On the Rho'd: The regulation of membrane protrusions by Rho-GTPases

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Abstract

Cell migration entails the formation of cellular protrusions such as lamellipodia or filopodia, the growth of which is powered by the polymerisation of actin filaments abutting the plasma membrane. Specific Rho-GTPase subfamilies are able to drive different types of protrusions. However, significant crosstalk between Rho-family members and the interplay of distinct Rho-effectors regulating or modulating actin reorganization in protrusions complicate the picture of how precisely they are initiated and maintained. Here, we briefly sketch our current knowledge on structure and dynamics of different protrusions as well as their regulation by Rho-GTPases. We also comment on topical, unresolved controversies in the field, with special emphasis on the interrelation of different protrusion types, and on the composition of the nanomachineries driving them.

Introduction

Cell motility is a fundamental feature of multi-cellular life, and formation of distinct actin-based membrane protrusions such as lamellipodia and ruffles or filopodia are at the core of this process [1] (see Figure 1). Initiation and turnover of these dynamic structures is intimately linked to assembly/disassembly cycles of actin filaments, which are thus tightly regulated in cells. Small GTPases of the Rho-family are key molecular switches regulating the formation of cellular protrusions [2]. As all small GTPases, they cycle between an active, GTP-bound, and an inactive, GDP-bound, state. The transition between the two states is catalysed by GTPase-activating proteins (GAPs) that accelerate the hydrolysis of bound GTP [3] and guanine nucleotide exchange factors (GEFs) that substitute GDP for GTP [4]. Rho-GTPases interact with their effectors mostly in their GTP-bound states, thereby relaying incoming signals to downstream signalling pathways, driving for instance membrane protrusion.

Here, we focus on discussing our current knowledge of signalling pathways to actin polymerisation in membrane protrusions, which are effected by Rho-GTPases of the Rac and Cdc42 or Rif subfamilies. The dynamic turnover of actin filaments is regulated in multiple ways, including factors driving nucleation or elongation, blocking filament growth (capping), mediating crosslinking/bundling or promoting depolymerisation [5-7]. All these activities appear to co-operate in cells allowing expansion, withdrawal and/or steady-state turnover of the plasma membrane protrusions mentioned above. We will attempt to specify the established factors behind these activities, including how they link to a given GTPase or pathway, and to pinpoint apparent gaps in our understanding of the molecular mechanisms of protrusion formation.

The lamellipodium – structure and function

Five decades ago scientists started to routinely analyse moving fibroblasts using light microscopy techniques. Vernon M. Ingram and Michael Abercrombie were among the first to systematically analyse the "leading edge" of motile cells [8,9]. The thin front part of the leading edge was termed "lamellipodium" as it stayed parallel to the substrate. When it detached from the substrate resulting in a crimping plasma membrane portion reaching vertically into the medium, it was referred to as "ruffling lamella" or "membrane ruffle" [10].

The front region of motile cells has subsequently been separated into the distal lamellipodium and the more proximal lamella (reviewed in [11]), although the analysis of actin speckles at the cell periphery has recently obscured this nomenclature [12,13]. Although not essential perhaps for all types of migration, and in all cell types or conditions (see also below), the lamellipodium still constitutes the motile organelle indispensable for and most effective in promoting the movement of rapidly migrating cells. Thus, in traditional terms, the lamellipodium covers a distal 1-5 μm (depending on cell type) region, which consists of a flat (0.1-0.2 μm in thickness) membrane-enclosed and highly dynamic leaflet of cytoplasm, passing into the longer and more voluminous lamella behind (Figure 1).

The lamellipodium is composed of a polar array of actin filaments, with their fast-growing ends abutting the plasma membrane [14]. By the 1990s, lamellipodium protrusion was generally established to be driven by polymerisation of the actin filament network, although the precise architecture of the network and the molecular mechanisms of its assembly have been matter of controversy (reviewed in [1,7,15]).

An interesting discussion concerning the dynamics of actin filament networks at the cell periphery has arisen from employment of different fluorescent microscopy techniques, such as speckle microscopy [16,17], photoactivation [18] or FRAP, fluorescent recovery after photobleaching [19,20]. Fluorescent speckles are derived from inhomogeneous incorporation of low amounts of fluorescent actin monomer, and their dynamics has been systematically

assessed by mathematical algorithms [21]. Interestingly, the majority of actin speckles that had emerged in the lamellipodium were observed to travel rearwards with the network more rapidly than those in the lamella [13]. The reclassification of the lamellipodium and the lamella based on the kinetics of these speckles has led to the conclusion that both actin-based networks overlap at the cell periphery, with lamella filaments extending into the very cell periphery within or even below the lamellipodial network ([12,13,15], reviewed in [22]). However, recent work has provided an alternative explanation of variable speckle kinetics within lamellipodia, based on the observation that lamellipodial filaments subtend a wide distribution of angles to the cell front, capable of accommodating to changes in protrusion rate, and revealing remarkable flexibility of different filament populations concerning individual assembly rates [23]. Thus, speckles that are slowly moving rearwards in the lamellipodium mesh are likely derived from actin incorporated into filaments with shallow angles relative to the front.

