Microtubules at focal adhesions – a double-edged sword

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REVIEW

SUBJECT COLLECTION: ADHESION

Microtubules at focal adhesions – a double-edged sword

Shailaja Seetharaman[1,2] and Sandrine Etienne-Manneville[1,*]

ABSTRACT

Cell adhesion to the extracellular matrix is essential for cellular processes, such as migration and invasion. In response to cues from the microenvironment, integrin-mediated adhesions alter cellular behaviour through cytoskeletal rearrangements. The tight association of the actin cytoskeleton with adhesive structures has been extensively studied, whereas the microtubule network in this context has gathered far less attention. In recent years, however, microtubules have emerged as key regulators of cell adhesion and migration through their participation in adhesion turnover and cellular signalling. In this Review, we focus on the interactions between microtubules and integrin-mediated adhesions, in particular, focal adhesions and podosomes. Starting with the association of microtubules with these adhesive structures, we describe the classical role of microtubules in vesicular trafficking, which is involved in the turnover of cell adhesions, before discussing how microtubules can also influence the actin–focal adhesion interplay through RhoGTPase signalling, thereby orchestrating a very crucial crosstalk between the cytoskeletal networks and adhesions.

KEY WORDS: RhoGTPases, Actin, Focal adhesion, Microtubules, Podosome

Introduction

Cell survival, proliferation and differentiation, as well as migration and invasion, require cell adhesion to the extracellular matrix (ECM). Several types of adhesive structures are involved in cell–ECM interactions, and the dysregulation of multiple genes in the adhesome has been implicated in pathological conditions, such as cancer and cardiovascular diseases (Byron et al., 2015; Winograd-Katz et al., 2014). Among the plasma membrane proteins contributing to cell–ECM interactions, α-integrin–β-integrin heterodimers play a crucial role in sensing the biochemical and mechanical properties of the ECM. Integrin-based structures, such as hemidesmosomes, focal adhesions (FAs) and podosomes, connect the ECM to the cytoskeleton, which is primarily composed of actin microfilaments, microtubules and intermediate filaments. Hemidesmosomes correspond to small adhesive structures that attach epithelial cells to the basal membrane. $\alpha_6\beta_4$ integrins form the core of hemidesmosomes, which mainly connect to the intermediate filament network (Walko et al., 2015), although some recent reports have suggested that microtubules can also be associated with hemidesmosomes (Quintin et al., 2016). In contrast to hemidesmosomes, FAs and podosomes vary in their integrin composition and are mainly associated with the actin cytoskeleton (Seetharaman and Etienne-Manneville, 2018). FAs and the smaller focal complexes are adhesive structures that are essential for cell spreading and cell motility. Functionally, podosomes and invadopodia help to degrade the ECM in normal and cancer cells, respectively (Linder et al., 2011; Murphy and Courtneidge, 2011; Saykali and El-Sibai, 2014). Podosomes form actin-rich, ring-like structures at the plasma membrane in several cell types, such as vascular smooth muscle cells, endothelial cells and monocyte-derived cells (e.g. macrophages, dendritic cells and osteoclasts). Podosomes can sometimes form rosettes, for instance in vascular smooth muscle cells, endothelial cells and Src-transformed fibroblasts (Murphy and Courtneidge, 2011). In osteoblasts, individual podosomes fuse to form a podosome belt (Destaing et al., 2003). Invadopodia are more localised structures that degrade the ECM and push through it or the vascular wall to promote invasion and transendothelial migration (Gould and Courtneidge, 2014).

Integrins bind to their respective ligands in the ECM and undergo conformational changes that can be triggered by either intracellular (inside-out signalling) or extracellular signals (outside-in signalling) (Calderwood et al., 2013). The clustering of ligand-bound integrins and recruitment of talin proteins leads to the formation of nascent adhesions (Klapholz and Brown, 2017). While most nascent adhesions are rapidly disassembled, intracellular signalling and recruitment of multiprotein complexes can promote their maturation into larger mature FAs (3–10 µm in length), which are defined by their association with contractile stress fibres (Ridley and Hall, 1992). Stress fibres, formed by long actin cables intertwined with myosin, are anchored at FAs through several molecular linkers, including mechanosensing proteins, such as talins or vinculin (Tojkander et al., 2012; Yan et al., 2015). FAs integrate several extracellular cues, including the biochemical nature of the integrin-bound ligand and the physical properties of the substrate, to influence the adaptation of cell behaviour to the microenvironment (Seetharaman and Etienne-Manneville, 2018). In particular, FAs modulate the acto-myosin network, which generates forces for cell contraction, shape changes and migration (De Pascalis and Etienne-Manneville, 2017). Although less-well characterised than FAs, podosomes and invadopodia are also tightly associated with actin. They are built around an actin core surrounded by a ring-shaped cluster of adhesion structures made up of proteins, including vinculin (Gimona et al., 2008; Murphy and Courtneidge, 2011; Saykali and El-Sibai, 2014). While the importance of the actin network in the structure and functions of FAs and podosomes has been vastly documented (Elosegui-Artola et al., 2018; Lee and Kumar, 2016; Paterson and Courtneidge, 2018), the contribution of microtubules and intermediate filaments is often overlooked (see LaFlamme et al., 2018; Leube et al., 2015).

Microtubules are hollow cylindrical structures made up of heterodimers of α- and β-tubulin. In mammalian cells, microtubules arise from microtubule-organising centres, including the centrosome and the Golgi (Petry and Vale, 2015), which stabilise the minus-ends of microtubules. The highly dynamic plus-ends of

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microtubules grow towards the cell periphery, where they undergo repeated phases of growth and shrinkage that characterise dynamic instability (Akhmanova and Steinmetz, 2008; Kirschner and Mitchison, 1986). The dynamics and functions of microtubules are regulated through microtubule-associated proteins (MAPs), which bind to the microtubule lattice, and microtubule plus-end/positive-end-tracking proteins (+TIPs), which specifically interact with the extremities of microtubules, as well as post-translational modifications of tubulins that decorate the microtubule network (Akhmanova and Steinmetz, 2015; Etienne-Manneville, 2010). One essential function of microtubules in interphase cells is to carry cargoes between intracellular compartments, as well as to and from the plasma membrane. Intracellular trafficking of vesicles, proteins and RNAs is mediated by two major classes of microtubule-associated motors: the kinesins and dyneins (Cross and Dodding, 2019; Reck-Peterson et al., 2018). Furthermore, microtubules interact with and regulate essential signalling molecules, including regulators of the Rho family of small GTPases (Wojnacki et al., 2014). The trafficking and signalling functions of microtubules, together with their interactions with other cytoskeletal networks, constitute the numerous ways by which microtubules can affect the dynamics and functions of FAs and podosomes (Etienne-Manneville, 2013).

