THE CO-WORKERS OF ACTIN FILAMENTS: FROM CELL STRUCTURES TO SIGNALS

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Cells have various surface architectures, which allow them to carry out different specialized functions. Actin microfilaments that are associated with the plasma membrane are important for generating these cell-surface specializations, and also provide the driving force for remodelling cell morphology and triggering new cell behaviour when the environment is modified. This phenomenon is achieved through a tight coupling between cell structure and signal transduction, a process that is modulated by the regulation of actin-binding proteins.

PHAGOCYTOSIS
An actin-dependent process by which cells engulf external particulate material by extension and fusion of pseudopods.

MICROVILLI
Small, finger-like projections (1–2 μm long and 100 nm wide) that occur on the exposed surfaces of epithelial cells to maximize the surface area.

STEREOCILIA
Tapered, finger-like projections from hair cells of the inner ear that respond to mechanical displacement with alterations in membrane potential, and thereby mediate sensory transduction.

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The integrity of the actin cytoskeleton is essential for cells to form and maintain their shape and structure. The remodelling of the cytoskeleton in dynamic cellular processes produces changes in cell shape and motility in response to external stimuli, and is therefore involved in signal transduction. These features of the actin cytoskeleton are regulated by a cohort of actin-binding proteins (ABPs), which were initially considered to be structural components that organize a stable actin cytoskeleton, but are now known to be regulators of cellular dynamics and key components of signalling processes.

Our increasing understanding of actin dynamics has revealed the biological complexity of cell behaviour and prompted us to investigate cell functions in a whole tissue or organ. For instance, actin dynamics are required for the absorptive function of the intestinal epithelium, for mechanosensing in the inner ear, for orientated nerve and capillary growth, and during defence processes that involve PHAGOCYTOSIS, migration and the activation of immunologically competent cells. The actin cytoskeleton is also essential for cell-motility events that are required for normal organogenesis and that occur in pathophysiological processes such as wound repair or tumour-cell migration (metastasis).

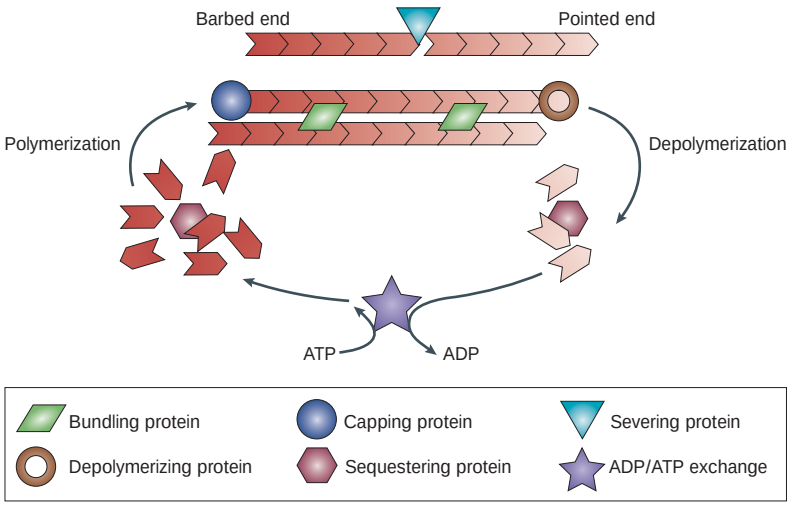
In this review, we tackle the issue of the complexity of the actin cytoskeleton in plasma-membrane specializations, as well as actin dynamics and their regulation by ABPs. We first describe the structure of different actin networks that support cellular specializations, and then focus on the ABPs that are implicated in these networks, illustrating their roles and regulation both in actin organization and in the integration of signals that lead to actin dynamics (BOX 1).

Surface specializations and underlying networks

Differentiated cells have morphological features that correlate with their specialized functions in the organs and organisms that they are part of. These features include membrane protrusions such as MICROVILLI, STEREOCILIA, bristles, FILOPODIA and LAMELLIPODIA, which are all formed and maintained by the underlying actin cytoskeleton (FIG. 1). Microvilli and microvilli-like structures are observed in a variety of polarized cells such as intestinal epithelial cells, kidney cells, hepatocytes and SCHWANN CELLS, but also are present in lymphocytes. Stereocilia and bristles, which are observed in the inner ear of vertebrates and the thorax of *Drosophila melanogaster*, respectively, are involved in mechanosensing. Lamellipodia and filopodia are membrane extensions at the LEADING EDGE of cells; their formation is involved in either motility or interactions with other cell types.

The study of the actin-filament networks in these different membrane processes has uncovered two main types of organization: parallel, unbranched bunches of filaments and highly branched, interlaced filament networks (FIG. 1).

Box 1 | Actin filaments: structure and dynamics



Globular (G)-actin monomers can associate to form a helical filament. As filaments are formed, the concentration of free monomers falls until it reaches the critical concentration at which monomers and filaments are at equilibrium. Below this concentration, no new filaments form and any that are present depolymerize.

Filamentous (F)-actin is, however, asymmetric and the two extremities retain different kinetic characteristics. Actin monomers assemble much more rapidly at the 'barbed end' compared to the 'pointed end' (these names correspond to the arrowhead appearance of myosin heads bound to actin filaments). The critical concentration of the pointed end is higher than that of the barbed end. When F-actin and G-actin are at equilibrium, the global critical concentration is intermediate between those of the two ends separately. So, at this stage, there is a net loss of molecules at the pointed end and a net addition at the barbed end. The two rates balance, which leads to treadmilling — a net flow of actin subunits through the filament.

Monomeric actin binds either ATP or ADP. ATP monomers assemble at a far higher rate than ADP ones. Following assembly on a treadmilling filament, ATP is hydrolysed to ADP and this induces a change in the filament conformation, resulting in a less stable form at the pointed end, which depolymerizes. A treadmilling filament therefore contains ATP-bound subunits at the barbed end, whereas the ones at the pointed end are ADP-bound.

Many proteins bind to actin and influence its dynamics or state. We refer to these proteins as actin-binding proteins (ABPs). Among ABPs, some link actin filaments in a loose network (crosslinking proteins) or in a tight bundle (bundling proteins), or anchor filaments to membranes. Others bind to the barbed end of the filament and prevent further elongation (capping proteins), whereas some cause fragmentation of filaments (severing proteins) or might favour the depolymerization of pointed ends. ABPs also regulate the addition of monomers by sequestering them or favouring ADP/ATP exchange. The figure is modified from REF. 136 © (2002) The American Physiological Society.

Parallel bundles of filaments. To illustrate the first group of actin filaments, we have chosen to focus on intestinal microvilli, hair-cell stereocilia and *D. melanogaster* neurosensory bristles. These apical cellular structures are thin membrane protrusions that are supported by one, or several, bundles of parallel actin filaments: a single axial bundle supports each microvillus or stereocilium, whereas 11 membrane-associated actin bundles maintain a bristle. Within a bundle, the filaments are straight and appear to be tightly packed into a hexagonal structure. They are uniformly polarized, with their barbed ends (BOX 1) towards the tip of the protrusion, and are linked to the membrane both at the bundle tip and laterally.

