Critical Reviews in Biochemistry and Molecular Biology, 2009; 44(6): 351–366

informa
healthcare

REVIEW ARTICLE

Actin filament nucleation and elongation factors – structure–function relationships

Roberto Dominguez

Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Abstract

The spontaneous and unregulated polymerization of actin filaments is inhibited in cells by actin monomer-binding proteins such as profilin and Tβ4. Eukaryotic cells and certain pathogens use filament nucleators to stabilize actin polymerization nuclei, whose formation is rate-limiting. Known filament nucleators include the Arp2/3 complex and its large family of nucleation promoting factors (NPFs), formins, Spire, Cobl, VopL/VopF, TARP and Lmod. These molecules control the time and location for polymerization, and additionally influence the structures of the actin networks that they generate. Filament nucleators are generally unrelated, but with the exception of formins they all use the WASP-Homology 2 domain (WH2 or W), a small and versatile actin-binding motif, for interaction with actin. A common architecture, found in Spire, Cobl and VopL/VopF, consists of tandem W domains that bind three to four actin subunits to form a nucleus. Structural considerations suggest that NPFs–Arp2/3 complex can also be viewed as a specialized form of tandem W-based nucleator. Formins are unique in that they use the formin-homology 2 (FH2) domain for interaction with actin and promote not only nucleation, but also processive barbed end elongation. In contrast, the elongation function among W-based nucleators has been "outsourced" to a dedicated family of proteins, Eva/VASP, which are related to WASP-family NPFs.

Keywords: *Actin filament; nucleation and elongation; Arp2/3 complex; Lmod; Spire; Cobl; VopL/VopF; Eva/VASP*

Introduction

The actin cytoskeleton is intimately involved in most cellular functions, including cell motility, cell adhesion, endo/exocytosis, intracellular trafficking and the maintenance of cell shape and polarity (Pollard and Borisy, 2003; Chhabra and Higgs, 2007; Le Clainche and Carlier, 2008; Galletta and Cooper, 2009). In addition, many pathogens disrupt or hijack the host cell actin cytoskeleton during infection (Gouin *et al.*, 2005; Bhavsar *et al.*, 2007; Cossart and Toledo-Arana, 2008). These processes are characterized by rapid oscillations of actin polymerization/depolymerization under tight temporal and spatial regulation. At its most basic level, the assembly of actin cytoskeletal networks depends on the regulated transition of cellular actin between its monomeric (G-actin) and filamentous (F-actin) states (Figure 1). Actin is an ATPase, and nucleotide hydrolysis by actin is a critical factor regulating the transition between the G- and F-actin states. Actin monomers join the fast growing barbed (or +) end of the filament primarily in the ATP state. Hydrolysis takes place in the filament, and ADP–actin monomers dissociate mainly from the pointed (or –) end. However, this simple steady state polymerization/depolymerization mechanism, known as actin filament treadmilling, cannot account for the vast variety of actin processes and actin networks observed in cells. Hundreds of G- and F-actin-binding proteins, along with signaling and scaffolding proteins, become involved in the regulation of actin dynamics (Pollard and Borisy, 2003). Actin-binding proteins (ABPs) have diverse functions, including actin monomer sequestration, filament barbed and pointed end capping, filament severing, and filament crosslinking. An important group of ABPs are those that regulate the *de novo* formation of actin filaments,

Address for Correspondence: Roberto Dominguez, Department of Physiology, 3700 Hamilton Walk, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6085, USA. Tel: 215 573 4559 Fax: 215 573 5851 E-mail: droberto@mail.med.upenn.edu

(Received 16 July 2009; revised 20 August 2009; accepted 21 August 2009)

ISSN 1040-9238 print/ISSN 1549-7798 online © 2009 Informa UK Ltd
DOI: 10.3109/10409230903277340

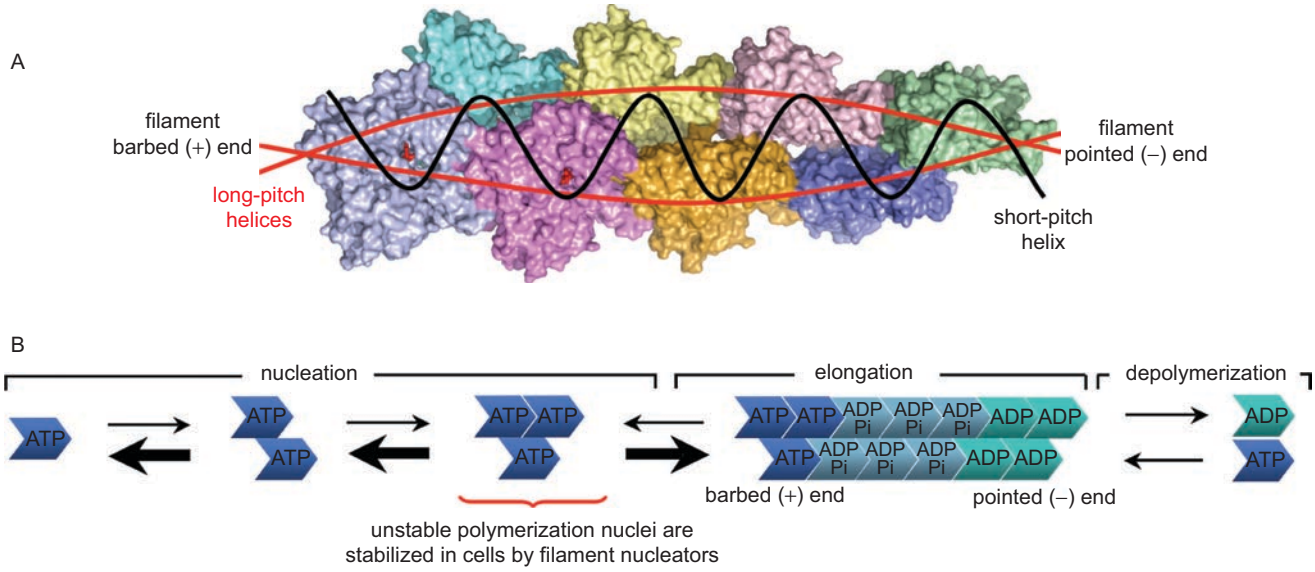

Figure 1. The actin filament is structurally and kinetically asymmetric. (A) Structurally, the actin filament can be described as either a single left-handed short-pitch helix, with consecutive lateral subunits staggered with respect to one another by half a monomer length, or two right-handed long-pitch helices of head-to-tail bound actin subunits (Holmes *et al.*, 1990). (B) Kinetically, ATP–actin monomers add faster at the barbed (or +) end, nucleotide hydrolysis takes place in the filament, and dissociation of ADP–actin monomers takes place mainly at the pointed (or –) end. In cells, the transition between monomeric and filamentous actin is controlled by numerous factors, including nucleotide hydrolysis by actin itself and proteins known as actin filament nucleators. Nucleation, i.e. the formation of small actin oligomers (dimers, trimes and tetramers), is rate limiting and is additionally inhibited by actin monomer-binding proteins. Therefore, the role of filament nucleators is to stabilize the formation of small actin oligomers, so that they can mature into actin filaments.

which include actin filament nucleation and elongation factors. A number of excellent reviews have been written recently about these proteins (Higgs, 2005; Faix and Grosse, 2006; Goley and Welch, 2006; Goode and Eck, 2007; Pollard, 2007; Renault *et al.*, 2008; Chesarone and Goode, 2009; Paul and Pollard, 2009; Qualmann and Kessels, 2009). This review differs in that it focuses on general structure–function principles of filament nucleation and elongation.

Actin filament nucleators

Actin is the most abundant protein in most eukaryotic cells, where its concentration (ranging from 100 to 500 μM in non-muscle cells) is much higher than the critical concentration for monomer addition at both the barbed end (0.1 μM) and the pointed end (0.7 μM) of the actin filament (Pollard and Borisy, 2003). Yet, actin monomer-binding proteins such as profilin and Tβ4 inhibit the spontaneous polymerization of actin filaments. Cells use filament nucleators to stabilize actin polymerization nuclei, whose formation is rate-limiting during actin assembly (Sept and McCammon, 2001) (Figure 1). Filament nucleators constitute a rapidly evolving and relatively recent field of investigation. The Arp2/3 complex was first purified more than 10 years ago (Machesky *et al.*, 1994), but it was not until the discovery of ActA as a nucleation promoting factor (NPF) at the surface of *Listeria monocytogenes* that the nucleation capacity of NPFs–Arp2/3 complex was fully recognized (Welch *et al.*, 1998; Goley and Welch, 2006). Almost simultaneously, eukaryotic NPFs belonging to the WASP/WAVE-family of proteins were identified (Machesky and Insall, 1998; Machesky *et al.*, 1999; Rohatgi *et al.*, 1999; Winter *et al.*, 1999; Yarar *et al.*, 1999). Subsequently, formins were shown to catalyze not only nucleation but also processive barbed end elongation (Pruyne *et al.*, 2002; Zigmond *et al.*, 2003; Higgs, 2005; Goode and Eck, 2007; Pollard, 2007). Recently, a series of new filament nucleators have been discovered, both in eukaryotic cells and bacterial pathogens, including Spire (Quinlan *et al.*, 2005), Cobl (Ahuja *et al.*, 2007), VopL (Liverman *et al.*, 2007), VopF (Tam *et al.*, 2007), TARP (Jewett *et al.*, 2006) and Lmod (Chereau *et al.*, 2008). These proteins control not only the time and location for actin polymerization, but also the specific type of actin filament networks that they generate.

The actin filament (Figure 1A) can be described as either a single left-handed short-pitch helix, where consecutive lateral subunits are staggered with respect to one another by half a monomer length, or two right-handed long-pitch helices of head-to-tail bound actin subunits (Holmes *et al.*, 1990; Holmes, 2009; Oda *et al.*,

2009). As discussed below, different actin filament nucleators work by different mechanisms, stabilizing small actin oligomers (dimers, trimers and tetramers) along either the long- or the short-pitch helices of the actin filament.

The W domain and actin filament nucleation

With the exception of formins, all known actin filament nucleation and elongation factors use the WASP-Homology 2 (WH2 or W) domain for interaction with actin, though in Lmod and the Arp2/3 complex other domains contribute as well. The W domain has a short size (17–27 aa) and is generally poorly conserved, making it difficult to identify based on sequence analysis alone (Dominguez, 2007). Other distinctive features of the W domain include its abundance and functional versatility. The N-terminal portion of the W domain forms a helix that binds in the hydrophobic cleft, or target-binding cleft (Dominguez, 2004), between subdomains 1 and 3 at the barbed end of the actin monomer (Hertzog *et al.*, 2004; Chereau *et al.*, 2005) (Figure 2). After this helix, the W domain presents an extended region that climbs toward the pointed end of the actin monomer. This region has variable length and sequence but comprises the conserved four-residue motif LKKT(V). The N-terminal helix and LKKT(V) motif constitute the conserved core of the W domain.

