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1 ***Nucleation, Stabilization and Disassembly of Branched Actin Networks***

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9

10 *Arp2/3 complex is an actin filament nucleation and branching machinery conserved in all*
11 *eukaryotes from yeast to human. Arp2/3 complex branched networks generate pushing forces*
12 *that drive cellular processes ranging from membrane remodeling to cell and organelle motility.*
13 *Several molecules regulate these processes by directly inhibiting or activating Arp2/3 complex*
14 *and by stabilizing or disassembling branched networks. Here, we review recent advances in our*
15 *understanding of Arp2/3 complex regulation, including high-resolution cryo-electron*
16 *microscopy structures that illuminate the mechanisms of Arp2/3 complex activation and branch*
17 *formation, and novel cellular pathways of branch formation, stabilization, and debranching. We*
18 *also identify major gaps in our understanding of Arp2/3 complex inhibition and branch*
19 *stabilization and disassembly.*

20

21 **Keywords:** Arp2/3 complex, nucleation-promoting factors, inhibitors, branched network

22 stabilization and disassembly, mechanosensation

23 **Arp2/3 complex and nucleation-promoting factors (NPFs)**

24 The actin cytoskeleton is a dynamic system of hundreds of proteins that helps maintain cell
25 shape and polarity and provides forces and directionality for a myriad of motile functions in cells.
26 At the core of this system is actin, the most abundant protein in the cytosol of eukaryotic cells.
27 Actin is in constant flux between monomeric and filamentous forms to produce networks with
28 distinct architectures. One such architecture is that consisting of branched actin networks (also
29 called dendritic networks), resulting from the filament nucleation and branching activities of
30 Arp2/3 complex. Activated by proteins known as nucleation-promoting factors (NPFs), Arp2/3
31 complex binds to the side of a pre-existing (mother) filament and nucleates the formation of a
32 new (branch) filament that grows at a $\sim 70^\circ$ angle relative to the mother filament.

33 The 7-subunit Arp2/3 complex comprises two actin-related proteins, Arp2 and Arp3, and
34 five scaffolding subunits, ArpC1 to ArpC5. The Arps (Arp2 and Arp3) act as a pseudo-actin
35 dimer during nucleation, whereas the scaffolding subunits hold the Arps together and mediate
36 key interactions with the mother filament in the branch [1]. In the basal state, Arp2/3 complex is
37 inactive, with the Arps splayed apart [2]. During activation, the NPFs perform three main
38 functions: *a*) they trigger a conformational change that repositions the Arps into a filament-like
39 (short-pitch) conformation [3], *b*) they recruit actin subunits to the barbed end of the Arps to
40 form the initial polymerization nucleus [4-6], and *c*) they promote binding of the complex to the
41 side of the mother filament [7]. Wiskott-Aldrich Syndrome Protein (WASP)-family NPFs, also
42 called class-I NPFs, are generally unrelated, but all share C-terminal proline (Pro)-rich and
43 WH2/Central/Acidic (WCA) domains (Fig. 1a). Recent work shows that the Pro-rich regions
44 play an essential role by mediating the recruitment of profilin-actin to feed the growth of newly
45 nucleated filaments via NPFs clustered on membranes (reviewed in [8]). The WCA region is

46 necessary and sufficient to activate Arp2/3 complex *in vitro* and comprises one to three WASP-
47 homology 2 (WH2 or W) domains that bind actin [4], and Central (C) and Acidic (A) domains
48 that bind to two distinct sites on Arp2/3 complex [9, 10].

49 The variable N-terminal domains of NPFs are typically involved in localization and
50 integration of upstream signaling, giving rise to a certain division of roles among NPFs, which
51 tend to fulfil specialized functions at specific subcellular locations (Fig. 1d). The NPF literature
52 is vast and not a focus of this review (reviewed in [11]). Only some of their better-documented or
53 recently-established functions are listed here. Thus, N-WASP is primarily implicated in
54 endocytosis, whereas WAVE (isoforms 1-3) forms part of a pentameric complex called the
55 WAVE regulatory complex (WRC) that is implicated in the formation of cell protrusions and cell
56 migration (reviewed in [12]). WASH, which forms part of a similar pentameric complex,
57 together with WHAMM and JMY are primarily implicated in remodeling of intracellular
58 membranes, including endosomes, ER, Golgi, and autophagosomes [13-15]. However, such a
59 division of roles among NPFs is an oversimplification. For example, N-WASP can substitute for
60 WAVE in the generation of membrane protrusions [16].

61

62 **Branched actin networks generate pushing forces at diverse subcellular locations**

63 Branched actin networks are present along the cell cortex and near most membranous
64 organelles in the cell (Fig. 1d). These networks exert forces during processes such as cell
65 motility, vesicular trafficking, and membrane scission. At the cortex, the pushing forces of
66 branched actin networks are counterbalanced by pulling forces from myosin motors on linear
67 actin filaments. This balance of forces is particularly evident at adherens junctions in epithelial
68 cells, where Arp2/3 complex-dependent protrusive forces push the membranes of neighboring

69 cells to maintain cadherin-mediated cell-cell adhesions [17]. If the Arp2/3 complex is inhibited,
70 myosin pulls the membranes of neighboring cells away from one another, resulting in the
71 formation of membrane bulges [18]. Imbalances in this “tug-of-war” between protrusive and
72 contractile forces results in defects in tissue mechanics and morphology, as observed during
73 epithelial folding in development [19] and the sprouting of blood vessels [20]. The forces exerted
74 by branched networks are also used to counteract membrane tension and separate transport
75 intermediates from the plasma membrane during clathrin-mediated endocytosis [21] and from
76 endosomes along the endocytic pathway [22]. Actin pushing forces with the help of BAR
77 domain proteins constrict the neck of clathrin-coated endocytic vesicles [23, 24] and elongate
78 endocytic invaginations [25, 26].