Each type of protrusion is precisely determined as assessed by the homogeneity of its morphology. However, protrusions vary in their number per cell, the number of filaments per bundle (which influences the width of the protrusion) and their length. These differences are thought to be correlated with the ABP content of the cell. Among ABPs, actin-bundling proteins are indeed implicated in the organization of the bundles (BOX 1). For example, intestinal microvilli range between 1 and 2 μm long and are 0.1 μm wide, and consist of 20–30 bundled actin filaments to which the bundling proteins villin[1], fimbrin[2] and, though less abundantly expressed, small espin[3] localize. Compared to microvilli, stereocilia have a tapered shape — owing to a pinching in of the end that is in contact with the apical surface of the cell — and are aligned into rows of increasing height, which resemble a staircase. The properties of stereocilia vary according to their location in the inner ear: there can be 50–450 stereocilia per HAIR CELL; the length of stereocilia ranges between 1.5 and 5.5 μm; and each actin bundle comprises up to 900 actin filaments[4]. The actin-bundling proteins that are found in stereocilia are espin and fimbrin[5,6]. A single *D. melanogaster* mechanosensory cell produces only one bristle that is 400 μm in length. Each of the 11 bundles that supports the bristle is composed of approximately 500–700 actin filaments, in which the bundling proteins fascin and forked are found[7].

At least two non-redundant actin-bundling proteins are present in the bundles that support these types of protrusion. This is true not only for the three structures described above, but also for other actin-bundle-based features. These include microvilli-like protrusions in sea urchin eggs (which contain fascin and a villin-like protein), filopodia (which contain α-actinin and fascin), *D. melanogaster* NURSE-CELL STRUT bundles (which contain the villin-like protein quail, and fascin) and ECTOPLASMIC SPECIALIZATIONS of SERTOLI CELLS (which contain espin and, potentially, a fimbrin-related protein). For a review of these structures, see REF. 8. The need for several actin crosslinkers to form the bundles has been studied in *D. melanogaster* bristles by Tilney and colleagues[9]. The crosslinking protein forked is first recruited to tiny bundles of actin and can cause their aggregation. However, the filaments remain loosely organized and twisted until the recruitment of fascin, which correlates with the tight packing of filaments into a hexagonal lattice and ensures that the bristle has a straight morphology. This scheme might be extended to other bundled cellular protrusions, as, on the whole, the generation occurs in two phases — the initial appearance of poorly ordered bundles and their subsequent packing into a straight functional structure (such as microvilli[10], stereocilia[11] or *D. melanogaster* nurse-cell struts[12]). The mechanism of nucleation of the initial actin bundles still needs to be unravelled. The bundles emerge from a dense patch on the plasma membrane and elongate further at barbed ends. They remain attached to this membrane nucleation site when

FILOPODIA
Thin cellular processes containing long, unbranched, parallel bundles of actin filaments.

LAMELLIPODIA
Broad, flat protrusions at the leading edge of a moving cell that are enriched with a branched network of actin filaments.

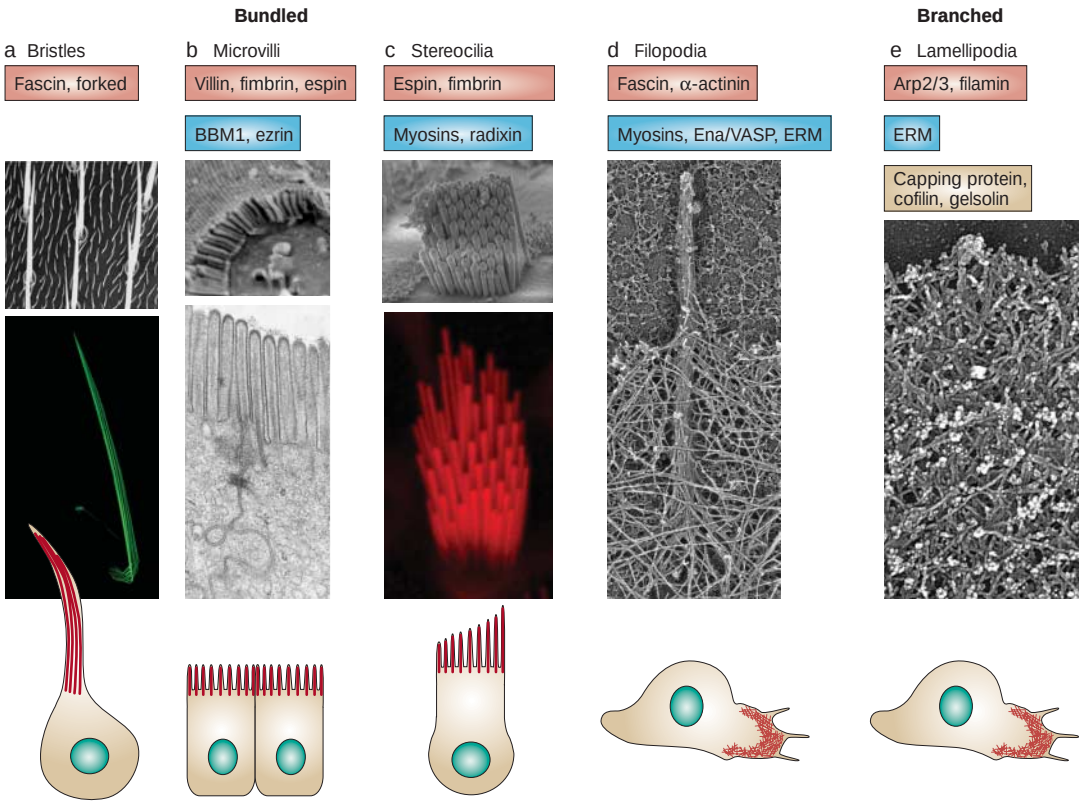


Figure 1 | **Actin networks in cellular protrusions.** Bristles, microvilli and stereocilia are supported by bundled actin filaments, whereas lamellipodia spread on a substrate with underlying branched actin networks. In between these two extremes are filopodia, which contain highly bundled filaments that emerge from branched networks. **a** | *Drosophila melanogaster* bristles are revealed by scanning electron microscopy (SEM; top; courtesy of Y. Bellaiche, Institut Curie, Paris, France). Animals expressing green fluorescent protein (GFP)–actin allow observation of the underlying actin bundles (bottom; courtesy of G. Guild, University of Pennsylvania, Philadelphia, USA). **b** | Intestinal microvilli are revealed by SEM (top) and associated actin bundles by transmission EM (TEM; bottom). **c** | Stereocilia in the inner ear are illustrated by SEM (top; courtesy of P. Gillespie, Oregon Hearing Research Center and Vollum Institute, Portland, USA) and actin staining (bottom; red; courtesy of I. A. Belyantseva and T. B. Friedman, National Institute on Deafness and Other Communication Disorders, Rockville, USA). **d**, **e** | Actin in filopodia and lamellipodia was observed using EM of platinum replicas. The image in part **d** was reproduced with permission from REF. 21 © (2003) the Rockefeller University Press. The image in part **e** was reproduced with permission from REF. 16 © (1999) the Rockefeller University Press. The structures of the actin filaments are also shown diagrammatically below the images. The main actin-binding proteins that are associated with these structures are included — red indicates crosslinking and bundling proteins, blue represents actin–membrane linkers, and yellow indicates proteins that are neither bundling proteins, nor actin–membrane linkers.

