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HYPOTHESIS

# The evolution of compositionally and functionally distinct actin filaments

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## ABSTRACT

The actin filament is astonishingly well conserved across a diverse set of eukaryotic species. It has essentially remained unchanged in the billion years that separate yeast, *Arabidopsis* and man. In contrast, bacterial actin-like proteins have diverged to the extreme, and many of them are not readily identified from sequence-based homology searches. Here, we present phylogenetic analyses that point to an evolutionary drive to diversify actin filament composition across kingdoms. Bacteria use a one-filament-one-function system to create distinct filament systems within a single cell. In contrast, eukaryotic actin is a universal force provider in a wide range of processes. In plants, there has been an expansion of the number of closely related actin genes, whereas in fungi and metazoa diversification in tropomyosins has increased the compositional variety in actin filament systems. Both mechanisms dictate the subset of actin-binding proteins that interact with each filament type, leading to specialization in function. In this Hypothesis, we thus propose that different mechanisms were selected in bacteria, plants and metazoa, which achieved actin filament compositional variation leading to the expansion of their functional diversity.

**KEY WORDS: Actin, Evolution, Filament, Tropomyosin**

## Introduction

The functions of biological filaments derive from their abilities to form linear polymers. These structures provide strength, architecture and location as scaffolding components within cells, which participate in ordering and shaping compartments and organelles, as well as localizing molecules to their appropriate physical locations. A second property of some polymers is that directed polymerization, and in some instances depolymerization, can provide pushing and pulling forces that can be integrated into biological processes in which movement is a necessary component (Pollard and Cooper, 2009). Essentially, if an elongating filament is pointed at an object, then a force will be exerted on that object from the growing filament. If enough filaments are arranged with their growing ends directed at that object, then the object will move, provided that the filament system is relatively immobile in comparison to the object – the filament system needs traction in order to push. Classic examples of the integration of the forces created from polymerization into biological systems include: (1) actin filaments polymerizing at the leading edge of a moving eukaryotic cell, driving the membrane forwards (Pollard and Cooper, 2009); and (2) during *E. coli* cell division, the bacterial actin-like filament ParM capturing an R1 plasmid at both ends, propelling the two copies apart to ensure faithful DNA inheritance (Salje et al., 2010).

The ability to form a protein polymer is a trivial feat in biology. The protein simply needs to possess a head-to-tail binding site for itself that allows for many copies of the protein to assemble into a geometry that is longer than it is wide. Several self-associating metabolic enzymes are known in bacteria, and it is from such origins that actins and tubulins are speculated to have evolved (Barry and Gitai, 2011). However in general, linear self-association interactions will have been selected against during evolution because polymerization is non-productive in most areas of protein function. Amyloid fibrils and sickle-cell hemoglobin are archetypal examples of the detrimental results of proteins self-associating without a biological justification.

To be a useful biological polymer, the protein needs to be dynamic in its self-association and dissociation so that it can be assembled and disassembled in an appropriate spatial and temporal manner. Tubulin and actin use mechanisms whereby they hydrolyze their bound nucleotides, which regulate the stability of the filaments by altering the conformation of their protomers through allosteric mechanisms (Mitchison and Kirschner, 1984; Pardee and Spudich, 1982). On polymerization, the ATPase activity of actin is activated, which creates a timing record of the progress of polymerization (Pardee and Spudich, 1982). The newly formed portion of the filament will still be mostly bound to ATP, the slightly older portion bound to ADP and phosphate, and the oldest portion is bound to ADP following phosphate release (Bugyi and Carlier, 2010). A similar mechanism exists for GTP bound to tubulin and its hydrolysis to GDP (Desai and Mitchison, 1997). The relative stabilities of these different nucleotide-bound forms within the filaments dictate the assembly and disassembly rates and disassembly mechanisms of actins and tubulins. Intermediate filaments use different and largely unknown mechanisms, which in part involve phosphorylation and ubiquitylation (Omary et al., 2006; Windoffer et al., 2011). The nucleotide hydrolysis mechanisms allow for the disassembly of actin and tubulin to be regulated on a filament-by-filament basis as opposed to phosphorylation and ubiquitylation, which work more on a bulk population basis. Thus the crucial properties of the actin polymer (and the microtubule) are the dynamic abilities to linearly self-associate, to be able to time the dissociation within single filaments and to recycle the monomers through nucleotide exchange. Furthermore, actins and tubulins generally form polar filaments. This adds directionality to the force generation during polymerization and directionality to the interactions with other proteins, such as eukaryotic motor proteins.

The roles of actin-like and tubulin-like filaments have been interchangeable during evolution. The contractile ring in mammalian cells consists, among other proteins, of actin nucleators (formins), myosin and actin filaments (Pollard, 2010). In contrast, the Z-ring in many bacteria is largely formed from the tubulin homolog FtsZ (Lutkenhaus et al., 2012). Conversely,

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tubulin is involved in chromosome segregation in mammals (Wittmann et al., 2001), whereas the actin-like protein ParM is involved in plasmid segregation in many bacteria (Salje et al., 2010). Thus, the biological functions of actins and tubulins have been somewhat transposable during evolution, which should not be surprising given that the filament systems share similar basic characteristics.