79 Lately, branched actin networks have been shown to push and squeeze the nucleus to
80 help cells migrate through constricted spaces [27] and to direct the movement of nuclei from the
81 center to the periphery of skeletal muscle myofibers during myogenesis [28]. Arp2/3 complex
82 also has functions inside the nucleus, where it was recently shown to facilitate DNA repair and
83 promote homology-directed recombination by moving and clustering double-strand DNA breaks
84 [29, 30]. In symmetrically dividing cells, a meshwork of actin filaments maintains the uniform
85 distribution of mitochondria around the mitotic spindle and shuffles mitochondria to ensure their
86 equal and random inheritance by daughter cells [31]. Mitochondria were propelled through the
87 cell by Arp2/3 complex-dependent actin comet tails, similar to those associated with the
88 movement of intracellular pathogens.

89

90 **Molecular mechanism of branched network formation and stabilization**

91 The role of NPFs in Arp2/3 complex activation was discovered in 1998 [32, 33] and for
92 the following 10 years, it was thought that activation resulted from NPF binding to a single site
93 on Arp2/3 complex. A breakthrough occurred in 2008, when it was discovered that activation
94 proceeded through binding of the Central-Acidic (CA) region of NPFs to two distinct sites on
95 Arp2/3 complex [9]. Several studies subsequently confirmed this finding, and models of CA-
96 bound Arp2/3 complex were proposed [5, 6, 34, 35]. While these models all captured the
97 generally accepted idea that one NPF binds to Arp2-ArpC1 whereas the other binds to Arp3, they
98 differed in important ways; they disagreed about the precise interactions of each NPFs, their
99 order of binding and role in activation, and the importance of actin binding to NPFs for
100 activation.

101 Recent cryo-electron microscopy (cryo-EM) structures of Arp2/3 complex substantially
102 advance our understanding of the mechanisms of nucleation and branch formation [1, 10, 36, 37].
103 The first of these structures was that of human Arp2/3 complex with the CA region of N-WASP
104 bound determined at 3.8-Å resolution [10]. This structure showed the precise binding paths of
105 NPFs on Arp2-ArpC1 and Arp3 (Fig. 2a). The ability to recombinantly express human Arp2/3
106 complex using a novel insect cell expression system further allowed for mutagenesis and
107 crosslinking studies that resulted in several new findings: *a)* actin binding to the W domains of
108 NPFs favors the transition of the Arps toward a filament-like short-pitch conformation, *b)* actin-
109 NPF binding to Arp2-ArpC1 precedes binding to Arp3 and is sufficient to shift the equilibrium
110 toward the short-pitch conformation, with no additional gain from binding to Arp3, *c)* while the
111 short-pitch transition is part of the activation pathway, it is insufficient to fully activate
112 mammalian Arp2/3 complex, which also requires NPF-mediated delivery of actin to the barbed
113 end of both Arps. As we discuss below, the latter finding is crucial to understand the difference

114 between actual NPFs that contain monomeric actin-binding sites and other molecules that
115 regulate Arp2/3 complex. The scaled-up expression of human Arp2/3 complex in Sf9 cells also
116 enabled the determination of the 4.2-Å resolution cryo-EM structures of the most and least active
117 isoform variants of Arp2/3 complex, containing respectively subunits ArpC1B and ArpC5L vs.
118 ArpC1A and ArpC5 [37]. This study, however, did not reveal substantial differences in structure
119 between variants, suggesting that their different activities may result from different energetic
120 barriers for activation. For instance, it has long been known that *Saccharomyces cerevisiae*
121 Arp2/3 complex has substantial background activity in the absence of NPFs, consistent with a
122 lower activation barrier [38].

123 An essential component of the Arp2/3 complex nucleation reaction is the mother filament
124 [33]. While bona fide WASP-family NPFs all contain actin monomer and profilin-actin binding
125 sites (Fig. 1a) that play essential roles in nucleation [10] and elongation [8], respectively, other
126 Arp2/3 complex regulators either bind to the mother filament or bypass the need for the mother
127 filament during nucleation. These include cortactin [39] and WISH/DIP/SPIN90 (WDS)-family
128 proteins [40]. These molecules are sometimes referred to as class-II NPFs, although their
129 function does not appear to be Arp2/3 complex activation. Indeed, on their own cortactin and
130 WDS-family proteins activate mammalian Arp2/3 complex only weakly [39, 40], requiring high,
131 micromolar concentrations, whereas nanomolar concentrations of WASP-family NPFs produce
132 very high polymerization rates [33]. It is also generally accepted that cortactin's major
133 biochemical activity is the stabilization of Arp2/3 complex branch junctions [39], and unlike
134 WASP-family NPFs that dissociate after nucleation [41], cortactin binds stably to these junctions
135 and may also accelerate the release of NPFs [42]. Moreover, contrary to WASP-family NPFs that
136 tend to have specialized roles and localization, cortactin is found rather ubiquitously in

137 association with branched networks (reviewed in [43]). These properties allow cortactin to fulfil
138 countless roles in cells by promoting the formation and stabilization of actin branched networks.

139 Although it has been proposed that WDS-family proteins (Fig. 1) act as bona fide NPFs,
140 capable of activating Arp2/3 complex while avoiding the need for the mother filament, actin
141 monomer binding and delivery to the Arps, and profilin-actin delivery to the newly formed
142 branch [40], their actual role and mechanism in branched network formation is only beginning to
143 emerge. Like cortactin [40], but unlike NPFs [41], WDS-family proteins remain bound to Arp2/3
144 complex at the pointed end of newly formed filaments, making them single-turnover regulators
145 [44]. Recent evidence offers a more nuanced role for these proteins, showing that yeast Dip1
146 works synergistically with Wsp1 (yeast WASP) to activate Arp2/3 complex during endocytosis,
147 without the need for preexisting filaments [45]. Of note, Wsp1 was required for both initiation
148 and propagation of endocytic actin networks, consistent with its role as the NPF in this process,
149 whereas Dip1 may have served as a substitute for mother filaments to kick-start endocytosis.
150 Another recent study in mammalian cells showed that SPIN90 (mammalian ortholog of Dip1)
151 synergizes with both Arp2/3 complex and the formin mDia1 to nucleate fast-growing filaments
152 leading to an increase in the density of linear actin arrays at the cell cortex [46]. A recent 3.9-Å
153 resolution cryo-EM structure of Dip1-bound yeast Arp2/3 complex at the pointed end of the
154 filament revealed the active conformation of Arp2/3 complex (Fig. 2b) [36]. The transition
155 toward the activated state was ascribed to the presence of Dip1 in the reconstruction, but this
156 conclusion is questionable. First, Arp2/3 complex has been long known to also act as a pointed
157 end-capping protein [47], and a previous EM reconstruction already showed Arp2/3 complex at
158 the pointed end in the absence of NPFs [48]. Although the lower resolution of this study
159 precluded a precise description of the structure of Arp2/3 complex at the pointed end, the authors

