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Review

Functional Actin Networks under Construction: The Cooperative Action of Actin Nucleation and Elongation Factors

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Cells require actin nucleation factors to catalyze the formation of actin networks and elongation factors to control the rate and extent of actin polymerization. Earlier models suggested that the different factors assemble actin networks independently. However, recent evidence indicates that the assembly of most cellular networks involves multiple nucleation and elongation factors that work in concert. Here, we describe how these different factors cooperate, directly or indirectly, to promote the assembly of functional actin network in cells, both in the cytoplasm and nucleoplasm. We show that, in many cases, multiple factors collaborate to initiate network assembly and growth. The selection of specific sets of key players enables the cells to fine-tune network structure and dynamics, optimizing them for particular cellular functions.

Cytoskeletal Actin Networks and Their Associated Nucleation and Elongation Factors

The actin cytoskeleton is a dynamic network of actin filaments (F-actin) and accessory proteins that have important roles in processes such as locomotion, cytokinesis, and morphogenesis. To accomplish such a diversity of tasks, the actin cytoskeleton constantly remodels itself, with the underlying processes being driven by the nucleation of filaments and by their assembly–disassembly dynamics. Actin localizes in the cytoplasm and in the nucleoplasm, although at lower concentrations in the latter [1].

While actin polymerization is favorable energetically, actin nucleation is not; thus, the rate-limiting step for F-actin polymerization is nucleation [2]. In many cases, network assembly involves the cooperative work of multiple factors, which enables spatiotemporal control of cytoskeletal networks. So far, three major classes of eukaryotic actin nucleator have been discovered: (i) the Arp2/3 complex, which functions with nucleation-promoting factors (NPFs) [3–5] (Figure 1A,B); (ii) the formins [4,6] (Figure 1C); and (iii) the tandem-monomer-binding (TMB) nucleators [7,8] (Figure 1E), which include Spire, Cordon-bleu (Cobl), *Vibrio parahaemolyticus* and *Vibrio cholerae* (VopL/VopF) factors, Leiomodin (Lmod), junction-mediating regulatory [JMY, which also functions as a NPF of the Arp2/3 complex (Figure 1A)], and adenomatous polyposis coli (APC). Cofilin also exhibits actin nucleation activity, although the mechanism responsible is unknown [9,10] (Figure 1F).

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Cells require actin nucleation factors to catalyze the formation of actin filament networks and actin elongation factors to control the rate and extent of polymerization.
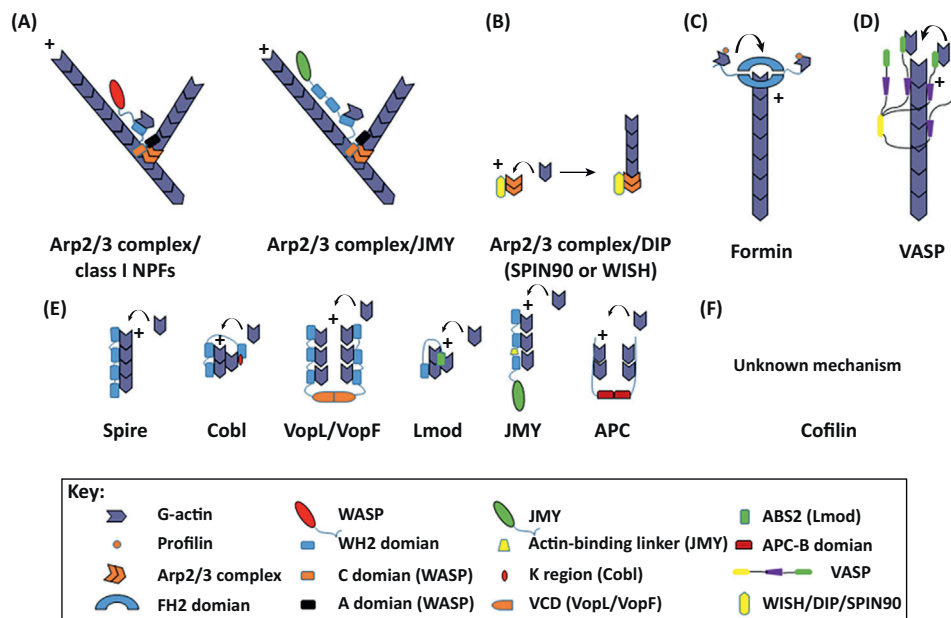
Nucleation and elongation factors cooperate, directly or indirectly, to control the formation of functional actin networks in cells.

Actin nucleation factors include *de novo* nucleation factors, which promote actin assembly from monomeric subunits, and those factors that require a supply of preformed F-actin to initiate network formation. The first group is involved in forming unbranched actin structures, while the second is mostly associated with dendritic network structures.

Preformed F-actin is supplied to the membrane surface by either nucleating F-actin seeds in the bulk cytoplasm, then recruiting them to the membrane surface, or by nucleating actin filaments directly at the membrane surface.

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Figure 1. Major Classes of Actin Nucleation and Elongation Factors. (A) Branched nucleation by the Arp2/3 complex with WASP and JMY functioning as NPFs. (B) DIP/SPIN90/WISH proteins activate Arp2/3 complex to create unbranched actin filaments in a non-WASP-like mechanism. (C) Formins promote actin nucleation and also function as elongation factors. (D) VASP proteins function as elongation factors but have no actin nucleation activity. (E) Tandem monomer-binding nucleators (TMBs) nucleate unbranched actin filaments using multiple WH2 domains. The Spire mechanism of nucleation is still under debate. (F) Cofilin promotes actin nucleation by an unknown mechanism. For additional definitions, please see the main text.

Once nucleated, the filaments elongate primarily from their barbed ends. *In vivo*, this process is rapidly terminated by capping proteins (CPs). The cell can counteract the elongation-limiting effects of CPs by expressing elongation factors, the best characterized of which are formins and Ena/VASP [6] (Figure 1C,D). These proteins compete with CPs for barbed-end attachment and control filament elongation rate. Many of the factors that regulate cytoplasmic actin nucleation are also found in the nucleus and cycle between the two compartments with the help of transport mediator proteins [1,11] (Figure 2A). These factors shuttle to the nucleus in response to external signals, such as cellular stress and DNA damage, and induce nuclear actin assembly [1,12].