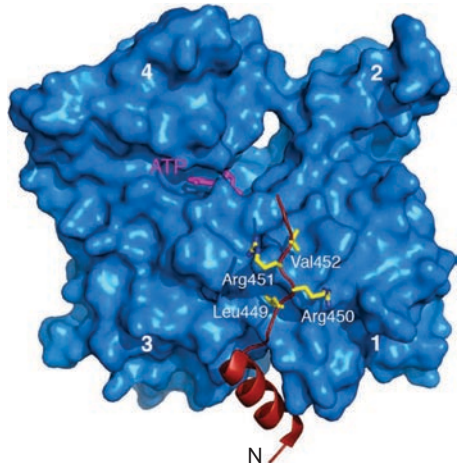

Figure 2. Structure of the W domain of the NPF protein WAVE2 (red) bound to actin (blue). Actin subdomains 1–4 are labeled. The W domain consists of an N-terminal amphiphilic helix that binds in the so-called hydrophobic or target-binding cleft (Dominguez, 2004) between actin subdomains 1 and 3, followed by a C-terminal extended region, featuring the conserved LKKT(V) motif (^{449}LRRV^{452} in WAVE2). This and other W-actin structures were determined as ternary complexes with DNase I, which prevents polymerization (Chereau *et al.*, 2005). For simplicity, DNase I is not shown.

However, the W domain displays remarkable plasticity. For example, the actin monomer sequestering protein Tβ4 is a unique member of the W domain family (Paunola *et al.*, 2002), featuring an additional helix C-terminal to the LKKT(V) motif that binds atop actin subdomains 2 and 4 and caps the pointed end of the actin monomer (Irobi *et al.*, 2004).

The W domain often occurs in tandem repeats, which is a common architecture among filament nucleators, found in Spire (Quinlan *et al.*, 2005), Cobl (Ahuja *et al.*, 2007) and VopL/VopF (Liverman *et al.*, 2007; Tam *et al.*, 2007). The protein TARP (translocated actin recruiting phosphoprotein) contains a single W domain, but forms large oligomers (Jewett *et al.*, 2006). The W domain also participates in filament nucleation by the Arp2/3 complex through NPFs, which can have between one and three W domains (Goley and Welch, 2006; Pollard, 2007; Zuchero *et al.*, 2009). Finally, one of the three actin-binding sites of Lmod is also a W domain (Chereau *et al.*, 2008).

It is important to note, however, that the presence of multiple copies of the W domain does not automatically mean that a protein is a filament nucleator. For example, amoeba actobindin (Hertzog *et al.*, 2002), *Drosophila* ciboulot (Hertzog *et al.*, 2004) and *C. elegans* tetrathymosin (Van Troys *et al.*, 2004) present two-and-a-half, three and four copies of the W fold, respectively, but do not nucleate actin filaments. Evolutionarily, these three proteins share more in common with Tβ4, in particularly the presence of Tβ4-related sequences C-terminal to the LKKT(V) motif, than with classical W domains of the kind found in WASP family proteins (Chereau *et al.*, 2005). However, ciboulot and actobindin do not sequester actin monomers like Tβ4, but rather promote filament barbed end growth in a way analogous to profilin (Hertzog *et al.*, 2002; Carlier *et al.*, 2007). These two proteins form 1:1 complexes with actin, suggesting that only one of their actin-binding sites is fully functional (Hertzog *et al.*, 2002; Hertzog *et al.*, 2004). In contrast, tetrathymosin appears to bind multiple actin monomers and has both monomer sequestering and filament-binding properties (Van Troys *et al.*, 2004). Tβ4, actobindin, ciboulot and tetrathymosin are thus examples of how changes in the sequence of the W domain and modular structure of the proteins in which it is found give rise to diverse functions in the regulation of actin cytoskeleton dynamics (Dominguez, 2007).

The Arp2/3 complex and nucleation promoting factors (NPFs)

The Arp2/3 complex consists of seven proteins, including two actin-related proteins, Arp2 and Arp3, and subunits ARPC1 to 5 (Figure 3). By itself, Arp2/3

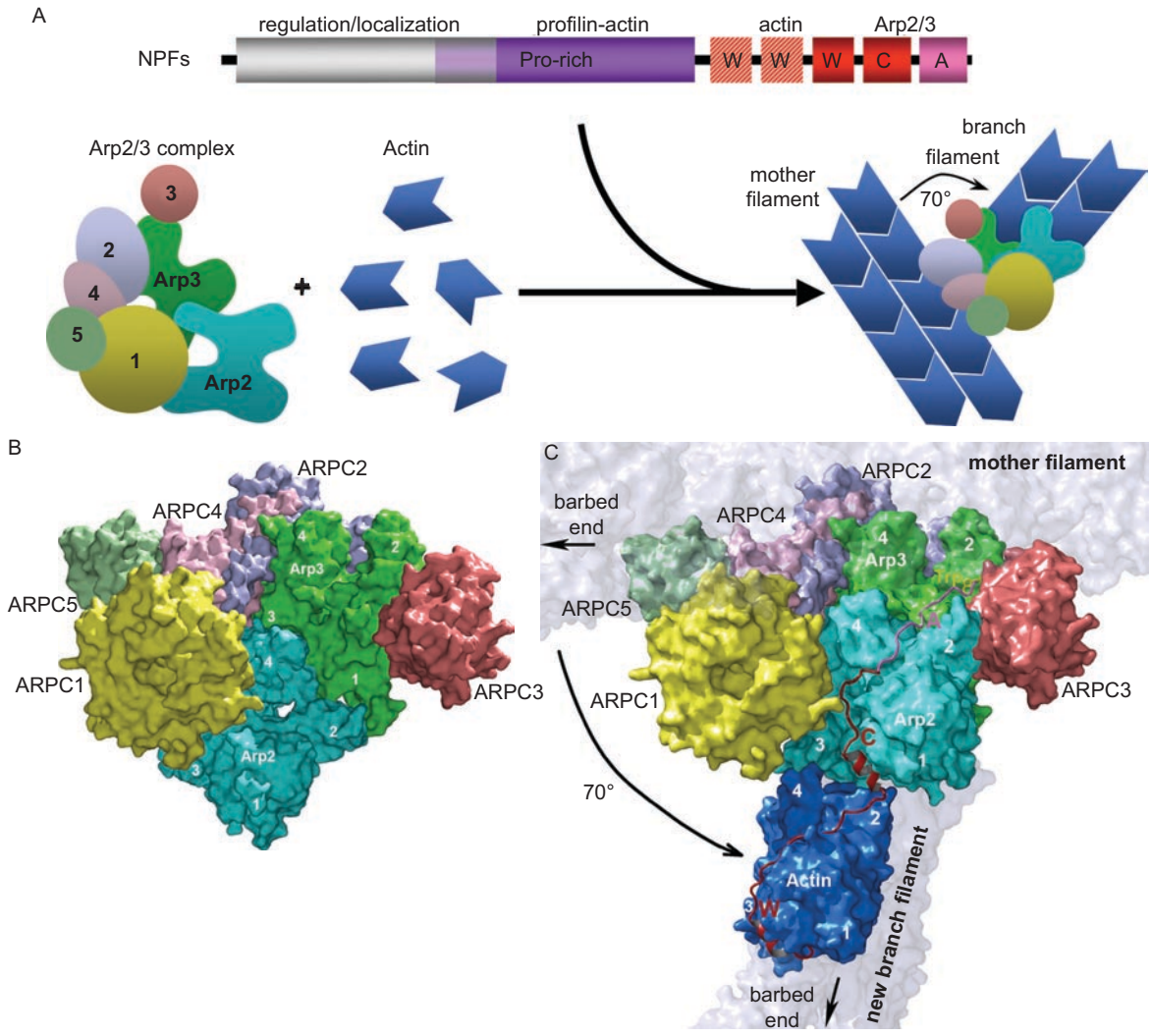

Figure 3. Actin filament nucleation by the Arp2/3 complex and NPFs. (A) The Arp2/3 complex consists of seven proteins, including the actin related proteins Arp2 and Arp3, and subunits ARPC1 to 5 (labeled 1 to 5). By itself, the complex has low nucleation activity, but it is activated by nucleation promoting factors (NPFs) (Goley and Welch, 2006; Pollard, 2007). NPFs are large multi-domain proteins. The figure illustrates a prototypical NPF protein, characterized by the presence of regulatory/localization domains, a Pro-rich region, and a WCA region that can have between one and three W domains. Two of the W domains are colored red/gray (striped) to indicate that their presence is not absolutely necessary for Arp2/3 complex activation. WCA is the smallest fragment capable of catalyzing the formation of a polymerization nucleus, consisting of the two Arps and one to three actin subunits, as well as a conformational change within Arp2/3 complex that allows for monomer addition to the branch and binding of the nucleus to the side of a preexisting actin filament (mother filament). The new filament (or branch) grows from the barbed ends of the Arps at a 70° angle with respect to the mother filament. (B) Structure of inactive Arp2/3 complex (Robinson *et al.*, 2001; Nolen and Pollard, 2007). Subdomains 1 and 2 of Arp2 are disordered in the structures, but were added here by analogy with actin. The Arp2/3 complex subunits are colored according to the diagram of part A. (C) SAXS-derived model of WCA-actin-Arp2/3 complex (Boczkowska *et al.*, 2008). The orientation is the same as in part B. The mother and branch filaments are shown for reference, although this work did not address branch assembly. Note that Arp2 moved up compared to its location in part B. This study placed the first actin subunit of the branch (bound to the W domain) at the barbed end of Arp2. The position of the crosslinked W domain, which is known precisely from its crystal structure with actin (Chereau *et al.*, 2005) (see Figure 2), imposes constraints on the location of the C motif. Thus, the hydrophobic cleft of Arp2 is in the path of the CA polypeptide as it progresses toward the Arp2/3 complex. The helical portion of the C motif may thus bind in this cleft, as supported by sequence similarity with W (see Figure 4C). The position of the A motif (pink) is less well constrained, but this model would be consisting with it binding at the interface between Arp3 and ARPC3, as suggested by biochemical studies (Zalevsky *et al.*, 2001; Weaver *et al.*, 2002; Pan *et al.*, 2004; Kreishman-Deitrick *et al.*, 2005; Kelly *et al.*, 2006).

complex has very low nucleation activity (Mullins *et al.*, 1998). Nucleation is activated by NPFs, the best known of which are members of the WASP/WAVE family of proteins (Goley and Welch, 2006; Pollard, 2007; Chesarone and Goode, 2009). These proteins recruit one to three actin subunits and promote a conformational change within the Arp2/3 complex. NPFs are themselves regulated by various factors,

in particular Rho-family GTPases. Thus, WASP and N-WASP function under the control of Cdc42, whereas WAVE forms part of a large complex that is regulated by Rac (Ma *et al.*, 1998; Kim *et al.*, 2000; Eden *et al.*, 2002; Bompard and Caron, 2004; Hall, 2005; Goley and Welch, 2006). Classical NPFs such as WASP/WAVE (Goley and Welch, 2006), WASH (Linardopoulou *et al.*, 2007), WHAMM (Campellone *et al.*, 2008) and JMY (Zuchero *et al.*, 2009) present a C-terminal WCA region, which constitutes the shortest polypeptide necessary for activation of nucleation with the Arp2/3 complex (Machesky *et al.*, 1999). This region consists of three distinct segments: W, C and A. W binds the first actin subunit of the new filament (Figure 2). The C (central or connecting) and A (acidic) motifs interact with various subunits of the Arp2/3 complex, helping to stabilize the activated conformation. However, the mechanism by which CA participates in Arp2/3 complex activation, and the specific interactions with subunits of the complex remain a mystery. The actin monomer bound to the W domain, together with Arp2 and Arp3, are thought to form a trimeric seed for the nucleation of a filament branch that emerges at a 70° angle from the side of a preexisting filament (Figure 3). According to this model (Robinson *et al.*, 2001), Arp2 and Arp3 are the first two subunits at the pointed end of the new filament branch, and are expected to adopt a short-pitch filament-like conformation.