Here, we use phylogenetic and structural arguments to form a hypothesis with regard to the specialization of different actins during evolution. The bacterial ParMs will have diverged due to the relative lack of constraints within these simple plasmid-segregating systems. In multi-plasmid settings, there might even have been positive selective pressures to diversify in order to faithfully ensure inheritance of each distinct plasmid. In contrast, eukaryotic actin is a universal force provider that is integrated into many biological processes. We invoke structural arguments to postulate that once the force of actin polymerization had been harnessed for more than one biological function, the central player, actin, had little chance to evolve without compromising one or more of those functions. We expand this hypothesis to explore the possibility that during eukaryotic multicellularization, different routes may have been taken that resulted in the expansion of functionality of actin. We identify two potential mechanisms: (1) the expansion in the number of closely related actin genes in plants, and (2) the introduction of tropomyosin, which forms a co-polymer with actin, in fungi and metazoa. Both mechanisms have expanded the composition of the actin filament through regulating the subset of actin-binding proteins that are capable of interacting with each filament type.

## Bacterial actins

Many bacteria contain three types of actin-like filaments, MreB, FtsA and ParM. MreB is involved in maintenance of cell shape and cell wall synthesis (Doi et al., 1988), FtsA aids FtsZ in forming the Z-ring (Addinall and Lutkenhaus, 1996), whereas ParM is the aforementioned polymerizing motor that segregates large DNA plasmids (Jensen and Gerdes, 1997). A fourth class of actin, MamK, forms the scaffolding component of the magnetosome in a few bacterial species (Komeili et al., 2006). Sequence analysis of these bacterial actins reveals that these types of bacterial actins cluster on different branches of the phylogenetic tree (Fig. 1A). ParMs are highly divergent, whereas FtsAs and MreBs have diverged to an intermediate extent with respect to ParMs and the highly conserved eukaryotic actins. This indicates that FtsA, MreB and ParM have diverged at different rates. The clustering of FtsAs and MreBs on separate branches of the phylogenetic tree suggests that the two proteins had already diverged in the common ancestor of these bacteria. However, ParMs (Fig. 1A) are less related than the whole genome sequences of their respective species (Fig. 1B), which is not surprising, because ParMs are plasmid-encoded proteins and thus might have been subjected to different selective pressures compared to genome-encoded proteins.

The reasons for these different rates of diversification might, at least in part, be attributable to function. One factor that will have affected the divergence of bacterial actin is the number of interacting partners. ParMs only need to interact with themselves and the filament end-binding proteins (ParRs) (Salje et al., 2010). This leaves large exposed surfaces on the sides of ParM filaments that are not subject to selective pressures from interaction partners. Thus ParMs have experienced relatively few constraints and will have diverged, at least in part, through genetic drift (Kimura, 1968). In contrast, MreB is a bacterial cell-shape-determining protein that recruits the machinery responsible for synthesizing the cell wall protein peptidoglycan and interacts with FtsZ during Z-ring contraction (Fenton and Gerdes, 2013). Similarly, FtsA is a second FtsZ-interacting protein that helps assemble the cell division machinery and coordinates cell wall synthesis during the process (Lutkenhaus et al., 2012; Szwedziak et al., 2012). These activities involve protein–protein and protein–membrane associations with the sides of the filaments. Such interactions will have placed greater restrictions on the divergence of the surfaces of MreB and FtsA filaments.

In addition, we speculate that in a multi-plasmid setting, the ParM segregating machineries might have been additionally subjected to positive selection to diverge in order for each distinct plasmid to be faithfully inherited within a single bacterium. One potential example of this are the two *Bacillus vietnamensis* ParMs, which are especially highly divergent (Fig. 1). Essentially, when each plasmid encodes a unique polymerizing motor (ParM) that can be harnessed by a distinct DNA-binding protein (ParR) bound to an exclusive DNA sequence on that plasmid (*parC*), then segregation of each distinct plasmid will be reproducible, irrespective of whether a second type of plasmid exists in the same cell. These highly divergent ParM amino acid sequences (Fig. 1) have recently been shown to translate into equally divergent filament architectures (Gayathri et al., 2013; Popp et al., 2010a; Popp et al., 2012; Popp et al., 2010b), which adds some weight to the hypothesis that an element of positive selection to diverge might have existed between selected ParMs.

The emergence of distinct bacterial actins, which co-exist in the same cell, is compatible with the interpretation that a single actin-like filament did not meet the functional requirements of the evolving bacterial cell. This might, at its simplest, reflect a need to be able to spatially separate, and therefore independently regulate, the assembly and function of different bacterial actin filaments in a 'one-filament-one-function' manner. For instance, the timing and location of assembly of the plasmid-segregation filament ParM will be different to those required for cell wall synthesis and cell division, and thus for MreB and FtsA. The divergence of the bacterial actins has ensured that they will form independent homopolymers, as protomer interfaces and helical parameters are variable among the classes of actin-like filaments (Gayathri et al., 2013; Popp and Robinson, 2011; Szwedziak et al., 2012; van den Ent et al., 2001). Thus accomplishment of a specific biological function has provided the context in which the host actins (MreB and FtsA) have diverged and have become optimized for their specialized function. This might also be the case for the ParMs that are encoded on plasmids that contain an essential trait, such as antibiotic resistance, where the interests of the cell and plasmid have to be coordinated.