SCHWANN CELLS
Supporting cells of the peripheral nervous system that associate with peripheral axons and can contribute to their myelination.

LEADING EDGE
The thin margin of a lamellipodium spanning the area of the cell from the plasma membrane to about 1 μm back into the lamellipodium.

HAIR CELLS
The sensory cells in the organ of Corti from the inner ear.

NURSE-CELL STRUT
D. melanogaster nurse cells are auxiliary cells that supply the oocyte with synthesized mRNAs and proteins during insect oogenesis. Nurse-cell struts are the cytoplasmic actin bundles in these cells that prevent the nucleus from blocking the canals that allow the delivery of cytoplasmic components to the oocyte.

ECTOPLASMIC SPECIALIZATION
An intercellular junction that tightly attaches the elongate spermatid head to the membrane of a Sertoli cell. It is characterized by the presence of a junctional plaque which contains a layer of tightly packed actin bundles under the Sertoli-cell plasma membrane.

SERTOLI CELLS
Non-dividing supporting cells that extend from the base of the seminiferous epithelium to the tubule lumen. They surround germ cells and provide a microenvironment in which germ cells develop and differentiate.

IMMUNOLOGICAL SYNAPSE
A junction that forms at the contact region between a T cell and its target cells. T-cell activation occurs here.

ANTIGEN-PRESENTING CELL
A cell, generally a macrophage or dendritic cell, that presents an antigen to activate a T cell.

forming short, bundled structures such as microvilli and stereocilia, whereas the head-to-tail alignment of these bundle units is needed for the long actin-bundled structures of bristles (for a review, see REF. 13). The aligned modules grow until they partially overlap and further elongation of the overlapping filaments smoothes the link and thickens the bundle — a process described as a graft by Guild and colleagues[14].

Branched networks of filaments. The second type of actin network, the branched network, supports lamellipodia and might structure the IMMUNOLOGICAL SYNAPSE at the site of interaction between a lymphocyte and an ANTIGEN-PRESENTING CELL (APC[15]; discussed in more detail below). Within lamellipodia, filament branches are separated by, at most, several tens of nm and they mainly consist of short actin filaments[16]. Numerous barbed ends of actin filaments face the leading edge of the lamellipodium. The pointed ends are associated with the side of a second filament, which results in branching[17]. Most of the branches occur at an angle of around 70° (referred to as Y-junctions) but some occur at higher angles (producing X-junctions). The Arp2/3 complex — the main crosslinker that is associated with lamellipodia — has been identified at Y-junctions[16] and is responsible for branching[18]. Another actin-crosslinking protein, filamin A, localizes at X-junctions all along the lamellipodium and is thought to stabilize the dendritic network[19]. Capping proteins localize at the leading edge of the lamellipodium and are responsible for the maintenance of short actin filaments[20,21]. The severing/capping protein gelsolin is also associated with actin filaments in lamellipodia[22]. Members of the ADF (actin-depolymerizing factor)/cofilin family are found associated with the rear of the lamellipodium, where Arp2/3 dissociates from the network[16].

From branched to bundled. As suggested by Svitkina and colleagues[21], we will consider that filopodia and microspikes (filopodia that have not protruded beyond the membrane) are interconvertible structures and will refer to them collectively as filopodia. They are observed at the edge of lamellipodia in moving cells such as fibroblasts, and are also associated with the GROWTH CONES of neuronal cells, with the sprouting of new blood vessels[23] and, probably, at immunological synapses[15]. Filopodia are thin cellular processes that are organized by a bundle of actin. The actin filaments within each bundle are of uniform polarity and are crosslinked by at least two proteins — α-actinin at the rear of the protrusion and fascin along its length. Fascin is the essential crosslinker that underpins the straightness and stiffness of filopodial actin bundles[24].

We could have included filopodia in the 'bundled-actin network' category. However, these structures form from a pre-existing lamellipodial actin network[21] — unlike the structures that were discussed above, which form anew[13]. Filopodia originate from actin filaments of the branched network that are prevented from capping and, as a result, can elongate at the leading edge of the lamellipodium. Because of the branched organization, some elongating filaments are likely to collide and seem to cluster on collision. Further elongation of those filaments that are linked at their tips and binding of fascin result in the formation of parallel actin bundles, which can be joined laterally by other elongating filaments and protrude at the leading edge of the lamellipodium[21]. The Ena/VASP (Enabled/vasodilator-stimulated phosphoprotein) proteins, which bind to barbed ends of actin filaments, are present at the tips of filopodia and are good candidates for protecting the filaments from capping[25]; through their oligomerization, these proteins might also cause clustering of actin filaments[26]. This model for the formation of filopodia from a branched network has been nicely documented by an *in vitro* approach using pure proteins in a reconstituted medium, which showed that a shift from a branched network to filopodia-like bundles could be produced merely by altering the concentration of capping proteins[27].

Controlling the morphology of a protrusion. Except in the case of filopodia, the length of actin bundles is very precisely determined, as is clearly illustrated by the homogeneity of intestinal microvilli, as well as by the positive correlation between the length of a stereocilium and the position of the hair cell in which the stereocilium is found. This regulation seems to be due, at least in part, to the type and concentration of the crosslinking protein that generates the bundle. A high level of expression of the ABP villin can induce the formation of microvilli on the dorsal surface of fibroblastic cells; the length of these microvilli was similar to the length of microvilli in intestinal epithelial cells[28]. Moreover, Loomis and colleagues[29] reported a lengthening of the microvilli of the kidney epithelial cell line LLC-PK1 by modulating the level of transfected espin and, to a lesser extent, by overexpression of villin and T-fimbrin. The correlation between the level of expression of espin and the length of the protrusion has also been analysed in inner-ear hair cells *in situ*, where a gradient of espin protein was found to correspond with the gradient in stereocilial length[29]. Overexpressed fascin in LLC-PK1 cells did not localize to microvilli but to filopodia-like protrusions, which indicates that different actin-bundling events might occur in filopodia compared to those in microvilli and stereocilia. It has nevertheless previously been reported that the microvilli in LLC-PK1 cells lengthen in the presence of fascin[24]. However, the influence of the level of expression of different actin-bundling proteins on the length of protrusions still remains to be explained.

Actin bundles and branched networks are linked to the plasma membrane, and this linkage is important for the formation and maintenance of protrusions, and therefore for their morphology. Members of the ERM (ezrin/radixin/moesin) family of proteins are widely distributed actin–membrane-protein linkers[30]. In fibroblast lamellipodia, ERM proteins bind actin filaments to the membrane Na–H exchanger isoform-1. The disruption of this interaction results in the extension of numerous small protrusions instead of one broad lamellipodium but also impairs the directionality of the cells and remodelling of focal adhesions[31]. This effect could be the result of defects in the tethering of actin filaments at the leading edge as well as perturbations in the clustering of membrane proteins and in the subsequent integration of polarization and guidance signals. In support of the relevance of this actin–membrane anchorage, laser inactivation of ezrin in fibroblast lamellipodia[32] or of radixin in growth-cone lamellipodia[33] resulted in the retraction or splitting of these structures. The existence of other actin–membrane linkers within lamellipodia remains controversial and requires further investigation.