160 concluded that the Arps were realigned into a filament-like conformation, consistent with the
161 addition of actin subunits at the barbed end of the Arps. Second, the Dip1-bound structure was
162 determined in the presence of 1 μ M Wsp1 WCA, presumably used to shift the equilibrium
163 toward the active conformation, although this was not discussed. Third, Dip1, a globular protein
164 consisting of 374 amino acids, was mostly disordered in the structure (only 132 amino acids
165 were observed, G235-E366), suggesting that it is weakly bound to Arp2/3 complex and thus
166 unlikely to be the source of the conformational change. Fourth, a previous crystal structure
167 showed Arp2/3 complex in the inactive conformation when bound to SPIN90 [49], suggesting
168 that on their own WDS-family proteins are unable to trigger the activating transition. The cryo-
169 EM structure is nonetheless informative in that it reveals the structural changes that take place
170 during Arp2/3 complex binding to the pointed end. The Arps are realigned into a filament-like
171 conformation, as previously suggested [48], and their outer and inner domains (referring to the
172 position of these domains in the filament, Fig. 1a) are rotated with respect to one another to
173 produce a flatter conformation analogous to that of actin subunits in the filament.

174 A recent breakthrough was the determination of the structure of the Arp2/3 complex
175 branch junction in cells using cryo-electron tomography (cryo-ET) [1]. While the technical
176 difficulties of this study limited the resolution to 9-Å, the main-chain backbone of all the
177 subunits of Arp2/3 complex, eight subunits of the mother filament and three subunits of the
178 branch filament were unambiguously defined. The transition of the Arps toward the short pitch
179 conformation is brought about by a $\sim 19^\circ$ rotation around an axis that coincides with that of the
180 coiled coil formed by ArpC2 and ArpC4, similar to what was observed when Arp2/3 complex
181 was bound at the pointed end [36]. This rotation divides Arp2/3 complex into two subcomplexes,
182 one comprising Arp2 (Arp2-ArpC1-ArpC4-ArpC5) and one comprising Arp3 (Arp3-ArpC2-

183 ArpC3). The rotation creates binding sites for the first two subunits of the branch filament at the
184 barbed ends of the Arps. On the opposite side, five subunits of Arp2/3 complex (Arp3, ArpC1,
185 ArpC3, ArpC2, ArpC4) contact five subunits of the mother filament, without this resulting in any
186 substantial change in the structure of the mother filament. As previously suggested [36, 48], the
187 conformation of Arp2/3 complex is very similar in the branch and at the pointed end, with some
188 differences. Notably, ArpC3 is positioned differently in the branch, allowing it to make contacts
189 with Arp3, Arp2 and the mother filament that help stabilize the short-pitch conformation and the
190 branch junction. Although the contact surface of Dip1 on Arp2/3 complex overlaps only with
191 one of the five subunits of the mother filament that interact with Arp2/3 complex (compare Figs.
192 2 b and c), it is now easy to see how Dip1 can compete with and possibly substitute for the
193 mother filament in certain cellular contexts.

194 Another twist on the source of mother filaments at cellular locations where these may be
195 lacking is a recent study that established dynactin as a seed for mother filament formation and
196 Arp2/3 complex activation on endosomes [50]. The dynactin complex, better known for its role
197 as a general scaffold of the dynein motor, is built around an actin-like minifilament comprising
198 eight actin-related protein-1 (Arp1) subunits and one β -actin subunit, and capped at the barbed
199 end by the capping protein (CP) $\alpha\beta$ heterodimer and at the pointed end by four subunits (Arp11,
200 p62, p25 and p27) [51]. The pentameric WASH complex, the NPF present on endosomes, has
201 evolved the necessary attributes to convert dynactin into a seed for the mother filament while
202 simultaneously activating Arp2/3 complex. One of the subunits of the WASH complex, FAM21,
203 contains a capping protein interaction (CPI) motif that binds CP $\alpha\beta$ and uncaps dynactin. Once
204 uncapped the dynactin minifilament elongates from the barbed end through the addition of actin
205 subunits to form the mother filament. Then, another subunit of the WASH complex (WASH)

206 recruits and activates Arp2/3 complex on the side of the dynactin-formed filament. This study
207 adds meaning to intriguing observations, such as the role of the actin minifilament in dynactin,
208 the role of some of the subunits of the WASH complex and the role of the CPI motif in FAM21.

209

210 **Mechanosensation, reinforcement and disassembly of branched actin networks**

211 In recent years, it has become increasingly clear that the actin cytoskeleton not only
212 generates forces but also senses extracellular forces [52-54], which in turn triggers actin network
213 remodeling and several cellular and tissue processes [55-57]. Branched actin networks placed
214 under mechanical load *in vitro* are strengthened through a force feedback mechanism that
215 increases their density, pushing power, and mechanical efficiency [52]. For actin filaments
216 buckling under load, Arp2/3 complex branching occurs preferentially on the convex than the
217 concave side of the bend, which may be one way in which the density of branched networks is
218 biased by force [58]. Increased debranching has also been observed under load [59], indicating
219 that both branching and debranching can be affected by force. Similar observations have been
220 made in cells, where dendritic actin networks adapt to mechanical load by transitioning toward a
221 configuration dominated by filaments growing perpendicularly to the plasma membrane [55]. In
222 another example *in vivo*, mechanical load was shown to trigger the formation of Rac1- and
223 Arp2/3 complex-dependent cell protrusions to perform the phagocytic clearance of apoptotic
224 cells by the surface epithelium of zebrafish and mouse embryos [57]. Cell cycle progression
225 through the G1/S transition also depends on mechanical inputs, such as substratum rigidity and
226 epithelial cell density [56]. These inputs are mechanotransduced via the Rac1-WAVE-Arp2/3
227 complex pathway and depend specifically on Arp2/3 complex subunit ArpC1B, which is the
228 predominant isoform in branch junctions at the cell cortex. Indeed, three of the Arp2/3 complex

229 subunits (Arp3, ArpC1, and ArpC5) have different isoforms, resulting in Arp2/3 complexes with
230 different properties. Thus, it was recently established that ArpC1B-containing Arp2/3 complexes
231 at the cortex display higher nucleation and branching activities and are more resistant to coronin-
232 mediated branch disassembly than complexes containing ArpC1A [56, 60].

