Diversity and evolution of actin-dependent phenotypes

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ABSTRACT

The actin cytoskeleton governs a vast array of core eukaryotic phenotypes that include cell movement, endocytosis, vesicular trafficking, and cytokinesis. Although the basic principle underlying these processes is strikingly simple—actin monomers polymerize into filaments that can depolymerize back into monomers—eukaryotic cells have sophisticated and layered control systems to regulate actin dynamics. The evolutionary origin of these complex systems is an area of active research. Here, we review the regulation and diversity of actin networks to provide a conceptual framework for cell biologists interested in evolution and for evolutionary biologists interested in actin-dependent phenotypes.

Complex regulation underlies actin phenotype diversity

Actin is among the most abundant proteins in eukaryotic cells and is often maintained at concentrations in excess of 200 μM [1]. At such high concentrations, actin monomers readily assemble into dynamic polymers. To avoid becoming a solid brick of polymerized actin, a cell must maintain tight control over its actin monomer pool (**Fig 1**). This control is mediated by a dizzying and ever-growing list of molecular regulators, including monomer-binding proteins that suppress spontaneous actin assembly, capping proteins that restrict polymer elongation, and polymer-severing proteins that promote disassembly [2,3●]. A key player among these regulators is profilin. Most actin monomers are bound to profilin, an association that impedes the formation of new polymers—a process called nucleation—yet can promote elongation (**Fig 1-2**) [3●].

Even at cellular concentrations of actin monomers, the inherent instability of actin dimers and trimers can create a kinetic barrier to nucleation. To overcome this barrier, cells typically employ three well-defined classes of actin nucleators: the Arp2/3 complex, formin family proteins, and tandem actin monomer-binding proteins of nucleation [4] (**Fig 2**). Distinct isoforms of Arp2/3 complex subunits and different classes of formins and tandem actin monomer-binders each have their own localizations and capacities for promoting actin assembly [5, 6●●, 7, 8]. Moreover, the efficient assembly of specific subcellular structures can require collaboration between multiple actin nucleators [8-11●]. Adding yet another layer of complexity, actin isoform diversity, post translational modifications, and profilin binding can each influence the assembly of actin networks [3●,12-14]. The resulting actin networks are extended and shaped through the activities of elongation

factors, crosslinkers, and bundling proteins, and by myosin motor proteins that mediate network contraction via filament sliding [15].

Collectively, actin networks give rise to a huge variety of dynamic cell phenotypes (**Fig. 1**), many of which are associated with membranes. For example, the membrane localization of Arp2/3 complex activators drives the rapid expansion of branched actin assemblies leading to cell movement [16,17]. In organisms without a cell wall, actin polymerization at the cortex can provide structure, support, and cell shape. Additionally, actin networks can act as tracks for myosin motors to transport cargo, often to and from various cell membranes. Beyond these cytoplasmic functions, actin assembly plays important roles in the nucleus including chromatin remodeling and DNA repair [18,19]. In addition to forming polymers, actin monomers themselves have important functions, including regulating the nuclear localization of proteins, altering chromatin methylation, and promoting transcription [20].

The pre-eukaryotic origins of actin

The ubiquity of phenotypes that are controlled by actin raises a seemingly simple question: “Where did actin come from?” The discovery of actin structural homologs in bacteria, and more recently in archaea, indicates that actin-like polymers are used by cells across the tree of life. These proteins are commonly referred to as “actins” despite no obvious sequence homology to eukaryotic actin, raising the possibility that this term may carry eukaryotic connotations that are misleading.

The genetic diversity of bacterial actins is greater than all eukaryotic actins and Arps (actin related proteins) put together and fittingly, bacterial actins contribute to a wide variety of basic cell biology [21, 22●]. In contrast to the diverse roles played by eukaryotic actin, individual bacterial actins appear to have distinct functions: FtsA helps organize the cell wall synthesis required for cell division [23], ParM segregates plasmids [24], MamK maintains organelle organization in magnetotactic bacteria [25], and MreB is important for determining rod shape [26,27]. The filaments formed by each of these actins have unique properties that are presumably optimized for their specific function (**Fig 3**). For example, MreB filaments contain two antiparallel strands with no helical twist [28]. This structure allows bending in a single direction, causing the filaments to orient around the circumference of rod-shaped bacteria [26]. In addition to their own actins, many pathogenic

bacteria (and even some viruses) encode actin regulators used to hijack eukaryotic actin assembly, typically for motility and/or cell-to-cell transmission [29-31]. Studying these host-pathogen interactions have made valuable contributions to defining the mechanisms of actin nucleation [32-34].