ERMs are also implicated in the growth and preservation of bundled protrusions, where they show different tissue distributions, the significance of which is not understood. Ezrin is associated with gut microvilli and filopodia, moesin is found in hepatocyte microvilli, and both are detected in kidney microvilli[30]. Radixin is found in the microvilli of the BILE CANALICULI and has recently been described in the lower shaft and the tapered end of stereocilia where it might help to anchor the pointed ends of actin filaments to the membrane[34].

Different MYOSIN proteins are essential for the proper formation and function of stereocilia. Mutations in the genes that encode these proteins are associated with alterations in the shape of stereocilia and are responsible for deafness. Among them, myosin XVa is important for the formation of the staircase pattern[35], and myosin VIIa, in association with the ABP harmonin b, ensures the structural integrity of stereocilia bundles, probably by mediating the anchorage of cadherin-23 crosslinks between adjacent stereocilia to their actin bundles[36]. Unconventional myosins are also implicated in actin–membrane linkage in other protrusions — for example, BBM1 (REF. 37) in intestinal microvilli and myosin X in lamellipodia, ruffles and filopodia[38].

GROWTH CONE
Motile tip of the axon or dendrite of a growing nerve cell, which spreads out into a cone-shaped outgrowth.

BILE CANALICULI
Tiny channels on the surface of liver cells that collect the bile that they produce.

MYOSIN
Molecular motors that move along actin filaments and have several cellular roles in contraction or protein transport.

Actin–membrane linkers are key components in controlling bristle shape[39], but the relevant proteins have not yet been identified in these structures. Finally, parameters that can be considered as physical constraints — such as the area of the cell surface, the availability of actin-filament precursors, and, for increased stability, the maximization of filament bridging by reducing the bundle surface:volume ratio — must also influence the localization, length, width and number of bundles[4,40].

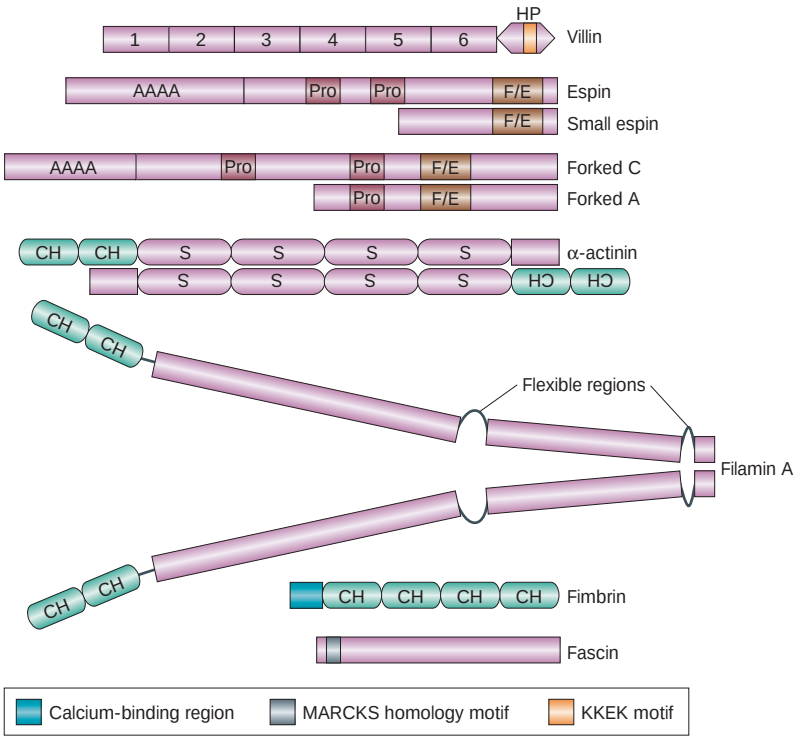
Dynamics of 'stable' structures. The actin cytoskeleton, whether it forms bundles or branched networks, is responsible for cell shape. However, actin filaments are never fixed molecular structures, despite the fact that cellular structures such as microvilli or stereocilia seem to be stable. Of course this becomes obvious when lamellipodia and filopodia, which are associated with cell movement, are studied. Within filopodia and lamellipodia, actin monomers are commonly described as cycling from the uncapped, pointed filament ends at the rear of the branched network, towards the barbed ends at the extreme leading edge, which results in the treadmilling of the whole network[41] (BOX 1).

The stable shape of actin-bundle-based protrusions is the result of a dynamic steady-state owing to a precise balance between actin polymerization and depolymerization. In fact, this turnover was described many years ago for microvilli[42] and stereocilia[4]. More recently, it has been shown that microvilli constantly incorporate actin monomers at their tips, as assessed by fluorescence recovery after photobleaching (FRAP) studies of cells that express actin labelled with green fluorescent protein[43]. This is also the case for stereocilial filament bundles, which treadmill towards the base of the stereocilium[44], where the stereocilium is attached to the cell. The rate of treadmilling differs between parallel actin bundles. The treadmilling rate of stereocilia bundles is 10–100 × slower than microvilli[43,45], which treadmill 1.5–20 × slower than filopodia in nerve growth cones[46]. The actin-bundling-protein content of the bundles is, in part, responsible for these discrepancies, as transfection of an intestinal cell line with espin correlates with a 5–6-fold increase in the actin-treadmilling rate in microvilli[29]. However, filopodia of a single growth cone show varying treadmilling rates that correlate with their dynamic behaviour[46] — which must be essential for their implicated role in pathfinding. Furthermore, the treadmilling rates in stereocilia are proportional to their length, which results in the synchronous turnover of a staircase[45]. Other factors are therefore essential for this regulation — they remain to be investigated, but myosins are already good candidates. The importance of the balance between polymerization and depolymerization has been demonstrated in the growth and breakdown of *D. melanogaster* bristles: in these structures, actin-filament depolymerization is essential for eliminating bundles that are not stabilized by tight packing and association with the plasma membrane[40,47].

Not lost, or produced, but remodelled

Regulation of ABPs. The dynamic steady-state nature of the actin cytoskeleton allows cells to adapt rapidly to external stimuli by perturbing the equilibrium between polymerization and depolymerization. This occurs through modulation of the activity of the ABPs that maintain actin filaments. Post-translational modifications,

Box 2 | Molecular features implicated in actin crosslinking and bundling



The diagram shows the organization of the main crosslinking and bundling proteins — the different actin-binding domains (ABDs) are highlighted.

Villin comprises six repeats (1–6) of a module that is common to those gelsolin-family members that retain an ability to sever and cap actin. Three Ca^{2+}-dependent ABDs have been identified: repeat 1 and repeats 4–6 bind actin monomers; repeats 2–3 bind actin filaments. Villin can bundle actin filaments because of an additional Ca^{2+}-independent filament-binding domain, the headpiece (HP), in which a cluster of basic residues, the KKEK motif, is essential.