## Eukaryotic actin regulation

Eukaryotes utilize actin as a universal scaffolding and force-providing molecule that is harnessed for a wide range of processes that require form and force. This approach has a distinct benefit in that a single pool of polymerizable actin can be maintained. In contrast, the bacterial 'one-filament-one-function' system requires a distinct pool of each actin-like protein to be sustained for each unique filament system and its associated biological process. Limitations in resources will restrict the number of actin-like protein pools a cell is able to simultaneously maintain, and in turn limit the number of processes in which filaments can participate. Thus, adoption of the eukaryotic 'universal-actin-pool' system allows force and scaffolding functions to be incorporated into a greater number of biological processes.

One requisite to adopting the universal-actin-pool system is that a more complex level of regulation is required, because actin needs to

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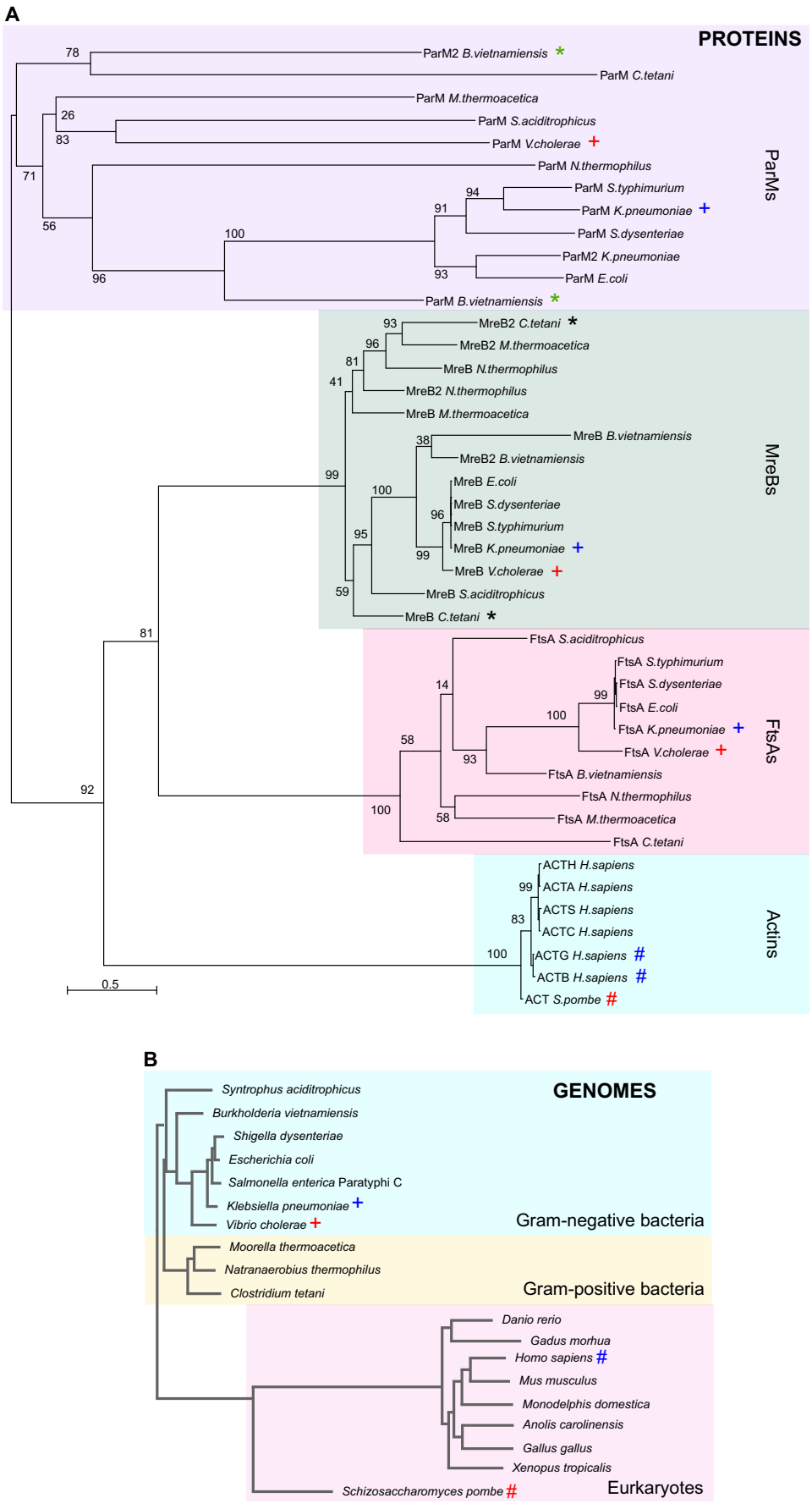


**Fig. 1. Maximum likelihood phylogenetic relationships between the actin-like bacterial proteins ParM, MreB, FtsA and eukaryotic actin.** (A) ParMs have diverged far more than the species in which they are found (B). In contrast, eukaryotic actins have hardly diverged in relation to their species. Horizontal branch lengths indicate the degree of divergence amongst the different proteins, and numbers indicate the branch support. The average numbers of substitutions per residue (SR) are 0.81, 1.16 and 4.10 for the MreB, FtsA and ParM branches, respectively, and 0.08 between *S. pombe* (red hash) and human (blue hashes) cytoplasmic actins. Relatedness within FtsAs and MreBs, but often not for ParMs, generally follows the relatedness of the species (compare blue and red plus symbols, SRs for these proteins between two species are 0.10, 0.45 and 5.4, respectively), except where two MreBs are found in a single species (black asterisks, SR 1.05), which suggests lineage-specific gene duplication. There, a likely diversification in function will have led to greater sequence variation. ParMs from the same host species can be highly divergent (green asterisks, SR 4.55). The sequences were aligned in MUSCLE 3.8 (Edgar, 2004), phylogenetic trees were constructed using FastTree 2.1.7 (Price et al., 2010) and visualized by FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Relationships between species were generated in SUPERFAMILY (Wilson et al., 2009). Accession codes for this figure, and subsequent figures, are given in supplementary material Table S1.