Here, we compare the biochemical mechanisms of actin nucleation and elongation factors. We first describe the factors that promote network assembly from monomeric actin ('*de novo*' nucleation factors) and then describe those that require preformed F-actin ('F-actin seed'-dependent factors). Finally, we discuss how these various factors cooperate, directly and indirectly, to control the formation, structure, and turnover of functional actin networks in cells (Figure 3).
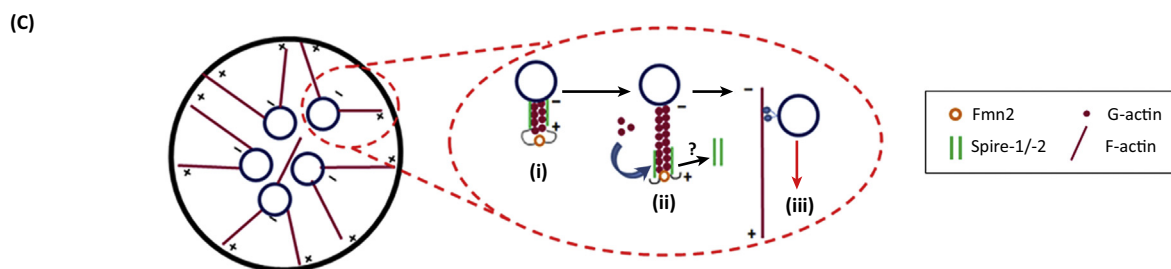
De novo Nucleation Factors
Formins
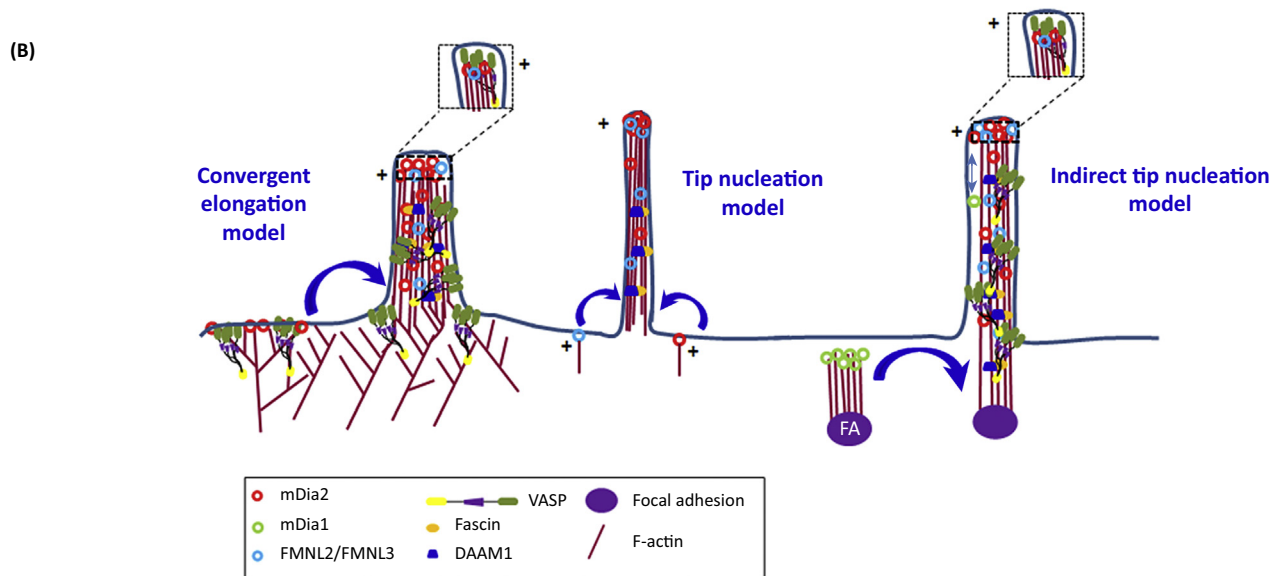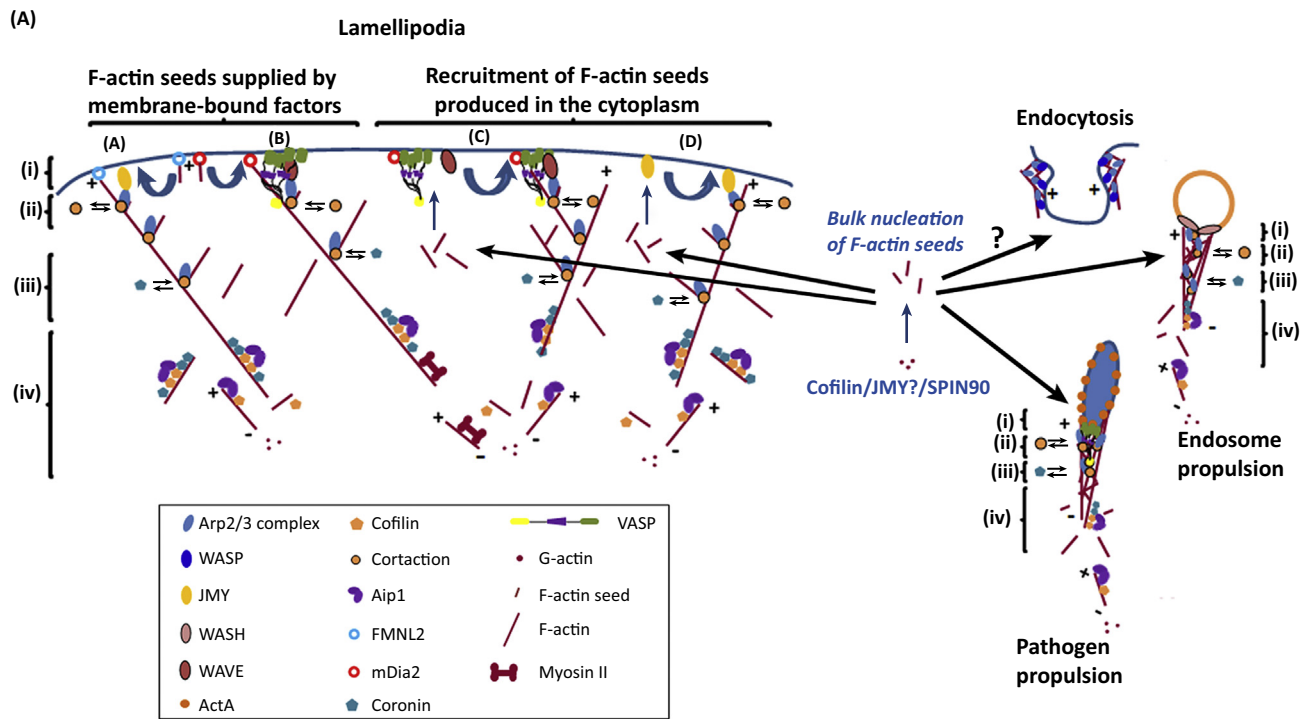Formins are involved in endocytosis, cytokinesis, morphogenesis, endosome motility [4,6], stress fiber formation [13], and filopodia [14–16] and lamellipodia [14] assembly. Besides their multiple functions in the cytoplasm, formins also localize in the nucleus, where they promote actin network assembly in response to DNA damage [17], serum stimulation [18], and integrin-dependent cell spreading [19].
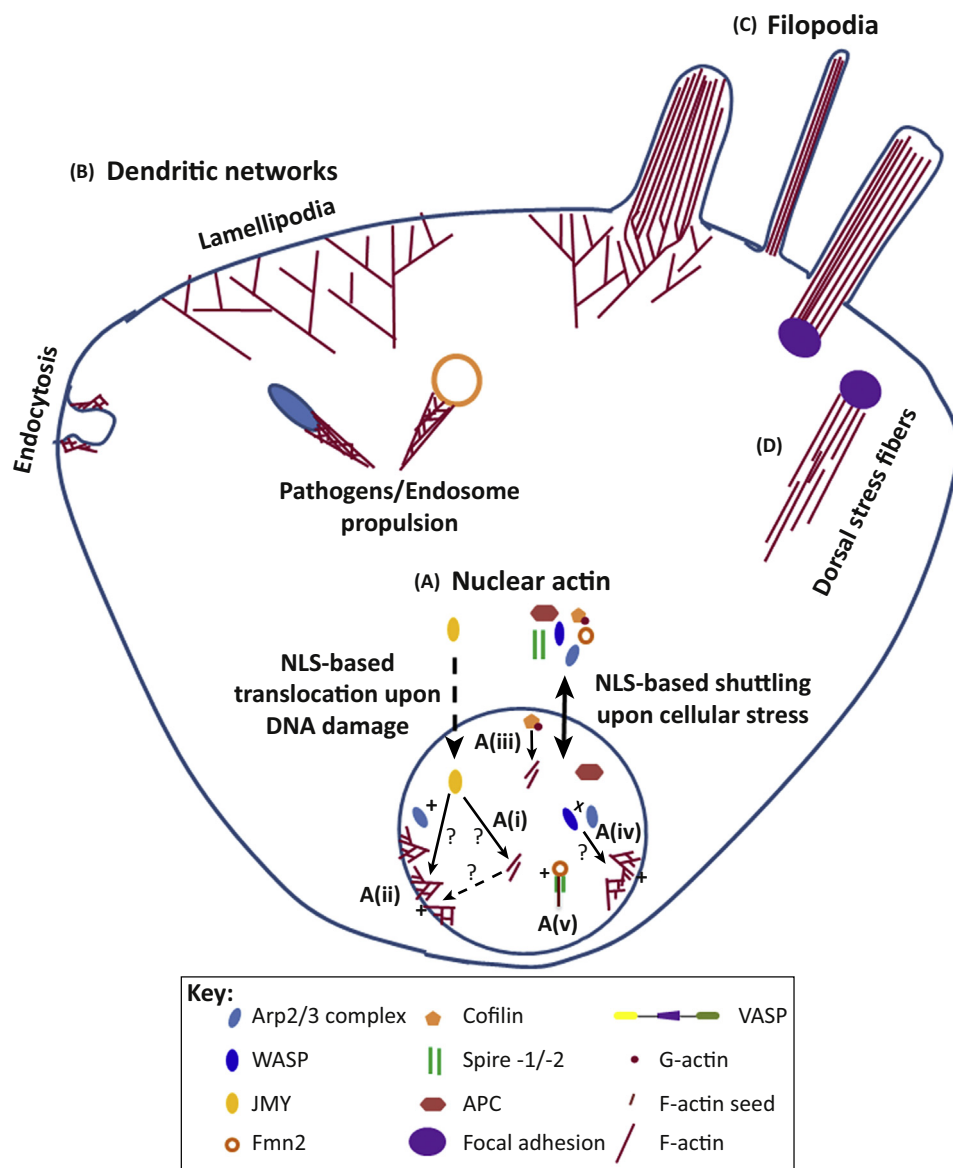
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Figure 2. Cytoskeletal Actin Networks Formed in the Cytoplasm and Nucleoplasm. (A) Actin in the nucleus. Nucleation and elongation factors shuttle to the nucleus in response to cellular stress and DNA damage, and induce nuclear actin assembly: (i) JMY-induced unbranched filament assembly (remains to be validated); (ii) Arp2/3 complex/JMY-dependent branched networks assembly (remains to be validated); (iii) cofilin-induced actin rods assembly; (iv) a branched actin 'shell' formed on the inner surface of the nuclear envelope by the Arp2/3 complex; the NPFs involved remain to be determined; and (v) Formin-2 (Fmn2) and Spire-1/Spire-2 collaborate to promote the assembly of unbranched actin networks in response to DNA damage. (B) Arp2/3-complex-dependent dendritic nucleation drives the formation of lamellipodia at the cell leading edge and the propulsion of endosomes and pathogens, and underlies endocytosis. (C) Filopodia protrusions comprise unbranched polar actin bundles formed via various mechanisms. (D) Dorsal stress fibers are unbranched apolar contractile actin bundles. For additional definitions, please see the main text.

