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Extracellular matrix internalisation links nutrient signalling to invasive migration

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Summary

Integrins are key mediators of cell-extracellular matrix (ECM) interaction, linking the ECM to the actin cytoskeleton. Beside localising at the cell surface, they can be internalised and transported back to the plasma membrane (recycled) or delivered to the late endosomes/lysosomes for degradation. We and others have shown that integrin can be endocytosed together with their ECM ligands. In this short review, I will highlight how extracellular protein (including ECM) endocytosis impinges on the activation of the mechanistic target of rapamycin (mTOR) pathway, a master regulator of cell metabolism and growth. This supports the intriguing hypothesis that ECM components may be considered as nutrient sources, primarily under soluble nutrient-depleted conditions.

Keywords

Extracellular matrix, integrins, vesicular trafficking, mTOR, nutrient signalling, cell metabolism

Abbreviation list

Extracellular matrix – ECM

Focal adhesions – FAs

Fibronectin – FN

Early endosomes - EEs

Early endosome antigen 1 – EEA1

Perinuclear recycling compartment – PNRC

Multivesicular bodies – MVBs

Mechanistic target of rapamycin – mTOR

Mechanistic target of rapamycin complex 1 – mTORC1

Mechanistic target of rapamycin complex 2 – mTORC2

Laminin – LN

Tricarboxylic acid – TCA

Hyaluronidase 1 – Hyal 1

Hyaluronan – HA

AMP-activated protein kinase - AMPK

All the changes in the revised manuscript are highlighted in red

Introduction

Integrins are a family of 24 transmembrane heterodimers, composed of a combination of 18 α and 8 β subunits, that link the extracellular matrix (ECM) with the actin cytoskeleton, mediating a plethora of cell functions, including adhesion, proliferation, survival, migration and differentiation (Streuli, 2016). Integrins are expressed in all cell types, except erythrocytes, and they are required for the maintenance of most tissue, as well as for adhesion related processes, like leukocyte activation (Manninen and Varjosalo, 2017). The ECM is a complex network of secreted proteins which on one hand provides physical support for tissues and organs and on the other hand has an active role in controlling cell behaviour, both in physiological and pathological conditions, such as in cancer (Daley and Yamada, 2013). During cancer progression, tumour cells acquire the ability to detach from the primary tumour mass, invade through the surrounding tissues and form secondary tumours at distant sites, known as metastasis. This process is the main cause of mortality in cancer patients. Several ECM components have been shown to be strongly upregulated in the stroma of invasive breast tumours compared to localised ductal carcinoma in situ (Lee *et al.*, 2012). This observation highlights the importance of deepening our understanding of the mechanisms controlling cell-ECM interaction, in order to elucidate how cancer cells exploit changes in the stroma to foster their invasive ability, eventually leading to metastasis formation.

Integrin-containing adhesive structures mediate the interaction between cells and the surrounding environment. Depending on the substrate composition (presence of laminin, collagen, fibronectin or vitronectin), different integrin heterodimers promote adhesion formation. Upon initial adhesion, small focal complexes are formed, which mature into

larger focal adhesions (FAs), mainly localised at the periphery of the cells. Fibrillar adhesion are longer adhesive structures predominantly located towards the middle of the cells, particularly enriched in active $\alpha5\beta1$ integrin and tensins. Their formation has been associated with fibronectin (FN) polymerisation (Clark *et al.*, 2005). Adhesive structures are continuously remodelled during cell migration and invasion, with formation of new adhesions at the leading edge and disassembly of mature adhesion at the rear of the cell.

Vesicular traffic of integrins

The endosomal traffic has a key role in controlling adhesion dynamics (Maritzen *et al.*, 2015). Vesicular trafficking is composed of a complex network of vesicular and tubular compartments, whose identity is defined by specific sets of proteins present on their surface, as represented in **figure 1**. These include members of the Rab and Arf family of GTPases. Besides characterising different compartments, these GTPases also control the movement of cargos along the endosomal system. Plasma membrane receptors are internalised, through a variety of different pathways, in early endosomes (EEs), characterised by the presence of Rab5 and early endosome antigen 1 (EEA1). From here, Rab4 coordinates the fast return of cargos back to the plasma membrane, via the so called "short loop" recycling pathway. Alternatively, internalised receptors can be transported to the perinuclear recycling compartment (PNRC) before being targeted to the cell surface, in a Rab11-dependent manner, via the "long loop" recycling pathway. Proteins destined for degradation are sorted into multivesicular bodies (MVBs) and Rab7-positive late endosomes. Fusion with the lysosomes dictates content degradation. Rather than only being the cell "waste compartment", recent findings highlight the multifaceted role of the

lysosomes in controlling cell behaviour. Indeed, lysosomes control secretion, plasma membrane repair, autophagy, signalling and energy metabolism (Settembre *et al.*, 2013)

