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ABSTRACT

How cells move is a fundamental biological question. The directionality of adherent migrating cells depends on the assembly and disassembly (turnover) of focal adhesions (FAs). FAs are micron-sized actin-based structures that link cells to the extracellular matrix. Traditionally, microtubules have been considered key to triggering FA turnover. Through the years, advancements in biochemistry, biophysics, and bioimaging tools have been invaluable for many research groups to unravel a variety of mechanisms and molecular players that contribute to FA turnover, beyond microtubules. Here we discuss recent discoveries of key molecular players that affect the dynamics and organization of the actin cytoskeleton to enable timely FA turnover and consequently proper directed cell migration.

INTRODUCTION

Directionality of adherent cells mostly depends on tight coordination of membrane protrusions at the leading edge, assembly of adhesive points known as focal adhesions (FAs), contraction of the cell body, and disassembly of mature FAs [1–3]. Decades of investigations have revealed that FAs are composed of a large repertoire of proteins that organize at the nanoscale level in three interconnected layers running parallel to the cell membrane, rather than in three independent layers [4–7]. Such FA organization starts with the association of integrin transmembrane receptors connecting the extracellular matrix (ECM) to the plasma membrane [8,9] (Figure1). Integrins bind to adaptor proteins, including the focal adhesion kinase (FAK) and paxillin which altogether compose the bottom or signaling layer. On top of those, forming the core or force transduction layer, lie vinculin and talin which link the actin cytoskeleton to the ECM [4–6,10,11]. Zyxin, vasodilator-stimulated phosphoprotein (VASP), and α-actinin, among other proteins are enriched at the top together with actin filaments (F-actin), forming the so-called actin regulatory layer [5,6,9,12–14] (Figure1). This complex nanoarchitecture of the FAs is assembled at the leading edge of migrating cells within minutes. Nascent adhesions grow in size and develop into focal complexes that will fully mature, then will disassemble, and eventually will reassemble (Figure1). Those association and dissociation cycles are key to permitting efficient and persistent motility [3,8,15]. The precise spatial and temporal control of FA turnover is achieved by multiple mechanisms, including changes in the cytoskeletal dynamics, the coordination of signaling pathways and trafficking of integrins [1,3,10,15,16].

In this review we highlight what we have learnt and what the puzzling pieces are related to two main questions:

(i) Which actin nucleation and elongation factors assemble actin filaments at FAs? How do they control FA turnover?
(ii) How does actin at FAs interplay with other components of the cytoskeleton such as microtubules and septins to control FA turnover?

Actin nucleation and elongation factors at focal adhesions

Several actin nucleation promoting factors have been found in close proximity to or at FAs, including the actin-related protein 2/3 (Arp2/3), formins, Ena/VASP, and the tumor suppressor Adenomatous polyposis coli (APC) (Figure 1).

The Arp2/3 complex nucleates branched actin filaments upon stimulation of the Wiskott–Aldrich syndrome protein (WASP)/Scar and WAVE (WASP family veroprolin-homologue), and this actin is coupled to integrin-mediated adhesion [17–19]. Depletion of the Scar/WAVE regulatory complex leads to FAK activation that results in fewer but more stable FAs [20]. Similarly, fewer FAs with reduced disassembly rates and longer lifetimes were found in a mouse embryonic fibroblast (MEF) model depleted of *Nckap1*, a conserved subunit of the WAVE regulatory complex [21]. The Arp2/3 complex can interact transiently with vinculin and/or FAK, and the 'hybrid' Arp2/3 complex is recruited to FAs [22–25]. For instance, Arp2/3-vinculin increases the levels of the Arp2/3 at FAs where it promotes FA formation [22,24]. The Arp2/3-FAK interaction [23] recruits Arp2/3 to the nascent adhesions where it 'tempers' the phosphorylation on Y397 of FAK, thereby reducing the formation of FAK-dependent nascent adhesions [23,25]. In addition, the formation of nascent adhesions at the leading edge is facilitated by the actin-binding protein lamellipodin (Lpd). Ldp has not been localized at FAs. Then, its effects on adhesions may be indirect, perhaps in part due to its interaction with the small GTPase Rac1 or WAVE regulatory complex, both of which associate with adhesions [26,27].