Biochemically, formins are defined by a unique and highly conserved C-terminal formin homology-2 (FH2) domain preceded by a formin homology-1 (FH1) domain. The best-studied formins are Diaphanous-related Formins (DRFs), direct effectors of Rho-family GTPases [6]. The DRFs include Dia, DAAM, and FRL in mammals, Bni1 and Bnr1 in yeast, and SepA in

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(A)

Lamellipodia

F-actin seeds supplied by membrane-bound factors

Recruitment of F-actin seeds produced in the cytoplasm

(A) (B) (C) (D)

(i)
(ii)
(iii)
(iv)

Endocytosis

Bulk nucleation of F-actin seeds   ?

Cofilin/JMY?/SPIN90

(i)
(ii)
(iii)
(iv)

Endosome propulsion

Pathogen propulsion

Legend:
- Arp2/3 complex
- WASP
- JMY
- WASH
- WAVE
- ActA
- Cofilin
- Cortaction
- Aip1
- FMNL2
- mDia2
- Coronin
- VASP
- G-actin
- F-actin seed
- F-actin
- Myosin II

(B)

Convergent elongation model

Tip nucleation model

Indirect tip nucleation model

FA

Legend:
- mDia2
- mDia1
- FMNL2/FMNL3
- VASP
- Fascin
- DAAM1
- Focal adhesion
- F-actin

(C)

(i) (ii) (iii)

Legend:
- Fmn2
- Spire-1/-2
- G-actin
- F-actin

(See figure legend on the bottom of the next page.)

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Aspergillus nidulans. The mechanism of regulation is based on intramolecular interactions between the N-terminal diaphanous inhibitory domain (DID) and the C-terminal diaphanous autoregulatory domain (DAD) to maintain the formin in an autoinhibited state [6,20–22].

Formins promote the *de novo* nucleation of actin and processively elongate filaments [6,23]. Generally, free G-actin is strongly favored for nucleation, while profilin–actin complexes are favored for elongation [24,25]. Specifically, the FH2 domain binds to G-actin to initiate filament nucleation and forms homodimers that act as a 'processive cap' enabling persistent filament elongation [25–27] (Figure 1C) with an efficiency that depends on profilin concentration [24,25]. Given that the bulk of G-actin in cells is bound to profilin, profilin is expected to have a strong influence on actin polymerization rate by formins in cells.

CPs also have a strong influence on filament elongation by formins. Initial studies suggested that formins and CPs bind to filament barbed ends in a mutually exclusive manner [28–30]. Yet, recent single-molecule studies revealed that formins and CPs form a complex at the barbed end that mutually weakens their binding to the barbed end, facilitating the displacement of formins by CPs, which terminates the elongation process [31,32]. Rebinding of formins was shown to restart the elongation process [31]. These findings explain how formins, which are highly processive elongation factors, can produce relatively short actin filaments in cells.