Together, the lamellipodium can be envisioned as a treadmilling network of flexible and crosslinked actin filaments (Figure 1), which is growing in front and not on top of the lamella. Lamellipodial filaments polymerise at the interface between their growing ends and the protruding plasma membrane, with their turnover being balanced by nucleation and capping at the front, and disassembly from the rear [19].

Regulation of lamellipodium protrusion by Rac GTPases

In the second half of the 1980s, small intracellular proteins with a molecular mass of 20-25 kDa exhibiting intrinsic GTPase activity were identified. They turned out to be members of a large superfamily of related small GTPases known as Ras proteins. In the following years, homologous proteins called Rac, and grouped into a larger family termed Rho (for Ras homology) have emerged as key molecules in stimulating the protrusion of lamellipodia and membrane ruffles [2,24,25]. The Rac subfamily of Rho-GTPases comprises four members, Rac1, Rac2, Rac3 and RhoG, showing a sequence identity of between 92% (Rac1 and Rac3) and 72% (Rac1 and RhoG) [25,26].

In 1992, microinjection of purified, constitutively active Rac1 was for the first time shown to induce the formation of lamellipodia and prominent membrane ruffles covering the entire cell surface [25]. This observation revealed the functional significance of this GTPase for actin cytoskeleton rearrangement and changes of cell morphology. The key role for Rac1 in tissue homeostasis and embryonic development was demonstrated by the fact that Rac1 knockout mice are embryonic lethal due to defects in formation of the three germ layers at gastrulation [27]. Multiple studies concerning actin remodelling by Rac subfamily GTPases were carried out subsequently, but Pontus Aspenström compared their activities and those of all other Rho-GTPases by ectopic expression in the same cell type. Interestingly, all members of the Rac subfamily triggered the formation of lamellipodia, and constitutively active Cdc42 and TCL, two members of the Cdc42 subfamily, induced lamellipodia as well, albeit smaller in appearance than those observed upon expression of Rac GTPases. Lamellipodia induction by Cdc42 subfamily proteins was presumed to be caused by indirect activation of Rac, as described previously (Figure 2) [2,28]. All these GTPases, except for RhoG, were also reported to induce the formation of bundles of actin filaments, which were most pronounced in cells expressing constitutively active Rac1, Rac2 and Cdc42 [24], indicating that GTPases

of the Rac and Cdc42 subfamilies have both specific and overlapping functions in signalling to actin remodelling, as confirmed by others (see e.g. [29,30,31]).

Several recent studies made use of conditional inactivation of *rac1* alleles in vitro. Removal of the gene in primary fibroblasts caused dramatic changes in actin cytoskeleton reorganization and cell morphology [32,33]. These data constituted an impressive confirmation of the key function of Rac1 in driving the formation of lamellipodia and ruffles (Figure 2). More surprisingly, spreading and migration in wound-healing assays of Rac1-deficient fibroblasts were less severely compromised than one might have anticipated [33]. However, since Rac1 removal in these cells by Cre-recombinase reflected a terminal treatment due to viability of treated cells of only approximately two weeks, remnants of protein in individual cells might have affected the final phenotypes. Nevertheless, the efficiency of Rac reduction was convincingly strong, indicating that Rac GTPases are helpful but not essential for migration and spreading. This conclusion is consistent with the observation that Rac GTPases are also not essential for chemotaxis and migration of macrophages [31]. Interestingly, Rac1-deficient fibroblasts as analysed by Vidali et al. (2006) formed filopodia in the absence of apparent lamellipodia, indicating that the presence of lamellipodial filaments is not a prerequisite for the formation of filopodia (see also below).