The Diaphanous-related formin actin nucleator, Dia1 (also known as mDia, DIAPH1 or DRF1) was reported to localize to FAs in mouse embryonic fibroblasts (MEFs) using fluorescent protein fusions [28]. Dia1 mediates actin polymerization at FAs to facilitate the formation and elongation of dorsal stress fibers (SF) [29–31]. Dorsal SFs are non-contractile actin bundles located at the cell front which connect to the substrate via a FA at one end, but can reorganize and generate ventral SFs, which are thick bundles connected to FAs at both ends [29–31] (Figure 1). Dia1-driven actin polymerization activity promotes the formation and maturation of FAs via Rho-associated protein kinase (ROCK) activation [28,29,32]. Dia2 formin has not been specifically found at FAs [28,33]. However, reports showed that Dia2 nucleates actin to facilitate FA assembly in migrating PtK1 epithelial cells and in MEFs [28,33].

In vitro work showed that the human FH1/FH2 domain-containing protein 1 (FHOD1) formin does not *de novo* polymerize actin filaments, but it binds dynamically to and bundles existing actin filaments [34]. In contrast, cell biology data suggested that FHOD1 nucleates actin filaments and bundles them in vivo [34–36]. The controversy between in vitro and in vivo data was resolved by a later study which showed that purified human FHOD1 does not nucleate actin filaments from rabbit skeletal muscle actin, in agreement with [34]; but it is capable to nucleate actin from non-muscle actin, (e.g. from *Acanthamoeba* actin*)* [37]. FHOD1 is expressed in both muscle and non-muscle cells although its localization differs and that might be important for effectively carrying out its cellular functions [37]. In many cell lines, including fibroblasts or osteosarcoma cells, FHOD1 has been found localized as clusters at integrin sites [34–36]. Targeting of FHOD1 to adhesions depends on direct interaction with Src family kinases. Src-dependent phosphorylation at Y99 of FHOD1 seems to bring FHOD1 in close proximity to ROCK1 at the plasma membrane to enable downstream FHOD1 activation by ROCK1 phosphorylation [34–36]. Activation of FHOD1 might induce actin SFs that generates force to sustain integrin cluster growth/maturation of FAs [34–36]. In short, FHOD1 seems to share similar actin nucleation activity than other formins [37]. Discrepancy between studies in number of actin filament number and/or organization using FHOD1 depleted MEF cells could be due to differences in knockdown efficiency, endogenous FHOD1 expression levels or experimental growth conditions that influence mechanical response (e.g. soft/stiff substrate) [28,36].

The major driver that elongates SFs at FAs seems to be Dia1 [28]. Supporting this is the fact that actin polymerization at FAs occurs in pulses which are coincident with Dia1-driven actin activity, which is stimulated by SF tension and was proposed to help prevent SF damage [28]. Moreover, both Dia1 knockdown MEF cells and MEFs treated with the pan-formin inhibitor SMIFH2 resulted in 75% suppression of the rate of SF elongation in photobleaching experiments [28]. Pharmacological treatment with the Arp2/3 inhibitor, CK666, did not show any effect on the rate of SF elongation in MEF cells [28]. However, it could be that Dia1 may not be the sole actin nucleation factor contributing to that safeguard SF mechanism at FAs [28].

Proteins from the Ena/VASP family - Mena, VASP or Ens/VASP-like (Evl) - are linked to the actin cytoskeleton through their association with vinculin and are known as

drivers of actin filament elongation [5,38]. It is unknown if any member of the family contributes to the Dia1-mediated safety mechanism against mechanical damage. Something perhaps worth to investigate since Puleo and coworkers reported that Evl polymerizes actin at FAs to promote mechanosensing in order to reinforce maturation of FAs, and consequently promote directed motility [39]. Although this study showed that SMIFH2 had no effect on the motility of MCF7 cells, the authors acknowledged that some level of formin could remain active, masking any potential collaborative role between formins and Evl [39]. Systematic re-expression of the individual Ena/VASP proteins in a B16-F1 triple knockdown (Mena, VASP, Evl) showed a higher rescue in FA size and vinculin levels after re-introduction of Evl than Mena or VASP [39]. This could seem unexpected since cellular levels of Mena and VASP are higher than Evl levels in B16-F1 [39]. However, FRAP assays showed that Evl is more stably associated at FAs, which could explain its important function at FAs [40]. Since Mena and VASP still rescued to some extent the defects found in the triple knockdown [40], future experiments will reveal how they cooperate with Evl to capture actin filament barbed ends to regulate force at FAs.