TMB Nucleators
Spire, Cobl, VopL/VopF, APC, and Lmod nucleate the assembly of unbranched actin filaments [7,8] (Figure 1E). JMY, which also belongs to this class, can nucleate both branched and unbranched actin filaments (Figure 1A,E). These nucleators have multiple WH2 domains that often appear in tandem repeats of up to four domains. The actin monomers bound to these WH2 domains are brought together to form an F-actin-like nucleus. All WH2 domains are intrinsically disordered peptides that fold upon binding to G-actin. Although the WH2 domains of the various TMBs are generally well conserved, there are significant differences in their nucleation activities, attributed to differences in the number of WH2 domains and the length of the linkers between them [33]. Also, the different TMBs use distinct arrangements and clustering of G-actin (along or across filament strands) to stabilize the formation of the F-actin nucleus [33]. Nevertheless, it was recently argued that, even in the form of tandem repeats, the WH2 domain is insufficient to drive efficient actin nucleation [34] and that it must cooperate with other factors [34–38]. These factors were shown to promote TMB dimerization, which dramatically stimulates the nucleation activity of TMBs [36,39,40].

Figure 3. Functional Actin Networks in Cells: The Cooperation of Multiple Actin Assembling and Disassembling Factors. (A) Arp2/3-complex-dependent dendritic nucleation drives lamellipodia protrusion and endocytosis, and the propulsion of endosomes and pathogens (shown for *Listeria monocytogenes*). The branched network is continuously generated at the membrane/endosome/pathogen surface by activation of the Arp2/3 complex by class I NPFs (and/or JMY in the case of the lamellipodia), with the barbed ends of the filaments pushing the membrane/endosome/pathogen forward. Ena/VASP interaction with class I NPFs enhances Arp2/3 complex-mediated branch nucleation. The branch nucleation process is initiated by the binding of preformed F-actin, recruited from the bulk and/or nucleated at the membrane surface by *de novo* nucleation factors [e.g., mDia2 and FMNL2 in the case of lamellipodia (a,b)] (i), followed by the cortactin-mediated release of NPFs (which triggers branch formation) and branch stabilization (ii). The branched network at the lamellipodia rear or comet tail end undergoes debranching by coronin (iii), followed by filament severing and depolymerization by the cooperative work of cofilin, coronin, and Aip1 and/or by myosin II motors (in the case of lamellipodia) (iv). (B) Filopodia form via different mechanisms. In the convergent elongation model, the barbed ends of filaments from the lamellipodia network captured by Ena/VASP and/or formins (e.g., mDia2, FMNL2, or FMNL3) elongate continuously and gradually converge into bundles mediated by mDia2, FMNL2, FMNL3, fascin, and DAAM1, which is recruited by fascin to the filopodial shaft. Ena/VASP proteins also localize to the filopodial shaft and connect the filopodia to the surrounding cytoskeleton and adhesion sites. In the tip nucleation model, membrane-bound formins (e.g, mDia2 and FMNL2) are activated at the membrane and promote *de novo* nucleation of actin filaments, which subsequently elongate (with or without Ena/VASP; shown is the case without Ena/VASP) and get bundled by, for example, mDia2, FMNL2, FMNL3, fascin, and DAAM1. In the indirect tip nucleation model, filopodia emerge from filaments nucleated by mDia1 at focal adhesions (FAs), with mDia2, FMNL2, and FMNL3 (and/or VASP) displacing the end-bound mDia1, and elongating the filaments into filopodia. Ena/VASP proteins also localize to the filopodial shaft and connect the filopodia to the surrounding adhesion sites. (C) Formin-2 (Fmn2) and Spire-1/-2 interact directly, forming a heterodimeric complex, to polymerize unbranched actin networks in oocytes required for radial transport of vesicles. In this process, Spire promotes actin nucleation (i). Filament elongation proceeds via Fmn2 and Spire -1/-2 cooperation ('ping-pong' mechanism) or by Fmn2 alone, preceded by Spire release (although this remains to be validated). For additional definitions, please see the main text.

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Spire

Spire was first described as a *Drosophila* polarity factor; it has since been shown to be essential to oogenesis in mammals [41] and flies [42]. The nucleation activity of spire is less than that of the Arp2/3 complex. Spire nucleates actin at low Spire:actin ratios and sequesters actin at high Spire:actin ratios [43]. Although Spire triggers actin polymerization, both its mechanism of nucleation and its localization on the filament end are a matter of debate. An electron microscopy study indicated that *Drosophila* Spire assembles an actin nucleus by coordinating the association of actin monomers along one strand of the two-start filament helix using its four WH2 domains [44], in accord with an X-ray scattering study [45]. In particular, the third and fourth WH2 domains, and their linker (linker-3), make a unique contribution to Spire nucleation activity [44,46]. Moreover, *Drosophila* Spire was suggested to cap filament pointed ends, inhibiting filament disassembly [44]. By contrast, human Spire binds four actin monomers cooperatively to form a tight complex [43] and was proposed to have activity at filament barbed ends based on Spire-mediated inhibition of profilin–actin assembly [43] and Spire–formin cooperation in filament assembly [47,48]. Further structural and multicolor single-molecule studies are needed to precisely define the configuration of the F-actin nucleus, the Spire location at the filament end, and which biochemical activity dominates under different physiological conditions.

Cobl

Cobl is predominantly expressed in the brain and is involved in neuromorphogenesis through its actin nucleation activity [49]. It localizes to axonal and dendritic growth cones and to F-actin ruffles in fibroblasts [49]. Cobl also localizes to the basal region of microvilli in epithelial cells, where it participates in their length regulation and in BAR protein recruitment [50]. Cobl promotes unbranched filament nucleation at low Cobl:actin ratios and actin sequestration at high Cobl:actin ratios. Cobl was proposed to nucleate actin filaments using all three WH2 domains to form a short-pitch actin trimer [49]. The length of linker-2, rather than its sequence, was proposed to be critical for the formation of the F-actin nucleus [49]. A more recent report proposed that a short lysine-rich sequence (K region), together with the three WH2 domains, is required for nucleation [51].

VopL/VopF

VopL and VopF are bacterial virulence factors that disrupt actin homeostasis during infection of the host. VopL induces the formation of, and localizes along, stress fibers when expressed in infected and transfected HeLa cells [52]. It also induces filopodia formation at the periphery of infected cells and localizes at the barbed ends of the filopodia tip [53,54]. VopF and VopL each form homodimers. VopL and VopF monomers comprise three WH2 domains, linked by a C-terminal dimerization domain (VCD), which are all essential for VopF and VopL nucleation activity [40,55]. Dimerization of the three WH2 domains stabilizes lateral contacts between the actin monomers in the nucleus [55], strongly enhancing VopF [54] and VopL [40,55] nucleation activity. VopL localizes at the filament pointed end and dissociates shortly after nucleation [40]. VopL nucleation activity is inhibited by profilin [40]. By contrast, VopF promotes actin assembly by binding to the filament barbed ends [54]. Moreover, the dimerization of the WH2 domains in VopF enables the uncapping of CPs from the barbed ends [54].