The expression of Rac2 is restricted to the haematopoietic system [34]. Rac2 regulates various signal transduction pathways in neutrophils, and its deletion or inactivation compromised cell migration and adhesion in different haematopoietic cell types, except for macrophages [26,31,35]. Interestingly, double deletion of Rac1 and Rac2 in macrophages resulted in even elevated migration as compared to the individual knockouts or wild-type cells alone, caused by an erratic type of migration based on cycles of spreading and rounding. Moreover, Rac2-deficient macrophages did show impaired migration on laminin, revealing that Rac proteins are required for effective migration under certain conditions even in macrophages, but this effect was abolished by additional removal of Rac1 [31]. Together, these data add to our

conviction that we are far from understanding both effector binding specificity by and the complexity of crosstalk between different members of the Rac subfamily.

Rac3 is less well studied than Rac1 or 2, and highly expressed in the brain [36,37]. Rac3-deficient mice are viable but show behavioural abnormalities in spite of co-expression of Rac3 in neurons with Rac1 [38,39], again highlighting specificity of Rac-isoform-mediated signalling pathways. Along this line, a recent study using RNA interference even described opposing functions for Rac1 and Rac3 in the neuroblastoma cell line N1E-115. In this study, both isoforms appeared to be required for normal morphology, but antagonised each other concerning the promotion of spreading or rounding in this cell line. The contracted, rounded morphology induced by Rac3 was mediated by the C-terminal polybasic residues 185-187 of Rac3, which are at variance to Rac1, and display additional effector and GEF (guanine nucleotide exchange factor) binding sites [29]. Thus, it will be interesting to see how these observations can be reconciled by apparent lamellipodia formation induced by both isoforms in other cell types [24]. Collectively, all these data as well as previously published observations [40] call for systematic comparison of effector binding capabilities of Rac isoforms, and establishment of their relevance for actin reorganisations induced in different cell types.

RhoG, the fourth member of the Rac subfamily, is expressed in multiple adult tissues like lung, heart and brain, but also in lymphocytes [41-43]. RhoG is thought to induce actin rearrangements and membrane protrusion by Rac activation through the ELMO-Dock180 pathway. Binding to its direct effector ELMO enables RhoG to link to Rac activation by the guanine nucleotide exchange factor Dock180, thought to result in the formation of lamellipodial protrusions [44-47]. Exciting recent publications indicated that this signalling cascade also plays an important role in host-pathogen interaction. Bacterial and other pathogens frequently hijack host cell signalling pathways to utilize them for their own benefit [48,49]. Bacterial effector proteins translocated into the host cytosol by *Shigella flexneri* and

other species were identified to functionally mimic several Rho GTPases [50]. For instance, the invasion plasmid antigen B1 (IpgB1) of *Shigella*, which induces the protrusion of prominent lamellipodia and was therefore originally considered a Rac1 mimic, is thought to activate Rac1 in a RhoG-like manner through its direct interaction with the ELMO-Dock180 complex ([51, reviewed in [52]). Certainty about whether RhoG (and its mimic IpgB1) is indeed unable to signal to actin reorganization in the absence of Rac1 may only come from confirmation of this issue in cells genetically depleted for this GTPase.

The machinery driving lamellipodia/peripheral ruffles

Due to the lack of direct interaction of Rac proteins with actin, additional molecules are required to relay external stimuli to actin cytoskeleton reorganization [2,53]. Multiple pathways, mediated by interaction of Rac GTPases with so-called effector proteins have been described, which are considered to drive the rearrangements of the actin cytoskeleton culminating in the formation of ruffles and lamellipodia. The proposed mechanisms include engagement of actin filament nucleation factors, e.g. Arp2/3-complex, but also of capping proteins like gelsolin [54] or the actin depolymerising factor ADF/cofilin (for a recent review see [55]). Future efforts should thus also aim at clarifying the relative relevance of all these pathways both in initiation and maintenance of these protrusive structures. A continuing challenge will be to juggle biochemical and cell biological observations, for instance indirect cofilin inactivation effected by Rac through the PAK/Lim-kinase pathway [56,57] with the established positive function of cofilin in lamellipodium protrusion and turnover [58-60] or actin-based motility in general (reviewed in [5]). Such discrepancies may be explained, at least in part, by considering spatial aspects of the function of a given protein *in vivo* [61].

Besides ADF/cofilin, two other factors are established as indispensable for lamellipodium protrusion, at least as suggested by RNAi studies, heterodimeric capping protein and Arp2/3-complex [62,63]. Interestingly, knockdown of capping protein not only abolishes lamellipodia

(at the expense of filopodia), but also removes Arp2/3-complex from the cell periphery [16,62] normally accumulating in the lamellipodia of non-treated cells [64], raising the issue as to whether the latter observation is cause or consequence of the loss of lamellipodia. The pathway studied most extensively in recent years is signalling by Rac1 to Arp2/3-complex-mediated actin assembly (Figure 2). The biochemical activity of this complex comprising two actin-related and five accessory proteins is most prominently characterized by its ability to amplify the numbers of fast-growing (barbed) actin filament ends through branching (for recent reviews see [5,6,64]).