Regarding FA disassembly, evidence shows that APC-driven actin nucleation activity promotes FA disassembly to facilitate directed cell migration, and this activity is inhibited by the microtubule end-binding protein EB1 [41–43]. Using as tool an APC mutant impaired in nucleating actin filaments (named APC-m4), it was shown that APC-driven actin nucleation is critical for sustaining levels, organization and dynamics of F-actin near/at FAs [41,42]. In addition, the APC-m4 mutant presented reduced density of FA components such as Zyxin and (phosphorylated)-Paxillin, -Src and -FAK [42]. That phenotype could be a consequence of the actin disorganization found in the APC-m4. Supporting this hypothesis is the fact that organized F-actin provides force that sculpts the molecular organization of integrins at focal adhesions which is critical for activating integrins to sense directional extracellular cues in fibroblasts and in leukocytes [12,13]. In addition, the FA phenotype found in APC-m4 cells agrees with an independent work linking actin-microtubules-Src at FAs [44]. Overexpression of MACF1/ACF7, a microtubule-actin crosslinker from the spectraplakin family, increases the levels of phosphorylated Src at FAs [44]. Phosphorylated Src binds to EB1 at FAs, resulting in less EB1 available to bind APC and promote FA turnover [44]. Together, it is tempting to propose that the arrival of MACF1 at FAs activates Src, dislodges EB1-

APC interaction and this relieves APC-mediated actin nucleation activity at FAs. The pool of actin filaments nucleated by APC may be key to organize/remodel F-actin at/near FAs which in turn may impact on the organization of FA components to facilitate timely disassembly. These scenarios are speculative, and others could be considered.

Actin interplay with other cytoskeletal components to control focal adhesion turnover

The speculative reduction in forces at/from FAs in the APC-m4 mutant could explain the FA's inability to sense and/or respond to captured microtubules and autophagosome delivery to promote timely FA disassembly [42]. Supporting this idea is the fact that forces within FAs may exert feedback on microtubules to complete FA turnover [45–47], and vicecersa: acetylated microtubules tune the mechanosensitivity of FAs to promote cell adhesion and migration of astrocytes [48].

Whilst there is some controversy on whether microtubules get captured at the vicinity or at the FAs, it is clear that microtubules play an important role in FA disassembly [15]. Pioneering live imaging experiments showed that microtubules use actin SFs as tracks to direct them to the vicinity of FAs [49]. Later works showed that microtubule guidance along SFs requires the crosslink protein MACF1/ACF7 to reach FAs [50,51]. Microtubules get captured and stabilized around FAs through their interaction with KANK1 and talin which in turn binds to actin [52,53]. Microtubules retract and regrow to contact several times FAs until FAs reach full maturation [15,49]. Fully mature FAs may be 'sensed somehow' by the microtubules which will deliver autophagosomes to the FA, and will concomitantly retract [54,55]. Autophagosomes contain LC3 and NBR1 that interact with Src-dependent phosphorylated Paxillin and Zyxin [55,56]. Those interactions activate signaling events that may be involved in the removal of phosphorylated FA components which may lead to their disintegration [56].

Another cytoskeletal element that interplays with actin to control FA maturation, is septin proteins. Mammalian septins are found as heterohexamers and heterooctamers that can self-assemble into septin filaments which are thought to be the functional form of septins in cells [57,58]. Knockdown of SEPT2 or SEPT9 in migrating MDCK cells led to a higher number of smaller FAs closer to the cell edge [59]. SEPT2 knockdown