The crystal structure of Arp2/3 complex was first determined in the absence of nucleotide and NPF (Robinson *et al.*, 2001) (Figure 3B). In the structure, Arp2 and Arp3 are separated (i.e. not in a filament-like conformation) and the nucleotide cleft of Arp3 is wide open, whereas subdomains 1 and 2 of Arp2 are disordered. Thus, this structure was described as the inactive conformation of the complex (Robinson *et al.*, 2001). Subsequently, Arp2/3 complex was crystallized in the presence of ATP or nucleotide analogs (Nolen and Pollard, 2007). Nucleotide binding favors closure of the nucleotide cleft of Arp3 and marginally stabilizes subdomains 1 and 2 of Arp2. However, the relative position of Arp2 and Arp3 was unchanged, indicating that, although necessary, ATP binding alone is insufficient to activate Arp2/3 complex. It is believed that the binding of nucleotide and WCA are thermodynamically coupled and that these two factors contribute together to activating Arp2/3 complex (Dayel *et al.*, 2001; Le Clainche *et al.*, 2001; Goley *et al.*, 2004). Preexisting filaments may help shift the equilibrium in favor of an activated complex (Pollard, 2007). However, Arp2/3 complex can bind to and cap filament pointed ends with high affinity outside the branch (Mullins *et al.*, 1998). Because pointed end binding requires an activated conformation (Robinson *et al.*, 2001; Boczkowska *et al.*, 2008; Rouiller *et al.*, 2008), side binding may not be necessary for activation, although it is probably favored by activation. Considering the high concentration of actin monomers in cells and typical affinities of the W–actin interaction ranging from ~0.05 to ~0.25 μM (Marchand *et al.*, 2001; Mattila *et al.*, 2003; Chereau *et al.*, 2005), it is likely that NPFs are actin-loaded prior to encountering the Arp2/3 complex. Thus, actin-loaded NPFs and nucleotide are probably the most important factors shifting the equilibrium in favor of an activated complex *in vivo*.

The structure of Arp2/3 complex in the branch and with bound WASP has been studied using electron microscopy (Egile *et al.*, 2005; Rodal *et al.*, 2005; Rouiller *et al.*, 2008). These studies agree that a major conformational change takes place upon activation, bringing Arp2 and Arp3 into a filament-like arrangement at the pointed end of the branch. Additionally, electron tomography of the branch junction reveals conformational changes in the mother filament at the interface with the Arp2/3 complex and suggests that all seven subunits of the Arp2/3 complex contact the mother filament (Rouiller *et al.*, 2008). None of the existing structures, however, resolves the location and interactions of the CA activator region of NPFs with subunits of the Arp2/3 complex. This question has mainly been addressed by crosslinking and NMR solution studies, showing that CA can be crosslinked to Arp2, ARPC1, Arp3 and ARPC3 (Zalevsky *et al.*, 2001; Weaver *et al.*, 2002; Kreishman-Deitrick *et al.*, 2005; Kelly *et al.*, 2006). Because of the short length of the WCA polypeptide (~73 aa), and considering that both the C (Panchal *et al.*, 2003) and W (Chereau *et al.*, 2005) motifs comprise regions of helical structure, it is difficult to rationalize how CA can span these four subunits in the complex. A recent study attempts a different approach to address this question.

Actin has a highly reactive cysteine residue at position 374. The structures of W-actin complexes (Figure 2) revealed that the N-terminal portion of the W domain faces directly actin Cys-374 (Chereau *et al.*, 2005). Based on this observation, a Cys residue was introduced by mutagenesis at the N-terminus of WCA, which was then crosslinked to actin Cys-374 (Boczkowska *et al.*, 2008). [In support of this approach, a crystal structure of crosslinked W-actin is now available and is nearly undistinguishable from the uncrosslinked structures (Rebowski *et al.*, in preparation).] Contrary to WCA alone, crosslinked WCA–actin forms a stable high affinity complex with the Arp2/3 complex, while also capping its barbed end so that the nucleus cannot elongate by addition of actin monomers. Importantly, the stoichiometry of this complex determined by various methods is precisely 1:1, and not 2:1 as it may be inferred from a recent study (Padrick *et al.*, 2008). This approach produced

a stable WCA–actin–Arp2/3 complex particle, whose structure in solution was analyzed by Small Angle X-ray Scattering (SAXS). The SAXS study indicated that the first actin subunit binds at the barbed end of Arp2, which additionally constrains the binding site of the C motif to subunit Arp2, near the interface with ARPC1 (Figure 3C). Less can be said about the location and interactions of the A region, except that it probably lies near the interface between subunits Arp3 and ARPC3, which is consistent with most of the biochemical evidence (Zalevsky *et al.*, 2001; Weaver *et al.*, 2002; Kreishman-Deitrick *et al.*, 2005; Kelly *et al.*, 2006). This study offers testable hypotheses and a new way to address the problem of activation, but because of its limited resolution it leaves unresolved the exact nature of the conformational change leading to activation and the precise role of WCA in this process.

Tandem W domain-based filament nucleators

The W domain often occurs in tandem repeats, which is the most common architecture found among known actin filament nucleators, observed in Spire (Quinlan *et al.*, 2005), Cobl (Ahuja *et al.*, 2007), and VopL/VopF (Liverman *et al.*, 2007; Tam *et al.*, 2007) (Figure 4). The actin monomers bound to the W repeats of these proteins are thought to come together to form an actin filament-like nucleus for polymer assembly. However, the specific nucleation mechanism of each protein appears to be different, as reflected by dramatic differences in their nucleation activities. For instance, Spire with the largest number of W domains (four) has relatively weak nucleation activity (Quinlan *et al.*, 2005), whereas VopL/VopF with just three W domains are even more efficient nucleators than the NPFs-Arp2/3 complex (our own observation). At least in part, the explanation may lie in the variable linkers between W domains, in particular linker-2 between the second and third W domains. Differences in the linkers may dictate the relative arrangement of actin subunits in the polymerization nucleus, and thereby the nucleation activities of each protein. When the linkers are short, as in Spire, only actin subunits along the long-pitch helix can be connected (Rebowski *et al.*, 2008). However, the brain-specific nucleator Cobl has strong nucleation activity and presents a long, Pro-rich linker-2 (Ahuja *et al.*, 2007). Shortening Cobl's linker-2 reduces dramatically its nucleation activity, whereas replacing this linker with an unrelated sequence of similar length restores most of the endogenous activity. Therefore, the length of the linker, but not necessarily its specific sequence, appears to be crucial for Cobl's activity. Because a longer linker may allow successive W domains to connect actin subunits laterally, it has been proposed that Cobl stabilizes a short-pitch actin nucleus (Ahuja *et al.*, 2007) (Figure 4B). However, the exact arrangement of actin subunits in Cobl's nucleus is unknown and two possibilities must be considered, with the actin subunit bound to either the first or the second W domain staggered forward at the pointed end. In any case, the examples of Cobl, the Arp2/3 complex and Lmod (discussed below) suggest that stabilization of a short-pitch actin trimer is a more effective way to promote nucleation than stabilization of a larger nucleus of four actin subunits along the long-pitch helix.

A recent study additionally suggests that some inter-W linkers present actin monomer-binding activity, and can as a result boost the nucleation activity of tandem W constructs (Zuchero *et al.*, 2009). Thus, for example, a fragment consisting of the two W domains of N-WASP had no nucleation activity, but a modest increase in nucleation was observed when the naturally occurring inter-W linker was replaced by Spire's linker-3 (Zuchero *et al.*, 2009).

Microbial pathogens often disrupt or hijack the host cell cytoskeleton for infection (Gouin *et al.*, 2005; Bhavsar *et al.*, 2007). A well known example is *Listeria monocytogenes*, whose surface protein ActA mimics eukaryotic NPFs and recruits both the filament elongation factor VASP and the Arp2/3 complex polymerization machineries at the surface of the parasite to propel its movement within and between cells (Cossart and Toledo-Arana, 2008). *Vibrios* are Gram-negative rod-shaped bacteria, comprising human pathogens that cause wound infections, gastro-intestinal disease and diarrhea, and are often associated with infection from consumption of raw seafood. *Vibrio parahaemolyticus* and *Vibrio cholerae* were nearly simultaneously shown to produce the type III secretion system (T3SS) virulence factors VopL (Liverman *et al.*, 2007), and VopF (Tam *et al.*, 2007), respectively. VopL and VopF display ~57% overall sequence identity. Similar proteins are also found among other *Vibrio* species. VopL/VopF disrupt actin homeostasis, and appear to be required for infection (Liverman *et al.*, 2007; Tam *et al.*, 2007). Both proteins present three W domains and Pro-rich sequences, and like Cobl, both are strong filament nucleators. It is, therefore, tempting to propose that like Cobl these two proteins stabilize a short-pitch polymerization nucleus. However, linker-2 in VopL/VopF is significantly shorter than in Cobl (Figure 4C), and because the length of the linker is such a critical factor for Cobl's activity (4), the reasons for the strong nucleation activities of VopL/VopF remain a mystery. A potential explanation is given next.