Lmod

Lmod (Lmod-2, cardiac leiomodin; C-Lmod, Lmod-1,smooth-muscle leiomodin; and SM-Lmod, Lmod-3, fetal leiomodin) is a tropomodulin (Tmod)-related protein expressed almost exclusively in skeletal and cardiac muscle cells [56]. Lmod uses a WH2 domain and an actin-binding site (ABS2) to recruit two or more actin monomers for nucleation, thereby stabilizing a trimeric actin nucleus, and all three are necessary for Lmod nucleation activity [56,57]. Lmod is a powerful nucleator, whose over- or underexpression has dramatic effects on sarcomeric

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structure and organization in rat cardiomyocytes [56] and actomyosin contractility in mature sarcomeres [58]. Lmod directly interacts with tropomyosin (TM), which modulates its nucleation activity [56]. In sarcomeres, Lmod localizes at the filament pointed ends [58,59], and TM was suggested to have an important role in this process [56,60]. The related protein tropomodulin-1 (Tmod1) also localizes to the filament pointed end, where both Tmod1 and Lmod fine-tune filament polymerization dynamics [59]. However, unlike Lmod, Tmod1 has no actin nucleation activity.

JMY

The multifunctional actin nucleator JMY contains three WH2 domains and an Arp2/3 complex-binding domain (CA domain). The third WH2 domain and a G-actin binding linker allow JMY to nucleate actin even in the absence of the Arp2/3 complex to form unbranched actin filaments [61]. Thus, JMY has dual nucleation capabilities: (i) direct nucleation of unbranched actin filaments; and (ii) acting as an NPF to the Arp2/3 complex to polymerize branched actin networks. JMY functions in cell motility, where the depletion or downregulation of JMY slows the migration rate, while its overexpression enhances the migration velocity [61,62]. JMY localizes at the cell leading edge, where it was proposed to promote the formation of branched networks using its dual actin nucleation activity, first to nucleate unbranched actin filaments ('seeds'), then to facilitate the Arp2/3 complex in using those seeds as substrates for dendritic nucleation [61] (Figure 3Ai). Future research is aiming to validate this model. JMY also affects oocyte asymmetric division via actin network regulation [62–64]. JMY localizes to tubular vesicles in the cytoplasm, where it drives actin-dependent anterograde trafficking away from the *trans*-Golgi face [65].

JMY shuttles between the cytoplasm and nucleoplasm, a process mediated by the WH2 domains in JMY, which contain a nuclear localization sequence (NLS) that binds to importins. G-actin regulates the nuclear import of JMY by blocking the interaction with the import machinery [66]. Conversely, JMY accumulates in the nucleus in response to DNA damage (Figure 2A) [62,66,67], because this induces cytoplasmic actin polymerization, consequently decreasing the cytoplasmic concentration of G-actin. In the nucleus, JMY was suggested to promote the assembly of unbranched filaments [61] (Figure 2Ai). Future research will have to validate this model. Moreover, it will be important to test whether JMY, alone or in cooperation with other factors, is involved in the nucleation of dendritic networks in the nucleus (Figure 2Aii).

APC

APC is a tumor-suppressor protein with a role in regulating microtubule and F-actin organization and dynamics [68]. APC forms a homodimer that recruits four actin monomers, thereby promoting unbranched F-actin nucleation [69]. APC shuttles to the nucleus (Figure 2A), where it affects the nuclear accumulation of β-catenin [70]. It remains to be seen whether DNA damage, cell density, or other factors influence APC subcellular localization.

WISH/DIP/SPIN90 Family Proteins

A distinct class of NPFs includes the WISH/DIP/SPIN90 family proteins, which directly activate the Arp2/3 complex in a non-WASP-like mechanism (i.e., without binding of preformed F-actin) [3] (Figure 1B). Specifically, the Dip1 (*Saccharomyces pombe*)-activated Arp2/3 complex creates linear instead of branched filament networks and, in contrast to WASP family proteins, oligomerization does not increase activity [71]. Dip1 was proposed to control the timing of actin patch assembly and endocytosis in *S. pombe* by providing F-actin seeds for Wsp1-activated branching nucleation [3,72]. The biochemical properties of Dip1 are conserved in SPIN90 [3], the mammalian ortholog, suggesting that WISH/DIP/SPIN90 proteins have a general role in providing seeds to initiate branching nucleation in cells [73] (Figure 3A).

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Cofilin

The cofilin/actin-depolymerizing factor (ADF) family proteins have a role in cell motility, cytokinesis, morphogenesis, and neuronal plasticity [74]. Cofilin stimulates actin network assembly in lamellipodia, invadopodia, and filopodia in motile cells and neuronal systems [75,76]. It is also required for actin network disassembly in cells [77], including the disassembly of *Listeria* actin comet tails [78]. Cofilin also functions as a co-factor in nucleocytoplasmic transport of actin (Figure 2A) and accumulates in the nucleus upon DNA damage [79] and cell stress, where it is involved in nuclear actin rod assembly [80] (Figure 2Aiii).

In mammalian cells, cofilin is present at concentrations of 5–20 μM [76,81]. Direct filament imaging revealed that micromolar cofilin concentrations promote actin nucleation [9,10], even under suppressed spontaneous nucleation conditions (reflecting the conditions in cells) [10], but not filament severing or disassembly [9,10]. Micromolar concentrations of cofilin are also required for comet tail assembly and motility of both pathogens [82] and beads coated with NPFs [83,84], Ena/VASP [10], or both [85]. The timing of tail assembly was shown to correlate with cofilin-dependent F-actin seed nucleation in the solution [10]. Although cofilin stimulates network assembly, it is insufficient to promote network disassembly [10,81–85]. For that, cofilin must cooperate with coronin and Aip1 [81]. Together, they promote actin filament severing and disassembly from pointed ends with high efficiency, even under assembly-promoting conditions [86]. Altogether, cofilin appears to have a dual role in cells: (i) supplying F-actin seeds to initiate branched and unbranched network assembly at membrane and/or pathogen surfaces; and (ii) promoting network disassembly from filament pointed ends together with coronin and Aip1 (Figure 3A).

In the above sections, we described the biochemical mechanisms of *de novo* nucleation factors. In the next sections, we describe dendritic nucleation factors and elongation factors that need preformed F-actin to initiate actin assembly.

Nucleation and Elongation Factors That Require Preformed F-Actin to Initiate Network Assembly

This section discusses Arp2/3 complex nucleation with Class I and II NPFs and the Ena/VASP elongation factor. Arp2/3 complex and Ena/VASP differ in their function, biochemical properties, and the types of network they form (branched versus unbranched, respectively); yet, both factors require preformed F-actin to initiate network assembly.

Arp2/3 Complex

The Arp2/3 complex participates in the formation of branched actin networks in cells [33,87] (Figures 2B, 3A), such as lamellipodia in migrating cells, endocytic vesicles, and invadopodia. The Arp2/3 complex comprises multiple isoforms of seven subunits, including the actin-related proteins Arp2 and Arp3, and five additional subunits (ArpC1–5), which impact the nucleation, organization, and stability of the network that the complex nucleates [88]. Several studies suggested that Arp2 and Arp3 interact with the pointed end of the daughter filament, while the remaining subunits, in particular ArpC2 and ArpC4, make substantial contacts with the mother filament. At equilibrium, the Arp2/3 complex is in an inactive conformational state [89,90], with Arp2 and Arp3 spatially separated. To influence this equilibrium, NPFs, a mother actin filament, and actin monomers must work in cooperation. Hydrolysis of ATP and binding to NPFs induce a major conformational change, stabilizing the active conformation and initiating a new filament branch [5].

