REVIEW ARTICLE

Signalling to actin assembly via the WASP (Wiskott–Aldrich syndrome protein)-family proteins and the Arp2/3 complex

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The assembly of a branched network of actin filaments provides the mechanical propulsion that drives a range of dynamic cellular processes, including cell motility. The Arp2/3 complex is a crucial component of such filament networks. Arp2/3 nucleates new actin filaments while bound to existing filaments, thus creating a branched network. In recent years, a number of proteins that activate the filament nucleation activity of Arp2/3 have been identified, most notably the WASP (Wiskott–Aldrich syndrome protein) family. WASP-family proteins activate the Arp2/3 complex, and consequently stimulate actin assembly, in response to extracellular signals. Structural studies have provided a significant refinement in our understanding of the molecular detail of how the Arp2/3 complex nucleates actin filaments. There has also been much progress towards an understanding of the complicated signalling processes that regulate WASP-family proteins. In addition, the use of gene disruption in a number of organisms has led to new insights into the specific functions of individual WASP-family members. The present review will discuss the Arp2/3 complex and its regulators, in particular the WASP-family proteins. Emphasis will be placed on recent developments in the field that have furthered our understanding of actin dynamics and cell motility.

Key words: actin, Arp2/3 complex, nucleation, suppressor of the cAMP receptor (Scar), WASP-family verprolin homologous protein (WAVE), Wiskott–Aldrich syndrome protein (WASP).

INTRODUCTION

Until the late 1990s there seemed to be a large gap in our understanding of how cells could trigger rapid actin assembly in response to signals. Some models postulated an actin nucleator, without actually identifying the protein(s) responsible. Many laboratories identified nucleation activity in proteins, but no proteins appeared to have the properties of a true nucleator that was likely to be linked to cell motility. Since that time, actin nucleation by the Arp2/3 complex, first isolated in 1994, and found to stimulate the assembly of actin filaments in 1997, has become central to most models describing signalling-induced actin assembly [1,2]. WASP (Wiskott–Aldrich syndrome protein)-family proteins mediate signals to activate the *de novo* nucleation activity of the Arp2/3 complex [3,4]. Other proteins interact with Arp2/3 complex and likely modulate its activity in synergy or in competition with WASP proteins, but genetic and loss-of-function studies suggest that WASP proteins are central to Arp2/3 regulation. Our review will focus on the structure, function and mechanism of action of the Arp2/3 complex and its modulation by WASP-family and other proteins. We will also discuss recent genetic studies that shed light on, and provide support for, the biochemical analysis that originally implicated Arp2/3 and WASP proteins in regulated actin nucleation. Overall it appears that this signalling pathway and the mechanism of Arp2/3-induced actin assembly is well conserved in evolution and is central to the dynamics of the actin cytoskeleton in many cellular processes.

ACTIN-FILAMENT ASSEMBLY

The ability of a cell to co-ordinate the assembly and disassembly of its actin cytoskeleton is essential for cell integrity, motility, membrane trafficking and shape changes. Within the cell, actin monomers polymerize into polar filaments, with fast growing (barbed) and slower growing (pointed) ends. The terms 'pointed' and 'barbed' come from the arrow-like appearance of actin filaments decorated with the motor domain of myosin as seen in electron micrographs. The actin monomer binds to and hydrolyses one molecule of ATP, which provides the energy to maintain the difference in affinity for monomer addition between the barbed and pointed ends. Biochemical data tell us that, under cellular conditions, the addition of ATP-actin monomers is favoured at the barbed end of the filament, while ADP-actin monomers are lost from the pointed end. This, together with the fact that barbed ends are orientated towards the plasma membrane in a cell, allows the barbed end to undergo rapid growth that drives protrusion of the cell membrane and thus cell motility.

The polymerization of pure actin monomers is unfavourable, due to the inherent instability of actin dimers and trimers; however, actin filaments grow rapidly once they are larger than trimers. Actin is the most abundant protein in many eukaryotic cells, with concentrations of over 100 μM. Pure actin has a critical concentration of only 0.1–1 μM at the barbed end, so the cell has evolved mechanisms to control the number of free barbed ends. Currently there are three proposed mechanisms for the generation of free

Abbreviations used: A, acidic; Abi-2, abl-interactor 2; ADF, actin-depolymerizing factor; Arp, actin-related protein; ARPC, Arp2/3 complex component; B, basic; C, central; cAR, cyclic AMP receptor; CARMIL, capping protein, Arp2/3 and myosin I linker; CRIB, Cdc42 and Rac interactive binding; EGF, epidermal growth factor; EVH1, Ena (*Drosophila* enabled)/VASP (vasodilator-stimulated phosphoprotein) homology 1; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; HSPC300, haematopoietic stem progenitor cell 300; IRS, insulin receptor substrate; Nap125, Nck-associated protein with an M_r of 125 000; N-WASP, neural Wiskott–Aldrich syndrome protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP5K, phosphatidylinositol phosphate 5-kinase; PIR121, p53-inducible mRNA with an M_r of 140 000; RNAi, RNA-mediated interference; Scar, suppressor of the cAR; SH2, src homology 2; SH3, src homology 3; SHD, Scar homology domain; WAVE, WASP-family verprolin homologous protein; WCA, WH2, central and acidic domains; WH1, WASP homology 1; WH2, WASP homology 2; WIP, WASP-interacting protein.

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Table 1 Arp2/3-complex nomenclature

The Arp2/3 complex composition of four commonly studied model organisms is shown together with that of *Homo sapiens*. The GenBank® accession numbers for the *Homo sapiens* Arp2/3 complex is also shown. N/A, not applicable.

	Molecular size in commonly studied species (kDa)				
ARPC nomenclature	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Dictyostelium discoideum*	*Acanthamoeba castellani*	*H. sapiens* (GenBank® accession number)
Arp2	44	44	44	44	44 (NM_005722)
Arp3	50	47	47	47	47 (NM_005721)
Arp3β	N/A	N/A	N/A	N/A	48 (NM_020445)
ARPC1a	40	42	41	40	42 (NM_006409)
ARPC1b	N/A	N/A	N/A	N/A	41 (NM_005720)
ARPC2	35	35	34	35	34 (NM_005731)
ARPC3	18	21	21	19	21 (NM_005719)
ARPC4	19	19	20	18	20 (NM_005718)
ARPC5a	15	15	16	14	16 (NM_005717)
ARPC5b	N/A	N/A	N/A	N/A	16 (AI680135)

barbed ends, and thus the regulation of actin filament assembly, in cells.

Firstly, cells regulate the growth of actin filaments via capping proteins, which bind to the barbed ends of filaments with nanomolar affinity and prevent further elongation. Examples are gelsolin and capping protein, which together are able to cap the majority of barbed ends within most animal-cell types. Caps can be removed when signals trigger actin assembly, leading to rapid filament elongation. One mechanism of uncapping is thought to be interaction with membrane polyphosphoinositides, which rapidly turn over when various types of cells are stimulated. Other mechanisms may exist, but have not been widely explored.

Secondly, filament severing provides a source of free barbed ends. ADF (actin-depolymerizing factor)/cofilin creates free barbed ends via a severing activity that is essential in cell spreading and lamellipodia formation in response to epidermal growth factor (EGF) [5,6] and other signals.

Finally, *de novo* actin filament nucleation produces new filament seeds. Since nucleation is kinetically unfavourable, other proteins have been postulated to accelerate the formation of actin dimers and trimers and stabilize them. We now accept that the Arp2/3 complex is such a nucleator and, together with uncapping and severing proteins, it nucleates actin networks in response to environmental cues.

SUBUNITS OF THE Arp2/3 COMPLEX

The Arp2/3 complex is composed of two Arps (actin-related proteins), Arp2 and Arp3, and five unique proteins, all with an apparent stoichiometry of 1:1 with each other [1,7]. In fibroblasts it was initially found to be localized to regions of dynamic actin assembly [1,9]. Early studies also provided evidence that the Arp2/3 complex was required for the motility and integrity of *Saccharomyces cerevisiae* actin patches [10]. However, the importance of this complex in actin assembly was first demonstrated in 1997, when Welch and colleagues isolated the Arp2/3 complex from human platelets and showed that it was sufficient to reconstitute actin tail formation and motility of the pathogenic bacterium *Listeria monocytogenes* [2].

Many different names have been proposed for the five non-Arp subunits of the complex, mostly based on the molecular size. However, the human genome nomenclature (HUGO) has been adopted by many groups, since sizes vary in different organisms, making naming confusing. Using this system, the name is written as ARPC1 (equivalent to p41-Arc), ARPC2 (p34-Arc), ARPC3 (p21-Arc), ARPC4 (p20-Arc) and ARPC5 (p16-Arc), with ARPC referring to Arp2/3 complex component (Table 1).

Mammals express isoforms of several of the Arp2/3-complex subunits. Humans express two isoforms of ARPC1, SOP2Hs (ARPC1a) and p41-Arc (ARPC1b). SOP2Hs functionally complements loss of the 41 kDa subunit [SOP2 (suppressor of profilin 2)], in the fission yeast *Schizosaccharomyces pombe*, as does p41-Arc [11]. Humans and mice also have at least two forms of Arp3 (Arp3 and Arp3β) and two of ARPC5 (ARPC5a and ARPC5b) [12,13]. As yet the functional relevance and tissue specificity of most of these different isoforms are unknown, but some details are emerging. In mouse, ARPC5a is highly abundant in haematopoietic tissue, whereas ARPC5b is more abundant in brain [13]. Peptide sequences obtained from purified Arp2/3 complex indicate that human platelets and neutrophils predominantly (or exclusively) contain ARPC1, but not SOP2Hs, and ARPC5a, but not ARPC5b [9], hinting again at specificity to haematopoietic cell expression. This may not be surprising, given that haematopoietic cells are often highly motile and participate in specialized functions such as phagocytosis and immune synapse formation.