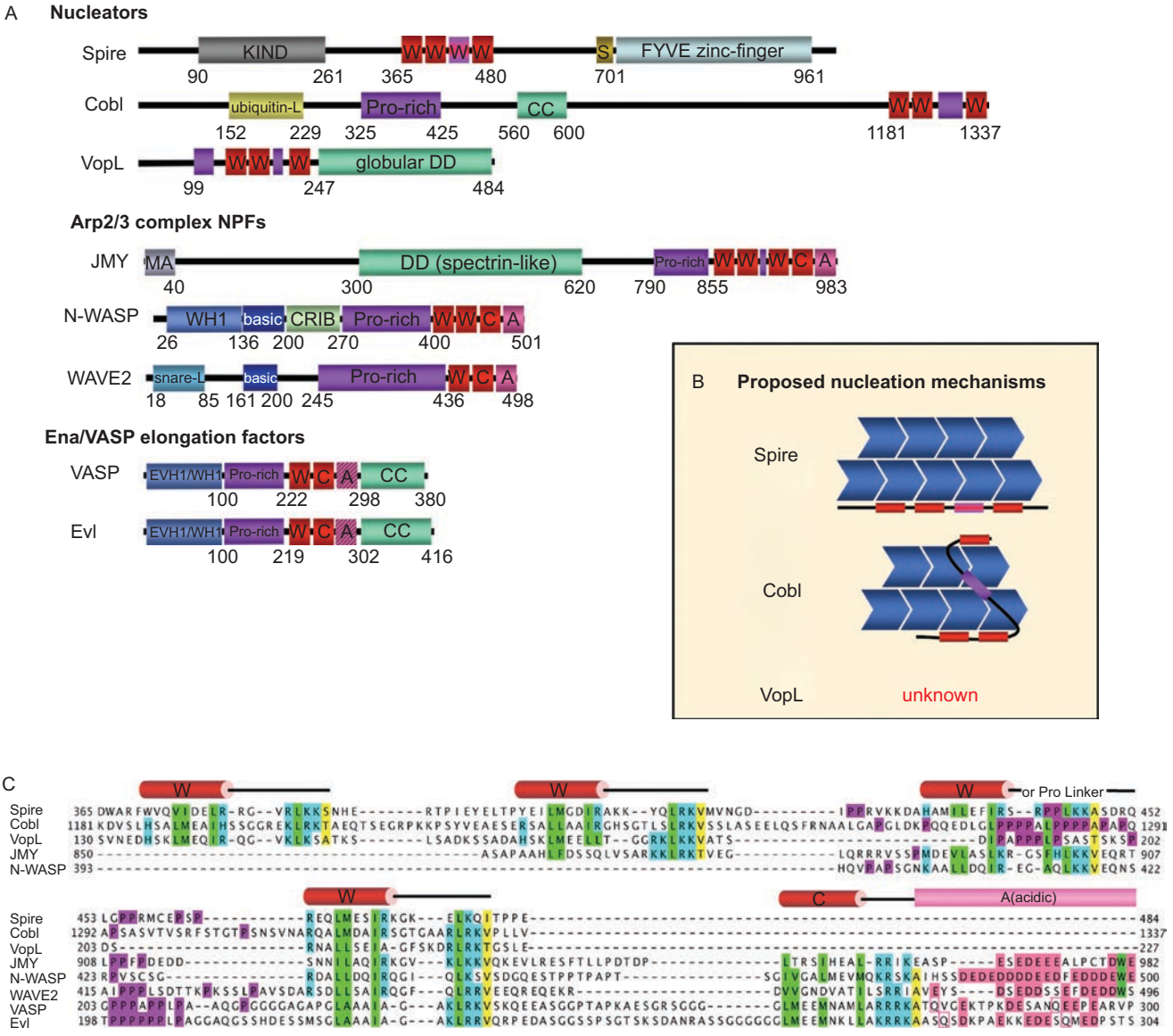

Figure 4. Relationship between tandem W-based filament nucleators, NPFs and Ena/VASP elongation factors. (A) Domain diagram. W and C are both colored red to highlight their relationship. Pro-rich regions (magenta) abound among these proteins, and bind regulatory proteins and profilin–actin complexes, which is the main source of polymerization-competent actin in cells. Coiled coils and other oligomerization domains (both known and predicted from sequence analysis) are also common (green). In Ena/VASP, the WASP-Homology domain 1 (WH1), W and C regions are respectively known as EVH1 (Ena/VASP homology 1), GAB and FAB (G- and F-actin binding) domains, but despite their different names these domains are related to their N-WASP counterparts. Ena/VASP also has an acidic region after the C motif, albeit less acidic than in NPFs and lacking the key tryptophan residue. An ubiquitin-like segment in Cobl (a potential Ras-binding site), a snare-like helix in WAVE2 (a potential multi-protein association site), a spectrin-like antiparallel dimerization motif in JMY and a globular dimerization domain in VopL/VopF are all predicted by bioinformatics analysis, but had not been previously reported. The third W domain of Spire (red/magenta) is non-canonical; it contains various Pro residues and, interestingly, occupies the position of the Pro-rich linker-2 of Cobl and VopL/VopF. (B) Proposed nucleation mechanisms of Spire (Quinlan *et al.*, 2005) and Cobl (Ahuja *et al.*, 2007). The lengths of the linkers determine whether neighboring W domains bridge actin subunits along a single strand (Spire) or across strands (Cobl) of the actin filament (i.e. long- versus short-pitch nuclei), which may in turn determine the nucleation activities of these proteins. Intriguingly, VopL has three W domains and strong nucleation activity like Cobl, but its shorter linker-2 would be inconsistent with stabilization of a short-pitch nucleus. (C) Sequence alignment of the W and CA regions of the proteins shown in part A. Conserved residues are colored according to their chemical properties. Uniprot codes: Spire (*Drosophila melanogaster*, Q9U1K1); Cobl (mouse, Q5NBX1); VopL (*Vibrio parahaemolyticus*, Q87GE5); JMY (mouse, Q9QXM1); N-WASP (mouse, Q91YD9); WAVE2 (human, Q9Y6W5); VASP (human, P50552); Evl (human, Q9UI08).

Role of oligomerization on the nucleation activities of W-based nucleators

In addition to the inter-W linkers, oligomerization may influence the nucleation activities of tandem W-based filament nucleators. For instance, Spire interacts with the formin Cappuccino (Rosales-Nieves *et al.*, 2006; Quinlan *et al.*, 2007; Quinlan and Kerkhoff, 2008; Renault *et al.*, 2008), and the two proteins appear to synergize to assemble actin filaments both *in vitro* (Bosch *et al.*, 2007) and *in vivo* (Rosales-Nieves *et al.*, 2006), where they may be involved in maintaining microtubule organization (Dahlgaard *et al.*, 2007). The interaction, which involves the kinase non-catalytic C-lobe domain (KIND) of Spire (Figure 4A) and the formin homology 2 (FH2) domain of Cappuccino, enhances the nucleation activity of Spire (Quinlan *et al.*, 2007). It is likely that the increased activity results from Spire dimerization mediated by the FH2 dimer. Another possibility for Spire to function as a dimer is through its C-terminal FYVE zinc-finger domain (Figure 4A), which in some proteins have been shown to dimerize (Dumas *et al.*, 2001). A recent report additionally finds that WASH, a WASP-family NPF (Linardopoulou *et al.*, 2007), interacts directly with Spire and synergizes with both Spire and Cappuccino to control actin and microtubule dynamics during *Drosophila* oogenesis (Liu *et al.*, 2009). WASH also appears to dimerize through its N-terminal WASH homology domain 1 (WHD1) (Liu *et al.*, 2009), providing yet another potential mechanism for Spire dimerization. Whether Spire dimerizes directly through its FYVE zinc-finger domain or indirectly through interaction with Cappuccino or WASH, dimerization is likely a contributing factor in Spire's nucleation activity. Indeed, an optimally assembled Spire dimer could stabilize the formation of a nucleus consisting of eight actin subunits, four on each side of the filament (or long-pitch helix), potentially resulting in a very powerful nucleator.

Another example is the T3SS protein TARP from *Chlamydia trachomatis*. Despite having a single W domain, TARP nucleates actin filaments, but this activity depends on the presence of the central Pro-rich domain, which in this protein appears to mediate oligomerization (Jewett *et al.*, 2006).

The existing relationship between NPFs–Arp2/3 complex and W-based nucleators is discussed below. In this regard, it is interesting to note that a recent study finds that the dimerization of WASP by external factors increases its affinity for the Arp2/3 complex and enhances its nucleation activity (Padrick *et al.*, 2008). It is still unknown whether WASP dimerization plays a role *in vivo*. However, as pointed out above, the WASP-related protein WASH appears to dimerize by itself. Although dimerization in this case does not seem to enhance Arp2/3 complex-mediated nucleation, it is likely to play a critical role *in vivo*, notably by mediating the bundling and crosslinking of F-actin and microtubules under the control of the GTPase Rho1 (Liu *et al.*, 2009). Finally, sequence analysis identifies potential oligomerization domains among other nucleators and NPFs (Figure 4A). Oligomerization is thus emerging as an important factor modulating the activities of W-based nucleators, which is analogous to formins (Copeland *et al.*, 2004). Whether oligomerization also contributes to the nucleation activities of Cobl and VopL/VopF remains to be demonstrated.

The Arp2/3 complex and NPFs as a specialized form of tandem W nucleator

Structural considerations suggest that NPFs–Arp2/3 complex can be conceptually viewed as a specialized form of tandem W-based nucleator (Boczkowska *et al.*, 2008). According to this view, the actual nucleators are the NPFs, and not the Arp2/3 complex as it has been traditionally described. The distinction is not merely semantic, but rather stems from a different structure–function understanding of how these proteins work. In isolation, neither the Arp2/3 complex nor the NPFs nucleate; they need each other for this activity. There is only one known exception to this rule, which actually reinforces the proposed relationship between NPFs and tandem W-based nucleators. It is the newly discovered NPF protein JMY, which presents three W domains N-terminal to its CA region and, in addition to activating the Arp2/3 complex, has some nucleation activity of its own (Zuchero *et al.*, 2009). More importantly, there is the undisputable fact that the newly discovered filament nucleators (Spire, Cobl, TARP and VopL/VopF) share far more in common with NPFs than they do with the Arp2/3 complex (Figure 4), including the presence of tandem W repeats and Pro-rich regions. The number of *bona fide* W domains in NPFs varies from 1 to 3, whereas the newly discovered nucleators contain between 1 and 4 W domains. However, as it has been pointed out by various investigators (Hertzog *et al.*, 2004; Aguda *et al.*, 2005; Chereau *et al.*, 2005; Boczkowska *et al.*, 2008), the C motif of NPFs is also related to the W domain, a relationship that can be further extended to the F-actin-binding (FAB) motif of Ena/VASP proteins (Ferron *et al.*, 2007) (Figure 4). Based on the location of the first actin subunit in the SAXS structure of WCA–actin–Arp2/3 complex, it was proposed that the C motif binds Arp2 (Boczkowska *et al.*, 2008). Like the W domain, the N-terminal portion of the C motif consists of an amphiphilic helix (Panchal *et al.*, 2003), which according to this proposal binds in the hydrophobic

cleft of Arp2 (Figure 3C), somewhat analogous to the binding of W to actin (Figure 2). As for the Arp2/3 complex itself, it can be thought of as an actin dimer that upon activation adopts a short-pitch conformation. The association of the Arps with five other proteins in the Arp2/3 complex probably emerged from a need to integrate nucleation and branching within a single system. Based on these considerations, NPFs can be described as tandem W-based filament nucleators, whose function is to recruit and realign the Arp2–Arp3 short-pitch heterodimer and one to three actin monomers to form a polymerization nucleus.

Certain proteins interact with the Arp2/3 complex and modulate its activity, but have only a modest effect (if any) on its nucleation activity. These proteins also have markedly different domain organization compared to classical NPFs such as WASP/WAVE (Goley and Welch, 2006), WHAMM (Campellone *et al.*, 2008), WASH (Linardopoulou *et al.*, 2007), and JMY (Zuchero *et al.*, 2009), and should probably not be considered as fully-fledged NPFs. In a recent review, some of these molecules were grouped into a separate category, identified as class II NPFs (Goley and Welch, 2006). A well-studied example is the protein cortactin (Ammer and Weed, 2008). Cortactin has only a limited effect on the nucleation activity of the Arp2/3 complex, but it plays a critical role by binding to the Arp2/3 complex at branch points, which stabilizes branch junctions and inhibits filament de-branching and network breakdown (Weaver *et al.*, 2001).