Class I NPFs

Class I NPFs are very efficient and include the pathogen proteins ActA from *Listeria monocytogenes*, RickA from *Rickettsia*, and BimA from *Burkholderia thailandensis* [91,92]. Mammalian class I NPFs include Wiskott-Aldrich syndrome protein (WASP), neural WASP (N-WASP), three

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WASP and verprolin homologs (WAVE/Scars), WHAMM, WASH, and JMY. Other class I NPFs include fungal type I myosins, and metazoan capping protein ARP2/3 and myosin-I linker (CARMIL) [5]. They have a conserved C-terminal verprolin-homology (WH2), cofilin-homology, and acidic regions (VCA) domain that interacts with G-actin and the Arp2/3 complex [93]. Arp2/3 complex activation involves the simultaneous binding of two WASP proteins that promotes binding of the Arp2/3 complex to the side of a mother filament to form a new filament branch [94]. WASP dimerization greatly enhances branch nucleation and was proposed to be a broadly used mechanism of activation [71]. Several regulatory and scaffolding proteins that dimerize WASP have been identified [95]. Nucleation of actin by N-WASP and the Arp2/3 complex also occurs inside the nucleus and is important for efficient transcription by RNA Polymerase II [96,97]. Recent evidence also suggests that the Arp2/3 complex drives the growth of a branched actin 'shell' on the inner surface of the nuclear envelope during chromosome segregation, leading to its fragmentation [12] (Figure 2Aiv).

Class II NPFs
Class II NPFs include *S. cerevisiae* actin-binding protein-1 (Abp1), Pan1, and cortactin [5]. Cortactin binds to and activates the Arp2/3 complex through its N-terminal acidic (NTA) domain [98,99], although, compared with Class I NPFs, it induces weaker activation of the Arp2/3 complex [100] because it cannot recruit G-actin to the Complex [101]. Cortactin interacts with F-actin through a set of central repeats, which help target the protein to Arp2/3 complexes at branch points [98,99,102,103]. Cortactin also colocalizes with class I NPFs at lamellipodia protrusions and to the propelling tails of various pathogens and endosomes (Figure 3A) [104]. Yet, in contrast to class I NPFs, cortactin is not essential for Arp2/3 complex-dependent branched network assembly. However, along with other factors, such as coronin, it enables cells to tune the formation and turnover of its branched network structures [88,105].

Ena/VASP Elongation Factor
Ena/VASP proteins are involved in the motility of eukaryotic and prokaryotic cells [106], axon growth and guidance [107], stress fiber formation [108], and filopodia assembly [109–111]. Unlike formins, Ena/VASP proteins do not nucleate actin under physiological salt conditions [10,112]. Ena/VASP proteins are further distinguished from formins by their (Ena) ability to bundle and elongate two filaments at once [113]. Ena/VASP contains several domains: an N-terminal EVH1 domain, a Pro-rich domain, and a C-terminal EVH2 domain. The VASP EHV1 domain mediates the direct interaction between VASP and different proteins, including formins [114] and class I NPFs, including ActA [115], WASP [116], and WAVE [85,117]. VASP cooperates with these factors to enhance Arp2/3 complex-mediated actin assembly and motility [85,115–117] (Figure 3). The Pro-rich domain binds profilin and profilin–actin complexes through a specific profilin-binding sequence. The EVH2 domain via a GAB-binding site (WH2 domain) binds G-actin or actin–profilin complexes [118,119]. Filament elongation depends on the ability of VASP to recruit and incorporate actin subunits onto filament barbed ends [10,120,121] while competing with CPs [that is, its anticapping activity (AC)] [10,112,121–125]. Under high profilin concentrations, reflecting the conditions in cells, VASP functions as a good anticapper at the expense of reduced elongation rates [10]. The GAB domain is followed by an F-actin (FAB)-binding site. Both domains are important for VASP-mediated filopodia reconstitution [10] and polymerase activity [10,121]. The EVH2 domain terminates with a coiled-coil region that mediates Ena/VASP tetramerization [118,126] and is important for F-actin binding, F-actin bundling, and Ena/VASP polymerase activity [118,121,124]. However, there is still no clear picture of how VASP might bind to the end of a filament and, thus, how it manages to promote its processive elongation.

Proteins that mimic Ena/VASP function also exist in pathogens. The bacterial Ena/VASP BimA from *Burkholderia mallei* and *Burkholderia pseudomallei* resembles eukaryotic Ena/VASP in that it mediates processive barbed-end elongation and has AC activity, but in contrast to Ena/

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VASP, it can also nucleate actin [92]. BimA oligomerization is required for its actin nucleation, polymerase, and AC activity.

The sections above focused on the biochemical mechanisms of individual nucleation and elongation factors. In the following section, we describe how the different factors cooperate, directly or indirectly, to promote the assembly of functional actin network in cells. We describe specific examples through which we demonstrate how, by choosing sets of players, cells can control the formation, architecture, mechanical properties, and turnover of their actin networks to accomplish a given cellular task.

Functional Actin Networks in Cells: A Cooperative Work of Multiple Actin-Assembling Factors

Branched Networks: Lamellipodia Protrusion, Endocytosis, and Comet Tail of Propelling Endosomes and Pathogens

The driving force for lamellipodia protrusion is the polymerization of a branched actin network at the plasma membrane. Arp2/3-complex-dependent dendritic nucleation also drives the propulsion of endosomes and pathogens, and underlies endocytosis (Figures 2B, 3A). In all of these cases, the branched network is continuously generated at the membrane or pathogen surface by class I NPFs, with the barbed ends of the filaments oriented towards the surface. The growth of the barbed ends pushes the membrane or pathogen forward, while the filaments at the rear (pointed ends) are severed and depolymerized to refill the pool of G-actin available for polymerization.

In lamellipodia, the branched nucleation process is initiated by activation of the Arp2/3 complex in the presence of F-actin by the Rac-activated WAVE molecule and possibly also by JMY [61] [in other cases by WASP (endocytosis), WASH (endosome), or ActA (*L. monocytogenes*)] (Figure 3Ai). Initiation of lamellipodia assembly is stimulated by the binding of preformed F-actin (Figure 3Ai), which is provided by factors such as formin-like 2 (FMNL2) [127,128] and mDia2 [14], that colocalize with WAVE (Figure 3Ab) and possibly JMY (Figure 3Aa) at the cell leading edge. Ena/VASP also localizes at the cell leading edge and negatively regulates mDia2 nucleation activity, enabling cells to control the density of barbed ends generated at the membrane surface [114] (Figure 3Ac). F-actin seeds nucleated by cofilin, and possibly also by JMY and SPIN90, and recruited to the cell membrane surface (Figure 3Ac,d) (or to the surface of endosomes and pathogens) provide another possible source of F-actin [10,61,73,76]. Lamellipodia assembly is enhanced by Ena/VASP, which directly interacts with WAVE at the cell leading edge [85,117] (or with WASP [116] and ActA [115]), assisting WAVE–Arp2/3 complex association with filaments, which is required for Arp2/3 complex activation [129]. In addition, Ena/VASP and formins (mDia2, FMNL2) function as elongation factors by providing additional sites for Arp2/3 branching events, which also enhances lamellipodia assembly. Lamellipodia can still form without Ena/VASP [123], but mDia2 is required [14].