THE Arp2/3 COMPLEX IS ESSENTIAL FOR MANY CELLULAR PROCESSES

The importance of the Arp2/3 complex is underscored by knockout and knockdown experiments showing that, among actin-binding proteins, it is one of the very few to be essential for life in many cell types or extremely important for actin organization in others. In *Schizo. pombe*, all of the Arp2/3 complex subunits are essential [11], whereas in *Sacch. cerevisiae* cells can surprisingly survive (albeit with various extents of cell-growth defects) without all subunits except ARPC1 [14]. In human cells, RNAi (RNA-mediated interference) of ARPC3 is lethal [15], whereas in *Dictyostelium* it has not been possible to knock out any of the Arp subunits (Robert Insall, personal communication), presumably on account of lethality.

The Arp2/3 complex appears to be important in a variety of specialized cell functions that involve the actin cytoskeleton. For example, the Arp2/3 complex is necessary for phagocytosis in mammals and the social amoeba *Dictyostelium discoideum*

[16,17]. It also has roles in endocytosis in yeast [18] and macropinocytosis [17]. Arp2/3 also appears to be involved in the establishment of cell polarity and the migration of fibroblast monolayers in a wound-healing model [19,20]. Studies utilizing loss of function mutations in the *Drosophila melanogaster* homologues of ARPC1 and Arp3 also reveal a requirement for Arp2/3 for ring-canal expansion during oogenesis, but not for formation of parallel actin bundles in the cytoplasm of nurse cells [21]. In *Drosophila*, Arp2/3 is also required for embryogenesis, as pseudocleavage furrow assembly, necessary for proper embryonic cell division, is disrupted when a mutation in the ARPC1 subunit is introduced [22]. Experiments using RNAi have revealed a crucial role for the complex in *Caenorhabditis elegans* development, as absence of any Arp2/3 subunit results in inhibition of cell migration during ventral closure [23]. The recruitment of Arp2/3 complex to the integrin-associated protein vinculin has also been reported [24] and may link actin polymerization, lamellipodial protrusion and integrin-mediated attachment.

Arp2/3-dependent actin polymerization is also necessary for shape changes during platelet activation, as addition of an anti-Arp2 antibody (which inhibits Arp2/3 nucleation activity *in vitro*) to permeabilized platelets causes them to 'freeze' and inhibits formation of filopodia and lamellipodia [25]. However, it should be noted that proteins such as gelsolin [26,27] have a critical role in regulating actin assembly as well, since, in gelsolin-null platelets, actin polymerization and cell spreading are poor [28,29]. Furthermore, evidence suggests that both the uncapping of filaments and nucleation by Arp2/3 complex contribute roughly equally to actin polymerization upon platelet activation ([30], but see [30a]).

Arp2/3 COMPLEX IN PLANTS

Plant cells contain a well-defined actin cytoskeleton that is important for many aspects of cell behaviour, including morphogenesis and organogenesis [31,32]. Recently, genetic studies suggest that plant Arp2/3 complex performs functions similar to those of the mammalian complex. To date, eight Arp genes have been identified in *Arabidopsis thaliana* (thale cress), including orthologues of mammalian Arp2 and Arp3 [33]. The Arp2 subunit from *A. thaliana* is expressed in all plant tissues at a very low level, especially when compared with Arp2 levels in mammalian tissues, but is most evident in cells surrounding the xylem and in pollen grains [34]. More recently, identification of eight essential genes necessary for actin-dependent processes of specialized leaf epidermal cells called trichomes has implicated Arp2/3 in plant development. The so-called 'distorted' complementation group of mutations (*alien, crooked, distorted1, distorted2, gnarled, klunker, spirrig* and *wurm*) encode components of the Arp2/3 complex [35–37]. *Wurm* and *distorted1* encode Arp2 and Arp3 respectively and are important in cell expansion during trichome development [35,37]. The most obvious feature of the mutants was a general distortion of trichome cells and the failure of cotyledon cells to develop their characteristic lobed, 'jigsaw-puzzle' shape [35–37]. Filamentous actin in these cells is densely bundled and incorrectly distributed compared with wild-type cells. The gene mutated in *crooked* encodes the *A. thaliana* ARPC5 homologue [38] and, like Arp2 and Arp3, is necessary for normal actin distribution, rapid cell expansion in developing trichomes and normal cell shape. Similar results were seen using T-DNA insertion mutants for Arp2, Arp3 and ARPC5 [36]. It is noteworthy that the T-DNA insertion plant mutants did not show major defects in development and were still viable [36], echoing the results in *Sacch. cerevisiae*, where only ARPC1 was essential [14].

THE DENDRITIC NUCLEATION MODEL

Since 1998, biochemical assays have provided major insights into the details of Arp2/3 function. Not only was the Arp2/3 complex shown to accelerate the rate of actin filament nucleation in solution, but, importantly, it bound to, and capped, the pointed end of filaments, leaving the barbed end free for elongation [3]. Additionally, Blanchoin and colleagues [4] discovered that inclusion of actin filaments in mixtures containing activated Arp2/3 complex accelerated the nucleation activity, likely by serving as a template for growth of new filaments and suggesting a branching method of growth. Branches were also evident in electron-micrographs of rapidly frozen specimens, suggesting that the Arp2/3 complex bound to the side of pre-existing filaments and induced the formation of end-side junctions between filaments by linking the pointed end of a newly nucleated filament to the side of a pre-existing one [3]. Recent structural analysis from cryo-electron-micrographs of Arp2/3 in actin filament branch junctions shows that the complex forms contacts with three actin subunits along the mother filament and provides evidence that the two Arps form the first subunits of the nascent daughter filament [40]. Structures identical with these are seen in the leading edge of the lamellipodia of both keratocytes and fibroblasts, indicating that the Arp2/3 complex promotes formation of an orthogonal Y-branched network of F-actin [41]. These studies have lead to the development of the now widely accepted dendritic nucleation model of actin polymerization (Figure 1) [3]. Elegant experiments coupling traditional actin polymerization assays with an *in vitro* optical-microscopy assay [42] demonstrate filament branching live during nucleation by the Arp2/3 complex activated by fragments of WASP proteins.

Exactly which subunits of the Arp2/3 complex bind to the side of the filament is not yet resolved. Although the crystal structure of the complex suggests ARPC2 and ARPC1 are the leading candidates, work using recombinant Arp2/3 *in vitro* reveals that ARPC1 is not required and that the anchoring subunits are in fact ARPC2 and ARPC4 [43]. An antibody raised to the C-terminus of ARPC2 also blocks Arp2/3 complex side binding and branching and, when microinjected into MTLn3 carcinoma cells, inhibits cell spreading, indicating that side-binding activity of Arp2/3 is necessary for protrusive force *in vivo* [44].

THE BARBED-END BRANCHING MODEL

Despite the popularity of the dendritic-nucleation model, data obtained by other groups have led to the development of an alternative barbed-end branching model. Pantaloni et al. [45] proposed that activated Arp2/3 complex binds to the barbed end of the actin filament, rather than at the side, and that the rate of Arp2/3-mediated actin polymerization is proportional to the number of free barbed ends, not the total length of the actin mother filament. The incorporated complex is proposed to initiate new branch formation while allowing continual growth of the old branch, and thus the Arp2/3 complex competes with capping protein in order to maintain the steady state of actin assembly [45]. Actin filaments that are capped by gelsolin are unable to promote Arp2/3-complex-mediated actin polymerization *in vitro*, but when filaments are uncapped, the characteristic lag in Arp2/3-complex-mediated nucleation is much reduced, further supporting the barbed-end-branching hypothesis [26].

Other studies support the concept for barbed-end branching, including one from the Condeelis laboratory, showing that severing by cofilin leads to the formation of new barbed ends, which are preferentially used for Arp2/3-mediated branching [6].

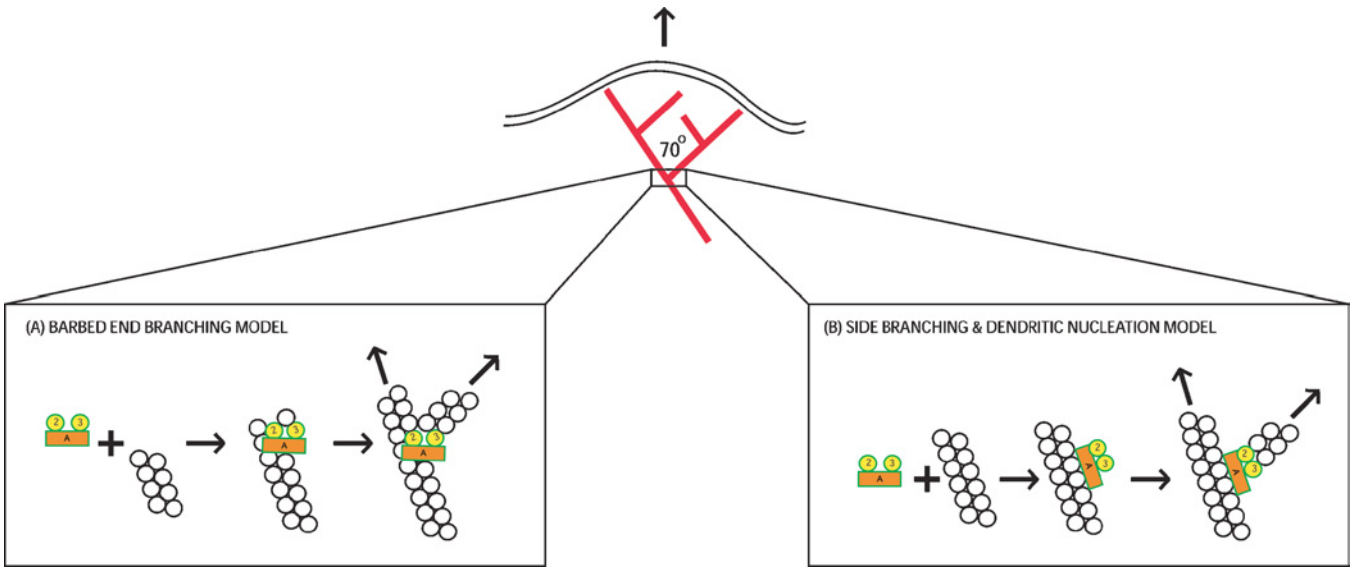

Figure 1 Barbed-end branching and dendritic nucleation models

(**A**) Barbed-end branching model. Activated Arp2/3 (for example, by binding to a WASP-family protein – this is not shown for clarity) competes with capping protein to bind to the barbed end of an actin filament. Arp2 remains bound to the mother filament, while Arp3 is outside. The two Arp subunits form the first subunits of each branch and the two branches continue to grow by addition of G-actin to each Arp. (**B**) Dendritic nucleation model. Activated Arp2/3 complex binds to the side of a pre-existing actin filament, and this side-binding activates the complex further. Both Arp2 and Arp3 form the first two subunits in the new filament. The Arp complex is indicated by the orange rectangle containing the bold letter 'A'.