Model of nucleation by NPFs–Arp2/3 complex

The model of filament nucleation by NPFs–Arp2/3 complex proposed here takes into account the purported relationship with tandem W-based filament nucleators. With the determination of the structure of inactive Arp2/3 complex it was proposed that the complex must undergo a major conformational change during activation that would bring Arp2 and Arp3 into a short-pitch filament-like arrangement, with Arp3 staggered toward the pointed end by half a monomer length relative to Arp2 (Robinson *et al.*, 2001). Nucleotide, the WCA region of NPFs and actin are all necessary ingredients of this conformational change (Pollard, 2007). However, the details of the activation mechanism remain a mystery, and one of the most pressing challenges in the field concerns the determination of a high-resolution structure of the activated complex. Part of the challenge is to make adequate guesses about the mechanism of activation so as to formulate strategies toward obtaining a structure of the activated complex. With this in mind, a model is proposed in Figure 5.

According to this model, the conserved Trp in the A region of NPFs, which contributes the most to the binding affinity of WCA to the Arp2/3 complex (Marchand *et al.*, 2001; Weaver *et al.*, 2002), works as a "hook", linking actin-loaded NPFs to the Arp2/3 complex. The SAXS study of WCA–actin–Arp2/3 complex suggests that after this initial encounter the first actin subunit binds at the barbed end of Arp2 (Boczkowska *et al.*, 2008). Arp2, which is partially disordered in the inactive structure (Robinson *et al.*, 2001; Nolen and Pollard, 2007), may transition between active/inactive states, but is stabilized in the activated structure by interaction with the C motif and the first actin subunit of the branch (bound to the W domain of NPFs). This model predicts that Arp2 moves mostly alone during activation, with minimal energetic cost, such as to occupy a filament-like position next to Arp3 (Aguda *et al.*, 2005; Boczkowska *et al.*, 2008). This is supported by flexibility of Arp2 in the inactive structure and the fact that it can be moved with minimal steric clashes. A different model had been initially proposed (Robinson *et al.*, 2001), predicting a more dramatic rearrangement of the complex, involving a rotation of Arp2, ARPC1, ARPC4, and ARPC5 relative to Arp3, ARPC2, and ARPC3. Although the latter model cannot be completely ruled out based on the available data, it appears less likely, because it would involve a large structural change and breakage of hydrophobic contacts along a large interface between the two halves of the complex. Yet, it is reasonable to expect

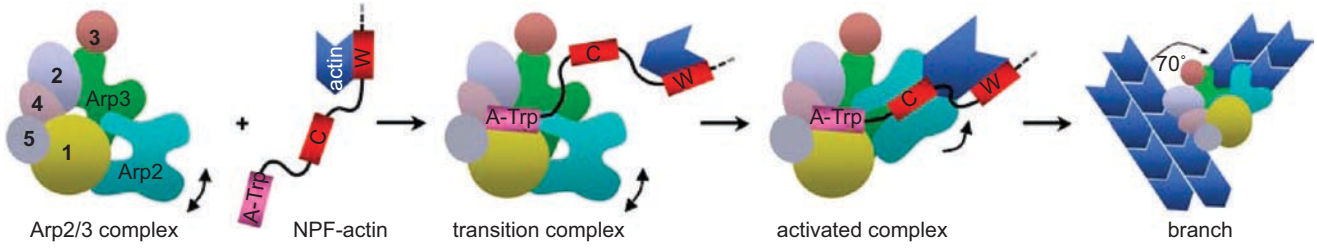

Figure 5. Model of nucleation by NPFs-Arp2/3 complex (see text for details).

that, in addition to movement of Arp2, other changes will occur in the complex during activation. Activation and branching (i.e. binding to the side of pre-existing filaments) may occur nearly simultaneously. Steric hindrance (discussed below) of the W domain with the actin subunits that begin joining the branch after activation (and possibly of the A motif with the mother filament) may help release NPFs after activation.

Leiomodin (Lmod) and the nucleation of actin filaments in muscle cells

The actin "thin" filaments in cardiac and striated muscle sarcomeres display regular length and spacing and are uniformly decorated with muscle-specific proteins such as the troponin complex, tropomyosin (TM) and the barbed and pointed end capping proteins CapZ and tropomodulin, respectively. Toward the center of sarcomeres, the actin filaments overlap with the myosin "thick" filaments, forming a tight hexagonal lattice. The appearance is that of a rigid structure, and it is not surprising that it has been traditionally thought that the actin filaments in sarcomeres are less dynamic than in non-muscle cells. This view is evolving (Wang *et al.*, 2005; Gunst and Zhang, 2008; Sanger and Sanger, 2008; Skwarek-Maruszewska *et al.*, 2009). The sarcomere may undergo constant dynamic remodeling (or repair), and actin filament nucleators may play a critical role in this process.

Leiomodin (Lmod) is a tropomodulin (Tmod)-related protein expressed almost exclusively in muscle cells. mRNA expression analysis indicates that there are three Lmod isoforms (Conley *et al.*, 2001): Lmod1 expressed at low levels in most tissues and at high levels in smooth muscle, Lmod2 expressed exclusively in heart and skeletal muscles and the fetal isoform Lmod3. The first ~340 amino acids of Lmod are ~40% identical to Tmod, a pointed end capping protein in muscles (Fischer and Fowler, 2003; Fowler *et al.*, 2003; Kostyukova *et al.*, 2007). In Tmod, the N-terminal portion is unstructured, except for three helical segments involved in binding TM and actin. Tmod has a second actin-binding site within the C-terminal Leu-rich repeat (LRR) domain (Krieger *et al.*, 2002; Fowler *et al.*, 2003). Lmod shares this domain organization, except for one important difference: only one of the two TM-binding sites of Tmod appears to be conserved in Lmod. More importantly, Lmod has a ~150 amino acid C-terminal extension featuring a third actin-binding site in the form of a W domain. With three actin-binding sites, Lmod could hypothetically recruit three actin monomers to form a trimeric polymerization nucleus, which led to the identification of Lmod as a potential filament nucleator (Chereau *et al.*, 2008). Consistent with this idea, the study of Lmod revealed a powerful nucleator, whose over- or down-expression had dramatic effects on sarcomeric structure and organization (Chereau *et al.*, 2008).

Compared to other nucleators, Lmod has one distinctive and important property: it directly interacts with TM. TM is a coiled coil dimer that associates end-to-end to form long helical strands that wind symmetrically along the two long-pitch helices of the actin filament (Holmes and Lehman, 2008). At the pointed end of the actin filament in muscle sarcomeres TM interacts with Tmod via two helical segments located within the N-terminal flexible domain of Tmod (Kostyukova *et al.*, 2007). As mentioned above, only one of these helices is conserved in Lmod. Yet, TM not only modulates the nucleation activity of Lmod, but more importantly it appears to determine Lmod's localization to filament pointed ends. Thus, $Lmod_{162–495}$, lacking the N-terminal flexible domain, retains approximately one third of the nucleation activity of full-length Lmod *in vitro*, but displays nuclear localization. A basic patch located within the long (and probably flexible) linker connecting the second and third actin-binding sites of Lmod is a predicted nuclear localization signal (NLS) and may be responsible for the nuclear localization of $Lmod_{162–495}$. While it is unknown whether trafficking through the nucleus forms part of Lmod's endogenous function, such an activity has been reported for Tmod (Kong and Kedes, 2004).

Perhaps reflecting its uniqueness as a muscle cell specific nucleator, Lmod shares little resemblance with other filament nucleators. With the presence of three actin-binding sites, Lmod is predicted to stabilize a trimeric actin seed for nucleation (Figure 6). However, the actual organization of actin subunits in the Lmod nucleus is a mystery. The W domain in Lmod seems to play an auxiliary role, somewhat analogous to its role in NPFs where the W domain contributes an actin subunit to complete a trimeric nucleus with the Arps. However, it is unknown which of the two actin subunits of the pointed end dimer in the Lmod nucleus is staggered forward. In other words, it is unknown whether the actin subunit bound to the N-terminal flexible domain of Lmod is staggered forward with respect to the one bound to the LRR domain or vise versa. This question also applies to Tmod, whose pointed end arrangement is still unknown. Lmod's linker-2 is also much longer than Cobl's linker-2, conferring significant freedom with respect to the relative positioning of the third actin subunit. Thus, the third actin subunit could be at the barbed end of either the first or the second subunit. Because Lmod contains a single TM-binding site, it can be predicted that in cells the Lmod nucleus is associated with a single TM dimer, but this has not been formally demonstrated. Finally,

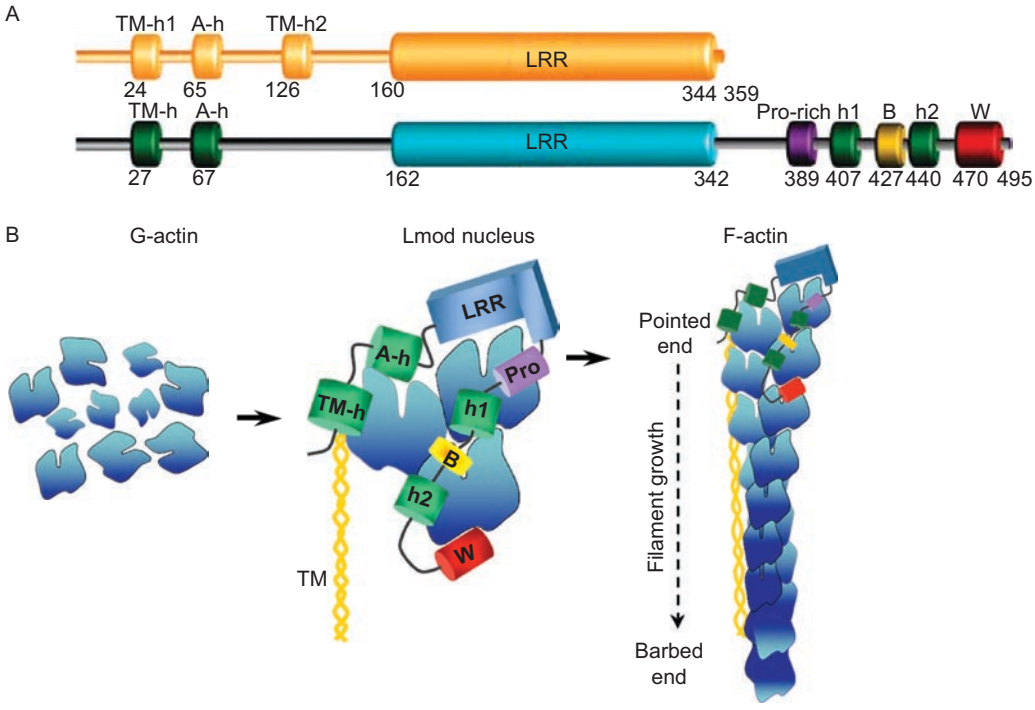

Figure 6. Domain organization of Lmod and proposed nucleation mechanism. (A) Domain organization of Lmod compared to Tmod. The first ~340 aa of Lmod are ~40% identical to Tmod, a pointed end capping protein in muscles. The N-terminal portion of Tmod is unstructured, except for three helical segments involved in binding tropomyosin (TM) and actin. Tmod has a second actin-binding site within the C-terminal Leu-rich repeat (LRR) domain (Krieger *et al.*, 2002; Fowler *et al.*, 2003). Lmod shares this domain organization, but has only one of the two TM-binding sites. More importantly, Lmod has a ~150 aa C-terminal extension featuring a third actin-binding site, a W domain, a basic patch and other predicted motifs. (B) Proposed nucleation mechanism; Lmod stabilizes a trimeric actin seed that grows from the barbed end. Importantly, Lmod's nucleation activity and localization are both modulated by interaction with TM (Chereau *et al.*, 2008).

one of the most intriguing questions about Lmod concerns the interplay with Tmod. The two proteins are clearly related and appear to have similar localization, but despite this similarity Lmod and Tmod probably have well-separated roles.