Actin assembly by Arp2/3-complex is activated by a family of proteins comprising the name-giving Wiskott-Aldrich syndrome protein (WASP), neural WASP (N-WASP) and three WASP family verprolin-homologous (WAVE) proteins, also called Scar in *Dictyostelium discoideum* [65,66]. Since they stimulate actin filament nucleation by Arp2/3-complex, these proteins are also called nucleation promoting factors (NPFs) type I, to be distinguished from a second class, type II NPFs (reviewed in [67]), comprising cortactin and the haematopoietic HS1 in mammals [68], and promoting Arp2/3-mediated actin assembly by a distinct mechanism.

WASP/WAVE family proteins are established to drive Arp2/3-complex activation *in vivo* in all eukaryotes, but evidence for an essential role in the formation of Rac1-induced lamellipodia and ruffles has only been convincing for WAVE subfamily members. Cortactin also prominently targets to lamellipodia, where it co-localizes with Arp2/3-complex, but its function in these structures is controversial [68], and comparison of its turnover in lamellipodia with that of Arp2/3-complex suggests its activity to be largely uncoupled from Arp2/3-complex activation [19]. Mammals express three WAVE isoforms, WAVE1, 2 and 3. Both WAVE1 and 2 are ubiquitous, but WAVE2 is more abundant in most tissues except for the brain. WAVE3 is largely restricted to the adult brain, at least in the mouse. Consistently,

wave2 knockout in this system is embryonic lethal and removal of the *wave1* gene was reported to cause strong, but more variable postnatal phenotypes [69,70].

Most relevant for the topic discussed here, WAVE2-deficient fibroblasts were defective in migration, spreading and ruffle or lamellipodia formation [71,72]. However, both WAVE3 and WAVE1, which was the first isoform shown to accumulate at the tips of lamellipodia [73], have also been implicated in contributing to actin assembly in these structures [74,75], suggesting functional redundancy for WAVE isoforms in lamellipodia protrusion, unlike previous suggestions ([76], see also below). As opposed to WASP and N-WASP; which are direct effectors of Rho-GTPases like Cdc42 (see also below), WAVE proteins lack an interaction surface with Rho-GTPases, so the link from Rac1 to WAVE requires additional components.

The first protein suggested to exert this function was the insulin receptor tyrosine kinase substrate of 53 kDa (IRSp53), which can bind both Rac and WAVE2 ([77], for recent review on IRSp53 family proteins see [78]). Although this protein also co-localizes with WAVEs at the tips of both lamellipodia and filopodia, it cannot link Rac1 to WAVE2 in the absence of additional WAVE-associated proteins (see below and [79]). Instead, IRSp53 was ascribed an optimising role in lamellipodium protrusion more recently ([66], reviewed in [80]), although it might be more relevant concerning WAVE positioning at the cell periphery in specific cell types [81] or downstream of specific signalling pathways. This view would be supported for instance by the observation that IRSp53 binds to the Rac-GEF Tiam1 and appears relevant for Tiam1-induced membrane ruffling [82]. Whatever the case, our understanding of the precise functions of IRSp53 and its family members in the protrusion of both lamellipodia and filopodia (see also below), as well as the relevance of their interactions with multiple actin binding proteins will certainly benefit from analyses of individual and/or combined gene knockouts [78].