Experiments from the Borisy laboratory reconstituting actin comet tails *in vitro* have also added support for barbed-end branching. Latex beads coated with the Arp2/3 complex activator ActA nucleated branches preferentially on the surface of the bead, and each branch was of similar length [46]. Thus new barbed ends might be the template for assembly. However, the authors suggested that these observations may simply result from the localized activation of Arp2/3 by the ActA that is immobilized on the bead surface.

Amann and Pollard [47] dismissed the barbed-end-branching model, arguing that if barbed-end branching occurred, both branches should elongate at similar rates and have equal lengths. They showed, using optical microscopy, that the lengths of mother and daughter filaments are very poorly correlated. Amann and Pollard [47] also demonstrated in real time, using imaging of fluorescent actin, that the Arp2/3 complex can nucleate actin filaments from the sides of pre-existing filaments.

Cooper and colleagues [48] used kinetic modelling to differentiate between the barbed-end and side-branching models, and they concluded that a side-branching model gives the best fit for the nucleation activity of Arp2/3 in the presence of a range of concentrations of capping protein. Interestingly, they favour a model with a factor included for the 'age' of the actin subunit, whereby older regions of a filament are less able to produce branches. This model provides a plausible mechanism to restrict new barbed-end growth near the periphery of a cell, unlike a straight side-branching model.

Arp2/3-COMPLEX STRUCTURE

Three years ago, a 2.0 Å (0.2 nm) crystal structure of bovine Arp2/3 complex was determined in a landmark study by Robinson and colleagues [49] (Figure 2). Perhaps unsurprisingly, the two Arp subunits are folded like actin, except that the central cleft characteristic to actin is more widely open for Arp2 and Arp3 (approx. 18° and 12° further than actin respectively). Unlike actin, however, which readily co-crystallizes with its bound nucleotide, the ATP bound to Arp2 and Arp3 was lost during the purification and crystallization processes, indicating that Arp2 and Arp3 have a lower affinity for ATP than actin, in agreement with previous results of Dayel et al. [50] (see below). ARPC2 and ARPC4 form the core of the complex and cradle the two Arp subunits by forming a C-shaped clamp. ARPC1 is a seven-bladed WD40 β-propeller protein and in the complex it has an extensive area of exposed basic residues. ARPC3 is a globular α-helical bundle that is structurally unrelated to any known protein and is the only member of the complex to contact only a single subunit, interacting with subdomains 3 and 4 of Arp3. Finally ARPC5, the smallest member of the complex, is also α-helical in structure and interacts with ARPC4, ARPC1 and Arp2 [49]. Reassuringly, these data agree with previous chemical cross-linking and yeast two-hybrid studies [51].

As the structure was obtained without ATP in the nucleotide-binding pockets of Arp2 and Arp3, the authors proposed that this was the conformation of the inactive complex. This is supported by the fact that, although the structure shows the two Arps to be orientated in a head-to-tail fashion, much like those in an actin filament, they are in fact rotated 180° around the filament axis relative to each other, so that, in this conformation, it is impossible for the Arps to form an actin helix [49]. Clearly in order to 'activate' the Arp2/3 complex a conformational change is required to orient the two Arps so that they assume the conformation of monomers in an actin filament to allow the rapid actin polymerization from the barbed end. WASP-family proteins (see below), pre-existing actin filaments and ATP have all been implicated as important in triggering an activating conformational change in the complex [52]. Robinson and colleagues propose that activation would need to promote a rotation of approx. 20° of Arp2, ARPC4, ARPC1 and ARPC5 relative to Arp3, ARPC2 and ARPC3, resulting in a translocation of Arp2 relative to Arp3 (Figure 2b) to position the Arp subdomains relative to each other

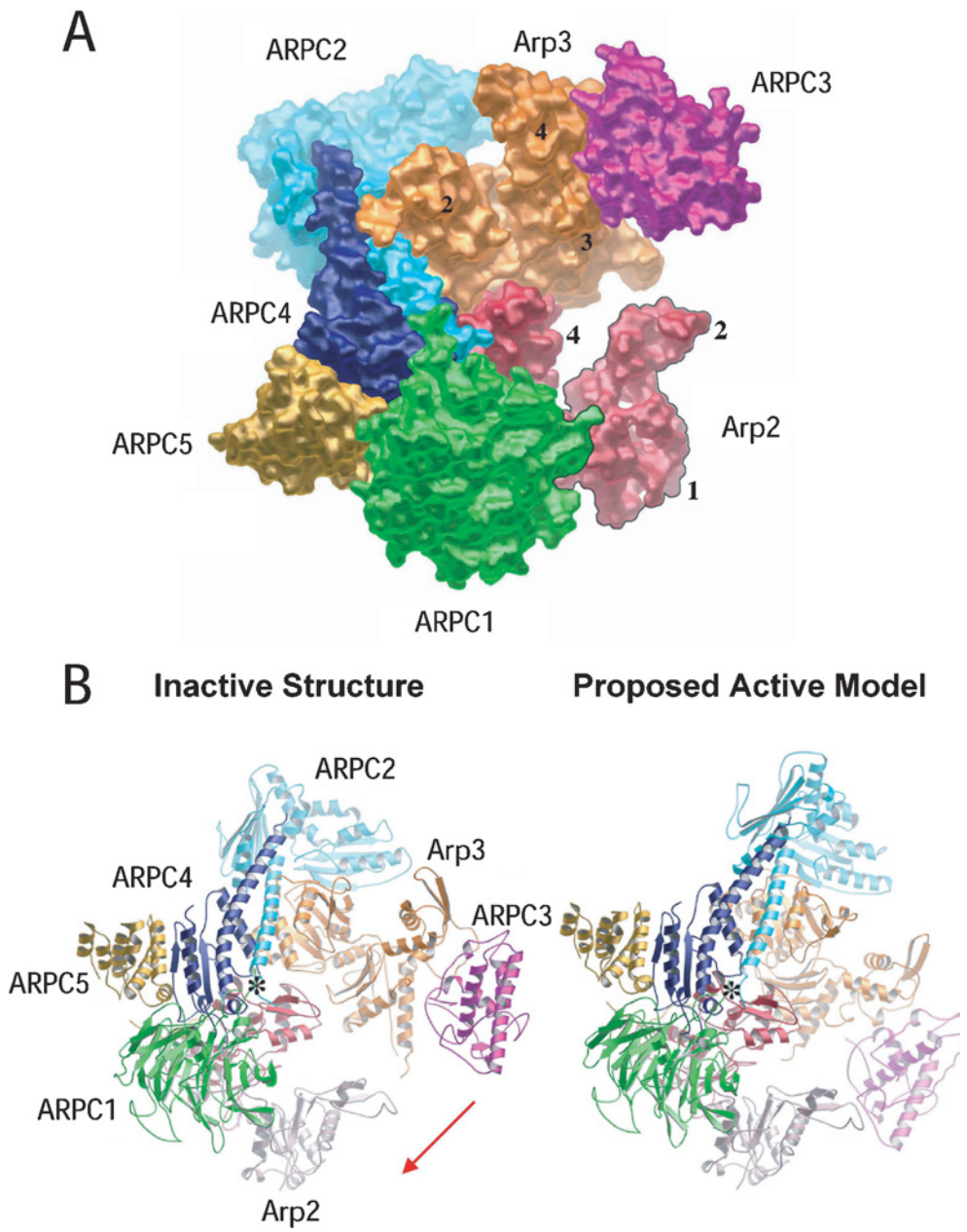

Figure 2 Structure of bovine Arp2/3 complex

(**A**) Space-filling model of the Arp2/3 complex solved by X-ray crystallography with the subdomains of Arp2 and Arp3 indicated. (**B**) Ribbon diagrams showing the model for activation of the Arp2/3 complex proposed by Robinson et al. [49]. In the inactive model the two Arps are positioned away from each other. On activation of the complex, a conformational change is proposed so that Arp2, ARPC4, ARPC1 and ARPCS are rotated (arrow) so that Arp2 and Arp3 are now in the same orientation, like two subunits of an actin-filament helix. Atomic structures are reprinted with permission from [49]. © 2001 AAAS.

such that nucleation could occur [49]. However, as yet there is no evidence that such a large conformational change does take place.

Despite conservation across species, it is interesting that, of the solvent-exposed residues of the Arp2/3 complex, only 45 % are conserved, and, of those, only 15 % are identical across seven of the most commonly used model organisms. Many of these conserved residues are presumably critical for Arp2/3 function [53]. Hopefully, the identification of these conserved residues and appropriate chemical and mutagenic studies will lead to new insights into the mechanism of Arp2/3 action [53].