Recent discoveries of actin and actin regulators in archaea have given support to an archaeal origin of the eukaryotic actin cytoskeleton [35-38]. Ettema et al. identified crenactin, an actin homolog in the crenarchaean *Pyrobaculum calidifontis* [38] that forms double stranded helical filaments, and can be depolymerized by its regulator, arcadin-2 [35]. More recently, homologs of actin and profilin were identified in Asgard archaea [36,37●●], which are more closely related to eukaryotes than *P. calidifontis*. Despite well over a billion years of evolutionary distance, profilins from Asgard archaea not only interact with mammalian actin, but also impair spontaneous actin nucleation, similar to eukaryotic profilins [37●●]. This early evolution of actin binding proteins is thought to have "locked in" actin's amino acid sequence and structure even prior to eukaryogenesis [39]. For instance, a core feature of eukaryotic actin, the hydrophobic groove, binds well-characterized proteins like gelsolin and ADF/cofilin [40,41], and mediates the interaction between crenactin and arcadin-2 [35].

Although actin is present in bacteria and archaea, the gene family has undergone rampant expansion during eukaryotic evolution. Due to various rounds of gene duplication, many organisms express several actin paralogs, including tissue-specific actins in multicellular organisms (**Fig 3**) that are commonly referred to as "isoforms" despite being encoded by distinct genes. For example, humans have two cytoplasmic and four muscle actin isoforms that, despite ≥93% amino acid identity, can vary in their localizations (as both mRNA and proteins) and post translational modifications [3●,42]. The complexity of isoform diversity and regulation among eukaryotic actins [3●,43] is an emerging theme that may change our fundamental understanding of actin network control.

Tracing the evolution of complex actin networks

Actin coordinates almost all cellular activities, and homologs of its major regulators have been identified in nearly every eukaryotic species. Phylogenetic analyses indicate that profilin [44], formins [45,46], the Arp2/3 complex and its upstream activators [47,48], major classes of myosin motors [49], and various other actin

regulators were most likely present in the genome of the last common eukaryotic ancestor. However, the presence of an individual actin binding protein does not tell much of a story; most actin-dependent cell behaviors are emergent properties of complex actin polymer networks. Tracing the evolution of actin phenotypes requires integrating the biochemical and phylogenetic information about the proteins that make up and control the underlying networks in the context of the rest of the cell and its environment.

Although recent analyses have begun to unravel the evolutionary history of actin structures [50] and behaviors like motility [51●●] and phagocytosis [52], there are three major complications that we must consider. The first complication is the large degree of overlap between networks that encode distinct phenotypes. For example, many of the branched actin network components used for phagocytosis are also used for branched-actin mediated cell crawling, *and* for endocytosis [53,54]. This raises the possibility that the capacity to perform one of these membrane remodeling behaviors automatically allows for the others. Alternatively, each behavior may have evolved separately. Differentiating these hypotheses requires identifying the genes specific to each phenotype and determining their phylogenetic history [55]. The second complication to tracing the evolution of actin-dependent phenotypes is the relatively few lineages for which we have direct evidence linking genotype to phenotype (**Fig 4**). We understand the mechanisms underlying the actin-based cell behaviors of a few closely-related species in great molecular detail. The problem comes when we assume that because a gene required for a behavior in a model organism is conserved, then the associated behavior must be, too. However, this is a hypothesis that should be tested by determining the function of actin and its regulators in organisms spanning eukaryotic diversity. The third complication is the possibility that there is no clear evolutionary history to trace for some actin-dependent phenotypes because they are controlled by outside factors, such as they extracellular environment.

Leveraging eukaryotic diversity to understand actin cytoskeletal phenotypes

A major bottleneck to understanding actin-dependent phenotypes is the complexity of the actin cytoskeleton at every level (**Fig 1**). At the sequence level, eukaryotes often have multiple actin isoforms. At the structural level, actin functions both as a monomer and as a polymer with diverse network architectures, each controlled by a wide variety of regulators. At the cellular level, actin monomers and networks interface with nearly every other

cell system, particularly membranes, organelles, microtubules, and septins. An obvious approach to this problem is to study organisms whose reduced cytoskeletal complexity and unique properties promise to reveal new actin biology, including:

Fungi: Much of our mechanistic understanding of actin networks is the result of rapid and inexpensive forward genetics screens in fungi, particularly budding and fission yeasts. These species continue to be a powerhouse for understanding dynamic actin networks, particularly those driving cytokinesis and endocytosis [56-58●] due to their vast repertoire of molecular and genetic tools, a relatively small number of actin regulators, and easily quantifiable actin phenotypes, particularly actin cables and patches (**Fig. 4A**). More diverse fungi, particularly the chytrids—deeply divergent motile fungi that include *Allomyces macrogynus* and *Batrachochytrium dendrobatidis* (**Fig. 4**)—have retained phenotypes lost in other fungal lineages. Such ancestral phenotypes include flagella/cilia, dynamic protrusions, and cell motility, making chytrid fungi poised to become model systems to study the evolution of these actin-based structures and processes [51●●].