Recent activities in lamellipodia research have centred around a stable protein assembly now generally referred to as WAVE-complex [65,66]. The complex comprises five stably associated constituents, the specifically Rac1-associated protein 1 (Sra-1; or its isogene PIR121), Nap1, Abl interacting (Abi), WAVE, and a small peptide called HSPC300 [83,84]. Biochemical analysis of the architecture of the ubiquitous complex revealed the core of Abi-1 and Nap1 subunits surrounded by WAVE2 and HSPC300, both binding to Abi-1, and the more peripheral Sra-1 tightly associated with Nap1 [85]. Importantly, the link to Rho-GTPase signalling is achieved by direct interaction of Sra-1 with Rac1 [79,86]. The complex is conserved in eukaryotes including *Dictyostelium* amoebae, and its indispensable function in lamellipodium protrusion and ruffling, presumably through linking Rac to Arp2/3-complex-mediated actin assembly is established not only for vertebrate, but also e.g. *Drosophila* S2 cells (Figure 2) [60,79,84,87]. Although individual RNAi-mediated downregulation of each complex component causes a severe reduction of WAVE protein levels, the remaining components are not just required to assure proper translational regulation of WAVE expression (mRNA levels are not affected). Instead, the intact complex is required to link WAVE to Rac, for instance because re-expression of functional WAVE2 is not sufficient to restore Rac1-induced actin reorganization in Nap1 knockdown cells [79]. Pressing questions for future research - at least in mammals - include the relevance of WAVE-complex-mediated actin reorganization downstream of Rac subfamily GTPases other than Rac1, or the characterization of WAVE-complex-independent functions of individual components. For instance, Abi-1 is well known for its association with EGF receptor substrate 8 (Eps8) and son of sevenless-1 (Sos-1) into a Rac-GEF-complex [55]. Furthermore, an exciting interaction of Abi-1 with the formin mDia2 was recently proposed to be required for the accumulation of this actin nucleator in lamellipodia of B16-F1 mouse melanoma cells [88]. The described importance of the observed interaction and of this formin in lamellipodia protrusion awaits confirmation by additional methods and in other cell types (see also below). Finally, the

recently observed differential turnover of WAVE2 and Abi-1 at the tips of lamellipodia also strongly speaks for non-exclusive interactions of these binding partners at the tips of lamellipodia [19], and deserves more in depth characterization.

Circular dorsal ruffles

Dorsal ruffles are distinct circular membrane protrusions raising upright from the 'dorsal' surface of cells *in vitro* (Figure 1). This term was first used for highly dynamic, F-actin-rich structures observed upon treatment with platelet-derived growth factor (PDGF) in 1983 [89], although other factors like epidermal (EGF) or hepatocyte growth factor (HGF) can also induce them [90,91]. Within a few minutes of treatment, flat, wide open ring-shaped ruffles develop and lift up and contract within 5-30 minutes, finally forming a chimney-like silhouette before disappearing again ([89,92,93], our unpublished observations]). These structures are perfectly suited to mediate processes like macropinocytosis [91,94,95] or ligand-induced internalization of receptor tyrosine kinases, e.g. epidermal growth factor receptor (EGFR) ([reviewed in [96]).

Studies on the molecular mechanisms of formation of these structures have so far been much less systematic than what we have seen for "peripheral" ruffles and lamellipodia. However, terminal Rac1 knockout fibroblast populations obtained through Cre-recombinase-mediated gene disruption recently appeared to largely lack circular dorsal ruffles, strongly suggesting a key role for this GTPase in driving actin polymerisation in these structures [33]. These observations and the structural similarities to lamellipodia may suggest a pathway downstream of Rac1 comparable to lamellipodia. However, conflicting results have been obtained concerning the contributions of WAVE isoforms (WAVE 1 *versus* 2) or N-WASP. Original observations implicated WAVE2 in driving actin assembly in dorsal ruffles [72,97], but a parallel study suggested, instead, WAVE1 to drive dorsal and WAVE2 to mediate peripheral ruffling [76]. More recently, WAVE1 was clearly shown not to be essential for this

process by re-examination of dorsal ruffling in WAVE1-deficient fibroblasts, but the same authors were also unable to establish a role for WAVE2 [98]. Instead, they proposed a contribution to Arp2/3-complex-mediated actin assembly in these structures for N-WASP, although dorsal ruffles appeared only diminished but not abolished in N-WASP-deficient cells [98]. Another study suggested at least one pathway operating independently of Rac, which employs the trafficking GTPase Rab5 connecting to actin filaments and to the actin-crosslinking protein actinin-4 through the Rab5-GAP RN-tre [99]. Finally, phosphorylation of the type II NPF cortactin by non-receptor tyrosine kinases such as Abl and Arg (Abl-related gene) has also recently been implicated in PDGF-triggered dorsal ruffle formation [100]. Clearly, more work is required to clarify the relative relevance of all these pathways and in particular of Arp2/3-complex and its activators in the formation of circular dorsal ruffles.