Starting from the early 1980s, several studies have hinted at a physical and functional connection between microtubules and adhesion structures. Microtubule depolymerisation induced by nocodazole increases the number and size of FAs, as well as contributing to the activation of the small G protein Rho, which, in turn, leads to the formation of numerous large FAs and to an increase in cell contractility (Bershadsky et al., 1996; Liu et al., 1998). Following nocodazole washout, microtubules regrow and FAs disassemble (Ezratty et al., 2005; Kaverina et al., 1998). In contrast to FAs, which enlarge upon nocodazole treatment, the podosome belt is disrupted (Destaing et al., 2003, 2005; Linder et al., 2000). Rho activation results in the disappearance or redistribution of podosomes, suggesting that the relationship between Rho activation and podosomes is certainly different from that with FAs.

Here, we will review the molecular links between microtubules and integrin-mediated adhesions, in particular, FAs and podosomes. We will then focus on the recent developments in elucidating the direct crosstalk between microtubules and adhesive structures that point to microtubules as essential participants in the actin–FA interplay, and therefore establish them as crucial regulators of cell responses to the biochemical and physical properties of the cell microenvironment.

Contacting FAs – touching and grabbing

Microtubules targeting FAs

The colocalisation of microtubule tips with focal contacts was first documented in 1984 by Geiger and colleagues, followed by another study in 1988 (Geiger et al., 1984; Rinnerthaler et al., 1988). A few years later, microtubule disruption using nocodazole or vinblastine was shown to result in an increased number of FAs together with an increased formation of stress fibres in serum-starved 3T3 cells, suggesting that microtubules participate in the control of FA dynamics and acto-myosin contractility (Bershadsky et al., 1996; Enomoto, 1996). Using live-cell fluorescence microscopy, Kaverina and colleagues later demonstrated that microtubules in migrating fibroblasts could pass through or target vinculin-containing FAs at the cell front (Kaverina et al., 1999, 1998). At the rear of migrating cells, microtubules repeatedly targeted large FAs, which were undergoing disassembly during the retraction of the trailing edge (Kaverina et al., 1999). However, since these cells have a considerably less-dense microtubule network as compared to several other mammalian cell types, the direct association of microtubules with FAs remains controversial (Fig. 1A,B). The accumulation of the growing plus-ends of microtubules near FAs has been described in goldfish fin fibroblasts (Ezratty et al., 2005; Krylyshkina et al., 2003). Indeed, by using higher-resolution microscopy techniques, such as total internal reflection microscopy (TIRFM), microtubules are observed to localise ∼100 nm away from FAs (Krylyshkina et al., 2003). Similarly, in migrating astrocytes, microtubules also extend towards FAs at the cell front. Furthermore, a proportion of these microtubules grow and bend to reach down towards the basal cell cortex, where they remain anchored (Etienne-Manneville et al., 2005). Also in this case, our group was able to show that microtubules run along FAs, without directly contacting them (Bance et al., 2019) (Fig. 1A,B).

Even if microtubules do not directly contact FAs, their close proximity suggests that a molecular connection exists between these two structures. In support of this, a proteomics approach demonstrated that +TIPs are well represented in complexes that result from integrin activation at the cortex (Byron et al., 2015). Microtubule-associated proteins, such as end-binding protein 1 (EB1; also known as MAPRE1) and actin crosslinking factor 7 (ACF7; also known as microtubule actin crosslinking factor 1, MACF1) are enriched in integrin complexes that contain FA proteins, such as talins, vinculin and kindlins. Although this study confirms the close association of microtubules with FAs (Byron et al., 2015), there is no clear evidence of the direct binding of microtubules to the core proteins of FAs. In fact, more recently, protein complexes localised at the periphery of FAs have been identified to act as cortical anchors of microtubules (Noordstra and Akhmanova, 2017).

In addition to their proximity to FAs, microtubules are also observed around podosomes. Growing microtubules can be seen targeting podosomes (Biosse Duplan et al., 2014). In osteoclasts, post-translationally modified microtubules are differently distributed within the cell to non-modified microtubules (Akisaka et al., 2011). In mature osteoclasts, both the tyrosinated and acetylated microtubules are seen at the cell periphery near podosomes (Akisaka et al., 2011; Destaing et al., 2005). All these studies suggest that microtubules do not necessarily contact FAs and podosomes, but are indeed observed in close proximity to these adhesive structures.

Microtubule capture and anchoring at the cortex

Three complementary steps promote the interaction of microtubules with FAs. In the first, microtubules are guided towards FAs by interacting with other cytoskeletal networks and crosslinking proteins. Once microtubules are near FAs, microtubule plus-ends are captured at the cell cortex. This eventually leads to stable microtubule anchorage by the recruitment of cortical protein complexes in the vicinity of FAs.

Microtubule guidance towards FAs

Since actin filaments are tightly associated with FAs, they can serve as tracks that direct growing microtubules to the core of FAs (Fig. 2A). The formins mammalian diaphanous (mDia)1 and mDia2 (encoded by *DIAPH1* and *DIAPH2*, respectively) have been shown to participate in the orientation and alignment of the microtubule and actin networks along the long axis of HeLa and 3T3 cells, respectively (Ishizaki et al., 2001; Palazzo et al., 2001). The spectraplakin ACF7 links the actin and microtubule networks and