Chlamydomonas: In contrast to budding and fission yeast, the two actin genes of the "green yeast" *Chlamydomonas reinhardtii* have clear roles in flagella/cilia assembly [59]. While one actin homolog, IDA5, resembles the major actins from other eukaryotic species, the second actin, NAP1, has diverged significantly [60,61]. NAP1 protein is insensitive to the broad-spectrum actin polymerization inhibitor latrunculin [43]. *Chlamydomonas* cells, which live in the soil among species known to produce actin toxins, presumably monitor actin polymerization and induce expression of the second, biochemically unique actin if polymerization of the first is inhibited [60]. The powerful forward genetic screens possible in their haploid cells make *Chlamydomonas* an unparalleled system with which to study actin's contributions to flagellar function and the evolution of biochemically unique actin isoforms [62].

Giardia: The genome of *Giardia lamblia* encodes a single actin gene, but no canonical actin binding proteins, including profilin, formins, Arp2/3, or myosins [63,64]. Although it represents the most divergent eukaryotic actin known to assemble into polymers, it has retained core actin functions; reducing *Giardia* actin protein

levels results in gross defects in exocytosis, endocytosis, and cytokinesis [65]. *Giardia* actin likely has its own collection of binding partners [66,67], and represents a unique system in which to probe the limits of actin sequence diversity.

Naegleria:

Cousin to the "brain-eating amoeba," *Naegleria gruberi* is profoundly different from other eukaryotes. *Naegleria* amoebae lack cytoplasmic microtubules [68,69], suggesting a heavy reliance on actin, especially considering that these cells can crawl at phenomenal speeds of >100 μm/min and divide in under 2 h [70]. Further, while myosin II is conserved in opisthokonts and related groups, organisms in all other major eukaryotic lineages lack myosin II, except *Naegleria* and its relatives (**Fig 4B**) [71,72]. Therefore, *Naegleria* is a unique system to study the evolution of actin networks that drive motility and cytokinesis in the absence of microtubule interactions.

Moving forward: using discovery-based science to shed light on "dark" actin biology

Most of what we know about actin comes from studying a handful of genetically tractable species, most of which belong to a single major eukaryotic group: the opisthokonts. This group encompasses animals, fungi, and related organisms, leaving entire major eukaryotic groups with nearly no experimental data, especially regarding the actin cytoskeleton (**Fig 4B**). Because of the massive numbers of genes gained and lost by major eukaryotic groups [71], there undoubtedly remain important and widespread actin biology that cannot be discovered using opisthokont models. Employing the following major approaches should identify completely new actin biology:

Comparative genomics: Because of its deep evolutionary conservation, comparative genomics is an obvious choice for studying actin. In particular, phylogenetic profiling is a conceptually simple method that can be used to identify the molecular underpinnings of actin phenotypes. Phylogenetic profiling works by identifying genes that are *only* conserved in species that display a given phenotype. This methodology has been successfully applied to flagellar motility, actin-based cell migration [51], and, at even finer resolution, to identify actin

regulatory complex subunits [73]. The power of this approach will only grow as we sequence more genomes spanning eukaryotic diversity.

Genetic screens: Although genetic screens may seem “old fashioned” in the age of CRISPR, many actin regulators were discovered using forward genetic screens. Developing forward genetics in emerging model systems could rapidly provide the information necessary to either verify the hypothesis that actin filament networks are generally conserved or lead to major discoveries that overturn this dogma.

Biochemistry: Biochemical techniques such as proteomics, fractionation, and *in vitro* reconstitution assays have been used to identify and characterize novel actin regulators [74,75●] and can be readily extended to new species. The recent development of methods that can identify transient associations, such as BioID and APEX [76], may prove even more fruitful for identifying regulators of inherently dynamic and ephemeral actin networks.

Outlook and Conclusions

The broad conservation of actin and actin binding proteins highlights their importance to cell biology. Much of our understanding of the evolution of the behaviors encoded by actin networks, however, relies on the assumption that their biochemistry, network properties, and higher-level phenotypes are all conserved. Given the billions of years of evolution separating the major eukaryotic lineages, it is almost certain that actin-dependent phenotypes have diverged at least to some level. A coherent understanding of the diversity, molecular underpinnings, and evolution of eukaryotic actin phenotypes must encompass associations with the other cell systems that interface with actin at every angle. Future investigations should focus on how actin phenotypes evolved in conjunction with microtubules, organelles, and cell membranes.

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Conflict of Interest:

The authors have no conflicts of interest to declare.