Ena/VASP proteins as dedicated elongation factors among W-based filament nucleators

A convenient way to introduce the Ena/VASP family of filament elongation factors (Drees and Gertler, 2008) is in contrast to formins. Formins have been reviewed extensively (Higgs, 2005; Faix and Grosse, 2006; Goode and Eck, 2007; Pollard, 2007; Chesarone and Goode, 2009; Paul and Pollard, 2009) and are only discussed briefly herein. Formins are the only proteins that do not use the W domain for nucleation or elongation, although there is at least one formin, INF2 (Chhabra and Higgs, 2006), that contains a W domain, but uses it for filament depolymerization and as a diaphanous autoregulatory domain (DAD) (Chhabra *et al.*, 2009). Formins use the dimeric formin-homology 2 (FH2) domain for interaction with actin, and like W-based nucleators, formins contain Pro-rich regions positioned N-terminal to the actin-binding domain (Higgs, 2005; Goode and Eck, 2007; Pollard, 2007; Paul and Pollard, 2009). But probably the most interesting property of formins is that in addition to nucleation they also promote processive barbed end elongation (or depolymerization).

So, the obvious question to ask is why do W-based nucleators not sustain processive barbed end elongation? The answer appears to be simple; because of steric hindrance of the W domain with intersubunit contacts in the actin filament. Indeed the crystal structure of a long-pitch actin dimer stabilized by a tandem repeat of two W domains has just been determined in our lab (Rebowski *et al.*, in preparation). The structure shows that although the two actins adopt a filament-like arrangement, they are somewhat more separated than in the actin filament. The separation occurs because the second W domain, bound in the hydrophobic cleft of the second actin subunit, interferes with filament-like contacts between the two actins. The implication is that tandem W-based nucleators cannot stay bound to filaments after nucleation, and therefore are unlikely to influence elongation. This is also likely to explain why

NPFs are ejected from the Arp2/3 complex once the branch filament begins to grow.

Part of the binding interface of the W-domain remains exposed in F-actin. So, tandem W domains may weakly (and non-specifically) co-sediment with F-actin and bind to (or cap) filament barbed ends, as discussed in a recent review (Renault *et al.*, 2008). But this also means that minor changes in the sequence of the W domain may give rise to an F-actin-binding domain. This appears to be the solution that nature has found to produce a filament elongation factor that is compatible with the W domain. Indeed, while formins participate in both nucleation and elongation, the elongation function among W-based filament nucleators has been "outsourced" to a dedicated family of proteins, Eva/VASP, which have a similar domain organization and may be evolutionarily related to WASP-family NPFs (see Figure 4 and legend for details). Eva/VASP and WASP/N-WASP both contain an N-terminal EVH1 (or WH1) domain, a central Pro-rich region and W-related sequences. A trace of their relationship can still be found in the acidic region C-terminal to the W-related sequences (albeit in Ena/VASP this region is less acidic than in NPFs and lacks the important tryptophan involved in binding to the Arp2/3 complex). The G-actin-binding (GAB) domain of Ena/VASP is not only related to the W domain of NPFs, but has also been shown to interact with actin in a similar manner (Ferron *et al.*, 2007) (compare Figures 2 and 7). Immediately C-terminal to the GAB domain is the F-actin-binding (FAB) domain, which is also related to the W domain. However, the FAB domain is more closely related to the C region of NPFs (Figure 4). Indeed, the FAB domain has evolved minor differences compared to the W domain, which probably reflect adaptation to bind F-actin in a way compatible with intersubunit contacts in the filament. A similar necessity may have arisen at the interface between Arp2 and the first actin subunit of the branch (Figure 3C), which probably explains the resemblance between the C and FAB domains. Another event in Ena/VASP's adaptation for processive barbed end elongation is tetramerization, which is mediated by the C-terminal coiled-coil domain (Bachmann *et al.*, 1999; Kuhnel *et al.*, 2004). Tetramerization may allow Ena/VASP to work cooperatively, by sequentially allowing each subunit of the tetramer to release and advance during monomer addition to the barbed end while the other subunits remain attached to the growing filament.

Profilin and the Pro-rich regions of nucleation and elongation factors

As mentioned above, two proteins, profilin and Tβ4, contribute to maintaining a large fraction (~50%) of the cellular actin in the unpolymerized pool. Tβ4 (Figure 7A) is a short 43-aa polypetide related to the W domain (Paunola *et al.*, 2002; Dominguez, 2007), but

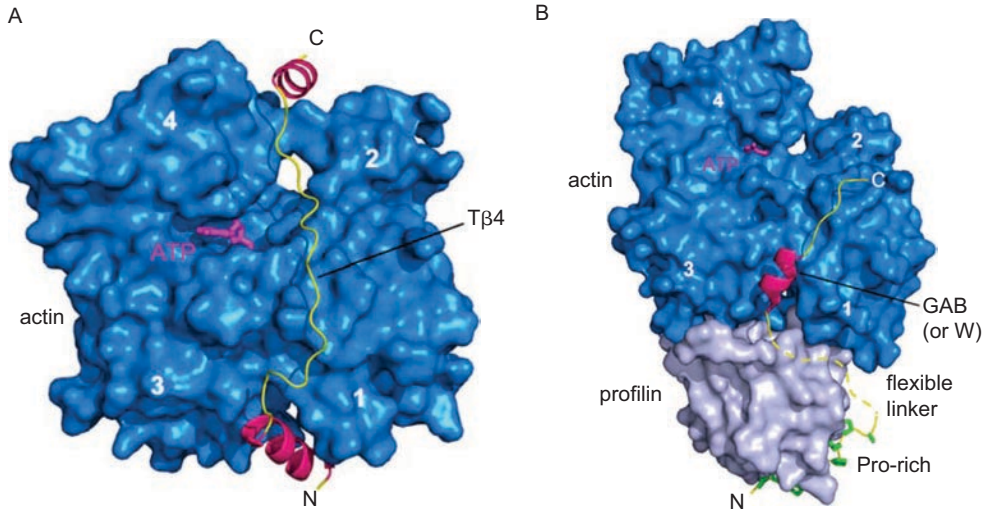

Figure 7. Tβ4 and profilin help maintain the pool of monomeric actin in cells. (A) Model of the structure of the complex of Tβ4-actin. This model was generated by combining the structures of W-actin (Chereau *et al.*, 2005) with that of a complex of actin with the C-terminal half of Tβ4 crystallized as a hybrid protein with gelsolin segment 1 (Irobi *et al.*, 2004). (B) Structure of the ternary complex of profilin–actin with human VASP fragment Gly-202 to Ser-244 (Ferron *et al.*, 2007). This fragment of VASP includes the last Pro-rich profilin-binding site and the G-actin-binding (GAB) domain, which is related to the W domain (see Figures 2 and 4). The Gly-rich linker between these two domains (^{213}QGPGGGGAG^{221}) was not visualized in the electron density map and is shown as a discontinuous line.

contains an additional C-terminal helix that binds atop actin subdomains 2 and 4 (Irobi *et al.*, 2004), making it an effective actin monomer sequestering protein (Safer *et al.*, 1990). As a result, Tβ4–actin complexes cannot participate in actin filament nucleation or elongation. Instead, Tβ4 is though to function as an actin buffer, losing actin in competitive equilibrium to profilin, which has higher affinity for actin monomers (Pollard and Borisy, 2003).

Multiple properties make profilin a crucial player in filament assembly (Pollard and Borisy, 2003; Witke, 2004; Paul and Pollard, 2009). Thus, profilin catalyzes the exchange of ADP for ATP on actin (which replenishes the pool of polymerization-competent ATP–actin), and inhibits nucleation and pointed end elongation, while having almost no effect on steady-state barbed end elongation. But probably the most interesting property of profilin is that it can bind simultaneously to Pro-rich sequences and actin (Figure 7B), and it binds both with higher affinity as a ternary complex than either one separately (Ferron *et al.*, 2007). This probably provides selectivity, so as to avoid non-productive interactions of profilin with Pro-rich regions in cells.

Indeed, a common feature of actin filament nucleation and elongation proteins is that they typically contain Pro-rich regions (magenta regions in Figure 4A). Pro-rich regions are most commonly positioned immediately N-terminal to the actin binding sites. These regions bind signaling and regulatory proteins, whose interactions are mediated by any of the existing Pro-rich binding modules, including SH3, WW, and EVH1 domains (Zarrinpar *et al.*, 2003). In addition, these regions frequently bind profilin–actin complexes, and could serve to increase the local concentration of actin monomers ready for assembly. As discussed above, W-based nucleators and NPFs are not expected to remain bound to newly formed filaments. So, any gain resulting from the initial recruitment of profilin–actin will be short-lived and probably difficult to detect using classical polymerization assays. The situation is far more interesting with processive barbed end elongation factors. The clearest evidence so far is for formins, whose elongation rates are increased by recruitment of profilin–actin (Kovar *et al.*, 2006). Perhaps not surprisingly the situation remains confusing with Ena/VASP. Various groups have demonstrated that profilin–actin stimulates Ena/VASP-dependent *Listeria* motility (Kang *et al.*, 1997; Geese *et al.*, 2000; Machner *et al.*, 2001; Dickinson *et al.*, 2002; Auerbuch *et al.*, 2003; Grenklo *et al.*, 2003). But, while a recent study established that Ena/VASP proteins accelerate barbed end elongation in a processive manner, it also concluded that profilin had no effect in this process (Breitsprecher *et al.*, 2008). This contrasts with the results of another group, which found that VASP accelerates filament elongation from a profilin–actin pool (Pasic *et al.*, 2008). Given the presence of various profilin-binding sites in Ena/VASP proteins, and the stimulatory effect that profilin has on formin-mediated assembly (Kovar *et al.*, 2006), this question deserves to be revisited. At least based on their domain architecture, Ena/VASP proteins appear to have evolved all the necessary adaptations to "process" profilin–actin complexes from the cellular pool onto the barbed ends of elongating filaments. Thus, the ternary structure of profilin–actin with a fragment of Ena/VASP consisting of the last Pro-rich segment and the GAB domain (Figure 7B) suggests that profilin–actin complexes may be delivered directly from the Pro-rich region to the barbed end of the growing filament (Ferron *et al.*, 2007). A similar monomer transfer mechanism may exist in formins, although this remains to be demonstrated.

Perspectives

Despite notable progress, many questions remain about filament nucleation and elongation. New Arp2/3 complex NPFs are being continuously discovered and each NPF redirects the Arp2/3 complex polymerization machinery into a different subcellular location and function. The exact function of Cobl and its role in neuronal morphology is still poorly understood and much remains to be learned about the Spire-Cappuccino-WASH connection. Concerning Lmod, an important question is the interplay with Tmod: which filaments are associated with Tmod in muscle cells and which are associated with Lmod? An issue that is catching attention is the crosstalk between different nucleation and elongation factors in the assembly of different actin cytoskeletal structures (Schirenbeck *et al.*, 2005; Chesarone and Goode, 2009). However, more directly connected with this review is the substantial gap of understanding of the structural aspects of filament nucleation/elongation, including the lack of a structure of activated Arp2/3 complex, and complexes of tandem W-based nucleators and Lmod with actin. Some of these questions are likely to dominate research within the actin cytoskeleton community during the following few years.