Arp2/3 COMPLEX AND ATP HYDROLYSIS

The role of nucleotide binding and hydrolysis on Arp2/3 is not yet clear, but nucleotide hydrolysis has been implicated in both branch formation and dissociation. Dayel et al. [50] report that ATP associates with Arp2 and Arp3 with micromolar affinity and that N-WASP (neural WASP, an Arp2/3 activator; see below) can increase this affinity 3-fold. Hydrolysis of bound ATP by the Arps is essential for the nucleation of new actin filaments, since, when bound to ADP or a non-hydrolysable ATP analogue, the Arp2/3 complex is inactive [50]. When the Arps are bound to an

ADP analogue that mimics an ADP–P_i conformation on actin, the nucleation activity of the complex is restored. This agrees with mathematical models of Arp2/3 activation, which require a hydrolysis step, so the authors argue that it is the ADP–P_i-bound Arp2/3 complex that is the active form. Dayel et al. [50] postulated that hydrolysis of ATP to ADP + P_i results in the closure of the nucleotide-binding clefts of the Arps and promotes a conformational change that brings Arp2 and Arp3 together, forming a stable nucleus that allows actin polymerization in the direction of the barbed end. Carlier and colleagues [54] agree that ATP hydrolysis is likely to be important for filament branching; however, in contrast with Dayel et al. [50], the Carlier group report a much higher affinity of ATP for Arp2. They also demonstrate that binding of WASP to Arp2/3 increases the affinity of ATP for Arp2 without affecting ATP binding to Arp3 [54]. The same group also report that ATP hydrolysis occurs exclusively on Arp2 and not Arp3 [55] and that ATP hydrolysis occurs after filament branching to promote debranching of the dendritic actin array [55]. This again contrasts with previous work of Blanchoin and Pollard [56–58], who argued that it is the hydrolysis of ATP bound to actin filaments that causes debranching. While ATP–actin, ADP–P_i–actin and ADP–actin could all support filament branching, branch formation from ADP–actin filaments is rare, suggesting that ATP hydrolysis and phosphate dissociation weaken the interaction of the Arp2/3 complex with the pointed end of the new filament, ultimately resulting in filament disassembly [42]. The ADF/cofilin protein actophorin was also shown to both promote the release of γ-phosphate from ADP–P_i actin filaments and promote debranching [58]. Clearly more work is needed to establish the details of how debranching occurs *in vivo*.

WASP family

The gene mutated in Wiskott–Aldrich syndrome, a rare X-chromosome-linked disorder, was identified in 1994 [59]. WASP is a 501-amino-acid proline-rich protein expressed exclusively in haematopoietic cells. An isoform was later isolated from brain and called N-WASP, although it is in fact widely expressed [60]. A protein related to WASP was identified in a genetic screen in *Dictyostelium* and named Scar, as disruption of the Scar gene suppresses abnormalities caused by loss of one of the cARs (cAMP receptors), namely cAR-2 [61]. In the same year, a mammalian homologue of Scar was isolated independently by two groups. One group retained the name Scar; the other named the protein WAVE (WASP family verprolin homologous protein), and both names are widely used [62,63]. Mammals express two further isoforms of Scar/WAVE, bringing the total number of WASP family members to five: namely WASP, N-WASP, Scar/WAVE1, Scar/WAVE2 and Scar/WAVE3 [64]. *Sacch. cerevisiae* expresses a single WASP family protein, more closely related to WASP than to Scar, while *Dictyostelium*, *C. elegans* and *Drosophila* express one WASP and one Scar [23,61, 65–67].

WASP was originally described as a protein that could induce actin polymerization in connection with Cdc42, a small GTPase already known to be an important actin regulator [68]. The precise nature of the link between WASP-family proteins and actin polymerization was established when it was discovered that human Scar binds to the Arp2/3 complex and that this interaction stimulates the actin-filament nucleation activity of the complex [4,62]. Soon after, it was demonstrated that binding of active Cdc42 enhances the ability of N-WASP to activate Arp2/3, and thus a signalling pathway leading to actin-filament nucleation was identified for the first time [69].

DOMAIN ORGANIZATION OF WASP-FAMILY PROTEINS

All WASP-family proteins have a conserved domain arrangement (Figure 3). The extreme C-terminus consists of a module responsible for binding to, and activating, the Arp2/3 complex [4]. This module is made up of a WH2 (WASP homology 2) sequence followed by a short C (central) sequence and an A (acidic) sequence. The C region is sometimes referred to as the 'cofilin homology domain'; however, it is likely that any homology with cofilin is coincidental. WH2 domains bind monomeric actin and are found in many proteins that regulate actin [70]. The C and A sequences bind to, and activate, the Arp2/3 complex. N-WASP is unique among the mammalian WASPs in having two, rather than one, WH2 domains. This C-terminal module variously termed 'WA', 'WCA' or 'VCA' [WCA (WH2, central and acidic domains) henceforth in this review] is the minimal region required to bind to, and activate, the Arp2/3 complex [4,62].

The N-termini of WASP-family proteins are more divergent among family members. Each possesses a proline-rich stretch adjacent to the WCA domain that acts as a binding site for various SH3 (src homology 3)-domain-containing proteins. A domain at the N-terminus of each family member defines whether it is classed as a WASP or a Scar: WASP and N-WASP contain the WH1 (WASP homology 1) domain, which is related to the EVH1 [Ena (*Drosophila* enabled)/VASP (vasodilator-stimulated phosphoprotein) homology 1] domain of the Ena/VASP family of proteins, while Scar proteins have a unique SHD (Scar homology domain). The WH1 domain of WASP and N-WASP bind to WIP (WASP-interacting protein); however, no interactions have yet been identified for the SHD [71]. WASP and N-WASP, but not the Scars, possess a CRIB (Cdc42 and Rac interactive binding) domain that binds the small GTPase Cdc42 [72,73]. Adjacent to the CRIB, WASP and N-WASP have a basic stretch that binds to the phospholipid PIP_2 (phosphatidylinositol 4,5-bisphosphate) [74]. The Scar proteins have a basic stretch adjacent to the SHD, but the function of this has not been established. Scar homologues in *Drosophila*, *C. elegans* and *Dictyostelium* share domain organization with the mammalian Scars. WASP homologues in *C. elegans* and *Drosophila* share domain organization with N-WASP, while *Dictyostelium* WASP is more similar to mammalian WASP in that it has one, rather than two, WH2 domain. *Sacch. cerevisiae* and *Schizo. pombe* WASP homologues are more divergent in having no CRIB or basic domains in addition to having a single WH2 domain.

ACTIVATION OF THE Arp2/3 COMPLEX BY WASP-FAMILY PROTEINS

The WCA domain of WASP-family proteins is necessary and sufficient for the activation of the Arp2/3 complex [4]. While the WCA domains of N-WASP, WASP and Scar1 bind to the Arp2/3 complex with similar affinities, they stimulate actin nucleation to different extents, with N-WASP WCA inducing nucleation 70-fold more quickly than Scar1 WCA [75]. This, in addition to data obtained using WASP point mutants, has led to the suggestion that, following the binding of WASP to Arp2/3, there is a separate activation step that involves a conformational change in the Arp2/3 complex [52]. Further interactions between WASP and Arp2/3, probably involving residues in the C domain, are required to induce this conformational change, and it is the rate at which this activation step occurs that determines the rate at which WASP proteins stimulate nucleation [52,73,75,76]. When not activated, N-WASP and WASP exist in an autoinhibited state in which the C domain interacts with CRIB domain [77,78]. When in the autoinhibited conformation, WASP and N-WASP are unable to induce activation of the Arp2/3 complex, as the WASP A domain

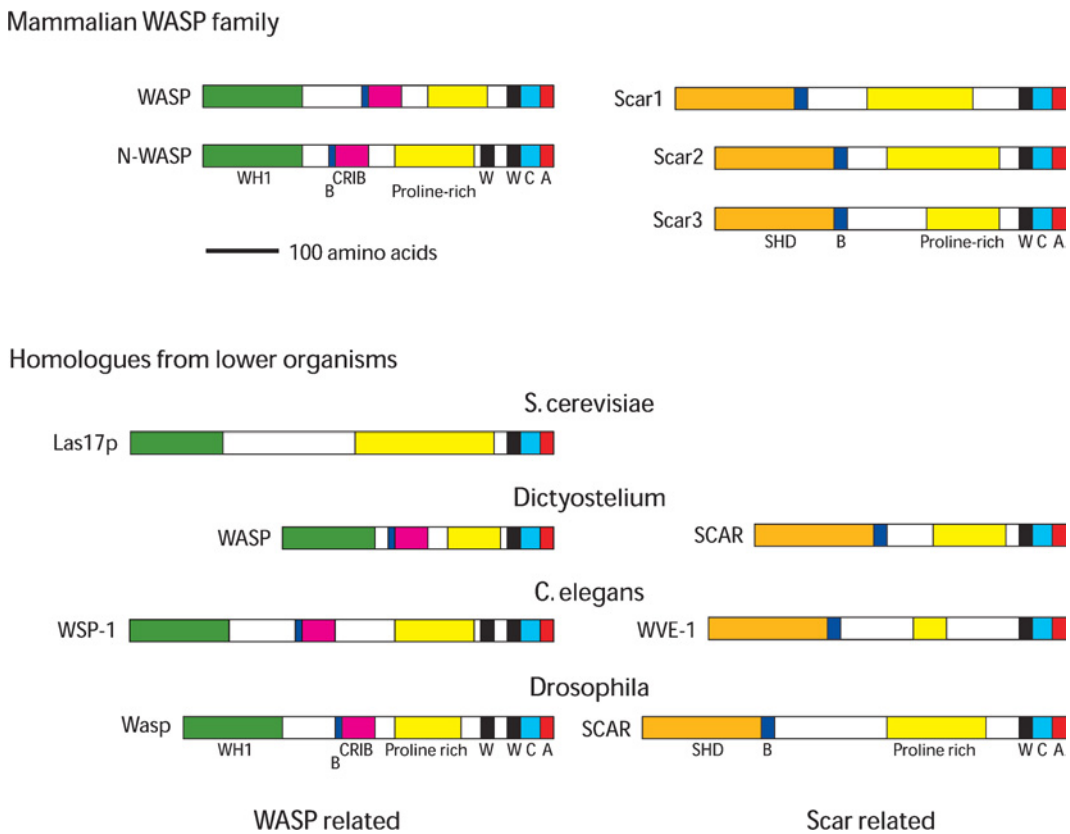

Figure 3 Domain organization of the mammalian WASP family and homologues from other organisms

Abbreviations: WH1, WASP homology 1 domain; B, basic domain; CRIB, Cdc42 and Rac interactive binding domain; W, WASP homology 2 domain; C, central domain; A, acidic domain; SHD, Scar homolgy domain.

cannot interact fully with the Arp2/3 complex [76]. Binding of activator proteins to the CRIB or proline-rich sequences of WASP destabilizes the interaction between the CRIB domain and the C domain, allowing full binding of the WASP A domain to Arp2/3 [78,79]. The Scar proteins, which do not possess a CRIB domain, do not form an autoinhibited state [62]. N-WASP is unique among the WASP family in having a second WH2 domain adjacent to the first. There are conflicting opinions as to the importance of the second WH2 domain of N-WASP. While one study found that the presence of a second WH2 domain enhanced the ability of N-WASP to stimulate Arp2/3-induced filament nucleation, another found it to have no effect [75,80,81].