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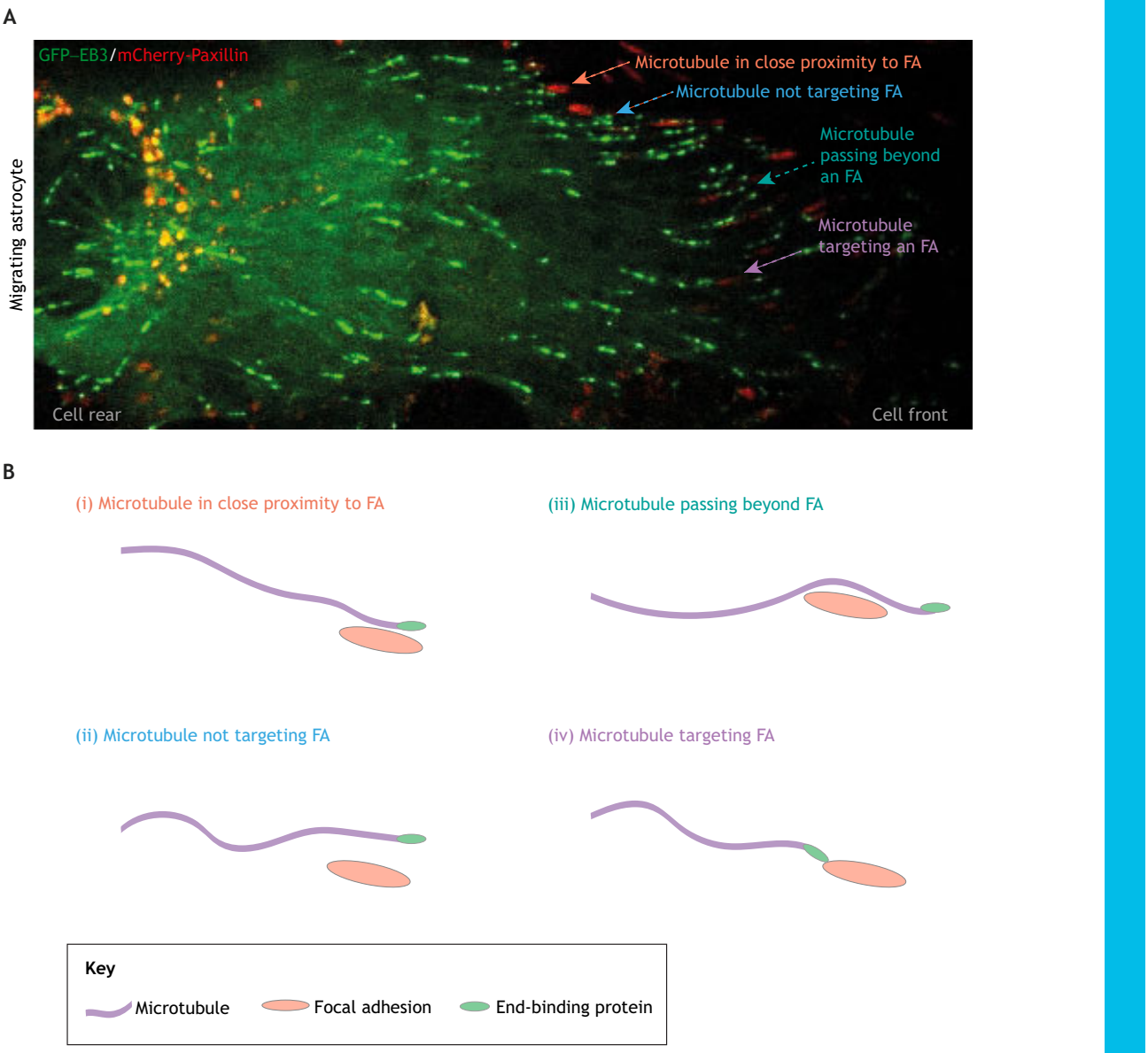

Fig. 1. Microtubule localisation to FAs. (A) Microtubule growth towards the cell periphery and their association with FAs. Confocal image of a migrating astrocyte transfected with GFP–EB3 (marking microtubule plus-ends in green) and mCherry–paxillin (marking FAs in red) showing the trajectories of growing microtubules compared to the position of FAs. Reproduced from Bance et al. (2019). (B) Schematic close-up view of different localisations that microtubules can take in the vicinity of an FA. (i) Microtubule in close proximity to an FA, without actually localising to an FA. (ii) Microtubule extending to the cell edge, but not necessarily near any FA. (iii) Microtubule passing by an FA, but continuing towards the cell edge without actually ending at FAs. (iv) Microtubule terminating at and localising to an FA at the cell edge.

also plays a key role in guiding growing microtubules along actin fibres towards FAs (Wu et al., 2008) (Fig. 2A). In keratinocytes, ACF7 localises near FAs, and its depletion results in reduced targeting of microtubules to FAs and slower FA turnover (Wu et al., 2008). In Caco2 epithelial cells, ACF7 interacts with calmodulin-regulated spectrin-associated protein 3 (CAMSAP3) to anchor non-centrosomal microtubules to actin filaments and align microtubules along the actin network (Ning et al., 2016). Microtubules might also be guided by intermediate filaments (Fig. 2A), which have been shown to interact with FA proteins (Gregor et al., 2014; Leube et al., 2015). However, it is also possible that intermediate filaments glide along microtubules in a kinesin-1-dependent manner (Robert et al., 2019). Intermediate filaments tightly associate with the microtubule lattice, in particular through the cytoskeletal linker plectin, the tumour suppressor protein adenomatous polyposis coli (APC) and also through kinesin and dynein microtubule-associated motors (Leduc and Etienne-Manneville, 2017; Sakamoto et al., 2013), and so control the precise orientation of growing microtubules (Gan et al., 2016).

Microtubule capture at the cell cortex

When microtubule plus-ends reach the cell cortex, they can be captured in close proximity to FAs. Cortical capture occurs mainly through the interactions of +TIPs, such as APC, ACF7, CAP-Gly-containing cytoplasmic linker protein of 170 kDa (CLIP-170; also known as CLIP1) and cytoplasmic linker-associated proteins (CLASPs) with cortical factors (Gouveia and Akhmanova, 2010). For instance, IQ motif containing GTPase-activating protein 1

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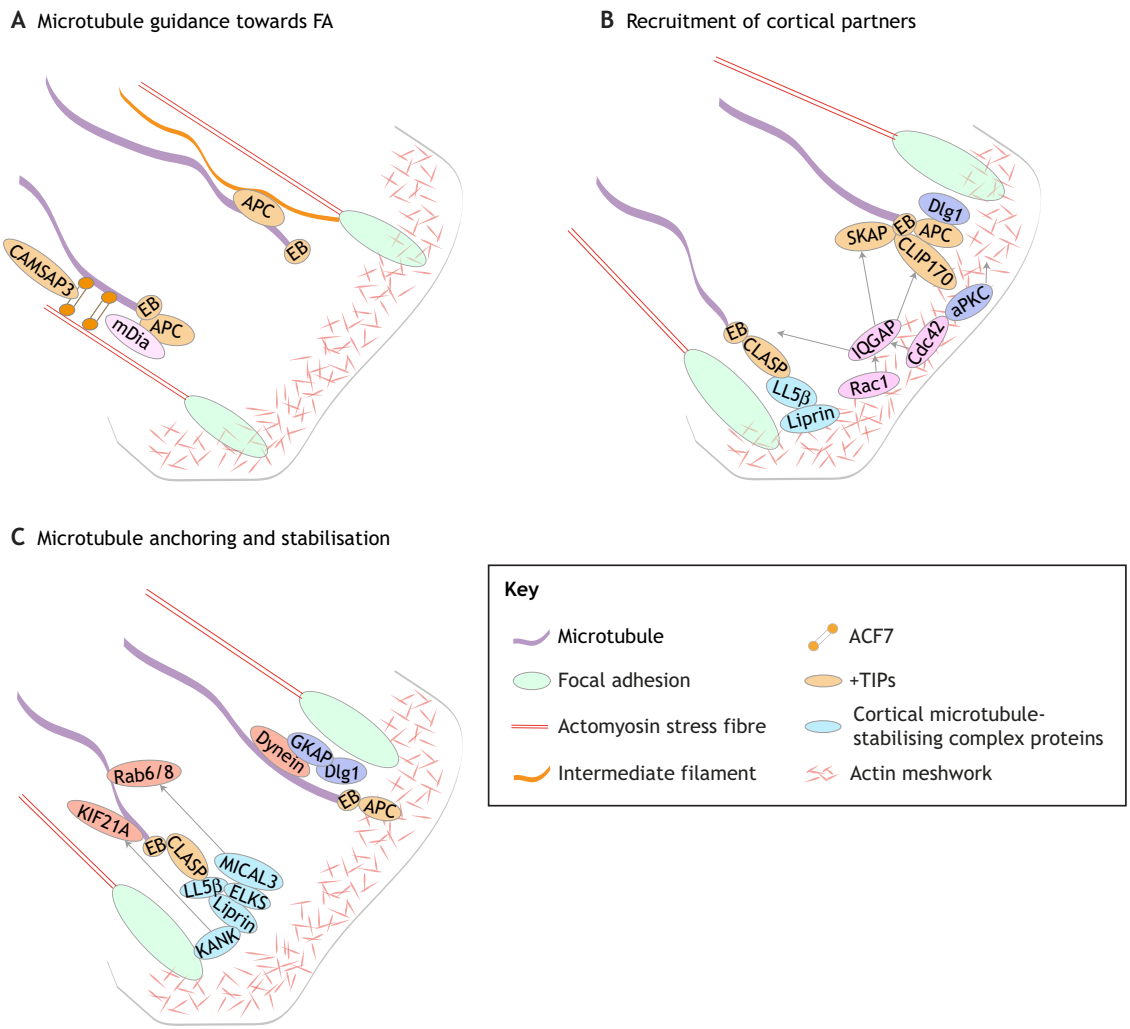