Acknowledgement

Supported by NIH grants GM073791 and HL086655.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Aguda AH, Burtnick LD and Robinson RC. 2005. The state of the filament. EMBO Rep 6:220–226.

Ahuja R, Pinyol R, Reichenbach N, Custer L, Klingensmith J, Kessels MM and Qualmann B. 2007. Cordon-bleu is an actin nucleation factor and controls neuronal morphology. Cell 131:337–350.

Ammer AG and Weed SA. 2008. Cortactin branches out: roles in regulating protrusive actin dynamics. Cell Motil Cytoskeleton 65:687–707.

Auerbuch V, Loureiro JJ, Gertler FB, Theriot JA and Portnoy DA. 2003. Ena/VASP proteins contribute to Listeria monocytogenes pathogenesis by controlling temporal and spatial persistence of bacterial actin-based motility. Mol Microbiol 49:1361–1375.

Bachmann C, Fischer L, Walter U and Reinhard M. 1999. The EVH2 domain of the vasodilator-stimulated phosphoprotein mediates tetramerization, F-actin binding, and actin bundle formation. J Biol Chem 274:23549–23557.

Bhavsar AP, Guttman JA and Finlay BB. 2007. Manipulation of host-cell pathways by bacterial pathogens. Nature 449:827–834.

Boczkowska M, Rebowski G, Petoukhov MV, Hayes DB, Svergun DI and Dominguez R. 2008. X-ray scattering study of activated Arp2/3 complex with bound actin-WCA. Structure 16:695–704.

Bompard G and Caron E. 2004. Regulation of WASP/WAVE proteins: making a long story short. J Cell Biol 166:957–962.

Bosch M, Le KH, Bugyi B, Correia JJ, Renault L and Carlier MF. 2007. Analysis of the function of Spire in actin assembly and its synergy with formin and profilin. Mol Cell 28:555–568.

Breitsprecher D, Kiesewetter AK, Linkner J, Urbanke C, Resch GP, Small JV and Faix J. 2008. Clustering of VASP actively drives processive, WH2 domain-mediated actin filament elongation. Embo J 27:2943–2954.

Campellone KG, Webb NJ, Znameroski EA and Welch MD. 2008. WHAMM is an Arp2/3 complex activator that binds microtubules and functions in ER to Golgi transport. Cell 134:148–161.

Carlier MF, Hertzog M, Didry D, Renault L, Cantrelle FX, van Heijenoort C, Knossow M and Guittet E. 2007. Structure, function, and evolution of the beta-thymosin/WH2 (WASP-Homology2) actin-binding module. Ann NY Acad Sci 1112:67–75.

Chereau D, Kerff F, Graceffa P, Grabarek Z, Langsetmo K and Dominguez R. 2005. Actin-bound structures of Wiskott-Aldrich syndrome protein (WASP)-homology domain 2 and the implications for filament assembly. Proc Natl Acad Sci USA 102:16644–16649.

Chereau D, Boczkowska M, Skwarek-Maruszewska A, Fujiwara I, Hayes DB, Rebowski G, Lappalainen P, Pollard TD and Dominguez R. 2008. Leiomodin is an actin filament nucleator in muscle cells. Science 320:239–243.

Chesarone MA and Goode BL. 2009. Actin nucleation and elongation factors: mechanisms and interplay. Curr Opin Cell Biol 21:28–37.

Chhabra ES and Higgs HN. 2006. INF2 Is a WASP homology 2 motif-containing formin that severs actin filaments and accelerates both polymerization and depolymerization. J Biol Chem 281:26754–26767.

Chhabra ES and Higgs HN. 2007. The many faces of actin: matching assembly factors with cellular structures. Nat Cell Biol 9:1110–1121.

Chhabra ES, Ramabhadran V, Gerber SA and Higgs HN. 2009. INF2 is an endoplasmic reticulum-associated formin protein. J Cell Sci 122:1430–1440.

Conley CA, Fritz-Six KL, Almenar-Queralt A and Fowler VM. 2001. Leiomodins: larger members of the tropomodulin (Tmod) gene family. Genomics 73:127–139.

Copeland JW, Copeland SJ and Treisman R. 2004. Homo-oligomerization is essential for F-actin assembly by the formin family FH2 domain. J Biol Chem, 279, 50250–50256.

Cossart P and Toledo-Arana A. 2008. Listeria monocytogenes, a unique model in infection biology: an overview. Microbes Infect 10:1041–1050.

Dahlgaard K, Raposo AA, Niccoli T and St Johnston D. 2007. Capu and Spire assemble a cytoplasmic actin mesh that maintains microtubule organization in the Drosophila oocyte. Dev Cell 13:539–553.

Dayel MJ, Holleran EA and Mullins RD. 2001. Arp2/3 complex requires hydrolyzable ATP for nucleation of new actin filaments. Proc Natl Acad Sci USA 98:14871–14876.

Dickinson RB, Southwick FS and Purich DL. 2002. A direct-transfer polymerization model explains how the multiple profilin-binding sites in the actoclampin motor promote rapid actin-based motility. Arch Biochem Biophys 406:296–301.

Dominguez R. 2004. Actin-binding proteins – a unifying hypothesis. Trends Biochem Sci 29:572–578.

Dominguez R. 2007. The beta-thymosin/WH2 fold: multifunctionality and structure. Ann NY Acad Sci 1112:86–94.

Drees F and Gertler FB. 2008. Ena/VASP: proteins at the tip of the nervous system. Curr Opin Neurobiol 18:53–59.

Dumas JJ, Merithew E, Sudharshan E, Rajamani D, Hayes S, Lawe D, Corvera S and Lambright DG. 2001. Multivalent endosome targeting by homodimeric EEA1. Mol Cell 8:947–958.

Eden S, Rohatgi R, Podtelejnikov AV, Mann M and Kirschner MW. 2002. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature 418:790–793.

Egile C, Rouiller I, Xu XP, Volkmann N, Li R and Hanein D. 2005. Mechanism of filament nucleation and branch stability revealed by the structure of the Arp2/3 complex at actin branch junctions. PLoS Biol 3:e383.

Faix J and Grosse R. 2006. Staying in shape with formins. Dev Cell 10:693–706.

Ferron F, Rebowski G, Lee SH and Dominguez R. 2007. Structural basis for the recruitment of profilin-actin complexes during filament elongation by Ena/VASP. Embo J 26:4597–4606.

Fischer RS and Fowler VM. 2003. Tropomodulins: life at the slow end. Trends Cell Biol 13:593–601.

Fowler VM, Greenfield NJ and Moyer J. 2003. Tropomodulin contains two actin filament pointed end-capping domains. J Biol Chem 278:40000–40009.

Galletta BJ and Cooper JA. 2009. Actin and endocytosis: mechanisms and phylogeny. Curr Opin Cell Biol 21:20–27.

Geese M, Schluter K, Rothkegel M, Jockusch BM, Wehland J and Sechi AS. 2000. Accumulation of profilin II at the surface of Listeria is concomitant with the onset of motility and correlates with bacterial speed. J Cell Sci 113:1415–1426.

Goley ED and Welch MD. 2006. The ARP2/3 complex: an actin nucleator comes of age. Nat Rev Mol Cell Biol 7:713–726.

Goley ED, Rodenbusch SE, Martin AC and Welch MD. 2004. Critical conformational changes in the Arp2/3 complex are induced by nucleotide and nucleation promoting factor. Mol Cell 16:269–279.

Goode BL and Eck MJ. 2007. Mechanism and function of formins in the control of actin assembly. Annu Rev Biochem 76:593–627.

Gouin E, Welch MD and Cossart P. 2005. Actin-based motility of intracellular pathogens. Curr Opin Microbiol 8:35–45.

Grenklo S, Geese M, Lindberg U, Wehland J, Karlsson R and Sechi AS. 2003. A crucial role for profilin-actin in the intracellular motility of Listeria monocytogenes. EMBO Rep 4:523–529.

Gunst SJ and Zhang W. 2008. Actin cytoskeletal dynamics in smooth muscle: a new paradigm for the regulation of smooth muscle contraction. Am J Physiol Cell Physiol 295:C576–587.

Hall A. 2005. Rho GTPases and the control of cell behaviour. Biochem Soc Trans 33:891–895.

Hertzog M, Yarmola EG, Didry D, Bubb MR and Carlier MF. 2002. Control of actin dynamics by proteins made of beta-thymosin repeats: the actobindin family. J Biol Chem 277:14786–14792.

Hertzog M, van Heijenoort C, Didry D, Gaudier M, Coutant J, Gigant B, Didelot G, Preat T, Knossow M, Guittet E and Carlier MF. 2004. The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. Cell 117:611–623.

Higgs HN. 2005. Formin proteins: a domain-based approach. Trends Biochem Sci 30:342–353.

Holmes KC. 2009. Structural biology: actin in a twist. Nature 457:389–390.

Holmes KC and Lehman W. 2008. Gestalt-binding of tropomyosin to actin filaments. J Muscle Res Cell Motil 29:213–219.

Holmes KC, Popp D, Gebhard W and Kabsch W. 1990. Atomic model of the actin filament. Nature 347:44–49.

Irobi E, Aguda AH, Larsson M, Guerin C, Yin HL, Burtnick LD, Blanchoin L and Robinson RC. 2004. Structural basis of actin sequestration by thymosin-beta4: implications for WH2 proteins. Embo J 23:3599–3608.

Jewett TJ, Fischer ER, Mead DJ and Hackstadt T. 2006. Chlamydial TARP is a bacterial nucleator of actin. Proc Natl Acad Sci USA 103:15599–15604.

Kang F, Laine RO, Bubb MR, Southwick FS and Purich DL. 1997. Profilin interacts with the Gly-Pro-Pro-Pro-Pro-Pro sequences of vasodilator-stimulated phosphoprotein (VASP): implications for actin-based Listeria motility. Biochemistry 36:8384–8392.

Kelly AE, Kranitz H, Dotsch V and Mullins RD. 2006. Actin binding to the central domain of WASP/Scar proteins plays a critical role in the activation of the Arp2/3 complex. J Biol Chem 281:10589–10597.

Kim AS, Kakalis LT, Abdul-Manan N, Liu GA and Rosen MK. 2000. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. Nature 404:151–158.

Kong KY and Kedes L. 2004. Cytoplasmic nuclear transfer of the actin-capping protein tropomodulin. J Biol Chem 279:30856–30864.

Kostyukova AS, Hitchcock-Degregori SE and Greenfield NJ. 2007. Molecular basis of tropomyosin binding to tropomodulin, an actin-capping protein. J Mol Biol 372:608–618.

Kovar DR, Harris ES, Mahaffy R, Higgs HN and Pollard TD. 2006. Control of the assembly of ATP- and ADP-actin by formins and profilin. Cell 124:423–435.