The espin and forked proteins share a carboxy-terminal peptide, the forked/espin-homology domain (F/E), which causes actin-filament bundling and must therefore contain two ABDs to bind to two different filaments. Proline-rich regions (Pro) and amino-terminal ankyrin repeats (AAAA) are found in the large espin isoform and the large forked protein, forked C (the *forked* gene encodes at least six different transcripts, *forked A* being the main one). A third espin ABD has been identified between the two proline-rich modules.

α-actinin, fimbrin and filamin ABDs are formed by a pair of calponin-homology (CH) domains. α-actinin forms an anti-parallel homodimer to crosslink actin filaments. A rod domain that is composed of four spectrin repeats (S), which is responsible for homodimer formation, separates the two ABDs, resulting in loose filament crosslinking. Filamins form parallel dimers that contain the same features: two ABDs separated by repeated, stiff, rod-like domains. The insertion of flexible regions could allow filamins to crosslink widely dispersed actin filaments at perpendicular angles. Fimbrin is a monomeric actin-filament-bundling protein that contains two tandem ABDs, with no spacer, which results in the organization of Ca^{2+}-dependent densely packed bundles.

Fascins are monomeric actin-bundling proteins. The carboxy-terminal half of fascins contains one ABD; a second ABD could be located in a highly conserved region that shares similarities with the ABD of the myristoylated alanine-rich C-kinase substrate (MARCKS).

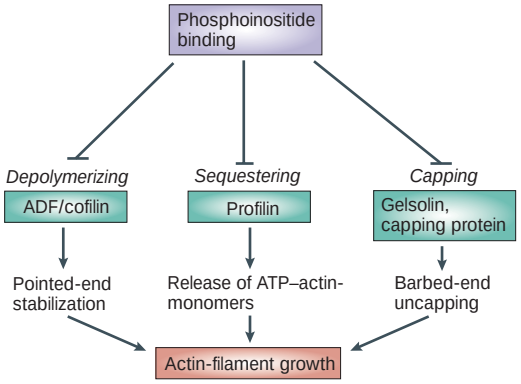


Figure 2 | **Phosphoinositides and actin filaments.** Binding to phosphoinositides inhibits pointed-end depolymerization by ADF/cofilin, actin-monomer-sequestration by profilin and the capping of barbed filament ends by gelsolin and capping protein. Inactivation of cofilin reduces pointed-end depolymerization. Inhibition of profilin releases ATP-bound actin monomers, which therefore become available for barbed-end polymerization. Inhibition of gelsolin and capping protein liberates free barbed ends, which allows filaments to elongate. So the binding of phosphoinositides to these actin-binding proteins favours the growth of actin filaments.

ion binding and interactions with co-factors are some of the molecular cues that regulate the actin-binding domains of ABPs (BOX 2), either by competing with actin for ABPs or by causing conformational changes in the ABPs. Such regulation can result in the acceleration of actin treadmilling, the breakdown of a specialized architecture to mobilize actin to another structure, or the creation of new cellular domains to complement the change in environment. Below we describe a few examples that illustrate the regulation of ABPs in some circumstances of cellular remodelling. However, the proteins presented cannot be reduced to a unique function but participate in several cytoskeletal processes even if each of them has been chosen only to illustrate a particular one here.

Increasing actin dynamics. Gelsolin has Ca^{2+}-dependent filament-capping and -severing activities[48]. The binding of Ca^{2+} *in vitro* induces a conformational change in gelsolin that leads to the exposure of its actin-binding site[49]. A low pH can also activate gelsolin — although to a lesser extent — in the absence of Ca^{2+} (REF. 50). The binding of gelsolin to actin filaments weakens bonds between actin subunits, which causes the filament to break. After severing, gelsolin remains attached to the barbed end of the actin filament, which therefore cannot re-anneal or elongate, and so the actin network is disassembled by pointed-end depolymerization[51]. The binding of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) to gelsolin induces a conformational change in gelsolin[52] and is required for its dissociation from the barbed ends[53,54]. In addition, lysophosphatidic acid has been shown to regulate gelsolin in a very similar manner to $PtdIns(4,5)P_2$ (REF. 55). In quiescent cells, gelsolin binds to $PtdIns(4,5)P_2$ at the plasma membrane. $PtdIns(4,5)P_2$ hydrolysis and the subsequent rise in Ca^{2+} levels, which occur when cells are stimulated with epidermal growth factor (EGF), release gelsolin and activate its severing activity[22]. *Gelsolin*-knockout mice have normal embryonic development and longevity[56]. However, in addition to a delayed HAEMOSTATIC RESPONSE and defects in the retraction of growth-cone filopodia, these mice show reduced motility of their neutrophils, fibroblasts and osteoclasts[56,57]. Motility normally requires highly dynamic actin. Severing and uncapping of actin filaments by gelsolin therefore increases the number of actin filaments and generates many ends that can polymerize and so increases actin treadmilling.

Phosphoinositides are also known to inhibit other ABPs, such as the capping protein[58], (see also the article by Paul A. Janmey and Uno Lindberg in this issue) and the severing/depolymerizing proteins of the ADF/cofilin family[59]. In addition, phosphoinositides dissociate actin from profilin[60], a small actin-monomer-sequestering protein that catalyses the exchange of ADP for ATP on actin monomers and thereby provides a pool of polymerization-competent monomers. This indicates that phosphoinositides might control the net growth of actin filaments by increasing the rates of uncapping and polymerization, while decreasing the rates of depolymerization (FIG. 2).

ADF/cofilin proteins increase the turnover of actin filaments. They bind to two longitudinally associated actin subunits along an actin filament, locally modify the twist of the filament[61] and disrupt the interaction between the two actin subunits[62,63]. ADF/cofilin proteins are regulated by the phosphorylation of a single serine residue[64], which inactivates their actin-depolymerizing/severing activities[65]. In response to various stimuli such as growth factors or chemotactic peptides, they are dephosphorylated and become active[66,67]. Slingshot proteins represent the only family of phosphatases that is known to dephosphorylate ADF/cofilin[68]. In unstimulated quiescent cells, ADF/cofilin proteins are diffusely distributed in the cytoplasm, whereas after stimulation, the activated proteins translocate to regions of the cells where actin filaments are highly dynamic[69]. Dephosphorylation of ADF/cofilin increases the turnover of actin filaments by either enhancing the release of actin monomers from filament pointed-ends for barbed-end polymerization, or by severing existing filaments and thereby creating new barbed ends for polymerization.