Different integrin heterodimers have been shown to populate the endosomal system, including α1β1, α2β1, α3β1, α5β1, α6β1, αvβ3, αvβ6 and α6β1. Their endocytosis and recycling pathways have recently been extensively reviewed and shown to promote cancer cell proliferation, invasion and metastasis (De Franceschi *et al.*, 2015). Interestingly, the way in which integrins are trafficked intracellularly is key in controlling how they affect cell behaviour (Caswell *et al.*, 2009). Direct interaction between integrins and trafficking regulators dictates the route that internalised integrins will follow. For instance, protein kinase D (PKD) directly binds to the C-terminal of integrin β3 and, through the phosphorylation of the Rab4 and Rab5 effector Rabaptin-5, promotes the Rab4-dependent short loop recycling of αvβ3. This pathway is required for fibroblast directed cell migration, as well as cancer cell invasion in the presence of αvβ3 ligands (Christoforides *et al.*, 2012). It is important to note that, at the plasma membrane, integrins can be found in a close or inactive conformation or bound to their ligand in an active conformation. Beside controlling downstream signalling outputs, the same integrin heterodimer is trafficked through distinct compartments depending on its activation status (Arjonen *et al.*, 2012). Different kinetics have been identified for active and inactive β1 integrin. After internalisation, while inactive β1 is rapidly recycled back to the plasma membrane in a Rab4- and Arf6-dependent manner, active β1 accumulates in recycling endosomes and late endosomes (Arjonen *et al.*, 2012). Moreover, in ovarian cancer cells, inactive α5β1 is trafficked to the perinuclear recycling compartment and then recycled to the plasma membrane through a Rab11-dependent mechanism (and not in a Rab4-dependent manner). It is possible that other β1-containing

integrin heterodimers are responsible for the observation that inactive β1 is mainly trafficked through the Rab4 compartment in (Arjonen *et al.*, 2012). In particular, the Rab11 effector RCP, which directly binds to β1 integrin, promotes the recycling of α5β1 to the tip of invasive protrusions when cells migrate in 3D environments, promoting their invasive ability through fibronectin-rich matrices (Caswell *et al.*, 2008). On the contrary, the traffic of ligand-bound α5β1 is controlled by a member of the Rab11 family, Rab25, and the putative chloride channel CLIC3. This pathway involves the recycling of active α5β1 from the late endosomal/lysosomal compartment towards the back of cells migrating in 3D environments (**figure 2**) (Dozynkiewicz *et al.*, 2012). The functions of this are not entirely clear, and evidence supports a role for integrin recycling in controlling cell rear dynamics during migration. The kinesin Kif1C has been reported to promote α5β1 delivery to the cell tail, and this is required to maintain rear adhesion stability and directional cell migration (Theisen *et al.*, 2012).

Integrins link ECM internalisation and mTOR signalling

Integrins have been shown to be internalised through a variety of endocytic mechanisms, including clathrin-, caveolin-, RhoA-dependent, macropinocytosis and clathrin-independent carriers. However, the spatial distribution of these endocytic events it is not entirely clear. We aimed to characterise the mechanisms specifically controlling ligand-bound α5β1 internalisation (Rainero *et al.*, 2015). The expression of Rab25 in ovarian cancer cells strongly induces the formation of fibrillar adhesions, mainly located underneath the nucleus, and promotes the internalisation of FN-bound α5β1, without affecting inactive integrin endocytosis. FN has been previously shown to be endocytosed in fibroblasts in a caveolin- and α5β1-dependent manner (Shi and Sottile, 2008). Interestingly, proteolytic