233 The positive feedback loop for branched network assembly and reinforcement is likely
234 stopped by Arp2/3 complex inhibitors such as Arpin at the cell cortex and other inhibitors at
235 other subcellular locations [61, 62]. Branched networks then need to be disassembled, which
236 primarily involves the coordinated action of cofilin-family members, with the nucleotide state on
237 actin and Arp2/3 complex acting as a molecular clock to signal disassembly (Figs. 1b and 3). A
238 characteristic feature of members of the cofilin family is to bind ADP-actin monomers and
239 filaments with higher affinity than ATP-actin monomers and filaments. This property allows
240 cofilin to specifically target older, ADP-actin segments of the filament for disassembly (Fig. 3).
241 Like with most actin-binding proteins, however, properly aligning biochemical and cell
242 biological data requires that proteins be analyzed at physiologically meaningful concentrations.
243 Case in point, cofilin; at saturating (equimolar) occupancy, cofilin stabilizes the actin filament by
244 forming additional contacts between subunits [63]. At low concentrations, however, clusters of
245 just two to three cofilin molecules efficiently sever filaments [64] by inducing structural
246 perturbations at the boundary between cofilin-bound and unbound subunits [63]. Recent studies
247 reveal that cofilin-mediated depolymerization can be further accelerated by novel mechanisms,
248 including functional synergy between cofilin and cyclase-associated-protein [65, 66] and actin
249 oxidation by MICAL [67, 68]. MICAL also oxidizes Arp2/3 complex subunit Arp3B,
250 accelerating the disassembly of branched networks enriched with this Arp3 isoform [69].

251 Although glial maturation factor (GMF) has been described as an inhibitor of Arp2/3
252 complex [70, 71], this view is controversial. GMF belongs to the cofilin family of proteins. Like
253 cofilin, GMF binds with higher affinity to ADP-Arp2/3 complex than ATP-Arp2/3 complex [72].
254 GMF's low affinity for ATP-Arp2/3 complex prevents competition with NPFs during nucleation,
255 whereas its preference for ADP-Arp2/3 complex is consistent with its role in branch disassembly.
256 Indeed, a new study reveals that older ADP-Arp2/3 complex branches are more sensitive to
257 mechanical force and GMF-mediated debranching [59]. There is also disagreement about the
258 mechanism of interaction of GMF with Arp2/3 complex. Both isothermal titration calorimetry
259 [72] and x-ray crystallography [73] reveal a single binding site for GMF on Arp2/3 complex. The
260 crystal structure shows GMF bound to the hydrophobic cleft at the barbed end of Arp2, which is
261 strikingly similar to the interactions of other cofilin-family members with actin [68]. However, a
262 secondary binding site on Arp3 has been proposed based on low-resolution EM of negatively
263 stained samples [71], and therefore the precise mechanism of GMF-mediated branch disassembly
264 remains incompletely characterized.

265

266 **Arp2/3 complex inhibition**

267 Like with most cellular processes, Arp2/3 complex inhibition is as important as activation.
268 Several inhibitors of Arp2/3 complex have been proposed, including coronins [74], Arpin [61],
269 GMF [70], gadkin [75], and protein interacting with C kinase (PICK1) [76]. However, while our
270 understanding of nucleation mechanisms has advanced substantially, inhibitory mechanisms are
271 poorly understood, and some of the existing evidence is controversial.

272 The first proposed and most extensively studied Arp2/3 complex inhibitor is coronin [74],
273 a protein found at sites of dynamic actin assembly in eukaryotes from yeast to mammals [77, 78].

274 Budding yeast has a single coronin (Crn1), whereas mammals have seven coronins subdivided
275 into three types: type-1 (coronins 1A, 1B, 1C and 6), type-2 (coronins 2A and 2B); and type-3
276 (coronin 7) (Fig. 1c). All coronins have a 7-bladed WD40 β -propeller domain, immediately
277 followed by a 40-amino acid conserved sequence that packs tightly against the β -propeller and is
278 required for its structural stability [79]. All type 1 and 2 coronins have a C-terminal coiled-coil
279 domain that mediates trimerization [80-82], but are distinguished from one another by the so-
280 called “Unique” region that separates the β -propeller and coiled-coil domains and varies
281 significantly in length and sequence. Coronin 7 is substantially different in that it has two β -
282 propeller domains and a C-terminal CA region similar to that of NPFs but lacks the coiled-coil
283 domain.

284 While coronins differ enough in sequence as to anticipate functional and mechanistic
285 differences among them [78], most studies have focused on type-1 mammalian coronins and
286 yeast Crn1, which more closely resemble one another. Some functions, however, are likely
287 conserved among all coronins, such as F-actin binding mediated by the most highly conserved β -
288 propeller domain and shown for both yeast [83] and type-1 mammalian coronins [84]. Coronins
289 bind with higher affinity to ATP/ADP•Pi- than ADP-actin filaments [84]. The β -propeller
290 domain has been also implicated in plasma membrane binding [81].