Fig. 2. Microtubule capture, anchoring and stabilisation at FAs. (A) Microtubule guidance to FAs. Both actin fibres and intermediate filaments can guide microtubules to the cell periphery and may contribute to microtubule localisation at the rim of FAs. Several protein partners involved in this process, such as CAMSAP3, mDia and APC, are depicted here. Intermediate filaments interact with APC, which associates with the microtubule lattice in an EB1-independent manner. (B) Recruitment of cortical partners. Local recruitment and accumulation of cortical factors, such as LL5β, liprins, IQGAP1 and IQGAP-binding proteins such as CLASPs, CLIP-170 and SKAP, in the proximity of FAs initiates microtubule capture at the cell cortex. Once recruited near FAs, these cortical proteins interact with +TIP proteins to promote microtubule capture at the cell cortex. Dlg1 can also serve as a cortical anchor for APC-positive microtubule ends. Dlg1 cortical recruitment is controlled by signalling cascades such as that mediated by the Cdc42–aPKC polarity complex, which also promotes the accumulation of APC at EB1-positive microtubule plus-ends to trigger microtubule capture. (C) Microtubule anchoring and stabilisation at the cortex. Microtubule stabilisation at the cortex occurs through factors, such as KANKs, ELKS, liprins, LL5β and CLASPs, which together form a cortical-microtubule-stabilising complex around the rim of FAs. KANKs recruit the kinesin-4 protein KIF21A. In the case of APC- and Dlg1-mediated microtubule capture, stable anchoring does not occur at the plus-end but further along the microtubule lattice and requires Dlg1-mediated recruitment of the microtubule-associated motor dynein. The Dlg1–GKAP complex recruits dynein which exerts forces required to anchor microtubules at the cell cortex.

(IQGAP1) and Disks Large 1 (Dlg1) localise at the front of cell protrusions during migration and are involved in anchoring microtubules at the cell cortex (Fig. 2B). IQGAP1 interacts with the EB1-binding protein small kinetochore-associated protein (SKAP, also known as KNSTRN) and thus may play a role in microtubule capture (Cao et al., 2015). In fibroblasts, CLIP-170 is involved in microtubule attachment at the lamella through its interactions with the dynein–dynactin complex, Rac1 and IQGAP1 (Fukata et al., 2002; Lansbergen et al., 2004; Watanabe et al., 2004) (Fig. 2B). Through mass spectrometry, two binding partners of CLASPs have been identified: pleckstrin homology like domain family B member 2 (LL5β, also known as PHLDB2) and protein rich in the amino acids E, L, K and S (ELKS, also known as ERC1); together CLASP, LL5β and ELKS form a complex around the rim of FAs where microtubules are anchored (Lansbergen et al., 2006). The authors showed that LL5β is essential for the recruitment of CLASPs to the cell cortex, where microtubules then become stabilised (Lansbergen et al., 2006). Liprins also associate with FAs and interact directly with ELKS, which is located at the periphery of FAs (Ko et al., 2003). Although ELKS does not bind microtubules directly, it plays an accessory role in clustering LL5β and CLASPs at the cortex, which is important for microtubule stabilisation (Lansbergen et al., 2006).

However, in migrating astrocytes, CLIP-170 and CLASPs are not necessary for microtubule capture. Instead, EB1-associated APC at microtubule plus-ends interacts with the PDZ domains of Dlg1, which localises at the basal cortex at the front of the protrusion (Etienne-Manneville et al., 2005). The direct interacting partner of

EB1, APC membrane recruitment 2 (Amer2; also known as FAM123) participates in the recruitment of APC to the cell membrane in order to direct microtubules to the proximity of FAs (Pfister et al., 2012). Membrane-bound APC then recruits ACF7 to the cortex, thus providing the means for an EB1-dependent guidance of microtubules to FAs (Akhmanova and Steinmetz, 2008; Zaoui et al., 2010). The given microtubule-anchoring machinery might be cell type dependent and cue specific, but crucial in regulating FA dynamics and cell migration in response to specific ECM components.

Microtubule capture is tightly regulated, which may explain why microtubules appear to localise to FAs so precisely. This spatio-temporal regulation of microtubule capture is essential for directed cell migration, during which the polarised organisation of microtubules defines cell polarity and the direction of migration (Etienne-Manneville, 2013). The exact localisation of microtubule capture, which requires the interaction between +TIPs and cortical partners, depends not only on the expression level of these proteins but also on their concomitant recruitment to the correct region of the cell cortex. It has been shown that signalling cascades affect the localisation of cortical proteins. Glycogen synthase kinase 3β (GSK3β) has recurrently emerged as a regulator of microtubule capture and anchoring in different systems (Beurel et al., 2015). Indeed, GSK3β negatively regulates the interaction between +TIPs and microtubule-associated EB1. For instance, in fibroblasts, inhibition of GSK3β by the phosphoinositide 3-kinase (PI3K) pathway promotes the recruitment of CLASPs specifically to the tip of microtubules at the cell front (Akhmanova et al., 2001). Similarly, in keratinocytes, expression of a constitutively active form of GSK3β disrupts microtubule–CLASP binding and, therefore, impairs microtubule anchoring at the lamella (Kumar et al., 2009). In astrocytes, the Cdc42–Par6–aPKC polarity pathway triggers the phosphorylation and inactivation of GSK3β at the cell front, which induces the interaction between APC and EB1 specifically in leading-edge microtubules to control the polarisation of the microtubule network (Etienne-Manneville and Hall, 2003). These observations suggest that the regulation of microtubule anchoring is essentially achieved by the control of the recruitment of the cortical anchors on one hand and of the +TIP-binding partners on the other hand, but does not seem to involve the direct modulation of the binding affinity of the +TIP proteins for their cortical partners.