The evidence that ADF/cofilin increases the rate of disassembly of pointed ends[70,71] and/or severs[67,69,72] actin filaments to enhance treadmilling is controversial. Zebda and colleagues[67] have proposed that ADF/cofilin activities could depend on the cellular context; in continuously motile cells, where globular (G)-actin is limiting, they would enhance the depolymerization of pointed ends, whereas in cells that are initiating movement in response to a stimulus, the massive production of new barbed ends that are needed for lamellipodial extension would be generated by severing. Moreover, Ghosh and colleagues[73] have recently proposed that active cofilin polymerizes actin and determines the direction of cell migration. Cofilin phosphorylation is mediated by LIM (for LIN11, ISL1 and MEC3) kinases,

FRAP
(fluorescence recovery after photobleaching). This technique involves the irradiation of a limited region containing fluorescent molecules, such that the fluorescent molecules inside the region become non-fluorescent. The subsequent reappearance of fluorescent molecules from the surrounding non-bleached region gives information on their dynamic parameters such as diffusion coefficient, immobile fraction and binding or residence time.

HAEMOSTATIC RESPONSE
The process that allows platelets to carry out coagulation.

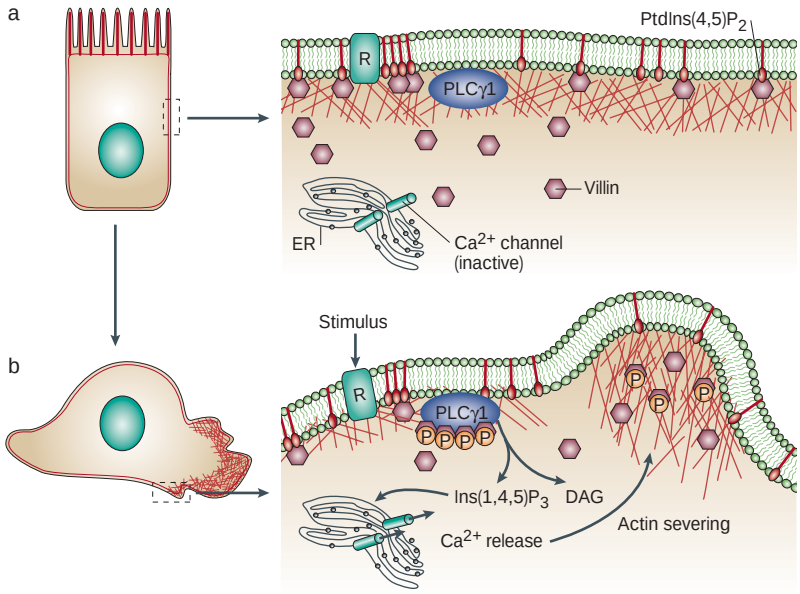


Figure 3 | **Model of villin regulation in actin remodelling.** The left-hand diagrams show cellular architecture. Part **a** depicts a highly polarized morphology whereas part **b** represents a motile cell; the diagrams on the right show the events that produce this change. **a** | In resting epithelial cells, villin is mainly associated with actin bundles in microvilli, and a small pool of villin binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at membranes. **b** | Following stimulation of membrane receptors (R), a pool of villin becomes tyrosine phosphorylated and associates with phospholipase Cγ1 (PLCγ1). This association triggers the activation of PLCγ1, which then cleaves PtdIns(4,5)P_2 into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3). Ins(1,4,5)P_3, in turn, releases Ca^{2+} from stores in the endoplasmic reticulum (ER). The increase in intracellular Ca^{2+} concentration induces actin severing by villin, which allows actin bundles within microvilli to dissociate, and dynamic protrusions that lead to cell migration to form.

which, in turn, are activated by signalling pathways that involve RHO-FAMILY GTPases and that also lead to lamellipodial extension, probably by stabilizing actin filaments[74–76]. Cofilin regulation in actin dynamics must therefore involve a complex balance between phosphatases and LIM kinases that leads to an equilibrium between actin-network maintenance and treadmilling enhancement.

Breaking down and rebuilding. The many activities of villin nicely illustrate how a cell can regulate an ABP to carry out a particular function. Villin belongs to the same family of ABPs as gelsolin and is the only ABP to show actin-bundling, -capping and -severing properties. *In vitro*, its filament-severing activity is observed at high Ca^{2+} concentrations, whereas actin bundling occurs at low Ca^{2+} concentrations[77]. Variations in the levels of intracellular Ca^{2+} occur in various cell systems following injury. A Ca^{2+}-triggered switch between the bundling and severing activities of villin could explain how, following injury to the intestinal epithelium, epithelial cells break down and redistribute their microvillar actin filaments so as to change from a highly polarized to a motile morphology[78]. PtdIns(4,5)P_2 negatively regulates the severing and capping activities of villin and enhances its bundling functions[79], whereas tyrosine phosphorylation is an additional way for cells to regulate the ability of villin to bind and sever actin filaments[80]. *In vitro* tyrosine phosphorylation of recombinant villin enhances its severing activity and overcomes the need for a high Ca^{2+} concentration[81]. Interestingly, phosphorylated villin has been shown to bind to phospholipase Cγ1 (PLCγ1)[82] and increase its enzymatic activity *in vitro*[83]. PLCγ1 activation *in vivo*, which occurs as a result of cellular stimulation with hepatocyte growth factor, correlates with an increase in the amount of villin that is bound to PLCγ1. The active PLCγ1 then cleaves PtdIns(4,5)P_2 into diacylglycerol and inositol-1,4,5-trisphosphate, which results in the release of Ca^{2+} from intracellular stores. This can again activate the severing activity of villin and leads to enhanced dynamic cellular processes such as remodelling and motility[84] (FIG. 3). This implies the existence of a regulation loop in which phosphorylation of villin induces villin-severing activation.

ERM proteins can exist in an inactive conformation as a result of an intramolecular association between their filament-binding domain and their FERM (protein 4.1/ERM) domain, which contains binding sites for several membrane-associated proteins[85,86]. Phosphorylation on an essential threonine residue induces the conformational change that is necessary to disrupt this association and to activate the proteins[87–89]. This phosphorylation is thought to be downstream of Rho kinase[87,90] and protein kinase C (PKC)[91], and, for ezrin, it occurs after the protein binds to PtdIns(4,5)P_2 at the plasma membrane[92,93]. Active ERMs allow the formation of microvilli-like structures[90] and filopodia[94]; microvilli break down following the dephosphorylation and consequent inactivation of ERMs[95]. ERM proteins, and moesin in particular, were described to maintain the polarity of epithelial cells by acting antagonistically to the Rho pathway[96]. This implies the existence of a negative-feedback loop between ERM activation and Rho-pathway activity. However, a positive effect of ERMs on the Rho pathway, through the binding of Rho guanine nucleotide-dissociation inhibitor, has previously been reported[97]. These results must therefore be interpreted with caution. PKC-mediated ezrin activation and its association at membrane protrusions is required for PKC-dependent cell migration[91]. By regulating the association between ezrin and the transmembrane receptor CD44, PKC controls directional cell motility[98]. Furthermore, in response to cellular stimulation with growth factors[99], ezrin is tyrosine phosphorylated and triggers cell motility and morphogenesis[100]. Ezrin is therefore not only responsible for the formation and maintenance of protrusions but is a key component in transducing signals from extracellular cues that lead to cytoskeleton remodelling, shape changes and motility.