291 Direct binding and inhibition of Arp2/3 complex has been reported for yeast [74, 82] and
292 mammalian [85, 86] coronins, although the role and mechanism of inhibition is debated. Studies
293 on Crn1, including low-resolution negative stain EM, support a model in which the trimeric
294 coiled coil domain unravels and Crn1 binds as a monomer via its C-terminal sequence to subunit
295 ArpC2 of Arp2/3 complex [71, 74]. Another study, however, found that low concentrations of
296 Crn1 do not inhibit but activate Arp2/3 complex [82]. These authors identified an Arp2/3

297 complex-binding site within a CA sequence, analogous to that of NPFs, located within the
298 Unique region of yeast Crn1. They mapped the binding site of this sequence to subunit Arp2 and
299 showed that Crn1 can activate Arp2/3 complex synergistically with NPFs. They also observed
300 Arp2/3 complex inhibition, but only at high Crn1 concentrations and saturating binding to the
301 mother filament, suggesting that inhibition results from competition with Arp2/3 complex for
302 binding to the mother filament. While this model is appealing, particularly because it does not
303 invoke the structurally unlikely event of coiled coil unraveling, it cannot be extended to
304 mammalian coronins, which lack a CA sequence within their Unique region. Moreover, this
305 mechanism of inhibition is indirect, analogous to competitive inhibition of Arp2/3 complex by
306 tropomyosin [87], and does not qualify coronin as a direct inhibitor of Arp2/3 complex. So, the
307 question remains, are mammalian coronins actual inhibitors of Arp2/3 complex?

308 Most of the data available is consistent with a role for type-1 coronins as branched
309 network destabilizers (Fig. 3). Thus, type-1 coronins destabilize actin filament branches *in vitro*
310 and in cells by inducing dissociation of Arp2/3 complex from the sides of actin filaments and by
311 competing with branch stabilization by cortactin [88]. Type-1 coronins specifically promote
312 disassembly of Arp2/3 complex networks containing subunit isoforms ArpC1A and ArpC5,
313 whereas cortactin preferentially stabilizes networks containing isoforms ArpC1B and ArpC5L
314 [60]. Both yeast [89, 90] and mammalian type-1 [91, 92] coronins have been also shown to
315 synergize with cofilin and actin-interacting protein 1 (Aip1) to promote actin network
316 disassembly. Mammalian coronin 7 remains virtually uncharacterized. A recent study of the *C.*
317 *elegans* coronin 7 ortholog POD-1 suggests that it induces filament debranching and plays a role
318 in cell migration and the establishment of cell polarity [93].

319 Arpin (Figs. 1 and 3) was discovered through a bioinformatics search for proteins
320 containing a C-terminal A domain analogous to that of NPFs, but experiments *in vitro* showed
321 that contrary to NPFs Arpin inhibited Arp2/3 complex [61]. Arpin depletion in cells and *in vivo*
322 resulted in more persistent lamellipodial protrusions, whereas injection of purified Arpin
323 destabilized lamellipodia protrusions [61]. Lamellipodial branched networks drive directional
324 and persistent cell migration through a Rac1-WAVE-Arp2/3 complex-dependent positive
325 feedback loop. Arpin also functions under Rac1 control, such that it does not appear to inhibit
326 Arp2/3 complex globally but rather acts locally to terminate the Rac1-WAVE-Arp2/3 complex
327 positive feedback loop [61]. It has now become apparent that the relative levels of Arpin and
328 WAVE critically control the growth and invasion of tumor cells and act as a prognosis factor for
329 multiple types of cancers [94, 95]. While Arpin is thought to function through direct competition
330 with WAVE [61], the exact mechanism of inhibition is unknown. A low-resolution negative
331 stain EM study suggested that two Arpin molecules bind through their A domains to Arp2/3
332 complex, one to each of the Arps [71]. However, the A domain accounts only partially for the
333 inhibitory capacity of full-length Arpin, suggesting that other regions of the protein participate in
334 binding and inhibition of Arp2/3 complex [61].

335 Gadkin is another proposed inhibitor that contains an A domain (Figs. 1 and 3), which
336 mediates binding to Arp2/3 complex and competition with NPFs [75]. Yet, *in vitro* gadkin does
337 not alter the nucleation activity of Arp2/3 complex. In cells, gadkin depletion leads to partial
338 redistribution of Arp2/3 complex to the plasma membrane and increased cell migration. Thus,
339 gadkin appears to function as a negative regulator of Arp2/3 complex by sequestering the
340 complex at intracellular sites without directly altering its activity [75]. Other inhibitors of Arp2/3
341 complex have been proposed, including PICK1 [76] and WD repeat-containing protein 63

342 (WDR63) [96], but remain mostly uncharacterized. Particularly, PICK1 does not bind or inhibit
343 Arp2/3 complex *in vitro* [97].

344 **Concluding remarks**

345 Recent advances in the cryo-EM field have led to substantial progress in our
346 understanding of the mechanisms of Arp2/3 complex nucleation and branch formation. We
347 anticipate this trend will continue in the near future, since many Arp2/3 complex interactors exist,
348 but their molecular mechanisms remain poorly understood. This concerns specifically the
349 mechanisms of Arp2/3 complex inhibition by Arpin, branch stabilization by cortactin and branch
350 destabilization by GMF and coronins, all areas of intense interest that should become major
351 topics of structural studies in the immediate future. Cryo-ET in cells is a still developing method
352 that offers great potential, with the promise to observe some of these regulatory pathways in
353 action at high resolution directly inside cells. Functional studies are also leading to new
354 understanding of the many ways in which the Arp2/3 complex and branched networks control
355 cellular processes from the nucleus to the cell cortex as well as undesirable activities such as
356 pathogen and cancer cell motility. We are also just beginning to understand how the cell uses
357 positive feedback loops centered on Arp2/3 complex to control processes such as cell-cell
358 communication, mechanosensation and cancer cell invasion. Owing to the combined power of
359 novel gene editing, structural, and cell visualization methods the research in the cytoskeleton
360 field and particularly Arp2/3 complex is undergoing a renaissance.