cells showed that assembly and disassembly rates of nascent adhesions were the same as in control cells, but that the stabilization phase was abolished resulting in short-lived FAs that fail to mature [59]. SEPT2 knockdown in Cancer-Associated Fibroblasts (CAFs) was further reported to lead to fewer FAs [60], whereas SEPT2 or SEPT9 knockdown in murine and human melanoma cells led to a decrease in FA size [61]. How septins affect FA maturation and thus the lifetime of FAs is not clear, in particular since septins are largely excluded from FAs, as shown by immunostainings in NRK [62], MDCK [59] and U2OS cells [63]. The role of septins in FA stabilization is most likely due to their association with FA-anchored actin SFs. Dolat et al showed that septins at the leading lamella of migrating MDCK cells localize to the interface of transverse arc SFs and dorsal SFs, getting recruited to the distal ends of dorsal SFs after the assembly of nascent FAs [59]. SEPT2 knockdown in these cells resulted in a more disorganized lamellar actin meshwork, with less, shorter and more short-lived dorsal SFs [59]. SEPT2 knockdown in CAFs led to a depletion of actomyosin ventral SFs, which are otherwise heavily decorated by septins in wild-type CAFs [60], whereas SEPT7 and SEPT9 knockdown in melanoma cells also affected the generation of ventral actin SFs, which are also decorated by septins in wild-type cells [61]. SEPT9 knockdown in U2OS cells, or impairing septins from polymerizing altogether, compromised both septin-SF association and actomyosin ventral SF integrity, emphasizing an essential role for septin heterooctamers, but not heterohexamers, in generating or/and maintaining SFs [63].

Given the importance of septins in the generation and/or maintenance of FA-anchored SF and the fact that purified mammalian septin hexamers and octamers cross-link actin filaments into bundles [64,65], the current hypothesis is that septins, through their actin filament cross-linking activity, maintain the integrity and organization of SFs, which in turn are required for the stabilization and maturation of FAs [66]. Septins could also impact FA maturation indirectly by affecting the accumulation of mechanical tension on SFs; whether septins directly affect SF actomyosin contractility is an ongoing matter of investigation. In addition to, or independently from their actin filament cross-linking activity, septins could stabilize SFs and thus contribute to FA maturation by anchoring SFs at the plasma membrane. Purified mammalian septin hexamers bind lipid membranes [67–69], purified human septin octamers can simultaneously bind lipid membranes and actin filaments [63], thus providing actin-

membrane anchoring, whereas ventral SF-associated septins are closely opposed to the plasma membrane [63], supporting altogether such a scenario. The identification of the actin- and membrane-binding domains of septins will be key for dissecting their role in FA maturation. A third way in which septins could affect FA turnover is through their direct association with microtubules leading to the stabilization of the latter [70]. There is no experimental evidence supporting this specific scenario, but the availability of mutants for disrupting or enhancing septin-microtubule association now enables this hypothesis to be tested [70]. Of note, specific SEPT9 isoforms can have different functions, as reported for cancer cell migration [71]. How the long SEPT9 isoform i1, which binds both microtubules and actin SFs, and the long SEPT9 isoform i3, which binds only actin SFs, compare with respect to their impact on FA dynamics, has not been tested given that the SEPT9 knockdowns in the mentioned studies did not differentiate among the two isoforms. The identification of the actin-binding domain of septins will further enable to test whether potential septin-mediated actin-microtubule cross-talk is relevant for their role in FA turnover. Finally, a recent study identified a complex between SEPT2, SEPT9 and the Rho-GAP, ARHGAP4, which impacts FA assembly and disassembly by regulating integrin-b1 levels [72], proposing a yet distinct mechanism on how septins affect FA turnover.

Conclusion and future directions

Our understanding on how FAs turn over has rapidly expanded using recent technologies. For example, advanced biochemistry and bioimaging, biophysical modeling, and quantitative analysis have been used to unravel novel players and/or decipher mechanisms underlying the FA dynamics.

However, many observations remain puzzling, in particular how septins contribute to FA maturation. Given that septins can bind both SFs and microtubules in cells, it is tempting to speculate that septin-mediated SF-microtubule cross-talk interplays with FA dynamics. Comparing how the long SEPT9 isoforms i1 and i3 differentially impact FA turnover is a promising avenue of research for future studies aiming to test this hypothesis. Identifying actin- and membrane-binding domains on septins, and thus being able to decouple actin-specific contributions, will be also key for testing the relevance of such a hypothesis. Simultaneous live imaging of septins, actin SFs,

microtubules and FA components as FAs assemble, mature and turn over offer further promises to provide new insights into the precise time window where septins act, including the potential co-existence of septin-actin and septin-microtubule interactions.