Figure Legends:

Figure 1. Distinct actin-driven phenotypes arise from diversity at both the molecular and network level. The diverse structures and processes orchestrated by actin polymers arise from overlapping network architectures, molecules, and regulatory pathways. **Molecular level (top)**: Small G-proteins, lipids, and kinases are among the upstream molecules that signal to Nucleation Promoting Factors (NPFs) to activate actin polymerization. NPFs vary in their localization and capacity to activate nucleators that include formins and the Arp2/3 complex. Distinct isoforms of Arp2/3 complex subunits impact the localization of actin assembly, and specific formin family proteins are often associated with discrete phenotypes. Finally, variability exists among actin isoforms themselves, and the actin monomer binding protein profilin influences actin nucleation and elongation. **Network level (middle)**: Branched actin assemblies are typically derived from Arp2/3 complex-mediated nucleation and their growth at the tips of the resulting polymers and addition of new actin branches provides outward, expansive, pushing forces. In contrast, actin bundles are frequently nucleated by formin family proteins, and can result in stable, crosslinked actin assemblies and/or contractile networks that exert force via myosin motor activity. **Phenotype level (bottom)**: Adding an additional layer of complexity, interactions can also occur between these and related actin networks, which cumulatively drive nearly every cellular function, from motility to cell division.

Figure 2. Actin assembly is driven by multiple actin nucleation pathways. Upper panels: The Arp2/3 complex, formin family proteins, and tandem actin monomer-binding proteins of nucleation are three well-characterized types of actin nucleators. **Top Left:** The Arp2/3 complex typically binds to the side of a pre-existing filament where it nucleates a new filament. Alone, the Arp2/3 complex is not an efficient nucleator, but NPFs such as WASP can activate the Arp2/3 complex, with two WASP molecules per Arp2/3 complex producing maximum activation [77]. The Arp2/3 complex can also be activated to form unbranched filaments by WISH/DIP/SPIN90 (not shown) [78]. **Top Middle:** Donut-shaped formin dimers nucleate actin, as well as elongate filaments by processively associating with and delivering profilin-bound monomers to the growing end of the filament [79]. **Top Right:** Tandem actin monomer-binders, including Cordon-Bleu (Cobl) and Spire, recruit multiple actin monomers to form an actin nucleus. Two of the various proposed models are shown [80-

83]. **Lower Panels**: There are several examples of direct and indirect collaboration between the pathways in the top panels [8]. **Lower Left:** Collaboration between the Arp2/3 complex and formin family proteins is critical for efficient actin assembly in structures such as lamellipodia [10,11●]. While the exact molecular mechanism for this collaboration remains elusive, the possibility that formins provide seed filaments from which the Arp2/3 complex can branch remains an attractive model. The actin nucleated by the Arp2/3 complex could also provide filaments that are elongated by formins. **Lower Right:** The *Drosophila* tandem actin monomer-binder Spire and the formin cappuccino have been shown to directly cooperate, with formin-mediated dimerization of Spire facilitating the nucleation step, followed by elongation by cappuccino [84]. There is also evidence for a "ping-pong" mechanism, where Spire binds the growing end when the formin dissociates, and vice versa [9].

Figure 3. Bacterial, archaeal, and eukaryotic actin homologs with unique properties drive diverse functions. Selected actins with distinct filament characteristics and cell functions are shown for Bacteria (**top panel**), Archaea (**middle panel**), and Eukarya (**bottom panel**). Examples of Eukaryotic and Archaeal cells that contain different actin isoforms and regulators are highlighted (**insets**). A "?" indicates unknown information. Detailed references supporting the data presented within each colored rectangle are displayed to the right.

Figure 4. Actin polymer networks generate diverse eukaryotic phenotypes.
(**A**) Selected organisms are stained with phalloidin to label polymerized actin (green), and a subset are also stained with DAPI or Hta1-mCherry (*Schizosaccharomyces pombe* only) to detect DNA (magenta). Scale bars, 5 μm. Notable structures include, (p) actin patches, (c) actin cables, (r) cytokinetic ring, (l) lamellipodium, (m) microvilli, (s) actin-filled pseudopod, (f) phragmoplast. Images were provided by: Alison Wirshing and Bruce Goode (*Saccharomyces cerevisiae*), Samantha Dundon and Thomas Pollard *(S. pombe*), Clinton Parraga (*Mus musculus*) Alexander Paredez (*Giardia lamblia*), Aoife Heaslip (*Toxoplasma gondii*) Qiong Nan (*Zea mays*), and Evan Craig and Prachee Avasthi (*Chlamydomonas reinhardtii*). (**B**) This diagram illustrates the phylogenetic relationships between the selected organisms in (A) and other groups (branch lengths have no meaning). Gray circles indicate the presence of a myosin II gene. Organisms for which there is abundant information available pertaining to actin are in bold. This was estimated by PubMed searches for the keyword

262 “actin” and the species name, genus name, or common name (whichever yielded the greatest number of
263 results). Organisms for which there were ≥250 results were considered to have abundant information available.