361

362

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367

368 **Declaration of Interest**

369 The authors declare no competing interests.

370

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583 **Figure legends**

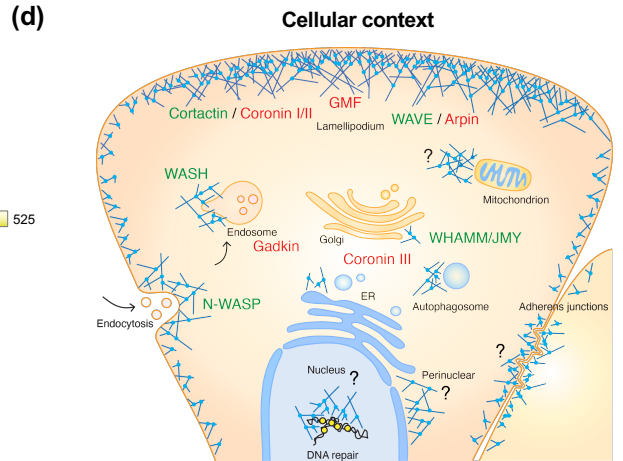
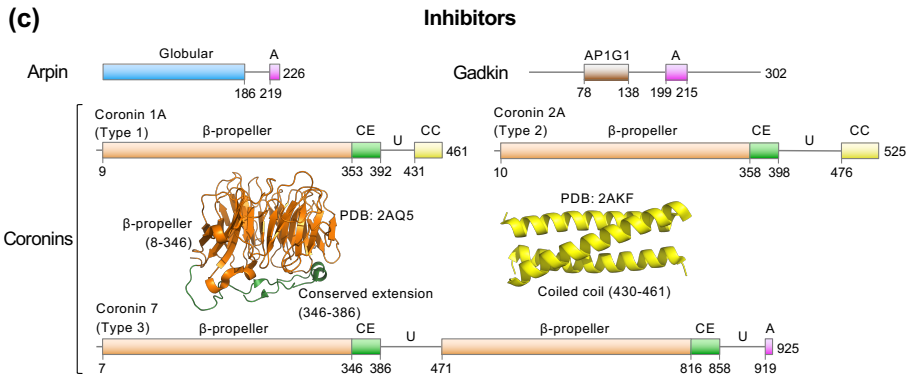
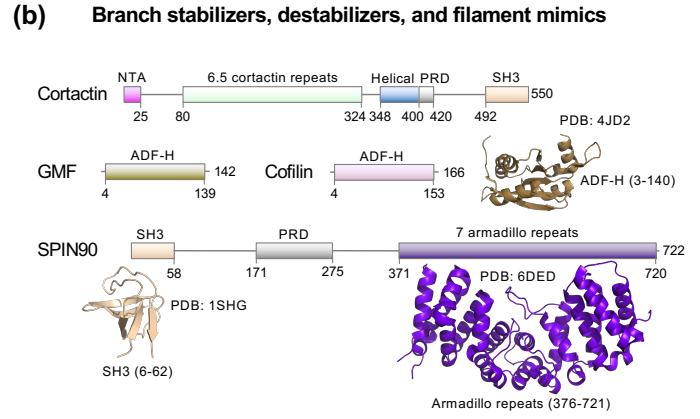
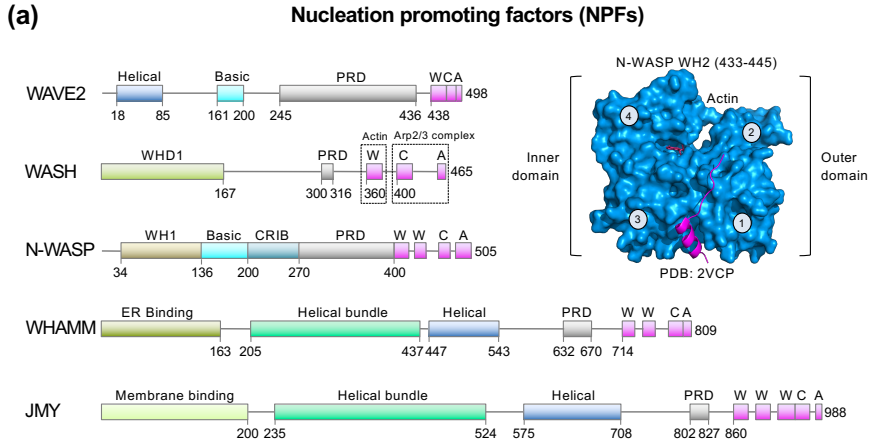
584 **Figure 1. Activators, inhibitors, and other regulators of Arp2/3 complex. (a – c)** Domain
585 diagrams of human NPFs (a), branch stabilizers and destabilizers and mother filament mimics
586 (b), and Arp2/3 complex inhibitors (c). WASP-homology 2 (WH2), central (C) and acidic (A)
587 domains are shown in magenta. Proline rich domains (PRDs) are shown in gray. Other domains
588 specific to each proteins include: WH1, WASP-homology domain 1; CRIB, Cdc42/Rac
589 interactive binding; AP1G1, adaptor protein-1 gamma-1 interacting domain; NTA, N-terminal
590 acidic domain; SH3, Src homology 3 domain; ADF-H, actin-depolymerizing factor homology;
591 CE, conserved extension; U, Unique region; CC, coiled-coil. The UniProt accession codes of the
592 human proteins shown are: WAVE2 (Q9Y6W5); WASH (A8K0Z3); N-WASP (O00401);
593 WHAMM (Q8TF30); JMY (Q8N9B5); Cortactin (Q14247); GMF (O60234); Cofilin (P23528);
594 SPIN90 (Q9NZQ3); Arpin (Q7Z6K5); Gadkin (Q63HQ0); Coronin 1A (P31146); Coronin 2A
595 (Q92828); Coronin 7 (P57737). Ribbon diagrams of determined structures of domains of these
596 proteins are shown along with the PDB accession codes. In the structure of the WH2 domain
597 bound to actin, the outer (subdomains 1 and 2) and inner (subdomains 3 and 4) domains of actin
598 are indicated. (d) Cellular context of NPFs and branch stabilizers (green text) and inhibitors and
599 branch destabilizers (red text). Question marks indicate Arp2/3 complex regulators yet to be
600 identified in connection with specific functions or subcellular locations.