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be maintained at higher levels than needed for many of the individual processes. This greater level of control will have resulted from the emergence of the actin-regulating proteins. Actin filament nucleation machineries, such as formins and Arp2/3 and its activators, allow the harnessing of the force of polymerization for specific processes. Steric obstruction of spontaneous non-productive polymerization from actin filaments and monomers are carried out by capping proteins and profilin, respectively, as failure to prevent unregulated polymerization would deplete the pool of polymerization-competent actin (Xue and Robinson, 2013), leading to intracellular chaos. The emergence of the actin-based motor myosin expanded the possibilities for force generation in biological systems.

For many of the actin-regulating factors, there are now crystal or electron microscopy reconstruction structures in their actin-bound state available. When their interacting-surfaces on actin are plotted against the actin sequence it becomes immediately apparent that almost every amino acid of actin is buried within a protomer or important for an actin–actin contact and/or for an actin–regulating-protein contact (Fig. 2). Actin surface residues for which no binding partner has been defined, might nevertheless have a role in the processive elongation of filaments by formins, as these proteins slide over the exterior of actin. Such interactions might not be revealed in the static crystal structures. Furthermore, many actin residues will be important for flexibility and function at other stages, such as during polymerization (Oda et al., 2009; Xue et al., 2014), ATP hydrolysis and transition from ATP- to ADP-bound filament structures (Pollard et al., 2000), as well as in the folding process of G-actin (Egelman, 2003). Human γ-actin and fission yeast actin share 91.2% overall identity. This subdivides into 93.8%, 91.2% and 87.0% identity for residues buried in a monomer, residues buried in the filament interface, and surface exposed residues, respectively (Fig. 2). This conservation of surface residues adds weight to the hypothesis that interactions between actin and its binding partners have dictated the evolution of actin in eukaryotes (Egelman, 2003; Galkin et al., 2002; Hightower and Meagher, 1986). Thus, a minimal set of actin-regulating proteins will have ‘locked-in’ the structure and sequence of actin as a highly connected hub during the evolution of eukaryotes (Carlson et al., 2006). In the background of these interacting proteins, there therefore has been little chance for actin to evolve further without compromising one or more of these interactions, which in turn would compromise one or more biological processes. Thus, we propose that interactions with the minimal set of proteins that are needed to maintain the actin-monomer pool and nucleate filament formation, which are present from yeast to humans, have maintained the remarkable sequence conservation of actin (Fig. 1). In support of this hypothesis, deletions of Arp2/3 (Madania et al., 1999; Winter et al., 1999), cofilin (Lappalainen et al., 1997), formins (Evangelista et al., 2002), profilin (Magdolen et al., 1988), myosin