Filopodia

As opposed to the criss-cross arrangement of actin filaments in lamellipodia [23], filopodia are relatively stiff rods filled with parallel bundles of actin filaments and protruding beyond the cell periphery (Figure 1). Bundles of actin filaments embedded into and polymerising with the lamellipodial mesh are also termed microspikes [1]. Filopodia have been implicated in functions as diverse as pathfinding and guidance towards chemo-attractants or cells, or probing the substrate for appropriate sites of adhesion [101]. In migrating cells, filopodia also contribute to the assembly of contractile structures in the lamella behind [102]. Although these dynamic structures are frequently formed in different motile systems, with explicit prominence e.g. in neuronal growth cones, they are not required for motility *per se*, since rapidly migrating cells such as the fish epidermal keratocyte lack them entirely [103]. The actin filaments in filopodia exclusively grow at their tips [104], and net forward movement depends on the balance between retrograde flow of filopodial filaments and rate of polymerisation. In depth reviews on the signalling pathways driving filopodia and the

potential molecular mechanisms of their formation have recently been published [101,105]. Interestingly, recent contradictory observations on the molecular mechanisms of filopodia formation have led investigators to propose specific types or classes of filopodia, with distinctive molecular requirements concerning their formation [101]. Clearly, induction of filopodia can occur downstream of multiple signalling pathways, but whether or not these signalling pathways all converge on the same core machinery of filopodial actin assembly still remains an open issue.

The seminal studies of Hall and co-workers employing microinjection of purified proteins and video microscopy established for the first time the prominent induction of filopodia by a Rho-family GTPase, Cdc42 (Figure 2) [28]. Surprisingly, today - more than a decade later - it is still unclear how Cdc42 induces these structures. The reasons for this can be summarized as follows: the discovery of direct interaction of Cdc42 with the Arp2/3-complex activator N-WASP provided a promising molecular mechanism linking Cdc42 activation to Arp2/3-dependent actin assembly [106]. However, a role for this potential N-WASP/Arp2/3-complex pathway in filopodia formation becomes increasingly unlikely [105]. First, neither N-WASP nor Arp2/3-complex prominently localize to filopodia ([107], our unpublished observations]). Second, genetic N-WASP removal does not abrogate Cdc42-induced filopodia formation [108]. Third, reduction of Arp2/3-complex expression by RNAi or functional inactivation of the complex in the cytosol does not interfere with filopodia formation [63,109]. Fourth, capping protein knockdown, which abrogates lamellipodia, but causes excess filopodia formation, is accompanied by complete removal of Arp2/3-complex from the cell periphery [62]. Thus, although the latter study was taken as supportive for the convergent elongation of lamellipodial filaments into filopodia (see below), it again demonstrates excessive filopodia formation in the absence of peripheral Arp2/3-complex accumulation.

How about alternative pathways? Interestingly, Cdc42 is now well established to bind to the formin mDia2 (also known as Drf3) [105,110]. Strikingly, this formin can accumulate at the

tips of filopodia, where actin polymerisation takes place [104], and its knockdown by RNA interference has recently been reported to abrogate filopodia formation in B16-F1 cells [88]. Moreover, genetic removal of the formin dDia2 in *Dictyostelium discoideum* also inhibited filopodia formation [111]. Together, it is not unreasonable to assume that, in mammals, activation of Cdc42 could employ mDia2 to drive actin polymerisation into filopodia, although formal prove for abolishment of this pathway by mDia2/Drf3 removal is lacking. Interestingly, the same formin was also implicated in filopodia formation triggered by Rif (Rho in filopodia) [112], so it bears the potential of more general functions than solely mediating Cdc42 signalling to actin assembly. Along these lines, Cdc42 is indeed not the only GTPase regulating filopodia, since Cdc42-deficiency did not abolish the formation of these structures [113], irrespective of observation of an apparently more severe phenotype in alternatively generated Cdc42-deficient cells [114]. Other members of the Cdc42 and Rif families, including Wrch-1 and Rho-D, respectively, have also been implicated in the formation of filopodia [24,115]. However, the effectors capable of driving or linking to actin polymerisation remain unknown.

Together, understanding of the precise molecular regulation of actin nucleation at filopodia tips requires additional studies. We should emphasize that polymerisation is just one issue. Other biochemical activities are also required, as evidenced by filopodia removal through RNAi in B16-F1 cells of the actin filament bundling protein fascin [116]. Additional biochemical activities currently discussed in the literature to potentially influence formation of these structures include outwards membrane deformation or spiral formation effected by IRSp53 family proteins [78,117], although the actin bundling activity of IRSp53, synergistically enhanced by Eps8, has also been implicated in promoting filopodia [118].