REGULATION OF WASP AND N-WASP

Cdc42 is an activator of WASP and N-WASP [69,72] and, in its active GTP-bound form, it binds to the CRIB domains of these proteins [82]. This results in destabilization of the autoinhibition and subsequent activation of WASPs [69,77]. Binding of PIP_2 to the basic region adjacent to the CRIB potentiates Cdc42 activation of WASP [69,72–74]. Cdc42 is a key regulator of the actin cytoskeleton, and a frequently observed effect of Cdc42 activation is the production of actin-rich protrusions or filopodia over the surface of the cell [83,84]. Cdc42 has numerous effectors, so it likely exerts its effects on the actin cytoskeleton via the co-ordinated actions of several proteins; however, WASP is noteworthy among Cdc42 effectors in that it is able to directly stimulate actin assembly. Cdc42 is activated downstream of receptor activation via the action of specific GEFs (guanine-nucleotide exchange factors) [85]. Two other GTPases, Tc10 and RhoT, both of which are closely related to Cdc42, have also been shown to activate N-WASP [86].

Two adaptor proteins, Nck and Grb2, activate N-WASP via binding of their SH3 domains to the proline-rich domain of N-WASP [79,87]. Nck and Grb2 possess SH2 (src homology 2) domains, which associate directly with activated tyrosine kinase receptors by binding to specific phosphotyrosine-containing motifs [88]. Activation of N-WASP by Grb2 and Nck therefore provides a potential direct link between ligand engagement of tyrosine kinase receptors and activation of Arp2/3-mediated actin assembly. WASP co-immunoprecipitates with activated EGF receptor and this association is enhanced by addition of exogenous Grb2 [60,89]. Nck is required for the recruitment of N-WASP to vaccinia virus, and this interaction is essential for motility of the virus [90]. *In vitro*, the activation of N-WASP by Nck can be further increased by the addition of PIP_2, but not Cdc42, suggesting that Nck and Cdc42 activate N-WASP by redundant mechanisms [79].

Various other SH3-domain-containing proteins have been identified as WASP interactors. These proteins include WISH (WASP-interacting SH3 protein), Tec family kinases, phospholipase Cγ, CIP4 (Cdc42-interacting protein 4), syndapin I, PSTPIP (proline/serine/threonine-phosphatase-interacting protein), endophilin A, intersectin and Src family kinases [91–99]. There are as yet insufficient data for us to have a true picture of the

physiological roles of these interactions; however, some of these interactors will be discussed below. Profilin and VASP also interact with the proline-rich domain of WASP, and both enhance the actin-based motility of WASP-coated beads in reconstituted systems; however, both of these proteins also interact with actin, and it is not known whether the observed effect is due to a direct interaction with WASP [100–102].

Recent studies have established that phosphorylation of WASP itself plays a role in regulating WASP activity. WASP is tyrosine-phosphorylated in response to B-cell-receptor cross-linking and, while neither the phosphorylation site or the kinase responsible has been identified, *in vitro*, Tyr^{291} of WASP is a substrate for Abl, Btk and Src family kinases [103–105]. Tyr^{291} lies adjacent to the CRIB domain of WASP, and phosphorylation of this residue destabilizes the interaction of the CRIB domain with the WCA region and thus increases the basal activity of the inactive form of WASP [104,105]. Interestingly, phosphorylation and dephosphorylation of this residue can only occur efficiently when active Cdc42 is bound to WASP [105,106]. A model is proposed whereby WASP is activated by Cdc42, allowing Tyr^{291} to become phosphorylated. This phosphorylated form of WASP will persist after Cdc42 has been released, and hence WASP will remain active even after removal of the stimulus. Phosphorylation of the equivalent tyrosine in N-WASP also increases basal activity, and there is evidence that Src family kinases are responsible for this phosphorylation *in vivo* [107]. In the presence of a proteasome inhibitor, ubiquitinated N-WASP has been detected, but this ubiquitination is lost if Src kinases are also inhibited. It has therefore been suggested that phosphorylation of N-WASP by Src family kinases might trigger the ubiquitination, and hence proteolysis, of N-WASP. If these data are taken together, a speculative model could be proposed whereby WASP and N-WASP are regulated in a cycle consisting of activation by Cdc42 binding, phosphorylation, then ultimately inactivation by proteolysis. WASP was recently found to be constitutively phosphorylated by casein kinase 2 on Ser^{483} and Ser^{484} in a number of cell lines [108]. These residues lie within the WCA region of WASP, and the phosphorylation increased the affinity of WASP for the Arp2/3 complex 7-fold.

WIP binds to the WH1 domains of both WASP and N-WASP [71]. Two proteins highly related to WIP, WICH (WIP- and CR16-homologous protein)/WIRE (WIP-related) and CR16, also bind to N-WASP [109–111]. *Sacch. cerevisiae* possesses a homologue of WIP, verprolin, which binds to Bee1p/Las17p, a WASP homologue, indicating that the relationship between WASP and WIP is evolutionarily conserved [112]. WIP possesses a WH2 domain and hence binds monomeric actin; however, it also binds to filamentous actin [71,113]. There is evidence that WIP can influence the activity of N-WASP, as WIP can prevent activation of N-WASP by Cdc42, but only in the absence of PIP_2 [113]. WIP is required for Nck-dependent recruitment of N-WASP to vaccinia virus in infected cells, and there is evidence that simultaneous binding of N-WASP and WIP to Nck occurs in this situation [114].

The *Sacch. cerevisiae* homologue of WASP, Bee1p/Las17p, does not appear to have a CRIB domain or bind to Cdc42p, although it does function downstream of Cdc42p [115]. Unlike WASP proteins in higher organisms, Las17p is not autoinhibited, and recent data have suggested that, instead, its ability to activate Arp2/3 is inhibited by the binding of regulatory proteins [116]. Sla1p and Bbc1p are two related SH3-domain-containing proteins that bind to the proline-rich region of Las17p and prevent Arp2/3 activation by Las17p *in vitro* [116]. It would be interesting to know whether Las17p behaves in the same way when complexed with verprolin, since Las17p appears to be constitutively associated with verprolin *in vivo* [115].

REGULATION OF Scar PROTEINS

One crucial difference between WASP/N-WASP and the Scars is that isolated Scar is constitutively active and lacks the autoinhibition of WASP/N-WASP [4,78]. Several lines of evidence have suggested a link between Scar and the small GTPase Rac. Like Cdc42, which binds to WASP, Rac is activated downstream of receptors by specific GEFs and has numerous effectors [83,85]. Activation of Rac results in the formation of broad, sheet-like projections from the cell edge known as lamellipodia [118]. These structures consist of a dense network of branched actin filaments, and activation of the Arp2/3 complex is essential for their formation; therefore an Arp2/3 activator presumably acts downstream of Rac (see Figures 4A and 4B) [119]. Scar1 translocates to Rac-induced lamellipodia, and evidence suggests that Rac activates Scar [63]. However, unlike WASP, Scar1 does not possess a CRIB domain, and there is no evidence that Rac binds directly to Scar1.

A number of recent publications have sought to establish a link between Scar proteins and Rac. IRSp53, originally isolated as an IRS (insulin receptor substrate), was identified as a Scar-binding protein that simultaneously binds active Rac [120]. IRSp53 contains an SH3 domain, and this associates with the proline-rich region of Scar2. However, IRSp53 and active Rac have little effect on the ability of Scar2 to activate the Arp2/3 complex *in vitro*. In fact there is no evidence that IRSp53 can activate Scar2 in response to Rac, although it is possible that IRSp53 recruits Scar2 to locations where Rac is active or participates in a complex associated with active Scar2. Scar1 purified from bovine brain is present in a complex with four other proteins, namely PIR121 (p53-inducible mRNA with an M_r of 140000), Nap125 (Nck-associated protein with an M_r of 125000), Abi-2 (abl-interactor 2) and HSPC300 (haemopoietic stem progenitor cell 300) [121]. Scar1 is inactive in this complex; however, addition of active Rac or the adaptor protein Nck leads to dissociation of Scar1 and HSPC300 from the rest of the complex and Scar1 activation. These data suggest a mechanism whereby Scar1 can be activated in response to activation of Rac or tyrosine-kinase receptors via Nck (Figure 5).