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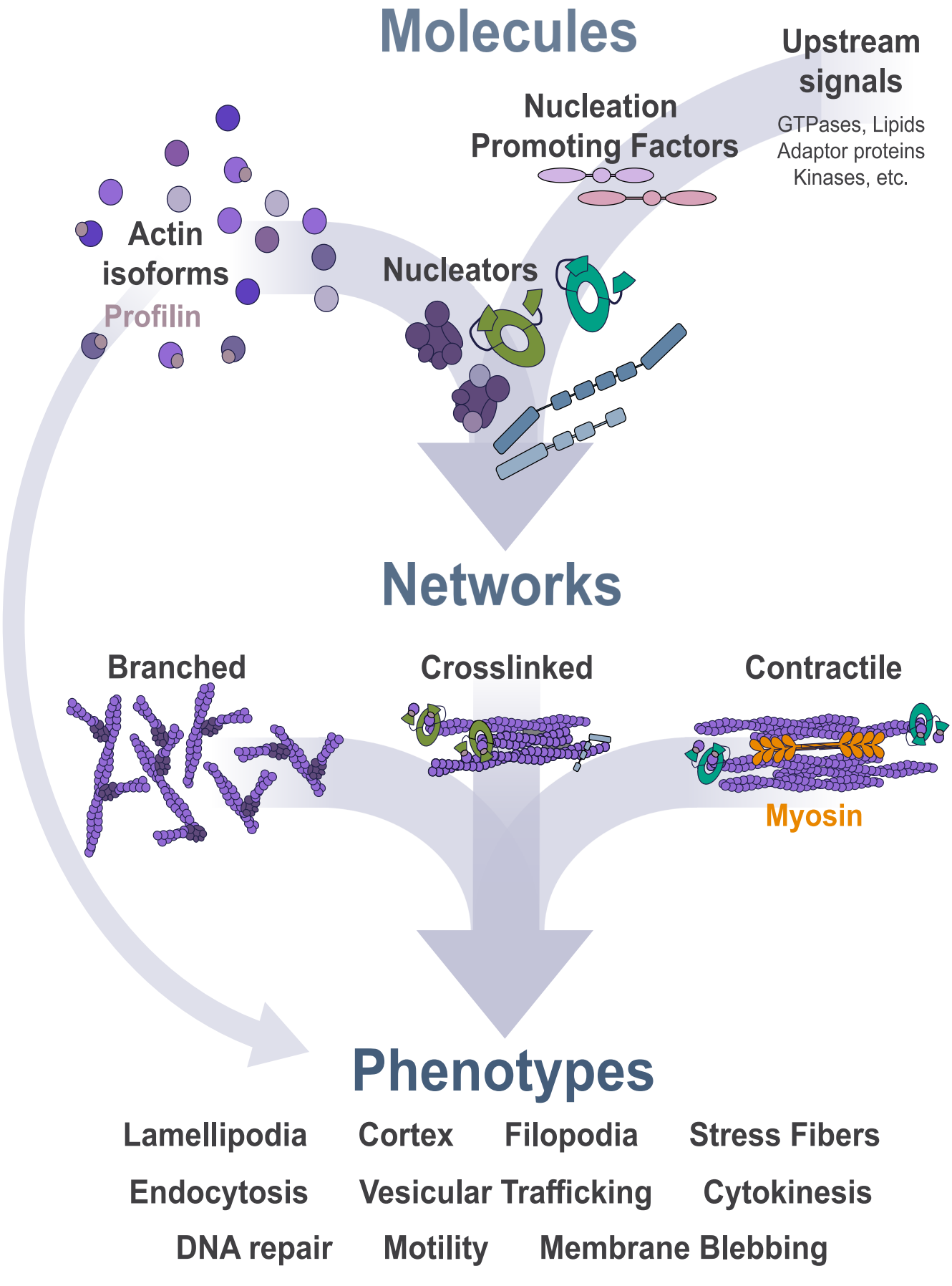
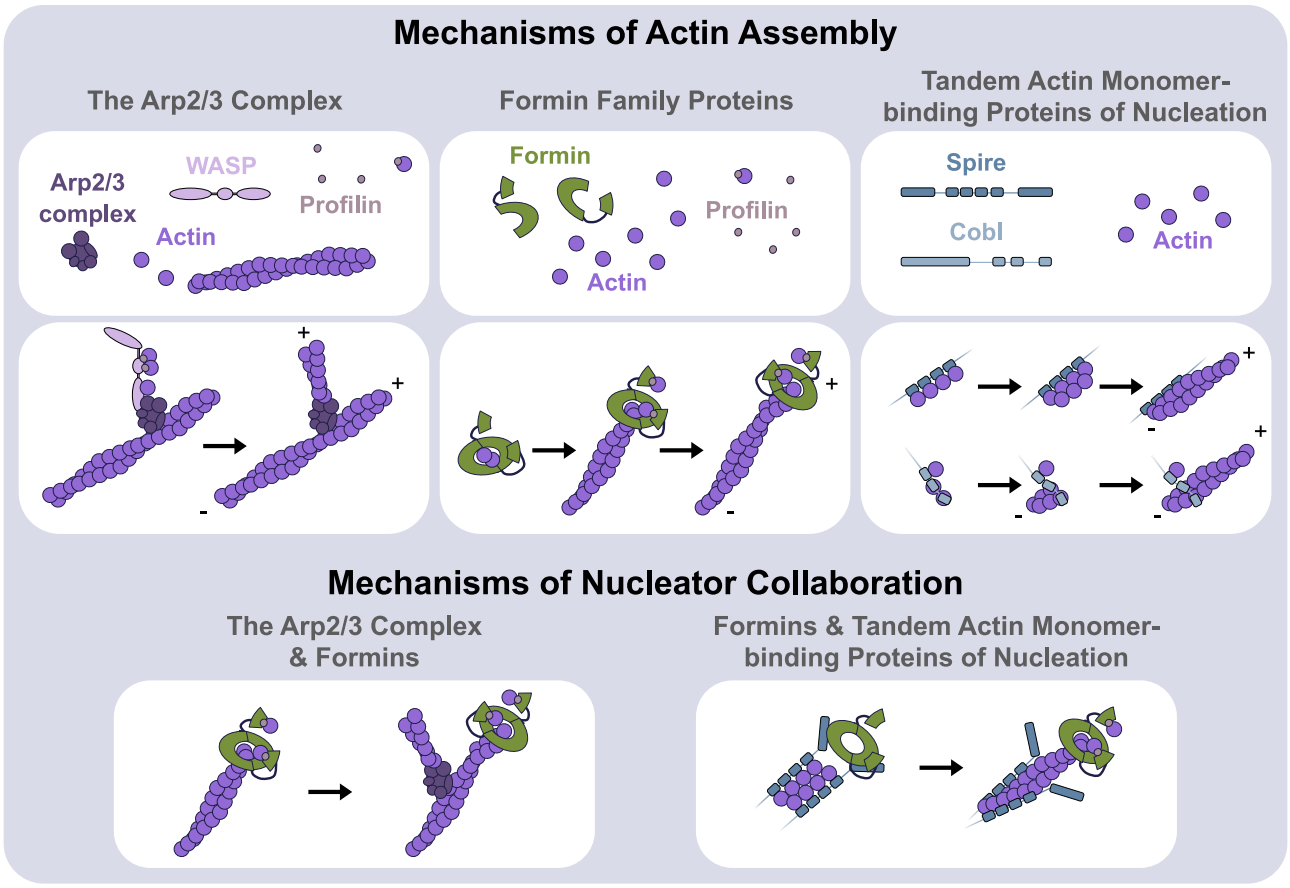