Co-existence of lamellipodia and filopodia

Both structures are frequently observed to form in co-incidence at the protrusive cell periphery. Indeed, filopodia/microspikes appear to frequently emerge from the meshwork of lamellipodial actin filaments, as prominently observed in movies of motile B16-F1 mouse melanoma cells [1,119]. The concept that filopodial filaments may thus originate from those generated in lamellipodia has a quite long tradition ([reviewed in [103,120]), and experienced a renaissance more recently as the "convergent elongation model" [119]. Recent data obtained upon Arp2/3-complex knockdown in neuronal cells again not only suggested defects in lamellipodia, but also in filopodia formation [121], unlike previous observations [63]. Surprisingly however, filopodia formation in the same study could be partially rescued with constitutively active Rac1, indicating signalling defects rather than a principal deficit in the core filopodial machinery operating in the absence of Arp2/3-complex. The fact that neither Rac1 deletion [33], interference with Arp2/3-complex or WAVE-complex function [63] nor capping protein knockdown, which also removed Arp2/3-complex from the cell periphery (see above, [62]), were reported to block filopodia formation strongly suggests lamellipodia and filopodia formation to be functionally separable. How can these observations be reconciled? Clearly, more experiments are required to resolve this issue, but constructive thoughts down this path might include consideration of the "real" origin of filopodial filaments. Can we exclude the possibility of *de novo* nucleation of filopodial precursors within the brush of lamellipodial filaments?

We are convinced that answers to these and additional pressing issues, as e.g. separation of the essential from modulating components engaged in the formation of the distinct protrusions downstream of the multiple GTPases mentioned above (see Figure 2), will be obtained in due time employing the toolbox of state-of-the-art genetic, imaging and biochemical methods available today.

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Figure legends

Figure 1. Schematic cell forming distinct plasma membrane protrusions discussed in this work. At the top, the main protrusive structures frequently found in migrating cells are depicted. Note the typical criss-cross arrangement of actin filaments building the lamellipodium [23], which assembles distal to the contractile actin network in the lamella behind [102]. Parallel bundles of actin filaments are found in the microspike embedded into the lamellipodium, or the filopodium protruding beyond the cell periphery (right). Lamellipodia can fold up- and backwards in a process termed ruffling, and can occur both as peripheral or circular dorsal ruffles (bottom), the latter of which are implicated into macropinocytosis (see text for details). The gradient of red intensity indicates concentration of dynamic actin filaments (F-actin) at the cell periphery, as indicated.

Figure 2. Signalling pathways addressed in this work. We propose that lamellipodium and filopodium protrusion is driven by Rac and Cdc42/Rif subfamilies, respectively, and by the effector pathways as indicated. mDia/Drf proteins designate family members of diaphanous related formins [122]. Proteins regulating these processes can be subdivided into different functional layers as indicated on the right, which are all essential for the output response (bottom). N-WASP, another prominent Arp2/3-complex activator and Cdc42 binding protein presumably operates in trafficking processes (not shown), but not in protrusion [65,108]. The precise function of IRSp53 is still controversial [78].