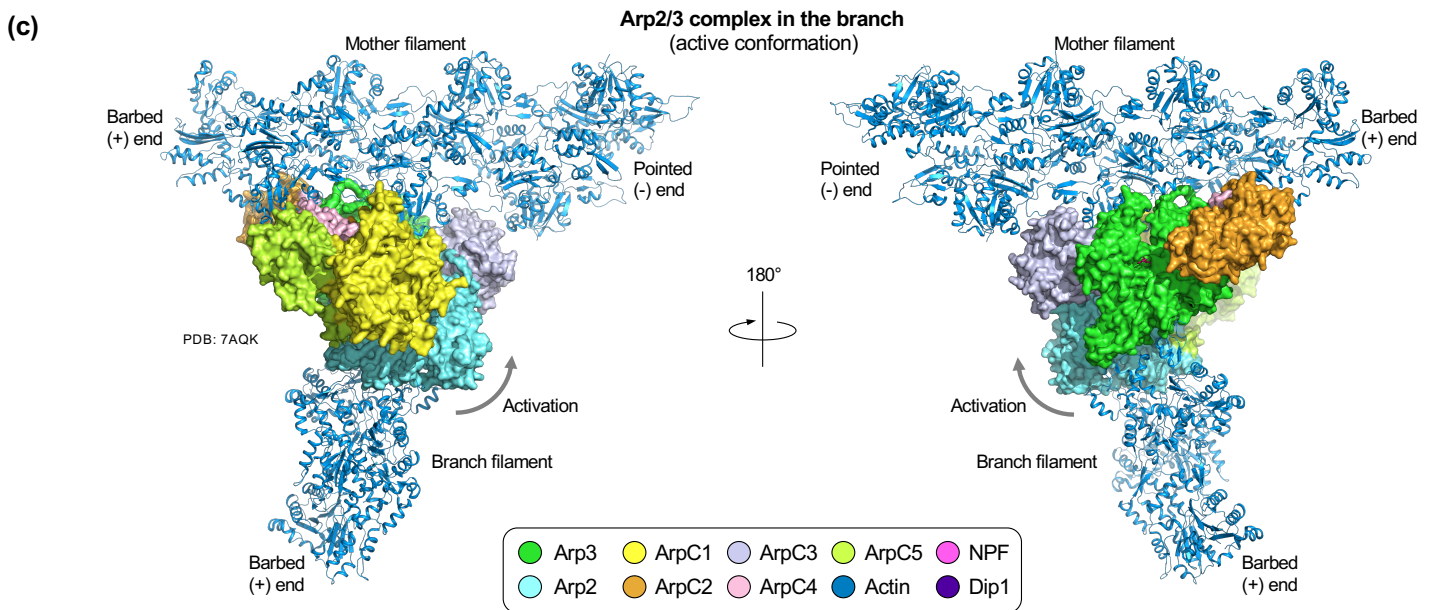
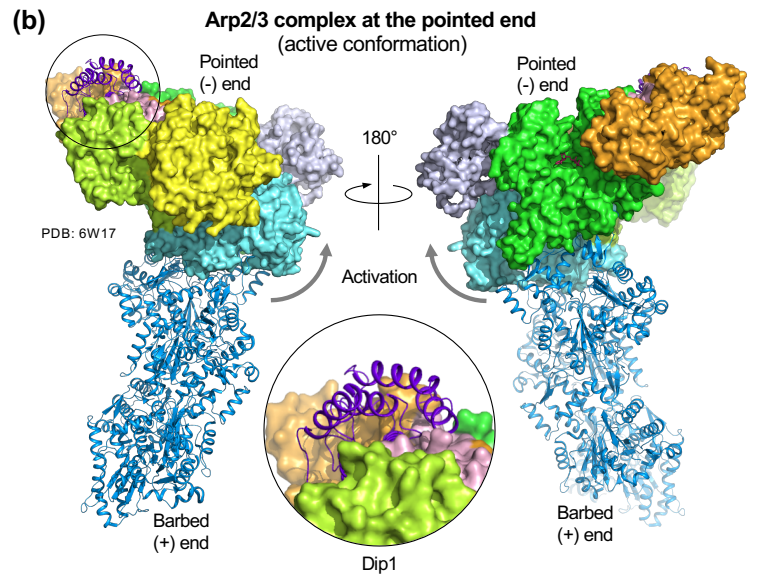
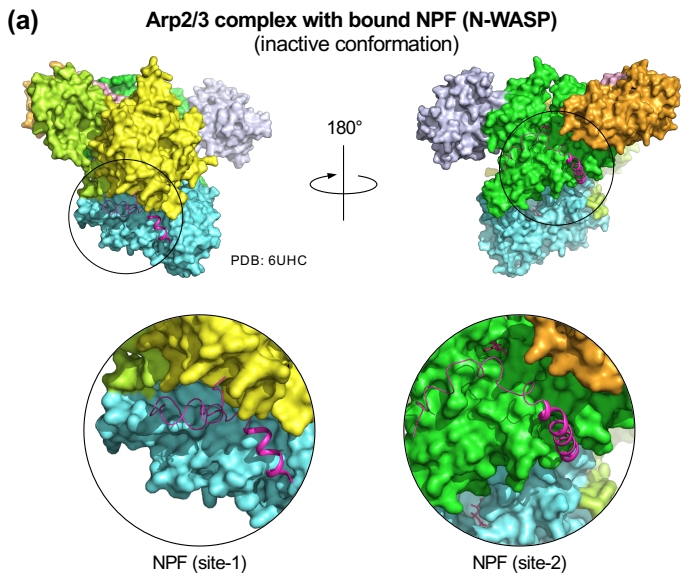
601 **Figure 2. Cryo-EM structures of Arp2/3 complex with bound NPFs, at the pointed end and**
602 **in the branch. (a)** Cryo-EM structure of human Arp2/3 complex with bound NPF N-WASP.
603 The coloring scheme is given at the bottom of the figure, and the PDB accession codes are listed