In *Dictyostelium* and *Drosophila*, Scar is regulated by proteins homologous with PIR121, Nap125 and Abi-2. Loss of Kette, a *Drosophila* homologue of Nap125, results in accumulation of cytosolic filamentous actin; however, this phenotype can be suppressed by reducing levels of Scar, suggesting that Kette antagonizes Scar function [122]. Loss of a PIR121 homologue in *Dictyostelium* results in uncontrolled pseudopod formation, consistent with increased Scar activity; however, levels of Scar were found to be far lower than in wild-type cells [123]. This has led to the suggestion that, following activation of Scar by dissociation of the inhibitory complex, Scar is ultimately inactivated by proteolysis. This hypothesis is supported by another study in which it was found that reducing levels of homologues of Abi-2, PIR121 or Nap125 by RNAi in the *Drosophila* cell line S2 also resulted in a reduction in the level of Scar [124]. In that study, reducing levels of these Scar inhibitor proteins produced a phenotype identical with that obtained when reducing levels of Scar by RNAi. This contrasts with the *Drosophila* Kette knockout and the *Dictyostelium* PIR121 knockout, which produced phenotypes opposing that of loss of Scar [122,123]. It is unclear why *Drosophila* S2 cells and *Dictyostelium* show different phenotypes for loss of PIR121 proteins or Scar. *Dictyostelium* does not lose lamellipodia in the absence of Scar, while S2 cells do. Perhaps it is due to different overall actin cytoskeletal regulation in these two cell types, which may not be surprising, since *Dictyostelium* has highly motile cells similar to

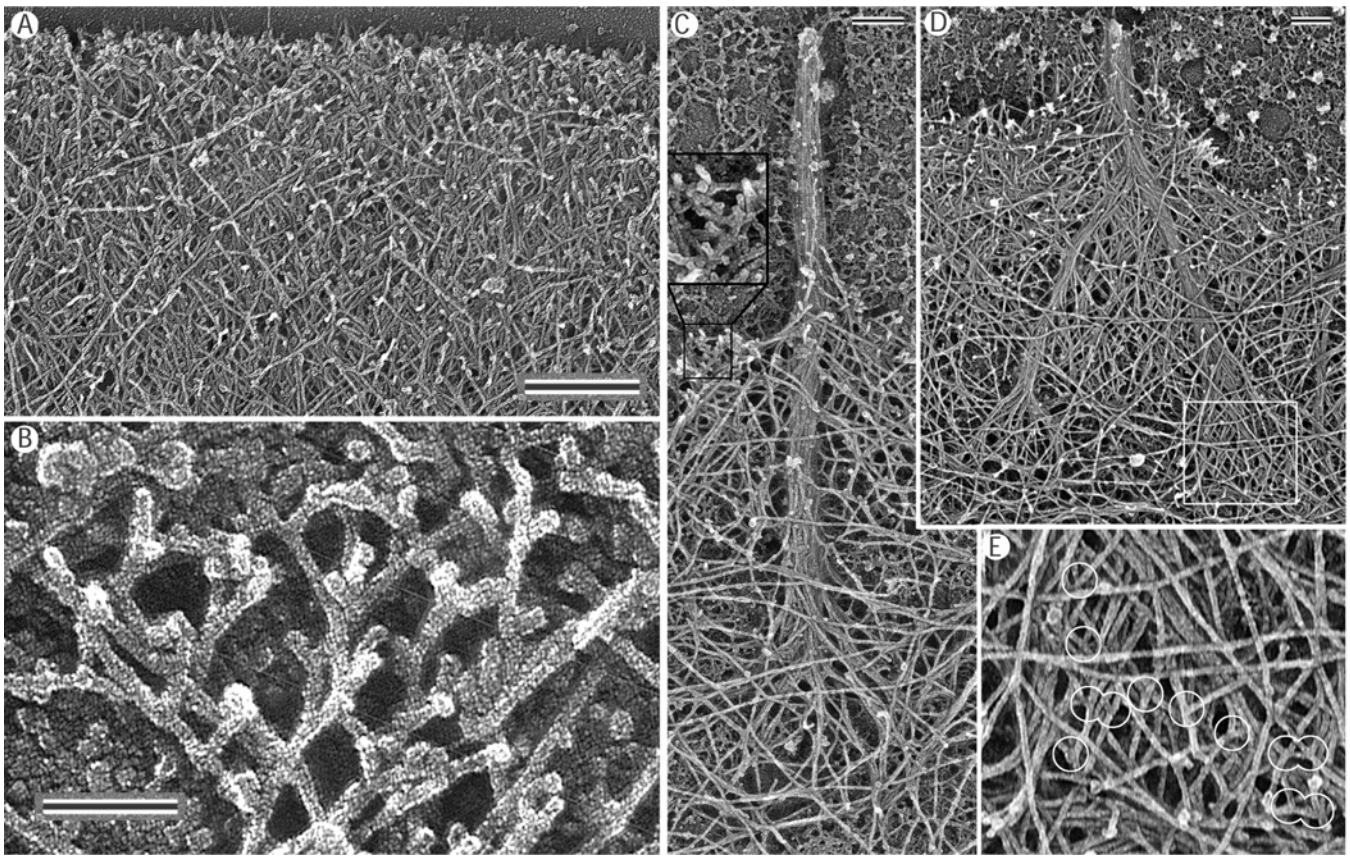

Figure 4 Electron-micrographic images of the actin cytoskeleton

(**A**) The actin cytoskeleton within a lamellipodium of a *Xenopus* keratinocyte. The scale bar represents 0.5 μm. (**B**) Lamellipodial actin cytoskeleton at greater magnification. The scale bar represents 0.1 μm. (**C**) Ultrastructure of a filopodium. The magnified box shows structure of actin cytoskeleton in the lamellipodium surrounding the filopodium. (**D**) Ultrastructure of newly formed filopodium. (**E**) Enlargement of the region boxed in (**D**) showing that the filaments within the filopodium are derived from the branched lamellipodial actin network. Circles indicate the branch point where filopodial filaments derive. The scale bars in (**C**) and (**D**) represent 0.2 μm. The Figure contains images reproduced from the Journal of Cell Biology (1999, volume 145, pp. 1009–1026 [119]; 2003, volume 160, pp. 409–421 [140]) with the permission of the publisher. Copyright © 1999 and 2003 The Rockefeller University Press.

neutrophils or macrophages and S2 cells are less motile and more closely resemble fibroblasts. Alternatively, the Scar-interacting proteins could serve a more complex role than merely acting as inhibitors. Clearly we are far from a complete understanding of Scar regulation.

A GTPase-activating protein (GAP) called WRP (WAVE-associated Rac-GAP protein) has been found to bind to mammalian Scar1. This provides a potential 'off switch' whereby Rac could be inactivated following Scar1 activation [125].

In contrast with WASP, little is known about the phosphorylation of Scar. It has been reported that Scar is phosphorylated downstream of mitogen-activated protein kinase, but the role of this phosphorylation is not known [126]. Scar binds to cAMP-activated protein kinase and Abelson kinase, although it has not been shown to be a substrate for either [127]. Given the current state of our knowledge, it seems likely that Scar proteins will be regulated by phosphorylation, but only future research will reveal the significance of these observations.

IDENTIFYING THE SPECIFIC ROLES OF WASP-FAMILY MEMBERS

To date, the fruitfly *Drosophila* is the only system in which both WASP and Scar have been successfully knocked out. WASP-deficient fruitflies mostly die as young adults. Surprisingly, those that survive do not have gross morphological abnormalities; however, a number of developmental defects are observed [67]. The most notable of these is a lack of neurosensory bristles, which results from an alteration in cell-fate decisions during embryonic development. A WASP expression construct recovers the wild-type phenotype; however, a WASP construct lacking the CA region does not [128]. This implies that WASP is acting through the Arp2/3 complex in controlling cell fate. Interestingly, a WASP construct unable to bind Cdc42 fully recovers the wild-type phenotype, suggesting that Cdc42 is not upstream of WASP in controlling cell fate. Complete loss of *Drosophila* Scar results in developmental arrest during oogenesis; however, weaker mutants have been used in which embryonic development can be studied [65]. These mutants display a range of abnormalities, including reduced cortical F-actin in the blastoderm and severe defects in axon morphology. Mutants were also generated in which Arp2/3 complex function was impaired by disruption of the ARPC1 gene [65,129]. All the defects observed in the ARPC1 mutants were common either to WASP or Scar mutants. This suggests that WASP and Scar are the only Arp2/3 activators required in these developmental processes and that the effects of WASP and Scar are mediated entirely through the Arp2/3 complex. Scar is required in more developmental processes than WASP, suggesting that Scar is the more significant Arp2/3 activator during *Drosophila* embryogenesis.

Loss of the *C. elegans* homologue of N-WASP, WSP-1, by RNAi resulted in embryonic lethality in 15 % of individuals [23]. The arrested embryos had failed to complete ventral enclosure, an actin-dependent process in which cells on either side of the embryo migrate towards one another to form a junction along the ventral midline. RNAi disruption of Arp2/3 complex subunits resulted in the same terminal phenotype.

Disruption of the gene encoding Scar in *Dictyostelium* results in small cells with movement defects and reduced levels of filamentous actin. This suggests that *Dictyostelium* Scar is involved in pseudopod formation [61].

THE MAMMALIAN WASP FAMILY

Determining the role of individual WASP-family proteins in mammals is complicated by the presence of two WASP genes and three Scar genes. Members of the mammalian WASP family have distinct tissue distributions. While WASP expression is limited to haematopoietic cells, N-WASP is ubiquitously expressed [59,60]. Of the Scars, Scar2 is the most widely expressed in adults [64,130]. Scar1 is expressed in many tissues, but its levels are far greater in brain than any other tissue [64,131]. Scar3 is also expressed in a variety of tissues, but most highly in brain, testis and lung. Levels of Scar1 and Scar2 mRNA are notably low in skeletal muscle, whereas Scar3 mRNA levels in skeletal muscle are comparable with its expression in most other tissues [130].

Of the five mammalian WASP-family proteins, four have been knocked out in mice, namely WASP, N-WASP, Scar1 and Scar2.

Loss of Scar1 in mice resulted in an increase in post-natal mortality and a reduction in animal size [132,133]. The anatomy of the Scar1-null mice appeared normal, although they showed reduced brain size and, in particular, a small cerebral cortex. Outgrowth of hippocampal neurons *in vitro* was found to be normal [133]. Scar1-null mice exhibited a range of defects in brain function, including reduced anxiety, sensorimotor retardation and reduced learning and memory [132]. These data indicate that Scar1 function is probably most important in the central nervous system, and this is consistent with its expression profile. The lack of significant effect of Scar1 deficiency outside the brain may indicate that other Scar isoforms can largely compensate for the lack of Scar1.