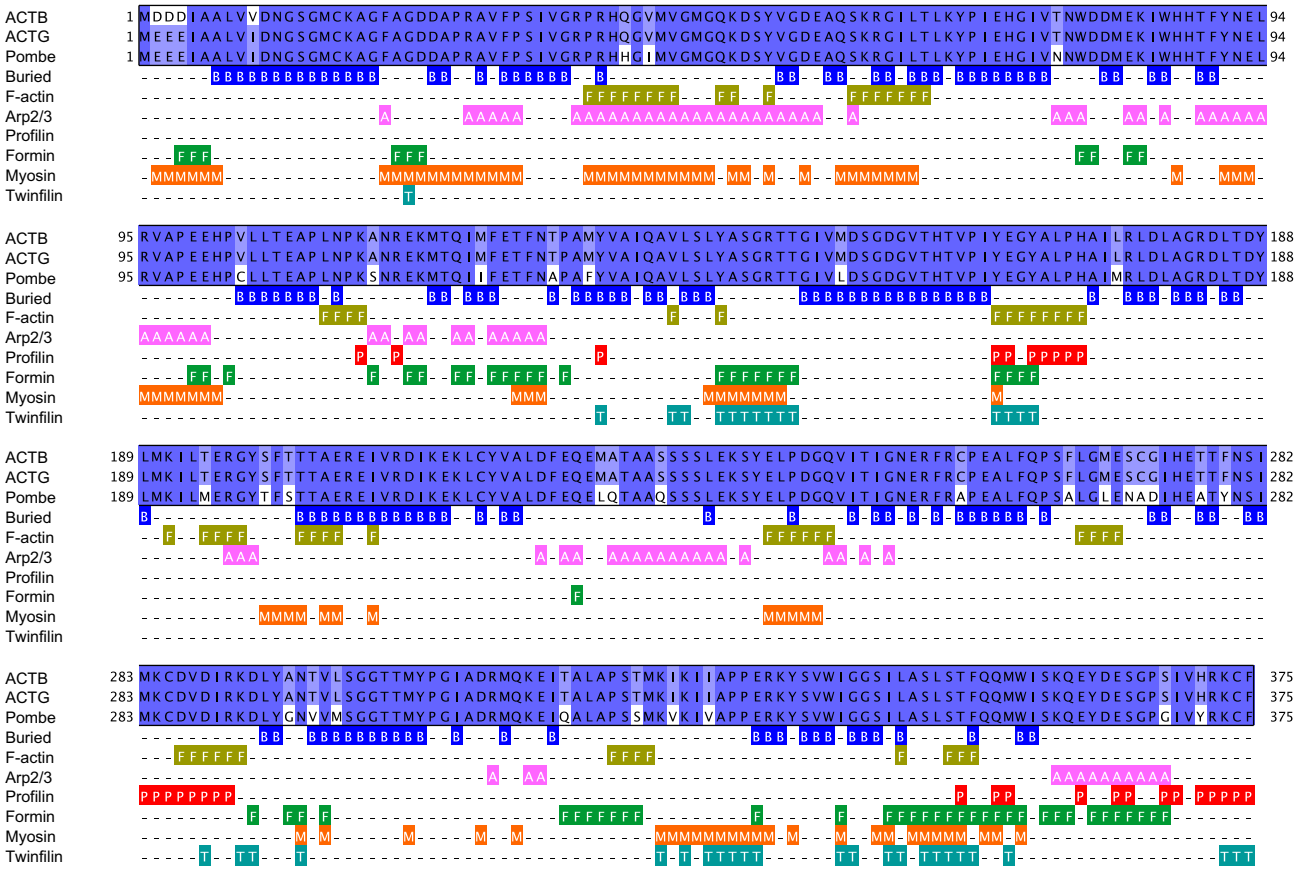


**Fig. 2. The majority of actin residues have a known function.** The alignment shows the human isoforms of cytoplasmic actin (ACTB and ACTG1) in comparison to fission yeast actin (Pombe), with the interaction properties of each amino acid indicated below the alignment. The majority of resides are involved in known actin–actin or actin–binding-partner contacts. Buried (B, blue) indicates buried residues in the G-actin structure (Wang et al., 2010), F-actin (F, mustard) indicates residues that are in the F-actin interfaces (von der Ecken et al., 2014), Arp2/3 (A, pink) (Robinson et al., 2001; Volkmann et al., 2001), Formins (F, green) (Otomo et al., 2005; Thompson et al., 2013), Myosin (M, orange) (Behrmann et al., 2012), Cofilin (T, cyan) (Paavilainen et al., 2008) and Profilin (P, red) (Schutt et al., 1993) indicate interacting residues with each protein. The twinfilin–actin structure is used here as a model for the cofilin–actin interactions. Protein interfaces were identified in CONTACT (Winn et al., 2011) and the figure produced in Jalview (Waterhouse et al., 2009).

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(Goodson et al., 1996) and tropomyosins (Balasubramanian et al., 1992) all show lethality in one or more yeast species (Costanzo et al., 2010; Moseley and Goode, 2006).

The two human cytoplasmic actins (β and γ) and four human muscle actins (α-cardiac, α-skeletal, α-smooth and γ-smooth) are extremely well conserved in sequence despite the muscle isoforms having to fulfill the highly specialized function of muscle contraction (Fig. 1; supplementary material Table S1). Nevertheless, comparison of vertebrate actins shows that there is a high level of conservation of the differences between the muscle and cytoskeletal isoforms (Fig. 3), which is not seen between the bacterial actins, MreB and FtsA (Fig. 1). Indeed, the amino acid sequences of the six avian and


**Fig. 3. Phylogenetic relationships of actins between diverse animal species.** Cytoplasmic and muscle actin isoforms are highly conserved between species. Actin protein sequences from human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), frog (*X. tropicalis*), zebrafish (*D. rerio*), cod (*G. morhua*), opossum (*M. domestica*) and lizard (*A. carolinensis*) are compared in a maximum likelihood phylogenetic tree. Sequences within the pink box indicate those found in the skeletal and smooth muscle actin classes and those within the blue box indicate those found within the cytoplasmic class. ACTA, ACTC, ACTH and ACTS refer to skeletal, cardiac, aortic smooth and γ-enteric smooth muscle actins, respectively. ACTB and ACTG are the cytoplasmic β- and γ-actin, respectively. Asterisks highlight the fish actins that show different patterns of isoform conservation. The tree-wide SR is 0.050, which breaks down into 0.020 and 0.019, for cytoplasmic and muscle actins, respectively. In comparison, the average substitutions per base (SB) in the equivalent DNA phylogenetic tree (not shown) is 0.47, indicating the restrictive pressure at the protein level.

mammalian actins are almost invariant for each isoform. This indicates that the selection pressure that has maintained actin sequence conservation has tolerated only a small degree of variation, which is under an even greater degree of selection pressure. This reflects the lack of functional redundancy of these isoforms (Schevzov et al., 1992), which have important functional consequences in terms of their interactions with specific actin-binding proteins and location to specific structures (Dugina et al., 2009; Perrin and Ervasti, 2010).