604 for each structure. The CA region of N-WASP binds to two sites on the complex (close-up
605 views). In site-1, the C helix binds in the hydrophobic cleft at the barbed end of Arp2 whereas
606 the A domain interacts with ArpC1. In site-2, both the C helix and the A domain bind to Arp3,
607 with the C helix interacting in the hydrophobic cleft of Arp3. Albeit NPFs drive the equilibrium
608 toward activation, the conformation of Arp2/3 complex in this structure is inactive. **(b)** Cryo-EM
609 structure of yeast Arp2/3 complex at the pointed end of the actin filament, also showing a
610 fragment of the armadillo repeat of Dip1 (residues G235-E366, close-up view). The two
611 orientations shows are the same as in part a. The conformation of Arp2/3 complex in this
612 structure is active, with the Arp2 subcomplex (Arp2-ArpC1-ArpC4-ArpC5) rotated up $\sim 19^\circ$
613 relative to the Arp3 subcomplex (Arp3-ArpC2-ArpC3), as indicated by the arrow. **(c)** Cryo-ET
614 structure of Arp2/3 complex in the branch. This structure contains only main-chain atoms (i.e. it
615 lacks side chains). To produce a comparable view to those shown in parts a and b, an all-atom
616 model of Arp2/3 complex was generated by superimposing individual subunits from a high-
617 resolution crystal structure onto the main-chain backbone of these subunits in the cryo-ET
618 structure. The orientation is the same as in parts a and b, and the conformation of Arp2/3
619 complex is active, and similar to that at the pointed end (part b). A notable difference is subunit
620 ArpC3, which in this structure moves substantially to make contacts with Arp2, Arp3 and the
621 mother filament.

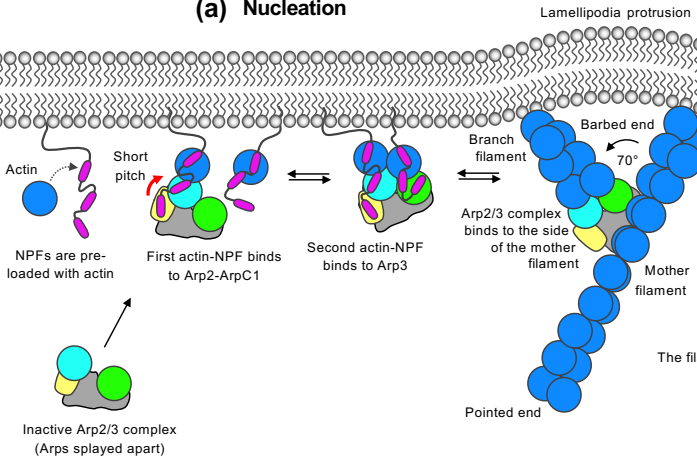
622 **Figure 3. Model of nucleation, stabilization, destabilization, and inhibition of branched**
623 **actin networks.** Proteins are colored and labeled according to figure 1. Boxed insets show
624 cartoon diagrams of the proteins and domains. **(a)** Nucleation is a multi-step process. NPFs
625 clustered at the membrane, with actin monomers pre-bound to their WH2 domains, recruit
626 Arp2/3 complex through site-1 (Arp2-ArpC1). This shifts the equilibrium toward the activated

627 conformation (indicated by a red arrow), allowing for NPF binding to site-2 (Arp3). The NPFs
628 deliver actin subunits at the barbed ends of both Arps. The complex then binds to the side of a
629 pre-existing filament (mother filament), which stabilizes the active conformation, prompts the
630 release of NPFs, and allows the branch to grow at a $\sim 70^\circ$ angle relative to the mother filament.
631 Growth of the branch and mother filaments is accelerated by profilin-actin delivery through the
632 Pro-rich regions of NPFs clustered at the membrane. **(b)** Proteins like cortactin stabilize the
633 branched network. Network reinforcement depends on positive feedback loops that converge on
634 signaling pathways, such as the Rac1-WAVE-Arp2/3 complex pathway. Older networks,
635 containing ADP-bound actin and Arp2/3 complex, are disassembled by members of the cofilin
636 family, a process that can be accelerated by other factors (see text). **(c)** Arp2/3 complex
637 inhibitors such as Arpin stop positive feedback loops for network reinforcement.

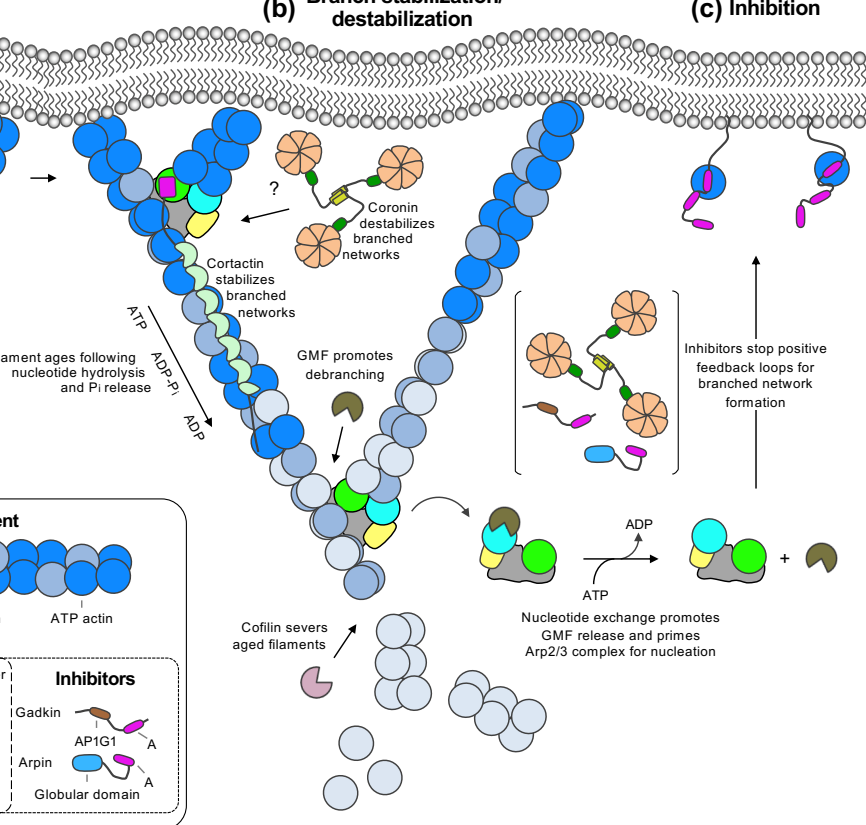




(a) Nucleation



(b) Branch stabilization/destabilization



(c) Inhibition