Disruption of the gene encoding Scar2 in mice resulted in embryonic death [134,135]. Scar2-null embryos possessed a range of developmental abnormalities, including defects in development of the brain and cardiovascular system. The widespread expression of Scar2 may indicate that it has a more general function than the other Scars and may explain why its deficiency is more severe than that of Scar1. In early embryonic development, all of the Scar isoforms are expressed evenly among tissues, but become more specifically expressed during latter stages [130,133]. At embryonic day 9, Scar1 is expressed throughout the embryo, whereas at day 12 its expression is more limited [133]. Scar2 knockout embryos died at around day 12, so it is possible that, until this time, Scar1 was able to compensate for Scar2 deficiency [134,135].

Disruption of the gene encoding N-WASP in mice resulted in embryonic death at around day 11 [136,137]. Defects in the neural tube and cardiac tissue were observed in the embryos. WASP knockout resulted in various abnormalities associated with the immune system, including a reduction in the numbers of lymphocytes, platelets and thymocytes [138]. The difference in phenotype for loss of N-WASP and WASP is presumably a reflection of their respective sites of expression. N-WASP is expressed ubiquitously and hence presumably acts ubiquitously, whereas WASP expression is limited exclusively to haematopoietic cells.

Of these knockouts, the severest phenotypes are associated with loss of N-WASP and Scar2, both of which are embryonically lethal [134–137]. This demonstrates that, in order to complete embryonic development, an individual must possess at least one Scar and one WASP, indicating that WASP and Scar are functionally distinct.

THE CELL BIOLOGY OF WASP-FAMILY KNOCKOUTS

A well-established consequence of Cdc42 activation is the production of actin-rich filopodia around the cell periphery [84,139]. When WASP/N-WASP were identified as promoters of actin polymerization downstream of Cdc42, it seemed logical that WASPs were responsible for filopodia formation. This hypothesis was supported by data showing that expression of N-WASP induced filopodia formation in a Cdc42-dependent manner [77]. A model was proposed whereby Cdc42 induced filopodia via N-WASP, while, in parallel, Rac induced lamellipodia via Scar. The recent generation of WASP-family knockouts has allowed this model to be tested.

Two groups independently generated N-WASP knockout mice and embryonic cell lines [136,137]. Surprisingly, both groups found that the resulting N-WASP-null cells were able to generate filopodia in response to Cdc42 activation. One group found a reduction in Cdc42-induced filopodia formation in N-WASP-null cells, while the other found no change. In fact little or no difference was found in terms of cell morphology or motility, including lamellipodium formation, in response to growth-factor stimulation. Since these cells do not express WASP, the results cannot be explained in terms of functional degeneracy between WASP and N-WASP.

A recent report examined the formation of filopodia using electron microscopy, a technique that allows individual actin filaments to be visualized [140]. This suggested that the filaments within filopodia originate from the dense network of highly branched Arp2/3-derived filaments found within lamellipodia (Figures 5C–5E). Unlike other filaments within the lamellipodia, which were short, owing to capping, the filaments forming filopodia were long and ultimately bundled together by cross-linking proteins. A model is proposed whereby filopodium initiation involves a stimulation of the elongation of a subset of the filaments within a lamellipodium. An Arp2/3-derived network of filaments would be a necessary prerequisite for filopodium formation, but filament nucleation would not need to be specifically activated in the initiation of filopodium formation. If this model is correct, then the factor that triggers filopodium formation acts by promoting filament elongation rather than filament nucleation. This model is consistent with the observation that N-WASP is not required for Cdc42-induced filopodia formation. It is conceivable that N-WASP could facilitate filopodium formation by providing a localized burst of filament nucleation, but ultimately other Cdc42 effectors must play a more significant role.

Studies in which Scar2 has been knocked out have supported the concept that Scar functions downstream of Rac in lamellipodium formation. Two groups that knocked out mouse Scar2 found that lamellipodia formation in response to growth-factor stimulation was severely impaired in fibroblast and endothelial cells derived from the knockouts [134,135]. Furthermore, Scar2-null cell extracts had lost the ability to activate Arp2/3 on addition of active Rac [134]. This is strong evidence that Scar2 does indeed lie downstream of Rac in lamellipodium formation. Interestingly, Scar1 expression could be detected in the Scar2-null fibroblasts,

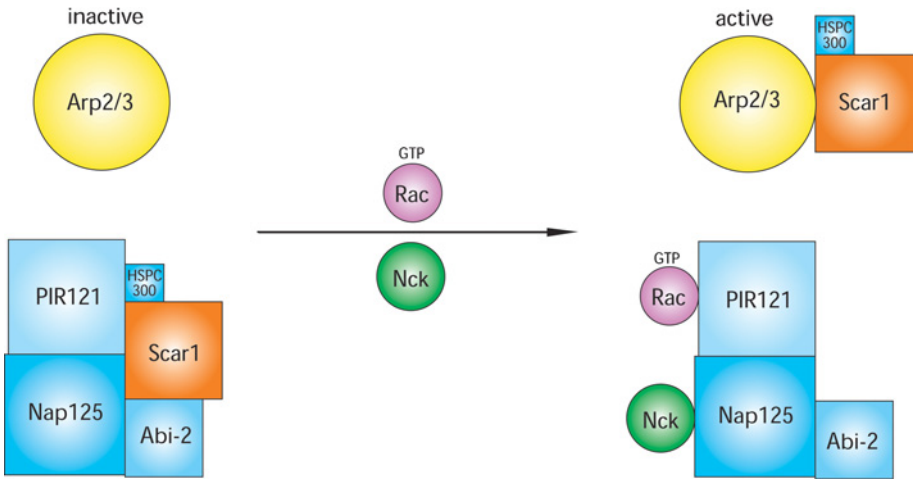

Figure 5 Model for the regulation of Scar1

Prior to stimulation, Scar1 is sequestered into a complex in which its activity is inhibited. A stimulus results in activation of Rac or Nck, which then bind to members of the inhibitory complex. Scar1 is then released from the complex, and the inhibition is relieved, allowing Scar1 to activate the Arp2/3 complex. Note that the binding site for Nck within the complex has not been established.

and yet its presence was unable to compensate for the lack of Scar2 in these experiments. Fibroblasts have also been isolated from Scar1-null embryos and their motility compared with that of Scar2-null fibroblasts. Unlike Scar2-deficient cells, the Scar1-null cells were able to generate lamellipodia in response to growth-factor stimulation; however, the Scar1-null cells did not generate dorsal ruffles, which are ruffles formed behind the leading edge of the cell [141]. Migration through medium was impaired by deficiency of Scar2, but not of Scar1; however, migration through the extracellular matrix was impaired by deficiency of either isoform. These data suggest that Scar1 and Scar2 may be regulated differentially, such that they trigger actin assembly in different cellular locations or in response to different extracellular environments.

Two recent studies in which WASP and Scar were depleted from *Drosophila* cell lines by RNAi found that Scar depletion severely inhibited lamellipodium formation, whereas no phenotype was observed on depletion of WASP [124,142]. Electron-microscopic analysis of lamellipodia in the Scar-depleted cells revealed a very sparse filament network. Interestingly, filopodia formation was also inhibited in the Scar-depleted cells. This may be evidence that filopodia do indeed derive from lamellipodia and, as such, require normal lamellipodial architecture in order to form. However, it has been previously shown that Cdc42 still induces filopodia in the presence of dominant-negative Rac and that a Cdc42 mutant that does not activate Rac also induces filopodia, suggesting that filopodia can form in the absence of detectable lamellipodia [84,143]. Therefore there is still doubt as to the precise relationship between filopodia and lamellipodia. It is likely that there are multiple mechanisms by which filopodia are formed and these could vary in significance between different cell types.

THE ROLE OF WASP IN MEMBRANE TRAFFIC

While knockout studies have failed to establish a role for N-WASP in the formation of filopodia or lamellipodia, N-WASP knockout cells were unable to support the motility of the pathogens *Shigella*, vaccinia virus and enteropathogenic *Escherichia coli* [136,137]. It is well established that these pathogens use their host cell's actin-assembly apparatus to move within and between host cells. For instance, vaccinia virus recruits N-WASP, which activates the Arp2/3 complex, resulting in the formation of an actin comet tail that propels the virus through the cytoplasm of the infected cell [90]. This has led to the suggestion that vesicle movement within cells could occur by a similar mechanism. Overexpression of phosphatidylinositol phosphate 5-kinase (PIP5K), an enzyme that generates PIP_2, induced the formation of actin comets on vesicles in fibroblasts [144]. This effect could be inhibited by expression of an N-WASP construct lacking the WCA domain, suggesting that N-WASP was involved in stimulating comet formation. Likewise, vesicle rocketing was abolished in N-WASP-deficient cells [145]. Interestingly, the recruitment of N-WASP to vesicles was found to involve WIP and Nck, which precisely mirrors the recruitment of N-WASP to vaccinia virus, suggesting that vaccinia-virus motility in host cells may be an exaggerated example of a genuine cellular means of vesicle propulsion [90,114,145].

In addition to vesicle motility, there is also evidence that WASP proteins play a role in clathrin-mediated endocytosis. Clathrin-mediated endocytosis involves the formation of an invagination or pit in the plasma membrane, the neck of which is then constricted and pinched off [146]. The constriction is controlled by a large GTPase called dynamin, and several proteins that bind dynamin have also been shown to bind N-WASP. Thus N-WASP may be recruited to sites of endocytosis, where it induces localized actin polymerization, providing a mechanical force for pinching off endosomes. This idea is supported by data showing that recruitment of dynamin to clathrin-coated pits is accompanied by transient actin assembly which is immediately followed by constriction of the pit neck [147]. Endophilin A, syndapin I and intersectin all bind to both dynamin and N-WASP and therefore could potentially recruit N-WASP to sites of endocytosis [96,98,99]. Lymphocytes from WASP knockout mice exhibit defective endocytosis, suggesting that WASP may also be involved in membrane trafficking [138].