Molecules
Upstream signals
GTPases, Lipids
Adaptor proteins
Kinases, etc.
Nucleation Promoting Factors
Actin isoforms
Profilin
Nucleators
Networks
Branched
Crosslinked
Contractile
Myosin
Phenotypes
Lamellipodia
Cortex
Filopodia
Stress Fibers
Endocytosis
Vesicular Trafficking
Cytokinesis
DNA repair
Motility
Membrane Blebbing


Mechanisms of Actin Assembly
The Arp2/3 Complex
Formin Family Proteins
Tandem Actin Monomer-binding Proteins of Nucleation
Arp2/3 complex
WASP
Profilin
Actin
Formin
Profilin
Actin
Spire
Cobl
Actin
+
-
Mechanisms of Nucleator Collaboration
The Arp2/3 Complex & Formins
Formins & Tandem Actin Monomer-binding Proteins of Nucleation

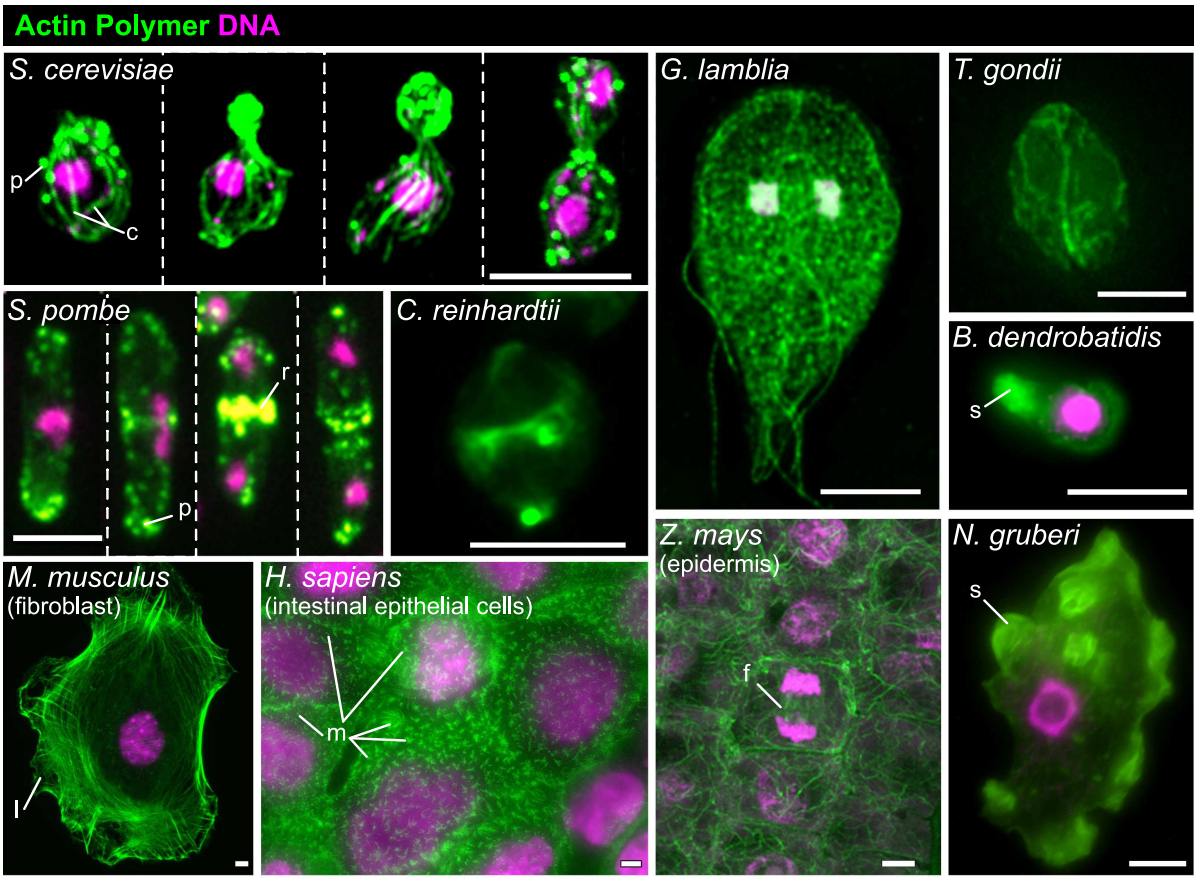
	Homolog	Filament Characteristics			References
Bacteria	FtsA (*T. maritima*)	single-stranded filaments			Szwedziak et al., 2012
	MreB (*C. crescentus*)	non-helical	antiparallel	non-staggered	van den Ent et al., 2014
	ParM (*E. coli*)	helical (left-handed)	parallel	staggered	Bharat et al., 2015
	MamK (*M. magneticum*)	helical (right-handed)	parallel	non-staggered	Löwe et al., 2016
Archaea	Crenactin (*P. calidifontis*)	helical (right-handed)	parallel	staggered	Izoré et al., 2016
	Actin (Lokiarchaea)	unknown			Spang et al., 2015

Organism	# Actins	# Profilins	References
Heimdall	1-7	1	Zaremba-Niedzwiedzka, 2017 (Table S7); Akıl and Robinson, 2018
Loki	4-5	2-3	
Thor	1-2	1	
Odin	1	1	