Muscle actins are likely to have experienced different selection pressures, in comparison to cytoplasmic actins, that have led to the optimization of their sequences for muscle contraction. So why have they diverged so little? In muscle cells, the usual cytoplasmic actin functions take place alongside the muscle-specific actin functions. Transgenic expression of γ-actin leads to substantial incorporation of γ-actin into the thin filaments (Jaeger et al., 2009), suggesting that some crossover of muscle actins and cytoplasmic actins can be tolerated. Indeed, biochemists routinely use rabbit skeletal muscle actin in characterizing cytoplasmic actin-regulating proteins *in vitro*. Thus we conjecture that muscle actins may have been subject to selection pressure, as drastic changes would lead to the impairment of normal cytoplasmic actin function through cross-contamination of isoforms. In other words, animal muscle actins may have experienced a high degree of negative selection pressure despite their specialization in function.

Thus eukaryotic actin, together with its repertoire of regulatory proteins, comprises an exquisitely conserved, universal force-generating polymerizing machine that is integrated into many biological processes. The actin polymerization machine is well conserved from single cellular eukaryotes, such as yeast, to multicellular organisms, such as man, which represents a period of about one billion years (Figs 1 and 2). In the background of rapidly evolving genomes, we next consider how the actin system might have evolved during the multicellularization process to allow for the expanding need of actin participation in an increasing number of processes, many of which are cell-type specific.

In all the multicellular organisms that are analyzed here, actin force generation appears to have been incorporated into an increased number of processes by enlarging the range of filament-nucleating complexes. For example, humans have many more formins than yeasts (Table 1). Furthermore, complicated actin filament geometries, which are comprised of protein-mediated crosslinked filaments, are found in filopodia, stress fibers, endocytotic structures, the contractile ring and root tips. The differential regulation of actin-filament side-binding proteins that are necessary for setting up defined actin geometries, and the regulation of filament lifetimes in each structure, appear to have evolved differently between eukaryotes and the plants, which we outline below.

## Plant actins and the evolution of multigene families

The most dramatic difference between the number of actins in bacteria, animals and plants is the considerable increase in the number of actins in plants (Table 1). The single cellular green alga *Chlamydomonas reinhardtii* has a single actin, whereas most

**Table 1. The numbers of genes with distinct protein products by species**

| Species | Muscle actin | Cytoplasmic actin | ADF | Profilin | Formin | Tmod or Lmod | Tm | Tm isoforms | Tubulin |
|---|---|---|---|---|---|---|---|---|---|
| Metazoa | | | | | | | | | |
| *Homo sapiens* | 4 | 2 | 3 | 3 | 13 | 7 | 4 | 20 | 24 |
| *Gallus gallus* | 4 | 2 | 3 | 2 | 11 | 7 | 4 | 12 | 17 |
| *Xenopus tropicalis* | 4 | 2 | 3 | 2 | 12 | 7 | 4 | 7 | 26 |
| *Danio rerio* | 10 | 2 | 3 | 3 | 26 | 8 | 4 (6) | 48 | 27 |
| *Ciona intestinalis* | 6 | 5 | 1 | 1 | 8 | 0 | 3 | 15 | 13 |
| *Drosophila melanogaster* | 6 | 2 | 1 | 1 | 5 | 0 | 2 | 22 | 7 |
| *Caenorhabditis elegans* | 4 | 1 | 1 | 3 | 4 | 0 | 1 | 1 | 13 |
| Plants | | | | | | | | | |
| *Arabidopsis thaliana* | – | 10 | 12 | 5 | 21 | 0 | 0 | – | 17 |
| *Musa acuminata* | – | 12 | 27 | 13 | 28 | 0 | 0 | – | 34 |
| *Glycine max* | – | 17 | 18 | 8 | 37 | 0 | 0 | – | 37 |
| *Brassica rapa* | – | 11 | 17 | 13 | 29 | 0 | 0 | – | 29 |
| *Chlamydomonas reinhardtii* | – | 1 | 1 | 1 | 1 | 0 | 0 | – | 7 |
| Fungi | | | | | | | | | |
| *Saccharomyces cerevisiae* | – | 1 | 1 | 1 | 2 | 0 | 2 | 2 | 4 |
| *Neurospora crassa* | – | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 4 |
| *Aspergillus nidulans* | – | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 7 |
| *Schizosaccharomyces pombe* | – | 1 | 1 | 1 | 3 | 0 | 1 | 1 | 4 |
| Protists | | | | | | | | | |
| *Entamoeba histolytica* | – | 1 (7) | 1 | 1 | 5 | 0 | 0 | – | 5 |
| *Phaeodactylum tricornutum* | – | 3 | 1 | 0 | 5 | 0 | 0 | – | 3 |
| *Trypanosoma brucei* | – | 2 | 1 | 1 | 2 | 0 | 0 | – | 11 |
| *Dictyostelium discoideum* | – | 8 (24) | 8 | 3 | 10 | 0 | 0 | – | 4 |

Lmod, leiomodin; Tmod, tropomodulin; Tm, tropomyosin. Multicellular plants have high numbers of actin, profilin and cofilin isoforms with no tropomyosins. Metazoa and fungi have fewer actin, profilin and cofilin isoforms but have evolved to have tropomyosins. Numbers in brackets signify the total number of genes, where two or more genes encode identical protein products. Archea generally do not possess actin. The Tm column shows the total number of isoforms of tropomyosin including known alternative splicing products.