Recruitment of cortical partners near FAs

Concomitantly with the recruitment of +TIPs to the cell cortex, microtubule anchoring requires the local accumulation of cortical proteins in the proximity to FAs. Here, IQGAP1 serves as a cortical interactor for both CLIP-170 and APC. IQGAP1, CLIP-170 and APC form a tripartite complex that facilitates the targeting of microtubule plus-ends to the leading edge of a cell (Fukata et al., 2002; Watanabe et al., 2004) (Fig. 2B). To that end, IQGAP is recruited to the leading edge through its interaction with Rac1 (Fukata et al., 2002), or that with the FERM domain of the leading-edge protein 4.1R (Ruiz-Sáenz et al., 2011). In migrating astrocytes, the Cdc42–aPKC polarity complex induces the recruitment of Dlg1 to the cell front, where it associates with APC-positive microtubule plus-ends and so promotes microtubule capture at the cortex (Etienne-Manneville et al., 2005). The APC–Dlg1 complex also promotes the recruitment of microtubule minus-end-directed motor dynein through the Dlg1-interacting factor guanylate kinase-associated protein (GKAP, also known as DLGAP1) (Manneville et al., 2010). The accumulation of microtubule-associated dynein at the cell cortex may help to exert the forces that are necessary to anchor microtubules and resist the actin-driven retrograde flow (Manneville and Etienne-Manneville, 2006).

Several additional cortical proteins are involved in regulating cell adhesion, including LL5β, CLASPs, ELKS, liprins and KN motif and ankyrin repeat domain-containing proteins (KANKs) (Fig. 2C). LL5β interacts with the PI3K product phosphatidylinositol 3,4,5-triphosphate (PIP_3) (Paranavitane et al., 2003). Experiments in HeLa cells using nocodazole suggest that LL5β recruitment to the cell cortex does not depend on intact microtubules, but mainly on the activity of PI3K (Lansbergen et al., 2006). However in epithelial cells, laminin-based cell adhesion, but not PI3K, regulates LL5β localisation and thus its control of microtubule anchoring at the cortex (Hotta et al., 2010). APC and CLASP2 can be recruited to the cell cortex upon GSK3β phosphorylation downstream of the ErbB2 receptor tyrosine kinase (Zaoui et al., 2010), suggesting that different external signals, such as those downstream of integrins (e.g. in astrocytes), NGF (e.g. in neurons) and EGFR (e.g. in breast carcinoma cells), can result in the recruitment of cortical proteins and polarisation via GSK3 regulation. CLASPs are also found closely associated with FAs, along with ELKS and liprins (Kumar et al., 2009; Lansbergen et al., 2006). CLASPs can be recruited to the vicinity of FAs through their interaction with LL5β (Lansbergen et al., 2006). In addition to their localisation near FAs, LL5β and ELKS are also observed at synaptic podosomes (Proszynski et al., 2009; Proszynski and Sanes, 2013). Amotl2, an actin regulator, interacts with LL5β, and the depletion of Amotl2 in fibroblasts disrupts invadopodia (Proszynski and Sanes, 2013), suggesting a possible way by which LL5β might also be recruited to the vicinity of invadopodia.

Microtubule stabilisation at the cortex

Cortical microtubules are generally stabilised upon anchorage (Etienne-Manneville, 2013). The stabilisation of microtubules typically refers to the lifetime of microtubules and their resistance to nocodazole or cold treatment, and not necessarily to microtubule dynamic instability. Following nocodazole treatment of goldfish fibroblasts, microtubules near FAs show very limited depolymerisation compared to microtubules localised at a distance from FAs (Kaverina et al., 1998). Upon nocodazole treatment, peripheral microtubules that do not interact with FAs shrink faster than those interacting with FAs (Kaverina et al., 1998).

MAPs, such as MAP2 and MAP4, can stabilise microtubules (by preventing depolymerisation and catastrophe events) to promote the formation of cell protrusions during migration (Dehmelt and Halpain, 2004). Moreover, some signalling cascades can indirectly participate in microtubule stabilisation. RhoA and formins, such as mDia2, control microtubule stabilisation, independently of their effect on actin (Bartolini et al., 2008). A screen for Rho-effector domains first identified the role of mDia in microtubule stabilisation in 3T3 fibroblasts; here, expression of constitutively active mDia2 or activation of endogenous mDia1 induced the detyrosination of microtubules, which is a classic marker of stable microtubules (Palazzo et al., 2001). Interestingly, in T cells, inhibition of Rho-associated protein kinases (ROCKs) and myosin stabilises microtubules, suggesting that the balance between Rho-mediated activation of ROCK proteins and mDia is crucial in controlling cortical microtubule stability (Takesono et al., 2010) (Fig. 3A). mDia1 and mDia2 might facilitate microtubule stabilisation downstream of Rho through their interaction with EB1 and APC at microtubule plus-ends (Wen et al., 2004). Another formin, inverted