The route to protrusions. Filamin, a large actin-crosslinking protein, is emerging as an important component in actin remodelling. *In vivo*, a direct interaction between filamin and the serine/threonine kinase p21-activated kinase-1 (PAK1) has been reported to occur after cellular stimulation by molecules that activate PAK1, such as the mitogen heregulin and sphingolipids[101]. The activation of PAK1, a downstream effector of the small GTPases Rac1 and Cdc42, induces polarized cytoskeletal

RHO-FAMILY GTPases
Ras-related small GTPases that function as GDP/GTP-regulated binary switches and regulate signal-transduction pathways that control actin organization. RhoGTPase function is required for both the establishment of a fully polarized state and a motile phenotype on epithelial–mesenchymal transition.

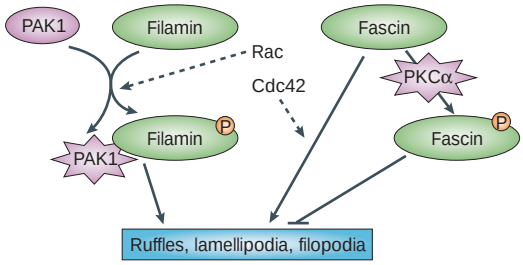


Figure 4 | **Filamin, fascin and the formation of protrusions.** In response to extracellular signals, an interaction between p21-activated kinase-1 (PAK1) and filamin is essential for PAK1 to mediate cytoskeletal rearrangements. This interaction allows filamin to be phosphorylated by PAK1 and PAK1 to be activated by filamin, which results in the formation of MEMBRANE RUFFLES and lamellipodia. Conversely, phosphorylation of serine residues in fascin by protein kinase Cα (PKCα) inhibits the binding of fascin to actin and its ability to organize filopodia and lamellipodia. Filamin- and fascin-mediated formation of protrusions both seem to occur downstream of Rac and Cdc42.

rearrangements such as ruffles, lamellipodia and filopodia[102], and regulates cell motility[103] (FIG. 4). The interaction between filamin and PAK1 is reciprocal — with filamin being phosphorylated by PAK1 and PAK1 being activated by filamin binding — and is necessary for PAK1-mediated ruffle formation[101]. Filamin also crosslinks actin filaments and binds numerous other proteins, such as transmembrane receptors and several small GTPases[104]. Consequently, it could be involved in the integration of external stimuli and function as a platform that would bring together downstream effectors such as small GTPases and PAK1.

Fascin induces dynamic protrusions such as filopodia and ruffles at the leading edge of a motile cell[24]. Fascin is phosphorylated on serine 39 by PKCα and this phosphorylation inhibits the actin-binding properties of fascin[105,106], leading to a loss of fascin from these actin-rich protrusions[107]. The phosphorylated form of fascin remains associated with PKCα and this interaction is important for full cell spreading; it also reduces cell migration[108]. Conversely, in melanoma cells, stimulation with the growth factor neurotrophin induces migration and a concomitant redistribution of fascin to actin-rich protrusions such as lamellipodia and filopodia — probably after fascin is dephosphorylated. A dominant-negative (constitutively phosphorylated) mutant of fascin inhibits this neurotrophin-induced cell migration[108]. The localization of fascin to filopodia and lamellipodia might occur downstream of Rac and Cdc42 (REF. 109; FIG. 4).

Organizing cell defence. The cytoskeleton is intimately involved in determining the efficiency and fidelity of the immune response. For instance, the actin cytoskeleton is essential for T-cell shape development, movement and polarization. Polarization includes the formation of the immunological synapse — a specialized and complex domain that results from the interaction of a T cell with an APC[15] (FIG. 5).

The actin cytoskeleton and ABPs are important for delivering membrane proteins to, and clustering them at, the interaction site, but are also responsible for the exclusion of other proteins from this site. For example, myosins and ERM proteins cluster receptors to the contact site, which might result in signal amplification and therefore participate in synapse formation[110,111]; moreover, ERM proteins are necessary for the removal of CD43 (a large molecule of the GLYCOCALYX that would prevent proper T-cell–APC interaction) from the forming synapse[111,112].

The interaction of T-cell antigen receptors with complementary antigen–MAJOR-HISTOCOMPATIBILITY-COMPLEX (MHC) molecules on an APC induces signalling cascades that allow numerous kinases and ADAPTOR PROTEINS, such as SLP76, VAV and NCK, to be recruited to the forming immunological synapse on the T-cell side[113]. This results in PAK1 activation. NCK, in turn, would recruit inactive Wiskott–Aldrich syndrome protein (WASP), and VAV activates Cdc42 and Rac[113,114]. Activated Cdc42 induces conformational changes in WASP that allow it to recruit and activate the Arp2/3 complex[115,41]. Other pathways that are independent of Cdc42 have recently been implicated in WASP activation[116]. This leads to actin polymerization at the interface and implies the formation of a branched network that supports the immunological synapse. Moreover, activation of LIM kinase by PAK1 (REF. 117) might, as described above, inhibit ADF/cofilin by phosphorylating it, and thereby increase the stability of the actin network. So, when a T cell and an APC interact, a set of ABPs is recruited and regulated to form a stable actin network that functions as a scaffold for the assembly of signalling complexes (FIG. 5).

Cells must also be able to defend themselves against mechanical stress. Their response to tension is mediated, in part, by cytoskeletal rearrangement — in particular, through an increase in the amount of filamentous actin and therefore a stiffening of the actin cytoskeleton. This allows membrane integrity and cell shape to be maintained[118]. The process is likely to be mediated by ABPs, and filamin A has been identified as an essential regulator. For instance, the appliance of a force through the integrins — transmembrane receptors for components of the extracellular matrix — induces the accumulation of actin filaments and of active filamin A at the site at which the force is applied. This mechanical stress induces PKC-mediated serine phosphorylation of filamin A, which results in its association with integrins[119,120]. The accumulation of actin filaments is probably due to the crosslinking properties of filamin.

How to study microfilament functions

Integrating the complexity of ABP regulation in the study of actin-filament functions is essential and it has therefore influenced the nature of experimental approaches. To illustrate this, we have chosen two examples of strategies that have allowed a better understanding of either the treadmilling of branched actin networks, or the role of villin in actin remodelling.

MEMBRANE RUFFLES
Processes that are formed by lamellipodia that have lifted from the substratum on which they previously extended.

GLYCOCALYX
The carbohydrate-rich region at the surface of many plant and animal cells that is rich in glycoproteins and glycolipids.

MAJOR HISTOCOMPATIBILITY COMPLEX
A complex of genetic loci in higher vertebrates that encodes a family of cellular antigens that allow the immune system to recognize self from non-self.

ADAPTOR PROTEINS
Proteins that usually have several protein–protein interaction domains and increase cellular responses by recruiting other proteins to a complex.