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multicellular plants have ten or more actins, with many having a substantially larger number. For example, *Arabidopsis thaliana* has 10 actin genes and *Glycine max* (soybean) has 17 (Table 1). Evolutionary analysis of the plant actins reveals that there are isoforms that are often more closely related to those of other species than to isoforms within the same species. For example, comparison of *Arabidopsis* and soybean actins reveals that many subgroups of *Arabidopsis* actins are interspersed among the soybean actins (Fig. 4). Thus, plant actins do not show the same high level of isoform conservation that is observed for animal actins (Fig. 3).

Phylogenetic evidence reveals two mechanisms underlying the expansion of actin genes in plants. Firstly, like MreB and FtsA from bacteria (and also like metazoa, see below), specific plant actin isoforms have been conserved between species. In other words, there are isoforms that have been under negative selection pressure between species (Fig. 4). This is most compatible with a conserved biological function for these isoforms, which has restricted the tolerated changes in amino acids over hundreds of millions of years. Secondly, there is also clear evidence of actin gene expansion within species. This is typified in the soybean gene family where actin genes within a subgroup are generally more highly related to each other than the genes from *Arabidopsis* (Fig. 4). This pattern of

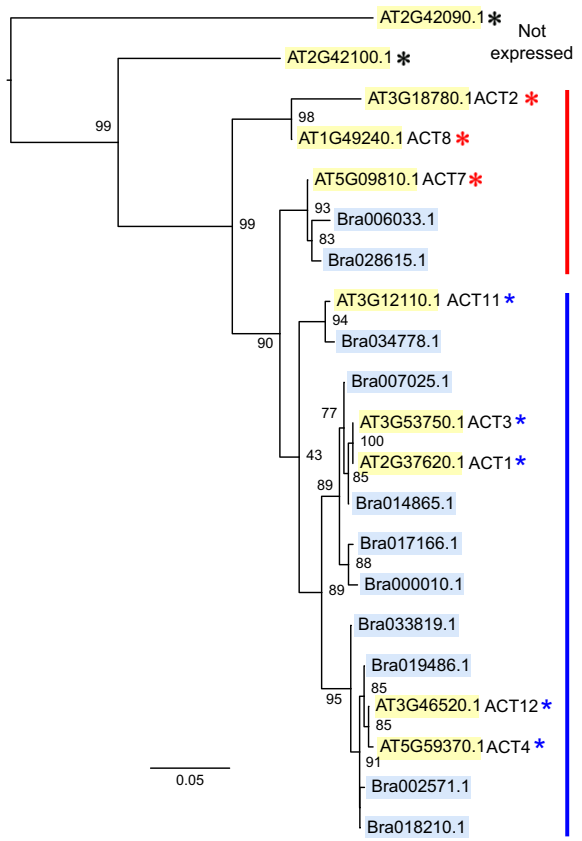


**Fig. 4. Phylogenetic relationships of actins between two plant species.** These two plant species show a conservation of isoforms between species and an expansion of isoforms within species. The relatedness of plant actin protein sequences is compared in a ML phylogenetic tree. Yellow and blue are used to indicate *Arabidopsis thaliana* (AT) and *Brassica rapa* (Bra) actins, respectively, and are identified by accession codes. Red, blue and black asterisks indicate vegetative, reproductive and not known to be expressed *Arabidopsis* actins, respectively. Red and blue vertical bars indicate probable vegetative and reproductive actins. The tree-wide SR is 0.10 after omitting the non-expressed actins, which are included for completeness as this is a sequence database analysis.

relationships is consistent with a model in which the evolution of a species is accompanied by multiple rounds of gene duplication to create an expanded gene family, which in plants, happened in part by a series of genome duplications (Lee et al., 2013; Lynch and Conery, 2000).

We propose that plant actins have a range of diverged functional properties based on the observation that within a plant species, the divergence between individual gene products is substantially greater that that seen in animals (An et al., 1996a; An et al., 1996b; Huang et al., 1997; Meagher et al., 1999). For example, the divergence of the human actins [substitutions per residue (SR)=0.01, Fig. 3] is smaller than that seen between the *Arabidopsis* actins (SR=0.09, Fig. 4). One significant observation is that the divergence in amino acid sequence in plant actins is more frequently associated with surface residues than that observed with animal actins (Kandasamy et al., 2007). This might be because there have been changes in the nature of interactions between the different plant actins and actin-binding proteins. In turn, this poses the question of why plants have greater divergence of their actins than vertebrate animals. Although it is possible that plants have a greater requirement for highly specialized actin filaments than vertebrates, we propose that it is not the actin itself that provides the greatest filament specialization in vertebrates (see below).

There are ten *Arabidopsis* actin genes, which display different expression patterns and are implicated in a range of cell functions (Šlajcherová et al., 2012). The eight expressed genes can be divided into two classes based on the cell types in which they are expressed, vegetative and reproductive, and the genes in each class are more closely related to each other than to the other class (Fig. 4) (Meagher et al., 1999). These two classes diverged about 400 million years ago. Induction of high levels of expression of a reproductive class of actin in vegetative tissue led to dwarfing of plants and disrupted cytoskeleton architecture, whereas similar expression of a vegetative actin had no effect. This result could be accounted for by isoform-specific differences in the interaction of actins with actin-binding proteins. Co-expression of both reproductive actin and actin-binding protein isovariants in vegetative tissue resulted in normal growth and eliminated the impact of expression of the reproductive actin alone (Kandasamy et al., 2007). This indicates the existence of co-evolution of actin and actin-binding protein isoforms, which perform specialized functions in a specific cellular context.