The branched nucleation process is followed by WAVE release, which triggers branch formation [130]. Cortactin acts synergistically with WAVE in the lamellipodia (or with WASP, N-WASP, WASH, or ActA in other cases) dissociating it from Arp2/3 [100–103] (Figure 3Aii); cortactin also stabilizes the actin branch by protecting the Arp2/3 complex from coronin 1B-mediated debranching [88,105]. Mechanical tension that develops between the growing filaments and the membrane-bound WAVE proteins can also stimulate WAVE release [131]. The release of WAVE from the newly formed branches has two major effects: (i) it increases the rate of branching per WAVE molecule; and (ii) it decreases the lifetime of WAVE-mediated connections between the polymerizing network and the membrane [102]. Overall, this mechanism enables WAVE to stimulate dendritic network assembly without restraining their growth [102], which is essential for rapid and persistent cell motility. NPF release is also essential for pathogen and

endosome propulsion: binding that is too strong not only reduces the propulsion velocity, but also gives rise to a stick-slip (hopping) motion [83,131,132]. To continuously refill the pool of G-actin available for polymerization, the rear of lamellipodia (and similarly, the ends of pathogens and/or endosome tails) undergoes debranching (Figure 3Aiii) followed by filament severing and depolymerization by the cooperative work of cofilin, coronin, and Aip1 [86] and/or by myosin II motors [133,134] (Figure 3Aiv). The actin monomers diffuse back to the front and assemble onto uncapped filaments of the protruding network.

Unbranched Polar Actin Bundles: Filopodia Protrusion

Filopodia are exploratory finger-like protrusions that dynamically extend and retract from the cell edge [135] and are used for sensing the cell environment and to guide cell migration [136]. Each filopodium contains polar-bundled unbranched filaments, which are elongated at filo-podial tips with their barbed ends pointing towards the membrane by formins and/or Ena/VASP proteins [14–16,110,114,127,137] (Figures 2C, 3B). Bundling of the filopodial filaments is critical to generate stiff filopodia that can produce protrusive forces against the membrane tension without buckling [138]. Fascin has a critical role in this process: it recruits DAAM1 to the filopodial shaft and collaborates with it to control filopodia structure and integrity [139]. mDia2, FMNL2, and FMNL3, which also have the ability to bundle filaments, also localize to filopodial shafts [16,139]. Filopodia can form via different mechanisms, either by nucleating filaments at the plasma membrane, by recruiting filaments to the plasma membrane, or both.

The Convergent Elongation Model

In the convergent elongation model, the barbed ends of the filaments from the lamellipodia network are captured by Ena/VASP and/or formins (e.g., mDia2, FMNL2, or FMNL3), elongate continuously, and gradually converge into bundles [16,109,128]. Filopodia form if the energy gained due to actin-bundling protein links between neighboring filaments exceeds the bending energy associated with bringing them into contact. This process is further facilitated because the filaments are longer and less branched [140–143]. Clustering of Ena/VASP (and/or formins) precedes filopodia formation. In the case of Ena/VASP, the proteins are recruited and clustered by IRSp53 BAR proteins that self-assemble at the membrane surface via a feedback mechanism mediated by membrane curvature [144,145]. Clustering increases Ena/VASP processive elongation efficacy [124], which is further enhanced with the addition of bundling proteins that maintain the cohesiveness of the bundle. Bundling proteins also provide an additional energy gain for elongating the filaments at the bundle tip, thereby enhancing filament elongation within a bundle [146]. When this bundle is large beyond some critical thickness, its buckling threshold overcomes the restoring force of the membrane, allowing the filopodia to grow persistently [147].

The Tip Nucleation Model

In the tip nucleation model [127,148], membrane-bound formins (e.g, mDia2 and FMNL2) are activated at the membrane, where they are clustered together and promote *de novo* nucleation of actin filaments, which subsequently elongate (with or without Ena/VASP; Figure 3B shows the case when Ena/VASP is not taking part in filopodia assembly) and become bundled together [127]. Filopodia formed by this mechanism localize to the cell edge and have no attachment with the surrounding lamellipodia. Formation of filopodia may also occur through Ena/VASP and other elongating factors if F-actin seeds are provided from the bulk. This mechanism drives filopodia-like formation *in vitro* [10], although its relevance *in vivo* remains to be demonstrated.

The Indirect Tip Nucleation Model

In the indirect tip nucleation model, filopodia emerge from filaments nucleated by mDia1 at focal adhesions (FAs), with formins (mDia2, FMNL2, and FMNL3) and/or Ena/VASP displacing the end-bound mDia1, and elongating the filaments [16]. In contrast to the first two mechanisms, filopodia emerging from FAs form only in adherent cells [16].

Generally, the different mechanisms described above are not mutually exclusive and can coexist in cells. Moreover, while filopodia can form by formins or Ena/VASP alone, the cooperation between the two proteins enables cells to control the formation, architecture, dynamics, and persistence of their filopodia [114,137]. Ena/VASP and formins form filopodia with distinct morphologies, dynamics, and protrusion efficacy. Ena/VASP generates short and thick protrusive filopodia, whereas mDia2 filopodia are long and thin and lack adhesion and protrusion persistence [137]. Cooperation between Ena/VASP and mDia2 rescues these adhesions defects, generating filopodia with morphology, dynamics, and adhesion properties resembling those of Ena/VASP filopodia [137]. Filopodia formed in the presence of Ena/VASP are more robust, persistent and better integrated into the rest of the cytoskeleton compared with those formed in the presence of formins, as a result of key differences in the structures and processive elongation mechanism between Ena/VASP and formins. Formins promote fast elongation of the growing filaments, while Ena/VASP interacts with the side of F-actin within filopodia and restrains its growth. In addition, Ena/VASP molecules can couple the filopodia to focal adhesion complexes [149] or to the lamellipodial network and, indeed, VASP is observed along the shaft and base of filopodia [16,137,149] (Figure 3B). Thus, by negatively regulating mDia2 nucleation but not elongation activity [114], Ena/VASP enables cells to regulate filopodia location along the cell edge, with dynamic and mechanical properties optimized for cell spreading and motility [114,137,149].

Unbranched Actin Networks

Spire/Formin Collaboration
Formins [Cappuccino, Formin-2 (Fmn2)] and Spire (dSpire, Spire-1/Spire-2) collaborate to form functional actin networks in mouse and *Drosophila* oocytes that are required for oocyte polarity [48,150,151] and for radial transport of vesicles [150] (Figure 3C). In the nucleus, Fmn2 and Spire-1/Spire-2 collaborate to promote the assembly of actin networks in response to DNA damage [17,152] (Figure 2Av). Fmn2 and Spire-1/-2 interact directly via the kinase-like non-catalytic domain (KIND) of Spire and the C-terminal sequence near the FH2 domain of formin [36,39,47,153,154] to form a heterodimeric complex at a stoichiometry of 2:2 (KIND:FH2) [36,39,47,153,155]. The formation of this complex, which promotes Spire dimerization, enhances Spire nucleation activity and inhibits formin nucleation activity [36,39]. After nucle-ation, Spire has been suggested to detach while formin retains association with the filament barbed end, promoting its processive elongation, suggesting that formin acts as an elonga-tion factor rather than as a nucleator [35]. By contrast, more recent reports suggest that both formin and Spire regulate filament elongation by binding alternatively at the filament barbed end, resulting in rapid processive assembly and arrested growth, respectively (the so-called 'ping-pong' mechanism) [47]. Future research will have to validate these models. In particular, single-molecule studies will enable the determination of the sequence of binding and unbind-ing events of each protein and their location at the filament ends during filament nucleation and elongation.