digestion of the FN-containing ECM has been shown to facilitate FN endocytosis, suggesting that FN fibrils degradation supports FN internalisation (Shi and Sottile, 2011). As mentioned above, tensins are a family of integrin and actin-binding proteins particularly enriched in fibrillar adhesions. In mammals, there are 4 tensin isoforms: tensin 1, 2 ,3 and CTEN. Knock-down of tensin1/2/3 impairs both fibrillar adhesion formation and ligand-bound integrin internalisation. In particular, the use of the novel photoactivation-in-TIRF approach highlighted how ligand-bound α5β1 is preferentially internalised from fibrillar adhesions (and not from focal adhesions) through a mechanism dependent on the small GTPase Arf4 and the SCAR/WAVE complex, a nucleator-promoting factor that controls Arp2/3-dependent actin polymerisation. Following endocytosis, α5β1 and its ligand FN are delivered to late endosomes/lysosomes. This flux controls lysosomal positioning and the activation of the mechanistic target of rapamycin (mTOR), a key regulator of nutrient sensing. The main role of mTOR consists of controlling the fine balance between cell growth and the environmental conditions, so that cell growth in only promoted when enough energy and nutrients are available to the cells. mTOR can be activated by several growth factors, glucose and amino acids (Saxton and Sabatini, 2017). mTOR exists in cells in two multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). While mTORC1 is a master regulator of metabolism and cell growth, mTORC2 controls cytoskeletal dynamics and AKT activation (Saxton and Sabatini, 2017). Intracellular concentration of amino acids is increased upon feeding and it has recently been elucidated that cytosolic and intra-lysosomal amino acids activate mTORC1 via different pathways. In particular, an increased in amino acid concentration within the lysosome drives mTORC1 recruitment to the lysosomal membrane, thus mediating its activation through direct interaction with the Ragulator complex (Bar-Peled *et al.*, 2012). Interestingly, ligand-bound integrin trafficking

specifically promotes mTORC1 activation, without affecting mTORC2. Moreover, this pathway is strongly activated by glucose starvation (Rainero *et al.*, 2015), suggesting a link between integrin trafficking and nutrient signalling (**figure 3**). In line with this, in melanoma cells mTORC2 inhibition has been shown to promote α2 integrin expression and focal adhesion reorganisation (Yoon *et al.*, 2017). Further work is required to elucidate the mechanisms through which mTOR activation impinges on integrin expression and adhesion remodelling.

In mammary epithelial cells, dietary restriction *in vivo* and growth factor starvation *in vitro* result in β4 integrin upregulation, and internalisation of the ECM component laminin (LN). β4/LN complexes are delivered to late endosome/lysosomes, where LN degradation into amino acids promotes mTORC1 activation, thus preventing starvation-induced cell death (Muranen *et al.*, 2017) (**figure 3**). It is hypothesised that mTORC1 activation is triggered by an increase in lysosomal amino acid concentration, upon digestion of ECM components, although further work is needed to specifically address the molecular mechanism(s) through which ECM internalisation promotes mTOR activation.

Altogether, these findings support the idea that cells are able to use extracellular proteins as nutrient sources. In agreement with this, extracellular albumin has been shown to be internalised and degraded in lysosomes, generating carbon intermediate to support the tricarboxylic acid (TCA) cycle. Interestingly, this pathway can support pancreatic cancer cell growth in glutamine-deprived condition and is strongly induced by the expression of oncogenic mutation of Ras (Commisso *et al.*, 2013). More recently, Ras-dependent extracellular protein internalisation has been shown to specifically support fibroblast growth in absence of essential amino acids (EAAs) or Leucine alone, with a minimal effect in

glutamine-free conditions (Palm *et al.*, 2015). This discrepancy could be ascribed to the use of different cell types (pancreatic cancer cells or fibroblast) in a different starvation protocol (0.1mM or complete glutamine deprivation). Albumin uptake, in absence of EAAs, results in mTOR lysosomal targeting and activation (Palm *et al.*, 2015), suggesting that both ECM components and soluble proteins can lead to mTOR activation upon internalisation and lysosomal degradation. Importantly, inhibition of mTORC1 (but not mTORC2) promotes extracellular protein degradation and cell proliferation in EAA-depleted conditions, without affecting macropinocytosis-dependent protein uptake (Palm *et al.*, 2015). On the other end, inhibition of mTORC1, but not mTORC2, strongly induces fibrillar adhesion formation and ligand-bound $\alpha5\beta1$ integrin endocytosis (Rainero *et al.*, 2015), suggesting that different endocytic/trafficking pathways are differentially controlled by mTOR signalling. Further studies are required to fully characterise the link between nutrient levels, mTOR activity and ECM component internalisation.