Additional questions that remain open relate to how microtubules are captured at FAs, how microtubule plus ends trigger FA disassembly and whether microtubule-FA interactions interplay with a specific pool of actin filaments or specific septin isoforms. Development of multi-wavelength imaging of photo-switched proteins, expressed at endogenous levels, at high spatial and temporal resolution are crucial. Real-time and superresolution microscopy using separation-of-function mutants and/or optogenetic switches will offer a powerful opportunity to address the dynamics, molecular organization, and interplay between the numerous players involved in FA turnover. Future research may provide a holistic model of the choreographed program that drives FA turnover and consequently directed migration in physiological and pathological environments.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

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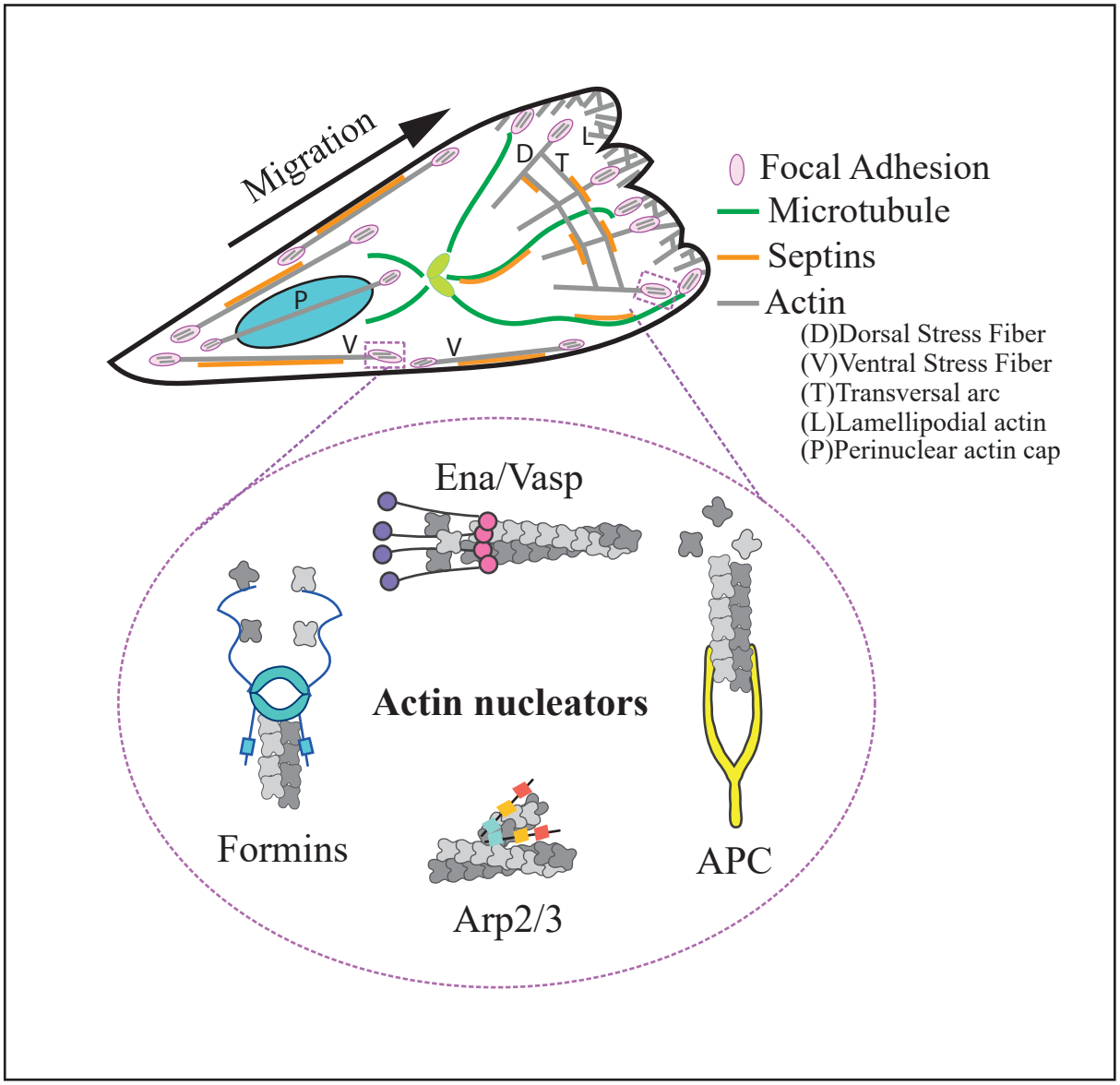
Migration
D
T
L
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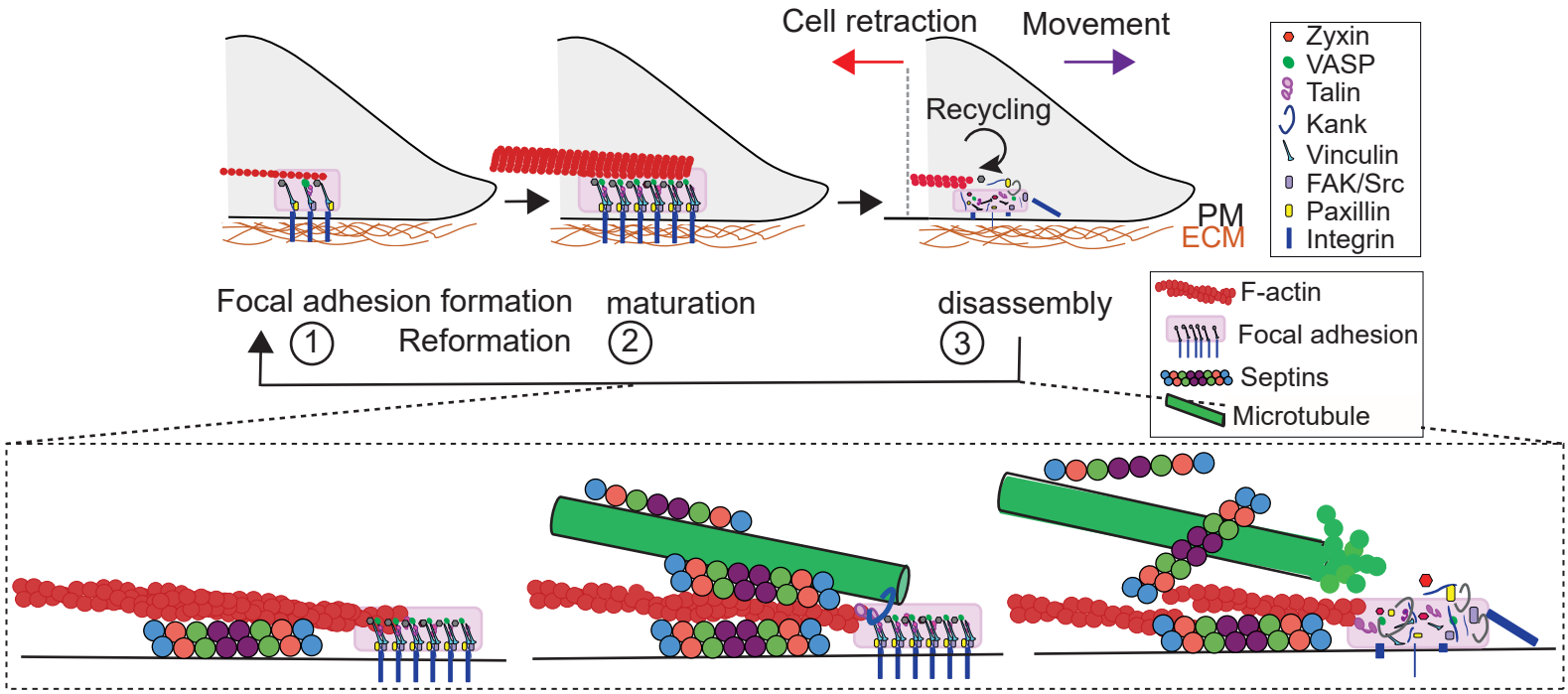
V
Focal Adhesion
Microtubule
Septins
Actin
(D)Dorsal Stress Fiber
(V)Ventral Stress Fiber
(T)Transversal arc
(L)Lamellipodial actin
(P)Perinuclear actin cap
Ena/Vasp
Actin nucleators
Formins
Arp2/3
APC


Cell retraction
Movement
Recycling
PM
ECM
Zyxin
VASP
Talin
Kank
Vinculin
FAK/Src
Paxillin
Integrin
Focal adhesion formation
Reformation
1
maturation
2
disassembly
3
F-actin
Focal adhesion
Septins
Microtubule