APC/mDia1 Collaboration
APC collaborates with the formin protein mDia1 in driving actin assembly [69,156]. This process involves dimerization of the APC 'basic' domain (APC-B), recruitment of multiple actin mono-mers, and binding of mDia1 to form a tripartite nucleation complex. APC has a central role in assembling the nucleation seed. Using single-molecule fluorescence microscopy, it was shown that APC and mDia1 separate following nucleation [157]. After separation, the APC dimer remains associated with the filament pointed end, whereas mDia1 promotes processive elongation of the filament barbed end (the 'Rocket-launcher mechanism') [157]. APC-Dia collaborations have a physiological role in *Drosophila* development [158]; the physiological role of the vertebrate APC-Dia collaboration has not yet been established. It remains to be seen whether APC-Dia also collaborates in the nucleus to promote nuclear actin assembly.

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Concluding Remarks

This review describes the major types of actin assembly-promoting factor and their mode of cooperation in promoting the assembly of functional actin networks. We have shown that direct and indirect collaboration between multiple factors enables cells to optimize the architecture and dynamics of their actin networks to accomplish a certain cellular function (Figure 3, Table 1).

Table 1. Direct and Indirect Cooperation between Specific Sets of Nucleation and Elongation Factors Involved in Given Cellular Processes

Nucleation and elongation factors involved	Functional roles	Refs
Cooperativity mediated via direct interaction		
Arp2/3 complex/class I NPFs (ActA, WASP, or WAVE)/Cortactin	Cortactin stimulates the release of class I NPFs from the Arp2/3 complex at a new branch site, enabling membrane-bound class I NPFs proteins to stimulate network formation without restraining its growth, which is essential for rapid actin-based motility	[100–103]
VASP/mDia2	VASP directly interacts with mDia2, negatively regulating mdia2 nucleation activity but not elongation activity. Cooperation between VASP and mDia2 enables cells to regulate lamellipodia and filopodia initiation, architecture, dynamics, and persistence	[114,137]
Arp2/3 complex/class I NPFs (ActA, WASP, or WAVE)/VASP	Direct interaction of VASP with class I NPFs enhances actin assembly and motility *in vivo* and *in vitro*	[85,115–117]
Spire-1,-2, spire/(Capu, Fmn2)	Spire directly interacts with formins to promote actin nucleation and elongation, required for oocyte polarity and radial transport of vesicles. Spire and formin also collaborate in the nucleus, where they promote the assembly of nuclear actin networks in response to DNA damage	[17,36,39,47, 48,150,153]
APC/mDia1	mDia1 and APC directly interact to drive actin assembly. APC–Dia collaborations have a physiological role in *Drosophila* development	[69,156–158]
Formin/CPs	Formin and CPs directly interact to form a complex at the filament barbed end used to control filament elongation rate and length	[31,32]
Cooperativity mediated by indirect interaction		
Formins (mDia2, FMNL2) + Arp2/3 complex/WAVE	mDia2 and FMNL2 supply fresh F-actin that serves as a substrate for dendritic nucleation by the Arp2/3 complex for lamellipodia formation	[14,127,128]
Arp2/3 complex/WAVE + formins (mDia2, FMNL2, FMNL3) and/or VASP	Filopodia formation via the convergent elongation model: filopodia emerge from the lamellipodial network with factors such as mDia2, FMNL2, FMNL3, and/or VASP, which elongate and bundle the filaments into filopodia	[16,109,128]
mDia1 + formins (mDia2, FMNL2, FMNL3) and/or VASP	Filopodia formation via the 'indirect tip nucleation model': filopodia emerge from filaments nucleated by mDia1 at focal adhesions with mDia2 (FMNL2 or FMNL3) and/or VASP displacing the end-bound mDia1, and elongating and bundling the filaments	[16]
Arp2/3 complex/WISH, DIP, or SPIN90 + Arp2/3 complex/WASP	WISH/DIP/SPIN90 proteins activate the Arp2/3 complex to create unbranched F-actin in a non-WASP-like mechanism for WASP-activated branch nucleation	[3,72,73]
Cofilin + Arp2/3 complex/ class I NPFs	Fresh F-actin seeds produced in the cytoplasm are recruited by surface-bound NPFs/Arp/3 complexes to drive dendritic nucleation of actin required for lamellipodia, comet tail assembly of endosomes and pathogens, endocytosis, as well as for *in vitro* reconstitution of actin-based motility	[76,82–84]
Cofilin + VASP	Fresh F-actin seeds produced in the solution are recruited by surface-bound VASP to reconstitute filopodia bundles *in vitro*	[10]
JMY (nucleator of unbranched F-actin seeds) + Arp2/3 complex/JMY	JMY localizing at lamellipodia protrusions was suggested to promote dendritic nucleation using its dual actin nucleation activity, to nucleate unbranched F-actin seeds that serve as substrates for dendritic nucleation by the Arp2/3 complex. This model remains to be validated	[61]

Outstanding Questions

How do cells control the differential distribution of nucleation and elongation factors between compartments (cytoplasm and nucleoplasm) and within each compartment?

How do cells choose the set of proteins that optimizes the dynamics and structural organization of their cytoskeletal networks?

How does the translocation of actin nucleators into the nucleus in response to cellular stress and DNA damage affect the reorganization of actin networks within the cytoplasm and vice versa?

Why do the proteins that initiate branched F-actin networks rely on existing F-actin seeds rather than directly *de novo* nucleating branches? What is the evolutionary advantage of this design?

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In many cases, cooperation is also required to initiate network assembly. While *de novo* nucleation factors can initiate network assembly with or without the aid of other factors, dendritic nucleation factors and elongation factors require preformed F-actin. As such, they must cooperate directly or indirectly with *de novo* nucleation factors, which supply them with preformed F-actin by: (i) nucleating F-actin seeds in the cytoplasm, which are then recruited by membrane-bound factors; and (ii) nucleating F-actin directly at the membrane surface where the different factors colocalize. The presence of F-actin seeds in the cytoplasm does not contradict the requirement to minimize spontaneous actin nucleation because seeds that are not recruited are rapidly capped and depolymerized. Furthermore, distribution of F-actin seeds in the cell, similar to G-actin, facilitates network formation throughout the cell. Finally, future studies should investigate how cells regulate the proportions and localization of their various actin assembly-promoting factors and how cells choose different sets of players to optimize the dynamics and structural organization of their cytoskeletal networks (see Outstanding Questions).

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