ECM traffic and metabolism

A few examples of crosstalk between ECM trafficking and cell metabolism have been reported. Hyaluronidases (Hyal) mediate the degradation of the ECM glycosaminoglycan hyaluronan (HA). High levels of Hyaluronidase 1 (Hyal1) correlate with poor prognosis in prostate cancer patients, while Hyal1 overexpression increases tumorigenesis and metastasis in mouse models. Recent evidence suggests that Hyal1 is required for clathrin-dependent internalisation of HA, promoting its delivery to Rab7-positive late endosomes/lysosomes. HA degradation within this organelle allows increased sugar access, which, through glycolysis, can generate the energy required to sustain tumour proliferation and invasion (McAtee *et al.*, 2015). Collagen I is the most abundant ECM protein in the

human body and contains large amount of proline. It is therefore being suggested to serve as a reservoir for this amino acid. Despite data indicating a role for matrix metalloproteases (MMPs) in proline release from collagen under conditions of nutritional stress (Phang *et al.*, 2015), further work is required to elucidate whether collagen endocytosis is required to support proline metabolism.

Energy stress promotes the activation of the metabolic sensor AMP-activated protein kinase (AMPK), which stimulates catabolic pathways. It has recently been demonstrated that AMPK is a negative regulator of β1 integrin activity, through the regulation of tensin 1 and tensin 3 levels. Loss of AMPK promotes tensin expression, in turn leading to β1 activation and FN fibrillogenesis in fibroblasts (Georgiadou *et al.*, 2017). It has been hypothesised that, within this FN-rich environment, cancer cells can take up the ECM deposited by cancer-associated fibroblasts, for the generation of the nutrients required to support cell proliferation and tumour growth (Georgiadou and Ivaska, 2017). Additional work is needed to define how this process is regulated and how it contributes to tumour growth *in vivo*.

Conclusions

The ECM is commonly viewed as “dead space” that provides a static scaffold for organ shape and acts as an obstacle for migrating cells. However, new data strengthen the idea that there is a dynamic interplay between cells and the ECM, which actively orchestrates cell behaviour. In particular, ECM receptors, including the integrin family members, promote the internalisation of ECM components. Recent evidence supports the intriguing hypothesis that ECM internalisation may represent a nutrient source for epithelial cells. Further research is needed in order to define the molecular mechanisms controlling ECM trafficking and fully elucidate how this pathway impinges on cellular metabolism.

Figure legends.

Figure 1. Schematic representation of endosomal trafficking and regulation by Rab small GTPases. EE, early endosome; RE, recycling endosome; MVB, multivesicular body; Lys, lysosome; EEA1, early endosome antigen 1; RCP, Rab coupling protein.

Figure 2. **Active α5β1 and inactive α5β1 are trafficked through different endocytic routes in cells migrating through complex 3D matrices**. Inactive α5β1 is internalised in early endosomes (EEs), transported to Rab coupling protein (RCP) and Rab11-positive recycling endosomes (REs) and recycled to the plasma membrane at the tip of invasive protrusions at the front of the cell. Fibronectin (FN)-bound α5β1 is preferentially endocytosed from sub-nuclear fibrillar adhesions and delivered to late endosomes (LEs)/lysosomes. From here, it is recycled to the plasma membrane at the back of migrating cells.

Figure 3. **Interplay between integrin trafficking and mTORC1 activity**. α5β1 integrin promotes the trafficking of fibronectin (FN) to late endosomes (LEs)/lysosomes, resulting in the recruitment of the mechanistic target of rapamycin complex 1 (mTORC1) on the lysosomal membrane and the activation of downstream signalling. This pathway is strongly promoted by glucose deprivation. α6β4 mediates the lysosomal delivery and degradation of laminin (LN), similarly leading to mTORC1 recruitment and activation. This is stimulated by growth factor starvation.