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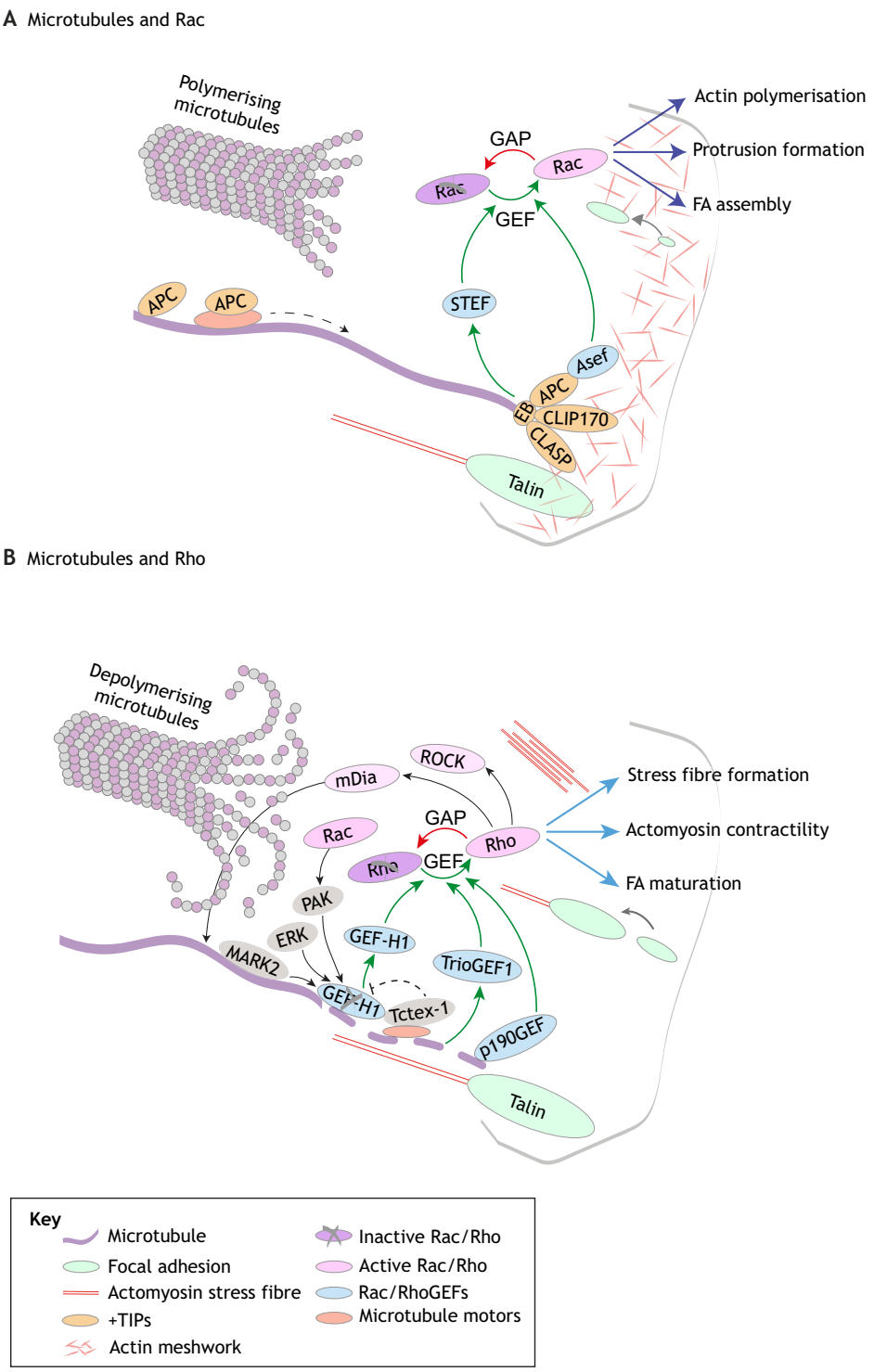

Fig. 3. RhoGTPase-mediated crosstalk between microtubules and actin at FAs. (A) Microtubule polymerisation and Rac signalling. Microtubule polymerisation (observed during microtubule growth) at the leading edge of the cell contributes to Rac activation, which involves the RacGEFs STEF and Asef. In turn, Rac activation promotes the formation of new cell adhesions and protrusion formation through its effects on actin polymerisation. (B) Microtubule depolymerisation and Rho signalling. RhoGEFs, such as GEF-H1, TrioGEF and p190RhoGEF, have been shown to activate (green arrows) Rho upon microtubule depolymerisation (observed during dynamic instability of microtubules or nocodazole-induced microtubule depolymerisation). GEF-H1, when inactive, localises on microtubules. PAK, ERKs and MARK2 and the dynein light chain protein Tctex-1 are a few of the proteins that regulate GEF-H1 activity or localisation, and they can induce the release of GEF-H1 from microtubules to activate Rho. Rho activation, in turn, increases acto-myosin contractility and FA maturation. Thus, the crosstalk between microtubules with actin through Rac and Rho is important for FA turnover, acto-myosin contractility and protrusion formation during cell migration. GAP activity inhibits (red arrows) Rho and Rac signalling.

formin 2 (INF2) is necessary for the formation of mDia1-dependent detyrosinated microtubules (Bartolini et al., 2016). Upon LPA treatment, microtubule acetylation and detyrosination increase, possibly through enhanced recruitment of mDia1 or INF1 and/or INF2 (Andrés-Delgado et al., 2012; Bartolini et al., 2016; Cook et al., 1998; Gaillard et al., 2011; Thurston et al., 2012). INF2 can promote microtubule acetylation through myocardin-related transcription factor (MRTF)/serum response factor (SRF)-dependent transcription of α-tubulin acetyltransferase 1 (αTAT1) (Fernández-Barrera et al., 2018). The APC-binding protein Amer2 recruits EB1-positive microtubule plus-ends to the cell membrane and promotes microtubule acetylation (Pfister et al., 2012). How microtubule stability, as characterised by microtubule detyrosination or acetylation, is controlled remains unclear. In fact, our recent observations indicate that acetylation and detyrosination are regulated independently and may have different roles that are possibly unrelated to microtubule stability (Bance et al., 2019).

Most importantly, microtubule stabilisation is also achieved through the regulation of microtubule plus-end dynamics. Although FAs have been shown to induce microtubule catastrophe to promote

the dynamic 'poking' behaviour observed during microtubule-induced FA disassembly (Efimov et al., 2008), more recent observations have focused on the inhibition of microtubule disassembly by the cortical microtubule-stabilising complex (CMSC) (Fig. 2C). The CMSC consists of proteins that are involved in microtubule capture, such as CLASPs, the scaffolding proteins liprin-α1 and liprin-β1, and the ELKS (Bouchet et al., 2016; van der Vaart et al., 2013). CMSC components accumulate at the periphery of FAs, but do not exactly colocalise with FAs (Lansbergen et al., 2006). In addition, the four KANK proteins, KANK1–KANK4, have been described as 'seeds' that initiate the formation of a cortical platform assembly for the stabilisation of microtubules (Bouchet et al., 2016; Sun et al., 2016). KANK1 and KANK2 localise around FAs and podosomes (Rafiq et al., 2019). KANK1 links the CMSC to FAs through its direct interaction with both talin and liprin-β1 (Bouchet et al., 2016; Sun et al., 2016). Furthermore, KANK1, which is enriched in the cortical complex formed by LL5β, ELKS and liprin, recruits kinesin-4 (KIF21A) to the cortex and so prevents microtubule catastrophe, thereby promoting cortical microtubule stability (Kakinuma and Kiyama, 2009; van der Vaart et al., 2013) (Fig. 2C).

Stabilisation of peripheral microtubules might also play a role in the formation of podosomes (Destaing et al., 2003). Overexpression of two adaptor proteins, Cbl and Cbl-b, which are present in osteoclasts, enhances microtubule acetylation and stability (Purev et al., 2009). Furthermore, depletion of the protein tyrosine kinase Pyk2 (also known as PTK2B), which is primarily confined to podosomes, results in higher RhoA activity and decreases microtubule stability (Gil-Henn et al., 2007). Therefore, the effect of RhoA activity on the stability of microtubules near podosomes in osteoclasts appears to be different from its effect on those in the vicinity to FAs, suggesting that there are different regulatory mechanisms that remain to be identified.