These ideas obtained from experiments in mammalian cells are supported by data obtained using yeast. Las17p/Bee1, the *Sacch. cerevisiae* homologue of WASP, is essential for endosome motility in yeast [148]. Las17p/Bee1 localizes to cortical actin patches – short-lived actin-rich structures that have been suggested to play a role in endocytosis [66,149]. Disruption of the Las17/Bee1 gene abolishes fluid-phase endocytosis as well as the formation of cortical actin patches [66,112]. Some of the clearest evidence for a role of WASP and Arp2/3 complex in endocytosis comes from live cell studies done in the Drubin laboratory [150], showing actin

assembly on nascent endocytic vesicles that appears to rocket the vesicles away from the cell cortex and then disassemble to allow complete internalization. That study showed that the composition of actin patches correlated with the spatial and temporal location of vesicles and that Arp2/3-dependent nucleation was an important component of endocytic internalization and patch disassembly.

IDENTIFYING THE SPECIFIC ROLES OF WASP-FAMILY PROTEINS: CONCLUSIONS

Genetic studies have confirmed some of the original models of WASP and Scar function, while contradicting others and forcing us to consider new ideas. There is increasing evidence implicating Scar proteins as Rac effectors promoting lamellipodium formation. There is surprisingly little evidence that WASP functions in the formation of lamellipodia or filopodia. By contrast there is increasing evidence that WASP acts in membrane-trafficking processes such as endocytosis, intracellular pathogen motility and vesicle motility. It is perhaps notable that yeasts, which are non-motile, only express a WASP-like protein, while higher, motile, organisms also express Scar-like proteins. Thus the original function of WASP may have been solely to stimulate actin assembly during membrane-trafficking events, and it was only when Scar evolved that Arp2/3-nucleated actin assembly was put to use in directed cell movement. Evidence is now starting to emerge that the different mammalian Scar isoforms have specific functions, possibly acting in different cellular locations or in response to different extracellular environments. A challenge will now be to identify how this specificity is achieved.

OTHER Arp2/3 ACTIVATORS

Several proteins that are not WASP-family members have also been shown to activate the Arp2/3 complex. The common structural feature linking all such proteins to the WASP family is a short stretch of largely acidic residues containing a tryptophan residue. The one exception to this is CARMIL (capping protein, Arp2/3 and myosin I linker), a *Dictyostelium* protein that weakly activates Arp2/3 [151]. CARMIL does possess an acidic stretch, but it lacks the conserved tryptophan residue found in all other Arp2/3 activators.

ActA

The intracellular pathogen *L. monocytogenes* uses the host cell's actin-polymerizing machinery to provide a means of propulsion through the cytoplasm similar to that described earlier for the vaccinia virus. Unlike vaccinia, which activates the Arp2/3 complex by recruiting N-WASP, *Listeria* expresses a protein, ActA, that directly binds to and activates the Arp2/3 complex [152]. ActA is a transmembrane protein, most of which is located outside the bacterium, exposed to the host cell's cytoplasm. This extracellular region contains an acidic region as well as one or two functional WH2 domains, and a region with some similarity to the C domain of the WASP family [153,154]. Thus ActA possesses all the sequences found to be essential for WASP proteins to activate Arp2/3, albeit rearranged, with the A domain N-terminal to the WH2 and C domains. ActA also binds VASP via a proline-rich sequence, and addition of VASP was found to increase the motility of *Listeria* in a reconstituted system [155].

MYOSIN I

Myosin Is from fungi possess a region at their C-termini related to the C and A regions of the WASP family. This region is absent from myosin Is of other eukaryotes [156–158]. Myo1p from *Schizo. pombe* binds to, and weakly activates, the Arp2/3 complex [156–158]. Interestingly, the *Sacch. cerevisiae* myosin Is, Myo3p and Myo5p, bind directly to Las17p and verprolin, homologues of WASP and WIP [156,157]. This suggests the presence of a large complex of proteins controlling actin assembly. Deletion of the acidic domains of Las17p, Myo3p and Myo5p is only lethal if all are simultaneously deleted, suggesting functional degeneracy between these proteins [157]. Unlike the WASP family and ActA, fungal myosin Is do not possess a monomeric actin-binding domain; however, it is possible that another protein in complex with myosin I, such as verprolin or Las17p, could serve this function.

Pan1p AND Abp1p

Two further yeast proteins have been shown to activate the Arp2/3 complex, Pan1p and Abp1p [159,160]. Interestingly, both of these proteins have links with endocytosis, adding to the evidence that, in yeast, Arp2/3-mediated actin assembly is principally involved in membrane trafficking. Pan1p is related to the mammalian protein Eps15 and is involved in the internalization step of endocytosis [161]. It is not known whether Eps15 possesses Arp2/3 activating activity, although, like Pan1p, it contains an acidic domain close to its C-terminus [159]. Abp1p also has a mammalian relative, Abp1, although this lacks the acidic domain found within Abp1p, suggesting that it likely does not activate Arp2/3 directly [160]. A feature Pan1p and Abp1p share with Las17p is the ability to bind Sla1p, suggesting that a general function of Sla1p may be the recruitment of Arp2/3 activators [18,163]. Unlike ActA and the WASP-family proteins, which bind monomeric actin, Abp1p binds filamentous actin and recruits Arp2/3 to filaments [160]. The filament binding of Abp1p is required for its enhancement of Arp2/3 activity, and provides a plausible mechanism for how Abp1p could recruit Arp2/3 to filaments to cause activation.

CORTACTIN

Cortactin is a mammalian protein originally identified as a prominent substrate for Src kinases and localizes to sites of dynamic actin assembly [164,165]. Two groups independently discovered that cortactin can activate the Arp2/3 complex [166,167]. In common with Abp1p, cortactin binds filamentous, but not monomeric, actin, and its ability to bind actin filaments is required for Arp2/3 activation. Compared with WASP-family proteins, cortactin is a weak activator of the Arp2/3 complex; however, WIP can bind to cortactin, and this interaction increases the ability of cortactin to activate Arp2/3 [168]. WIP possesses a WH2 domain, and therefore it may increase cortactin activity by acting as a source of actin monomers. In addition to activating Arp2/3, cortactin stabilizes Arp2/3-induced filament branch points [167]. Despite the fact that cortactin has a lower affinity for the Arp2/3 complex than the WASP WCA domain, cortactin still increases filament assembly and branching in the presence of saturating quantities of WASP WCA [167,169]. This suggests that WASP and cortactin stimulate actin assembly in a synergistic manner, which is surprising, since both interact with Arp2/3 through acidic domains. Cross-linking studies have shown that the interaction of Arp2/3 with WASP and cortactin is not identical, raising the possibility that WASP and cortactin could interact simultaneously with Arp2/3 [170]. An alternative model is that interaction is sequential, with WASP activating Arp2/3 and then being replaced by cortactin, acting to stabilize branch points. Cortactin interacts with dynamin, and there is evidence to suggest that this

interaction plays a role in regulating actin assembly at membranes [171–173].

INHIBITION OF THE Arp2/3 COMPLEX

Electron-microscopic analysis suggests that the orthogonally branched actin network in a cell is restricted to its leading edge [41,174], whereas those filaments further from the edge are longer and infrequently branched (Figure 4A). Recently several proteins that can inhibit Arp2/3-complex activity and function have been identified that may be important in regulating the localization of Arp2/3 activity in cells. Firstly, *in vitro*, the actin-filament-binding protein tropomyosin is able to inhibit Arp2/3-complex-mediated actin polymerization and reduce the formation of filament branches by approx. 50%, presumably by reducing the ability of the filaments to stimulate nucleation by the Arp2/3 complex by competing with the complex for binding sites on the sides of actin filaments [175]. Thus newly formed actin filaments at the leading edge of the cell are able to form branches, the authors argue, because, unlike the filaments deep within the cell's cortex, they have not yet bound to tropomyosin [175]. Immunolocalization studies support this hypothesis as, *in vivo*, tropomyosin is relatively absent from the leading edge of cells, whilst this area is rich in free barbed ends [176]. Conversely, tropomyosin is present towards the base of the lamellipodium and in the deeper cortex of the cell, where it is associated with actin structures and stress fibres [176]. Secondly, in the budding yeast *Sacch. cerevisiae*, the protein coronin (Crn1) binds directly to the Arp2/3 complex via the ARPC2 subunit *in vivo* and inhibits activated Arp2/3 complex actin nucleation *in vitro* [177]. Finally, the actin-binding protein caldesmon, which co-localizes with Arp2/3 complex at membrane ruffles, has also been identified as an inhibitor of Arp2/3-complex-mediated actin nucleation *in vitro* [178]. Like tropomyosin, caldesmon binds to the side of actin filaments and is able to inhibit Arp2/3 complex binding [178]. This inhibition is reversed by addition of Ca^{2+}/calmodulin or phosphorylation of caldesmon by cdc2 kinase, both of which regulate the interaction of actin with caldesmon. This suggests that caldesmon could potentially relay a variety of cellular signals to the actin cytoskeleton.

CONCLUSIONS

In the last few years there has been significant progress in refining our understanding of how the Arp2/3 complex nucleates actin assembly. There have also been major advances in identifying how the WASP family, in particular the Scar proteins, are regulated. Gene disruption and RNAi, in combination with other techniques, are now allowing us to identify the precise roles of individual WASP-family members. We are starting to appreciate the diversity of the cellular roles that the Arp2/3 complex undertakes and the subtlety with which its action can be regulated. Inevitably, advances in our understanding have thrown up many new questions. It is now apparent that the Arp2/3 complex is regulated by a number of different activators, each of which is subject to complicated regulation. A challenge for the future will be to attempt to understand the interplay between the numerous cellular factors that influence Arp2/3 activity. Another challenge will be to understand how Arp2/3 co-ordinates with other actin regulators to produce a highly organized and yet dynamic actin cytoskeleton. The discovery that formins can nucleate actin filaments has added an intriguing new element to the field, and doubtless there will be many further surprises in coming years.

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