PI3P positive endosomes to inactivate Rab22a. Aids integrin delivery to the cell front of fibroblasts and promotes polarized migration via α5β1-integrin.	Fibroblasts	[126]
APPL1	Decreases internalization and promotes recycling of α5β1-integrin in a Rab5-dependent manner. Inhibits cell migration on fibronectin, but not collagen by decreasing Rac1 and PAK activity at the leading edge.	Fibrosarcoma and breast cancer cells	[64]
Cullin-3 & ANKFY1	Together, they inhibit integrin recycling and mediate cell spreading and angiogenesis *in vitro*.	Endothelial cells and HUVECs	[127]
GGA2	Arf-binding protein 2 (GGA2) associates with β1-integrins and promotes receptor recycling via Rab13, which boosts active cell migration.	Breast cancer cells	[65]
LRP1	Mediates internalization of β1-integrins. In thyroid cancer cells, it binds to SNX17 and directs active and inactive integrins to the Rab11 recycling pathway. In fibroblasts, LRP1 bridges kindlin2 to β1-integrin, promoting its activation and lysosomal degradation.	Thyroid cancer, melanoma, glioblastoma, breast cancer cells, HUVECs, fibroblasts	[128, 129]
Rab7a	Controls the localization of β1-integrins to filopodia via Rac1 activation and in this way promotes cell migration.	Lung cancer cells	[130]
Rab34	Mediates endocytosis of β3-integrin and prevents its lysosomal degradation, promoting cell adhesion, migration and invasion.	Breast cancer cells	[54]

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Molecule/modification	Mechanism and/or phenotype	Model system	Reference
Rab11-FIP5	Controls Rab11-mediated recycling of α6β1 (but not α3β1) integrins and directs these integrins to cell-cell adhesions to promote migration on laminin.	Prostate cancer cells	[131]
Reggie-1/Flotillin 2	Delivers integrins to new adhesions via Rab11. Involved in focal adhesion turnover, cell spreading and modulation of Rac1 activity.	HeLa and squamous cell carcinoma	[132]
Retriever & CCC complex	Together WASH, retriever and the CCC complex mediate recycling of α5β1-integrin.	RPE-1 and HeLa cells	[60]
Vps3 & Vps8	Mediate fusion of early endosomes as part of CORVET and deliver integrins to recycling endosomes via association with Rab4-positive endosomes. Contribute to cell adhesion, spreading and migration.	HeLa cells	[67]