Kreishman-Deitrick M, Goley ED, Burdine L, Denison C, Egile C, Li R, Murali N, Kodadek TJ, Welch MD and Rosen MK. 2005. NMR analyses of the activation of the Arp2/3 complex by neuronal Wiskott-Aldrich syndrome protein. Biochemistry 44:15247–15256.

Krieger I, Kostyukova A, Yamashita A, Nitanai Y and Maeda Y. 2002. Crystal structure of the C-terminal half of tropomodulin and structural basis of actin filament pointed-end capping. Biophys J 83:2716–2725.

Kuhnel K, Jarchau T, Wolf E, Schlichting I, Walter U, Wittinghofer A and Strelkov SV. 2004. The VASP tetramerization domain is a right-handed coiled coil based on a 15-residue repeat. Proc Natl Acad Sci USA 101:17027–17032.

Le Clainche C and Carlier MF. 2008. Regulation of actin assembly associated with protrusion and adhesion in cell migration. Physiol Rev 88:489–513.

Le Clainche C, Didry D, Carlier MF and Pantaloni D. 2001. Activation of Arp2/3 complex by Wiskott-Aldrich Syndrome protein is linked to enhanced binding of ATP to Arp2. J Biol Chem 276:46689–46692.

Linardopoulou EV, Parghi SS, Friedman C, Osborn GE, Parkhurst SM and Trask BJ. 2007. Human subtelomeric WASH genes encode a new subclass of the WASP family. PLoS Genet 3:e237.

Liu R, Abreu-Blanco MT, Barry KC, Linardopoulou EV, Osborn GE and Parkhurst SM. 2009. Wash functions downstream of Rho and links linear and branched actin nucleation factors. Development 136:2849–2860.

Liverman AD, Cheng HC, Trosky JE, Leung DW, Yarbrough ML, Burdette DL, Rosen MK and Orth K. 2007. Arp2/3-independent assembly of actin by Vibrio type III effector VopL. Proc Natl Acad Sci USA 104:17117–17122.

Ma L, Rohatgi R and Kirschner MW. 1998. The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. Proc Natl Acad Sci USA 95:15362–15367.

Machesky LM and Insall RH 1998. Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. Curr Biol 8:1347–1356.

Machesky LM, Atkinson SJ, Ampe C, Vandekerckhove J and Pollard TD. 1994. Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. J Cell Biol 127:107–115.

Machesky LM, Mullins RD, Higgs HN, Kaiser DA, Blanchoin L, May RC, Hall ME and Pollard TD. 1999. Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. Proc Natl Acad Sci USA 96:3739–3744.

Machner MP, Urbanke C, Barzik M, Otten S, Sechi AS, Wehland J and Heinz DW. 2001. ActA from Listeria monocytogenes can interact with up to four Ena/VASP homology 1 domains simultaneously. J Biol Chem 276:40096–40103.

Marchand JB, Kaiser DA, Pollard TD and Higgs HN. 2001. Interaction of WASP/Scar proteins with actin and vertebrate Arp2/3 complex. Nat Cell Biol 3:76–82.

Mattila PK, Salminen M, Yamashiro T and Lappalainen P. 2003. Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its C-terminal WH2 domain. J Biol Chem 278:8452–8459.

Mullins RD, Heuser JA and Pollard TD. 1998. The interaction of Arp2/3 complex with actin: nucleation, high affinity pointed end capping, and formation of branching networks of filaments. Proc Natl Acad Sci USA 95:6181–6186.

Nolen BJ and Pollard TD. 2007. Insights into the influence of nucleotides on actin family proteins from seven structures of Arp2/3 complex. Mol Cell 26:449–457.

Oda T, Iwasa M, Aihara T, Maeda Y and Narita A. 2009. The nature of the globular- to fibrous-actin transition. Nature 457:441–445.

Padrick SB, Cheng HC, Ismail AM, Panchal SC, Doolittle LK, Kim S, Skehan BM, Umetani J, Brautigam CA, Leong JM and Rosen MK. 2008. Hierarchical regulation of WASP/WAVE proteins. Mol Cell 32:426–438.

Pan F, Egile C, Lipkin T and Li R. 2004. ARPC1/Arc40 mediates the interaction of the actin-related protein 2 and 3 complex with Wiskott-Aldrich syndrome protein family activators. J Biol Chem 279:54629–54636.

Panchal SC, Kaiser DA, Torres E, Pollard TD and Rosen MK. 2003. A conserved amphipathic helix in WASP/Scar proteins is essential for activation of Arp2/3 complex. Nat Struct Biol 10:591–598.

Pasic L, Kotova T and Schafer DA. 2008. Ena/VASP proteins capture actin filament barbed ends. J Biol Chem 283:9814–9819.

Paul AS and Pollard TD. 2009. Review of the mechanism of processive actin filament elongation by formins. Cell Motil Cytoskeleton 66:606–617.

Paunola E, Mattila PK and Lappalainen P. 2002. WH2 domain: a small, versatile adapter for actin monomers. FEBS Lett 513:92–97.

Pollard TD. 2007. Regulation of actin filament assembly by Arp2/3 complex and formins. Annu Rev Biophys Biomol Struct 36:451–477.

Pollard TD and Borisy GG. 2003. Cellular motility driven by assembly and disassembly of actin filaments. Cell 112:453–465.

Pruyne D, Evangelista M, Yang C, Bi E, Zigmond S, Bretscher A and Boone C. 2002. Role of formins in actin assembly: nucleation and barbed-end association. Science 297:612–615.

Qualmann B and Kessels MM. 2009. New players in actin polymerization – WH2-domain-containing actin nucleators. Trends Cell Biol 19:276–285.

Quinlan ME and Kerkhoff E. 2008. Actin nucleation: bacteria get inSpired. Nat Cell Biol 10:13–15.

Quinlan ME, Heuser JE, Kerkhoff E and Mullins RD. 2005. Drosophila Spire is an actin nucleation factor. Nature 433:382–388.

Quinlan ME, Hilgert S, Bedrossian A, Mullins RD and Kerkhoff E. 2007. Regulatory interactions between two actin nucleators, Spire and Cappuccino. J Cell Biol 179:117–128.

Rebowski G, Boczkowska M, Hayes DB, Guo L, Irving TC and Dominguez R. 2008. X-ray scattering study of actin polymerization nuclei assembled by tandem W domains. Proc Natl Acad Sci USA 105:10785–10790.

Renault L, Bugyi B and Carlier MF 2008. Spire and Cordon-bleu: multifunctional regulators of actin dynamics. Trends Cell Biol 18:494–504.

Robinson RC, Turbedsky K, Kaiser DA, Marchand JB, Higgs HN, Choe S and Pollard TD. 2001. Crystal structure of Arp2/3 complex. Science 294:1679–1684.

Rodal AA, Sokolova O, Robins DB, Daugherty KM, Hippenmeyer S, Riezman H, Grigorieff N and Goode BL. 2005. Conformational changes in the Arp2/3 complex leading to actin nucleation. Nat Struct Mol Biol 12:26–31.

Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, Takenawa T and Kirschner MW. 1999. The interaction between N-WASP and the

Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell 97:221–231.
Rosales-Nieves AE, Johndrow JE, Keller LC, Magie CR, Pinto-Santini DM and Parkhurst SM. 2006. Coordination of microtubule and microfilament dynamics by Drosophila Rho1, Spire and Cappuccino. Nat Cell Biol 8:367–376.
Rouiller I, Xu XP, Amann KJ, Egile C, Nickell S, Nicastro D, Li R, Pollard TD, Volkmann N and Hanein D. 2008. The structural basis of actin filament branching by the Arp2/3 complex. J Cell Biol 180:887–895.
Safer D, Golla R and Nachmias VT. 1990. Isolation of a 5-kilodalton actin-sequestering peptide from human blood platelets. Proc Natl Acad Sci USA 87:2536–2540.
Sanger JM and Sanger JW. 2008. The dynamic Z bands of striated muscle cells. Sci Signal 1:pe37.
Schirenbeck A, Arasada R, Bretschneider T, Schleicher M and Faix J. 2005. Formins and VASPs may co-operate in the formation of filopodia. Biochem Soc Trans 33:1256–1259.
Sept D and McCammon JA. 2001. Thermodynamics and kinetics of actin filament nucleation. Biophys J 81:667–674.
Skwarek-Maruszewska A, Hotulainen P, Mattila PK and Lappalainen P. 2009. Contractility-dependent actin dynamics in cardiomyocyte sarcomeres. J Cell Sci 122:2119–2126.
Tam VC, Serruto D, Dziejman M, Brieher W and Mekalanos JJ. 2007. A type III secretion system in Vibrio cholerae translocates a formin/Spire hybrid-like actin nucleator to promote intestinal colonization. Cell Host Microbe 1:95–107.
Van Troys M, Ono K, Dewitte D, Jonckheere V, De Ruyck N, Vandekerckhove J, Ono S and Ampe C. 2004. TetraThymosinbeta is required for actin dynamics in Caenorhabditis elegans and acts via functionally different actin-binding repeats. Mol Biol Cell 15:4735–4748.
Wang J, Shaner N, Mittal B, Zhou Q, Chen J, Sanger JM and Sanger JW. 2005. Dynamics of Z-band based proteins in developing skeletal muscle cells. Cell Motil Cytoskeleton 61:34–48.
Weaver AM, Karginov AV, Kinley AW, Weed SA, Li Y, Parsons JT and Cooper JA. 2001. Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr Biol 11:370–374.
Weaver AM, Heuser JE, Karginov AV, Lee WL, Parsons JT and Cooper JA. 2002. Interaction of cortactin and N-WASp with Arp2/3 complex. Curr Biol 12:1270–1278.
Welch MD, Rosenblatt J, Skoble J, Portnoy DA and Mitchison TJ. 1998. Interaction of human Arp2/3 complex and the Listeria monocytogenes ActA protein in actin filament nucleation. Science 281:105–108.
Winter D, Lechler T and Li R. 1999. Activation of the yeast Arp2/3 complex by Bee1p, a WASP-family protein. Curr Biol 9:501–504.
Witke W. 2004. The role of profilin complexes in cell motility and other cellular processes. Trends Cell Biol 14:461–469.
Yarar D, To W, Abo A and Welch MD. 1999. The Wiskott-Aldrich syndrome protein directs actin-based motility by stimulating actin nucleation with the Arp2/3 complex. Curr Biol 9:555–558.
Zalevsky J, Grigorova I and Mullins RD. 2001. Activation of the Arp2/3 complex by the Listeria acta protein. Acta binds two actin monomers and three subunits of the Arp2/3 complex. J Biol Chem 276:3468–3475.
Zarrinpar A, Bhattacharyya RP and Lim WA. 2003. The structure and function of proline recognition domains. Sci STKE, 2003, RE8.
Zigmond SH, Evangelista M, Boone C, Yang C, Dar AC, Sicheri F, Forkey J and Pring M. 2003. Formin leaky cap allows elongation in the presence of tight capping proteins. Curr Biol 13:1820–1823.
Zuchero JB, Coutts AS, Quinlan ME, Thangue NB and Mullins RD. 2009. p53-cofactor JMY is a multifunctional actin nucleation factor. Nat Cell Biol 11:451–459.

Editor: Michael M. Cox