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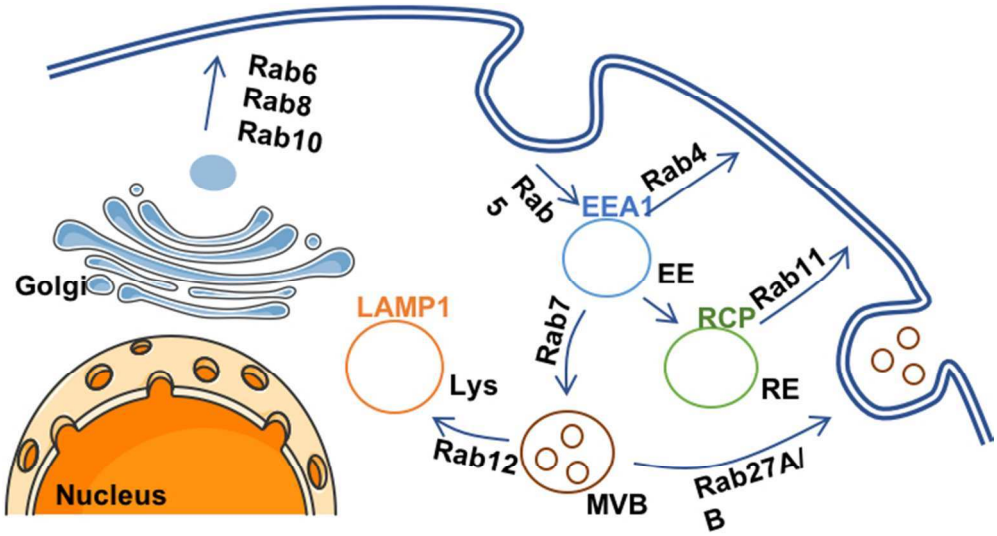

Figure 1. Schematic representation of endosomal trafficking and regulation by Rab small GTPases. EE, early endosome; RE, recycling endosome; MVB, multivesicular body; Lys, lysosome; EEA1, early endosome antigen 1; RCP, Rab coupling protein.

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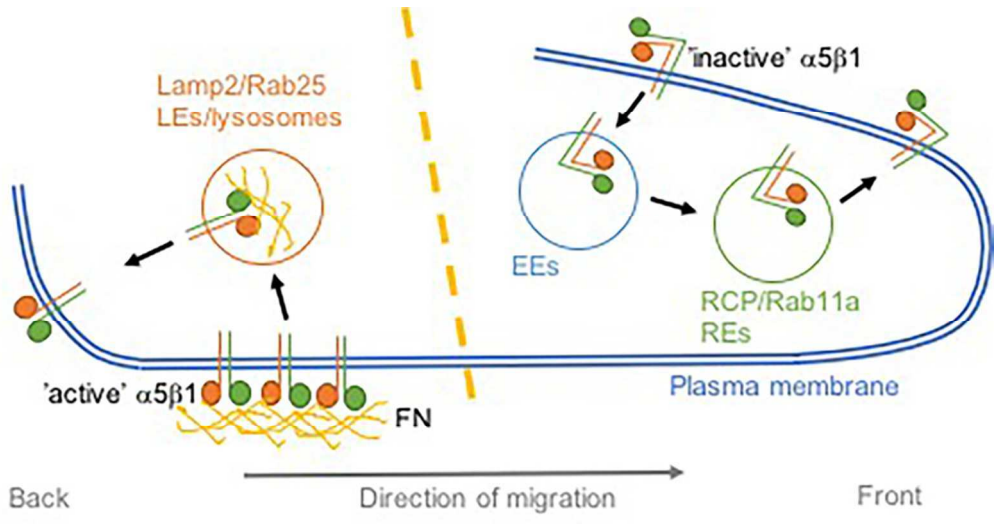

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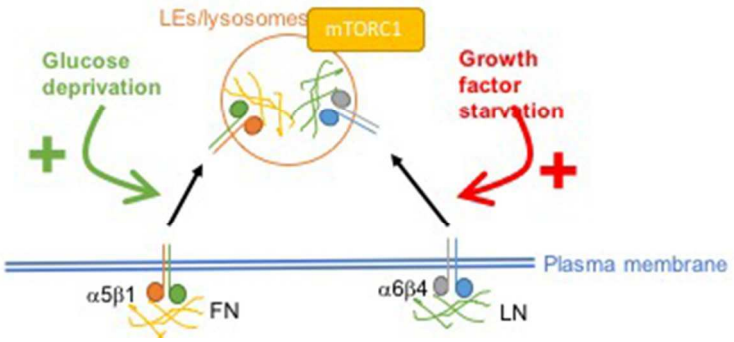

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133x59mm (72 x 72 DPI)