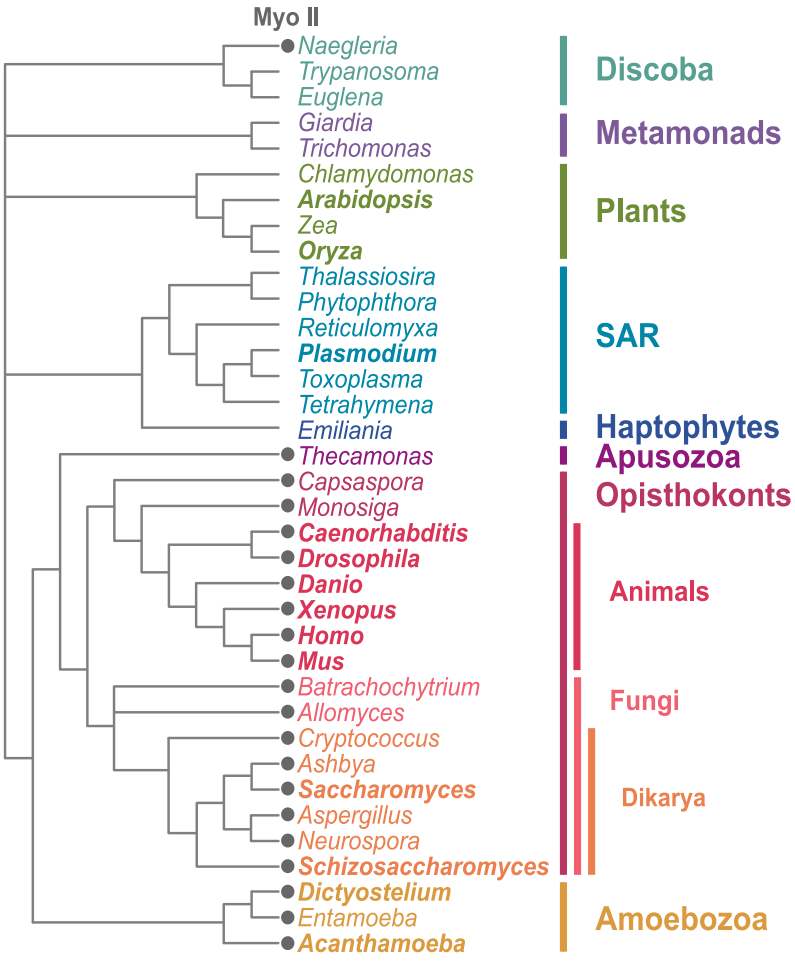
Eukarya	Eukaryotic Actin	helical (right-handed)	parallel	staggered	Oda et al., 2009

Organism	# Actins	# Profilins	# Formins	References
Animals				Skruber et al., 2018; Pandey et al., 2017; Chalkia et al., 2008; Verheyen et al., 1994;Fyrberg et al., 1980
H. sapiens	6	4	15	
D. melanogaster	6	1	7	
Fungi				Mertins & Gallwitz, 1987; Balasubramanian et al., 1994; Chalkia et al., 2008; Ng & Abelson, 1980; Pandey et al., 2017
S. pombe	1	1	3	
S. cerevisiae	1	1	2	
Amoebozoa				Joseph et al., 2008; Arasada et al., 2007; Manich et al., 2008; Chalkia et. al, 2008
D. discoidium	17	3	10	
E. histolytica	8	1	6	
Plants				Kandasamy et al., 2010; Pandey et al., 2017; Cvrčková et al., 2004; Avasthi et al., 2014; Avasthi (personal communication)
A. thaliana	8	5	21	
C. reinhardtii	2	1	4	
SAR				Aumeier et al., 2015 (Table S1); Chalkia et al., 2008; Schüler & Matuschewski, 2006; Glöckner et al., 2014
T. pseudonana	1	0	6	
P. falciparum	2	1	2	
R. filosa	5	?	5	
Discoba				Fritz-Laylin et al., 2010 (Fig S4); Vizcaino-Castillo et al., 2019; El-Sayed et al., 2005 (Table S5)
N. gruberi	≥24	4	14	
T. cruzi	4	1	3	
Metamonads				Morrison et al., 2007; Chalkia et al., 2008; Carlton et al., 2007 (Table S15)
G. lamblia	1	0	0	
T. vaginalis	12	9	5	

A


Actin Polymer DNA
S. cerevisiae
p
c
G. lamblia
T. gondii
S. pombe
r
p
C. reinhardtii
B. dendrobatidis
s
Z. mays
(epidermis)
f
N. gruberi
s
M. musculus
(fibroblast)
l
H. sapiens
(intestinal epithelial cells)
m

B


Myo II
Naegleria
Trypanosoma
Euglena
Giardia
Trichomonas
Chlamydomonas
Arabidopsis
Zea
Oryza
Thalassiosira
Phytophthora
Reticulomyxa
Plasmodium
Toxoplasma
Tetrahymena
Emiliania
Thecamonas
Capsaspora
Monosiga
Caenorhabditis
Drosophila
Danio
Xenopus
Homo
Mus
Batrachochytrium
Allomyces
Cryptococcus
Ashbya
Saccharomyces
Aspergillus
Neurospora
Schizosaccharomyces
Dictyostelium
Entamoeba
Acanthamoeba
Discoba
Metamonads
Plants
SAR
Haptophytes
Apusozoa
Opisthokonts
Animals
Fungi
Dikarya
Amoebozoa