Actin treadmilling allows cells to move, by generating lamellipodia and filopodia. It also allows bacteria such as *Listeria monocytogenes* and *Shigella flexneri* to be propelled around inside cells, by forming actin 'comets'. These actin-based movements were first observed in cell cultures, but important steps that have advanced our understanding of the underlying mechanisms have been made as a result of *in vitro* reconstitution assays. These assays have allowed the determination of the minimal set of purified proteins that is required for actin-based motility of *L. monocytogenes* and *S. flexneri* [121]. Further progress with these reconstitution assays has been achieved by the use of beads that are coated with an activator of the Arp2/3 complex (such as ActA[122] or the VCA — verprolin homology, cofilin homology, and acidic — domain of WASP). These beads, when added to cell extracts or reconstituted media, can recruit and polymerize actin networks and even form actin comets that propel the beads in the solution[123]. Using these strategies, the question of the impact of a protein on actin-based movement or on the structure of actin networks can be addressed directly[27,124,125], and the physical parameters that explain the generation of the force that is necessary for movement can be modified and studied[126,127].

Our second example concerns villin, which was first discovered to be associated with actin bundles in intestinal microvilli[1]. Subsequent *in vitro* studies allowed the determination of the activities of villin towards actin and the characterization of its functional domains[77,128]. At the time, villin was the only protein of the gelsolin family that was known to possess a domain that is responsible for actin bundling, and so was thought to be preponderant in the formation of core bundles in microvilli. This role was further studied in cell-culture models, by transfecting *villin* genes into cell lines that do not normally express villin[28,129] or by silencing *villin* in cell lines that develop BRUSH BORDERS[130]. This seemed to confirm the essential role of villin in the formation and maintenance of microvilli.

The challenge, however, was to demonstrate this role *in vivo*. Unexpectedly, *villin*-knockout mice did not show a detectable deterioration in the morphology of microvilli[78]. Nevertheless, they did seem to be unable to repair their intestinal epithelium — a process that, as described above, requires polarized epithelial cells to change to a motile form that can move towards a wound. This indicated an *in vivo* role for villin in the remodelling of the actin cytoskeleton. It was interpreted to be the result of the actin-severing activity of villin, which would enhance actin remodelling and dynamics. This role of villin in the enhancement of actin dynamics was further demonstrated in transfected cell lines and in primary cultures of intestinal epithelial cells, in which expression of villin induced enhanced motility and morphogenesis following stimulation with hepatocyte growth factor[84]. Finally, this understanding of villin function allowed abnormalities in its expression to be identified as the cause of human liver diseases that are characterized by malformed microvilli in the bile canaliculi[131].

By greatly simplifying the system that is being studied and allowing a precise control of the components that are present, *in vitro* approaches are of great value in the analysis of complex phenomena. However, the importance of both post-translational modifications and interactions with several cellular components urges the use of cell-culture models. Such models allow the study of cytoskeletal rearrangements and cell movements in response to controlled external stimuli, or after the expression of exogenous proteins or silencing of endogenous proteins. These models also allow the biochemical analysis of subsequent signalling pathways. By establishing primary cultures, we can obtain conditions that more closely resemble those found *in vivo*, although such cultures remain cumbersome to use routinely. Numerous transgenic animal models, particularly mice, have been established for *in vivo* analysis. Several ABPs have been knocked out in mice, including villin, gelsolin and espin. These models allow the *in vivo* analysis of the role of the protein in question in development and in organ/cell function, and are becoming especially promising as *in vivo* imaging techniques are developed.

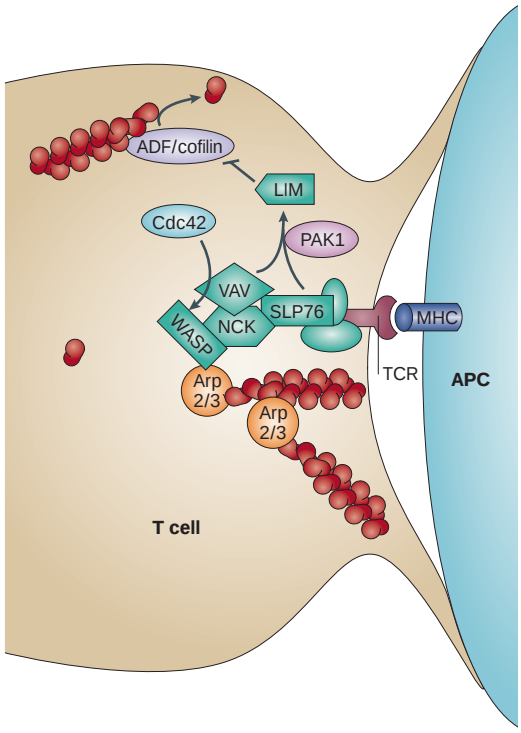


Figure 5 | **Regulation of Arp2/3 and cofilin in the formation of immunological synapses.** The interaction between a T-cell antigen receptor (TCR) and an antigen-major-histocompatibility-complex (MHC) molecule from an antigen-presenting cell (APC) leads to the recruitment of SLP76, NCK and VAV to the TCR. NCK recruits Wiskott–Aldrich syndrome protein (WASP), which, after activation by Cdc42, binds to and activates the Arp2/3 complex. This results in the polymerization of actin filaments. At the same time, VAV and SLP76 activate LIM kinase through PAK1, which leads to the inhibition of the severing/depolymerization activities of ADF/cofilin. This increases the stability of the forming actin network.

BRUSH BORDER
The apical surface of epithelial intestinal or kidney cells. It is involved in absorption, and is characterized by the presence of abundant microvilli.

Conclusions and perspectives

In this review we have tried to illustrate the extent of the regulation of ABPs in cell protrusions, and how ABPs are implicated in cellular dynamics and signalling. ABPs organize the actin cytoskeleton to maintain stable cellular protrusions or dynamic processes. However, they also mediate the formation or breakdown of these structures by regulating and adjusting actin dynamics according to stimuli in the environment. ABPs are indeed highly regulated downstream of numerous signalling pathways and integrate signals that drive actin remodelling. However, these features are part of a very broad research field and other important aspects such as the formation of phagocytic cups or bacterial infectious processes have not been discussed here. Neither did we discuss neuronal synapses or growth cones as, to our knowledge, there is currently no information regarding the regulation of ABPs that are specific to these cellular specializations. Formins, which are newly identified capping proteins of actin (although they are slightly 'leaky') and which potentially also nucleate actin[132] have not yet been definitively implicated in the formation and regulation of the cellular protrusions that we have focused on, but they might quickly emerge as important regulatory components. Furthermore, new functions of ABPs are also being identified. For example, the ABPs that organize microvilli are also involved in nutrient uptake or protein secretion and in the modulation of ion transport across membranes, and it will be interesting to investigate how they do this. ABPs are also emerging as co-activators of gene transcription[133], which could be consistent with a role in transducing signals from membrane receptors. Filopodia also seem to be cellular sensors that are involved in contact-mediated inhibition of neuronal differentiation in *D. melanogaster*[134], and in orientated cell growth, in response to extracellular signals, at the tips of sprouting blood vessels or nerves[23]. This actin-dependent orientation hints at a finely tuned integration of signal gradients by ABPs that warrants further investigation. Finally, the interaction between actin filaments and another cytoskeletal component, microtubules, is fundamental in diverse dynamic processes including cell motility and growth-cone guidance. The analysis of the mechanisms that underlie this interaction will certainly shed light on highly regulated actin and microtubule-binding proteins[135].

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Competing interests statement

The authors declare that they have no competing financial interests.

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