Figure 1

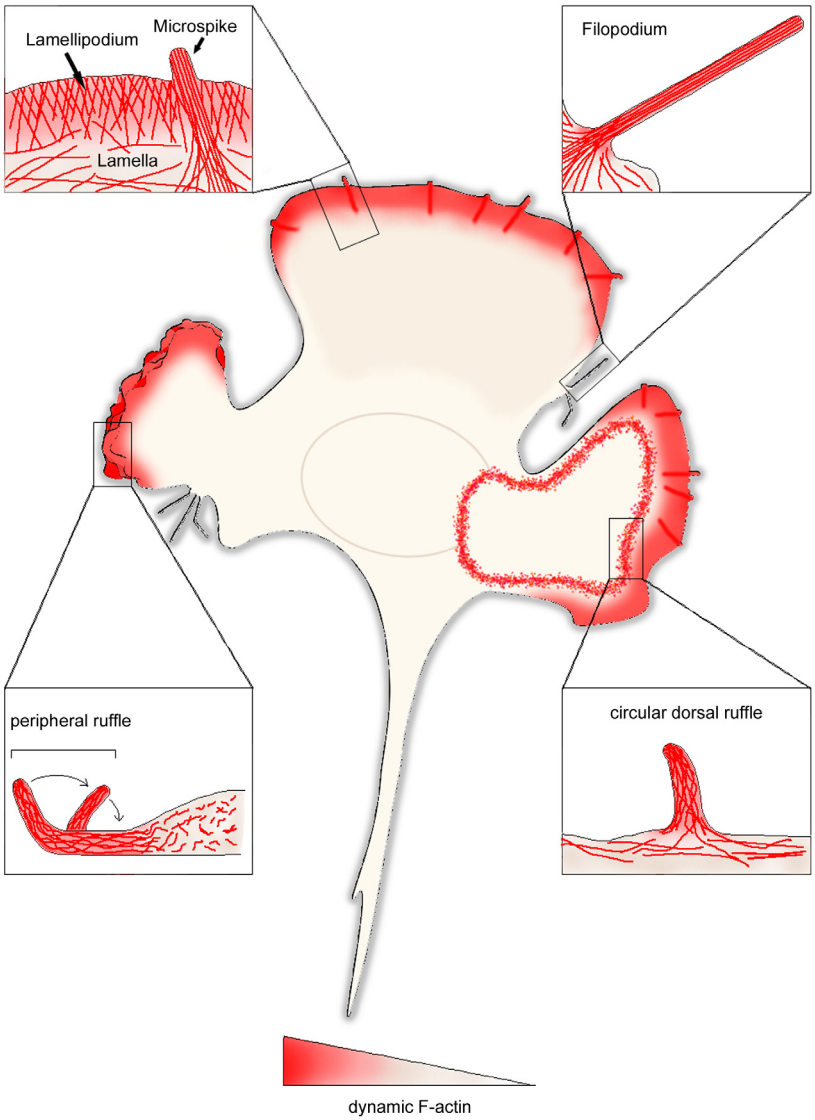
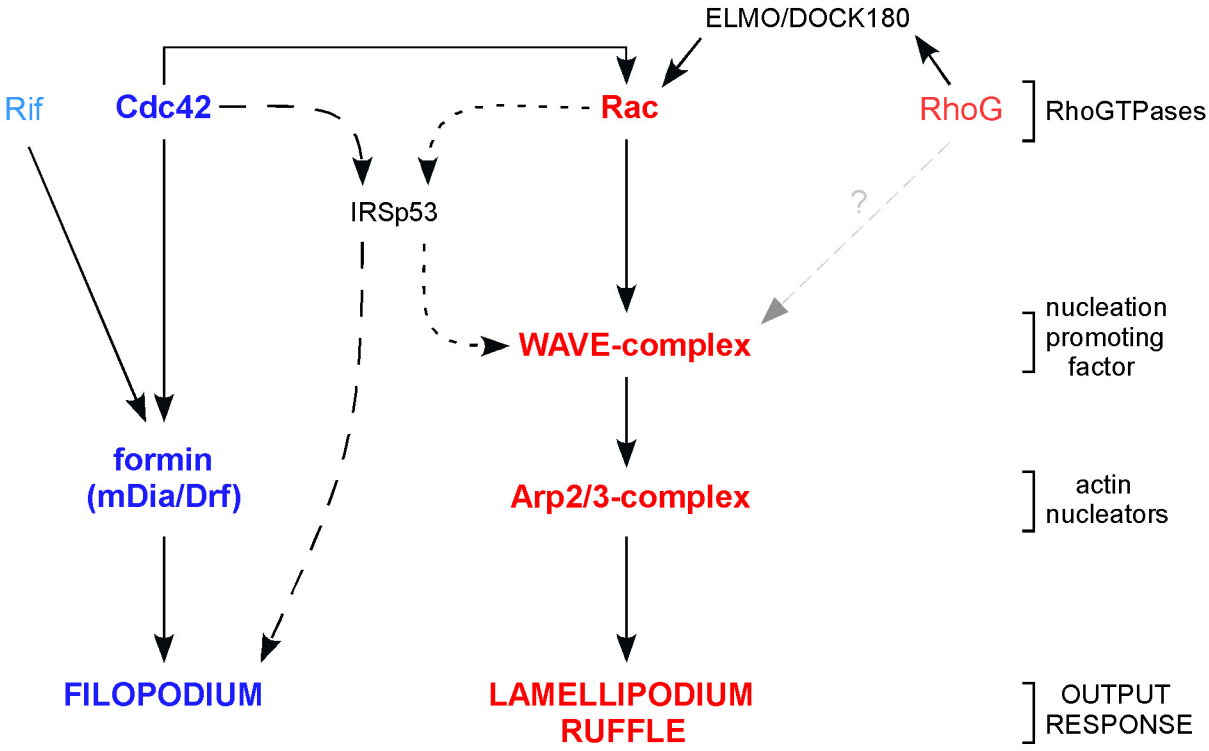

Lamellipodium
Microspike
Lamella
Filopodium
peripheral ruffle
circular dorsal ruffle
dynamic F-actin

Figure 2


ELMO/DOCK180
Rif
Cdc42
Rac
RhoG
RhoGTPases
IRSp53
?
WAVE-complex
nucleation promoting factor
formin (mDia/Drf)
Arp2/3-complex
actin nucleators
FILOPODIUM
LAMELLIPODIUM RUFFLE
OUTPUT RESPONSE