Thus, multiple protein complexes that appear to be cell type specific and may associate with FAs dependent on distinct integrin subtypes (Seetharaman and Etienne-Manneville, 2018), are not only involved in recruiting microtubules to the vicinity of FAs, but also in keeping them anchored at these sites by preventing their depolymerisation or removal by the actin retrograde flow. In turn, FA-associated microtubules can also contribute to intracellular transport and signalling to alter FA dynamics as discussed below.

Giving and taking back: microtubule-driven vesicular trafficking at FAs

The spatio-temporal regulation of FA turnover is crucial for cells to move forward and to counterbalance traction forces exerted on the substrate during migration. Microtubules control the turnover of FAs by regulating both FA assembly and disassembly during migration (Etienne-Manneville, 2013). Microtubules also serve as cargo tracks within the cell and are, therefore, key players in the regulation of molecular trafficking of proteins to and from the plasma membrane. Microtubule motors also carry cytoskeletal regulators, such as APC or Cdc42 and Rac small GTPases, cytoskeletal elements, such as intermediate filaments (Leduc and Etienne-Manneville, 2017), and mRNAs encoding proteins involved in actin polymerisation, such as subunits of the Arp2/3 complex (Mili et al., 2008; Mingle et al., 2005). For instance, the plus-end-directed microtubule motor kinesin KIF3A transports APC to nascent axon tips (Nishimura et al., 2004; Shi et al., 2004). At the tip, APC associates with Par3 and helps in the recruitment of atypical protein kinase C (aPKC) (Nishimura et al., 2004), which may then interact with, and phosphorylate GSK3 to promote microtubule anchoring and stabilisation (see above; 'recruitment of cortical partners near FAs') (Etienne-Manneville and Hall, 2003). Such microtubule-based intracellular trafficking contributes to cell polarisation, protrusion formation and the turnover of FAs during migration (Etienne-Manneville, 2013).

Microtubule-dependent transport to FAs

Integrin exocytosis plays a role in FA assembly (Gu et al., 2011; Gupton and Gertler, 2010). Microtubules anchored to the plasma membrane serve as tracks for the transport of exocytic vesicles to FA sites where they can disassemble FAs and in turn, promote FA turnover (Noordstra and Akhmanova, 2017). Retention using selective hooks (RUSH) and specific protein immobilisation (SPI) assays helped to show that Rab6-dependent exocytosis of vesicles occurs along microtubule tracks and at specific hotspots around FAs (Fourriere et al., 2019). Rab6 associates with most post-Golgi vesicles [for instance, vesicles containing CD59, TNF or ColX (also called Col10A)], irrespective of the cargoes transported to the plasma membrane; these are all delivered to the plasma membrane in close proximity to FAs, although not directly at FAs (Fourriere et al., 2019). ELKS has been shown to play a crucial role in the exocytosis of Rab6-positive vesicles around FAs (Del Nery et al., 2006; Grigoriev et al., 2007). Rab8, another binding partner of ELKS, interacts with MICAL3, a protein of the MICAL family of flavoprotein monooxygenases (Grigoriev et al., 2011). This interaction between Rab6, Rab8 and ELKS is key for the docking and fusion of vesicles at cortical adhesion sites to promote FA turnover. We also recently demonstrated a potential role for microtubule acetylation in controlling FA dynamics in astrocytes, as αTAT1, the enzyme responsible for microtubule acetylation, promotes Rab6-mediated vesicular fusion at FAs, which regulates FA turnover and astrocyte migration (Bance et al., 2019). While it is easy to speculate that microtubules play a key role in delivering integrins and possible membrane-associated partners to FAs, their involvement in the precise control of vesicle delivery remains unclear. Whether anchored microtubules merely serve as tracks for vesicular traffic or can directly control vesicle docking and fusion needs to be further explored.

Microtubule-dependent disassembly of FAs

The role of microtubules in FA disassembly has been mainly characterised by using nocodazole washout-induced FA disassembly. Nocodazole-induced microtubule depolymerisation causes an accumulation of FAs and phosphorylation of FAK and paxillin (Bershadsky et al., 1996); however, following nocodazole washout, microtubules grow again and localise to FAs as these simultaneously disassemble (Kaverina et al., 1999). This is in agreement with the finding that dynamic microtubules disassemble FAs from the rear of migrating cells and a higher degree of FA disassembly corresponds to regions with microtubules in close proximity to FAs (Efimov et al., 2008; Kaverina et al., 1999; Rid et al., 2005; Waterman-Storer et al., 1999).

A number of observations point to the role of microtubules in controlling endocytosis, which contributes to FA disassembly (Chao and Kunz, 2009; Ezratty et al., 2009, 2005). During nocodazole washout-induced regrowth of microtubules, clathrin-mediated endocytosis is involved in FA disassembly (Ezratty et al., 2005), suggesting that microtubules targeting FAs can directly trigger endocytosis. More recent studies strongly suggest a direct role of microtubules in endocytosis. By using quantitative proteomics, two studies have shown that the protein MAP4K4 binds to EB2 (also known as MAPRE2) at microtubule plus-ends to promote integrin internalisation (Gu et al., 2011; Margadant et al.,

2011). The recruitment of MAP4K4 near FAs appears to also involve the cytoskeletal linker ACF7 (Ning et al., 2016; Yue et al., 2014). Moreover, localisation of CLASPs near FAs facilitates FA disassembly (Stehbens et al., 2014). In addition, microtubules can also trigger endocytosis in an indirect manner. Stehbens et al. have shown that the matrix metalloprotease MT1-MMP is exocytosed in the vicinity of FAs through CLASP-stabilised microtubule tracks, thereby promoting ECM degradation and facilitating integrin internalisation (Stehbens et al., 2014).

Podosomes are also influenced by microtubules; in particular, acetylated microtubules are known to regulate podosome patterning. More generally, proteins, such as Cbl, Cbl-b and Pyk2, which help stabilise microtubules, promote podosome formation (Gil-Henn et al., 2007; Purev et al., 2009). Acetylation of tubulin, which characterises stable microtubules, affects the distribution of the kinesin KIF1C and thereby controls the delivery of matrix proteases and podosome activity in macrophages (Bhuwania et al., 2014; Castro-Castro et al., 2012). In addition to KIF1C, KIF9 also contributes to the matrix-degrading capacity of macrophages and is thus likely to participate in the role of microtubules in stabilising podosomes (Cornfine et al., 2011). The contribution of microtubules to the regulation of podosomes is confirmed by the fact that microtubule plus-end proteins CLASP and EB1 have been shown to be involved in podosome maturation, possibly by promoting microtubule-mediated podosome belt stability (Biosse Duplan et al., 2014).

Thus, the microtubule-mediated transport of cargoes to and from FAs is now regarded as a pivotal process in the regulation of FAs. The transport of this wide range of molecules by microtubules also raises speculations of several different roles for microtubules at the cortex, other than the regulation of FAs.

Meddling with the FA–actin duo

It has become increasingly evident over the years that the crosstalk between microtubules and actin is crucial in regulating FA dynamics, cell adhesion and migration. Key proteins involved in this tripartite crosstalk are the family of RhoGTPases, which are well known for their role in regulating actin and microtubules, as well as FAs (Fig. 3A,B). RhoGTPases act as molecular switches that cycle between a GDP-bound inactive form and a GTP-bound active form. RhoGTPases bound to GTP can interact with downstream effectors and trigger signalling cascades that regulate various cellular processes (Lawson and Ridley, 2018). Guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP, whereas, GTPase-activating proteins (GAPs) catalyse the hydrolysis of GTP to GDP.

All the evidence converges towards a role for polymerising microtubules in the activation of the Rho family member Rac, which in turns promote actin polymerisation, membrane protrusion and FA turnover (Fig. 3A) (Stehbens and Wittmann, 2012; Waterman-Storer et al., 1999). Microtubule regrowth after nocodazole washout activates Rac at the leading edge of the cell to promote actin polymerisation and membrane protrusion (Waterman-Storer et al., 1999). During microtubule regrowth, the Rac GEF Sif and TIAM1-like exchange factor (STEF, also known as TIAM2) activates Rac1 (Fig. 3A). Accordingly, STEF-depleted cells exhibit impaired FA disassembly and decreased Rac1 activity (Rooney et al., 2010). Furthermore, microtubule regrowth after nocodazole washout in STEF-depleted cells, does not result in Rac1 activation, suggesting a role for the RacGEF in microtubule regrowth-induced FA disassembly (Rooney et al., 2010). Another Rac1-specific GEF, Asef, binds to APC, which associates with microtubule plus-ends at the leading edge of migrating cells. In MDCK cells, this interaction between APC and Asef activates the GEF and might be important in inducing local Rac1 activity to promote the formation of protrusions (Kawasaki et al., 2000) (Fig. 3A).

In contrast to the role of growing microtubules in Rac-mediated actin polymerisation, depolymerising microtubules activate Rho to increase acto-myosin contractility (Fig. 3B). Activation of Rho leads to the formation of stress fibres and, in turn, facilitates FA maturation (Fig. 3B) (Ezratty et al., 2005; Liu et al., 1998; Ren et al., 1999). The RhoA-specific GEF p190RhoGEF (also known as ARHGEF28) interacts with microtubules and might play a role in microtubule-dependent FA turnover (van Horck et al., 2001).

Another RhoGEF, named GEF-H1 (also known as ARHGEF2 or Lfc) has emerged as a key mediator of the effects of microtubules on Rho activity, acto-myosin contractility and FA maturation. When inactive, GEF-H1 associates with microtubules. Upon release from microtubules, GEF-H1 activates Rho in the cytoplasm. This, in turn, contributes to increased acto-myosin contractility, similar to what is observed upon nocodazole-induced microtubule depolymerisation (Chang et al., 2008; Krendel et al., 2002; Ren et al., 1998) (Fig. 3B). Expression of a mutated form of GEF-H1 that cannot bind to microtubules displays similar effects with regard to RhoA activation and cell contractility (Krendel et al., 2002). How the GEF-H1 interaction with microtubules is regulated is still a matter of debate. Uncoupling microtubules from FAs, by depleting KANKs, releases GEF-H1 from microtubules (Rafiq et al., 2019), which indicates that anchoring microtubules at FAs inhibits GEF-H1 and may act as a negative-feedback loop to prevent excessive acto-myosin-mediated forces at FAs. Signalling cascades involving the serine threonine kinase Par1b/MARK2, as well as p21-activated-kinase 4 (PAK4), phosphorylate GEF-H1, which promotes its release from microtubules (Callow et al., 2005; Yoshimura and Miki, 2011). Microtubule-mediated regulation of GEF-H1 might also involve its association with the dynein light chain protein Tctex-1, which directly binds to GEF-H1 and inhibits its activity (Meiri et al., 2012). Moreover, activation of GEF-H1 is triggered by force-induced integrin stimulation through a mechanism that involves phosphorylation of GEF-H1 by extracellular signal–regulated kinases (ERKs) (Guilluy et al., 2011). Once released from microtubules, GEF-H1 activates protein kinase D (PKD), a serine/threonine kinase localised at the trans-Golgi, which stimulates Rab6 delivery to FAs (Eisler et al., 2018), thus hinting at an additional role of GEF-H1 in the vesicular traffic along FA-associated microtubules. Taken together, all these studies suggest that a crucial crosstalk exists between the actin and microtubule networks for the spatio-temporal regulation of RhoGTPases and microtubule-dependent FA dynamics.

Conclusions and perspectives

The role of microtubules in the regulation of FAs and, in particular, the control of FA turnover has become indisputable. Surprisingly, the molecular basis of the interaction between microtubules and FAs still remains obscure, but it appears to be indirect and mediated through the recruitment of large molecular complexes, whose regulation also needs to be further explored. Differences in the molecular machineries involved in microtubule capture and anchoring are likely to be responsible for the apparent discrepancy in both the localisation of microtubules at or surrounding FAs and the role of microtubules in promoting assembly and disassembly of FAs. Apart from the classical role of microtubules in vesicular trafficking, it is now also clear that microtubules can affect FAs through both the transport and

regulation of signalling molecules, such as RhoGTPases. Moreover, microtubules indirectly impact FAs by modulating actin dynamics and acto-myosin contractility. In addition to microtubule-mediated regulation of FAs and actin, which is achieved through RhoGTPases, there exists a feedback mechanism, whereby RhoGTPase activity, which is regulated by integrin signalling, alters microtubule stabilisation, organisation and dynamics (Wojnacki et al., 2014). This crosstalk highlights the necessity of systematically considering the potential impact of microtubules on acto-myosin-mediated cell adhesion and migration. FAs have emerged as major mechanosensitive structures, which help the cell sense extracellular cues to alter the cytoskeleton and force transmission (Jansen et al., 2017). Given that microtubules function together with FAs to regulate cell migration, it will be interesting to explore the effect of mechanosensing at FAs on microtubule structure and organisation, as well as their capture, anchorage and stability. The role of microtubules in the crosstalk between mechanosensing, FA and actin regulation, together with the emerging idea that cancer cell motility is strongly affected by the physical properties of their microenvironment, points to microtubules as potential targets to prevent cancer cell invasion and metastasis.

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