A comparison of the numbers of actin genes with the numbers of genes encoding actin-binding proteins in plants is compatible with preferential interactions between specific actin isoforms and specific actin-binding protein isoforms (Table 1). For example, *Arabidopsis* has ten actins, 12 actin-depolymerizing factors (ADFs) and five profilins, whereas humans have only two cytoplasmic actins, three ADFs and three profilins. Plants are known to have larger gene numbers than metazoans, with *Arabidopsis thaliana*, *Glycine max* and humans having ~27,400, 56,000 and 19,000 genes, respectively. These large numbers of plant genes have arisen from genome duplications. However, the plant genome sizes are not sufficient to attribute the even more highly elevated numbers of actins and actin-binding proteins to genome duplications (Lee et al., 2013).

A simple explanation for the diversity of plant actins might be provided by the expanded numbers of formins (Table 1) – if different formins are able to discriminate between different actins, possibly through recruiting dedicated profilins, then we hypothesize that each formin might be able to control the formation of homopolymers of different actin isoforms. If this is true, each type of actin might have co-evolved with a corresponding set of

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actin-binding proteins, such as a preferred profilin and cofilin based on similar isoform numbers in each plant (Table 1). Thus, plant cells might be capable of making multiple distinct and functionally specialized actin filament systems (Kandasamy et al., 2007). This would parallel the strategy that evolved in bacteria as discussed above.

### Integration of tropomyosins into actin filaments

Perhaps the most surprising observation of actin filament evolution is that only one or two actin isoforms are required to perform the wide array of functions required of cytoplasmic actin in fungi and metazoa (Table 1). We hypothesize that the introduction of tropomyosin into the actin filaments of fungi and metazoa provided a simple mechanism to diversify the functional capacity of actin filaments in these kingdoms without expanding the number of actin isoforms (Gunning et al., 2008). Tropomyosin forms two continuous co-polymers with actin that lie along the major grooves on either side of the actin filament (Phillips et al., 1979). Fission yeast, which has one actin and one tropomyosin gene, generates three compositionally distinct types of actin filaments: one class of actin filaments without tropomyosin (Kovar et al., 2011), a second type containing a co-polymer of actin with N-terminal acetylated tropomyosin and a third comprising a co-polymer of actin with non-acetylated tropomyosin (Coulton et al., 2010). These three classes of actin filaments are spatially segregated in the cell and the two tropomyosin-containing filaments are able to discriminate between and segregate different myosin motors (Clayton et al., 2010; Coulton et al., 2010). This tropomyosin-based selectivity is important because, to our knowledge, all myosins that have been isolated from yeast, fungi and plants productively interact with mammalian actin filaments containing just skeletal muscle actin. Recent data indicate that tropomyosin regulates the functional capabilities of the MyoV motor (Hodges et al., 2012).

The increasing complexity of metazoa is paralleled by increasing numbers of tropomyosin genes and larger numbers of alternately-spliced isoforms. Phylogenetic analysis of the tropomyosin genes shows that they are under a high degree of selection pressure and this is most dramatically seen in the vertebrate tropomyosins (Barua et al., 2011; Ochiai et al., 2010) (Fig. 5). The four vertebrate genes have undergone limited changes over the last 500 million years (Schevzov et al., 2011) and, moreover, the specific vertebrate genes are highly conserved suggesting that the entire surface of the coiled-coil is under strong selection pressure (Schevzov et al., 2011). The expansion of the number of tropomyosin isoforms, rather than of actin isoforms, that accompanies the vertebrate radiation leads us to hypothesize that it is the compositional diversity of actin filaments that has been the subject of selection (Table 1).

It is an axiom of evolution that if you spatially segregate isoforms you will inevitably select for specialized function that is based on the spatial context of isoform location (Gunning, 2003). It is therefore not surprising that the tropomyosin isoforms of mammals have acquired specialized functions. For instance, cytoplasmic isoforms of tropomyosin have been shown to be functionally distinct in mice (Hook et al., 2004; Hook et al., 2011). This functional specialization of tropomyosin was first suggested by the tropomyosin-isoform-dependent protection of actin filaments from gelsolin-mediated severing (Ishikawa et al., 1989a; Ishikawa et al., 1989b; Nag et al., 2013). Subsequent studies have shown that different tropomyosin isoforms allow actin filaments to functionally discriminate between myosins (Bryce et al., 2003; Fanning et al., 1994; Tang and Ostap, 2001). Tropomyosin allosterically increases the hydrophobic and stereospecific interactions between myosin and actin, providing a possible basis for recruitment and selection of particular myosins (Lu et al., 2006). Furthermore, post-translational acetylation and phosphorylation of mammalian tropomyosin increases the variation in the system through altering the association with actin and changing actin–myosin dynamics, respectively (Hitchcock-DeGregori and Heald, 1987; Nixon et al., 2013). Tropomyosins also respond

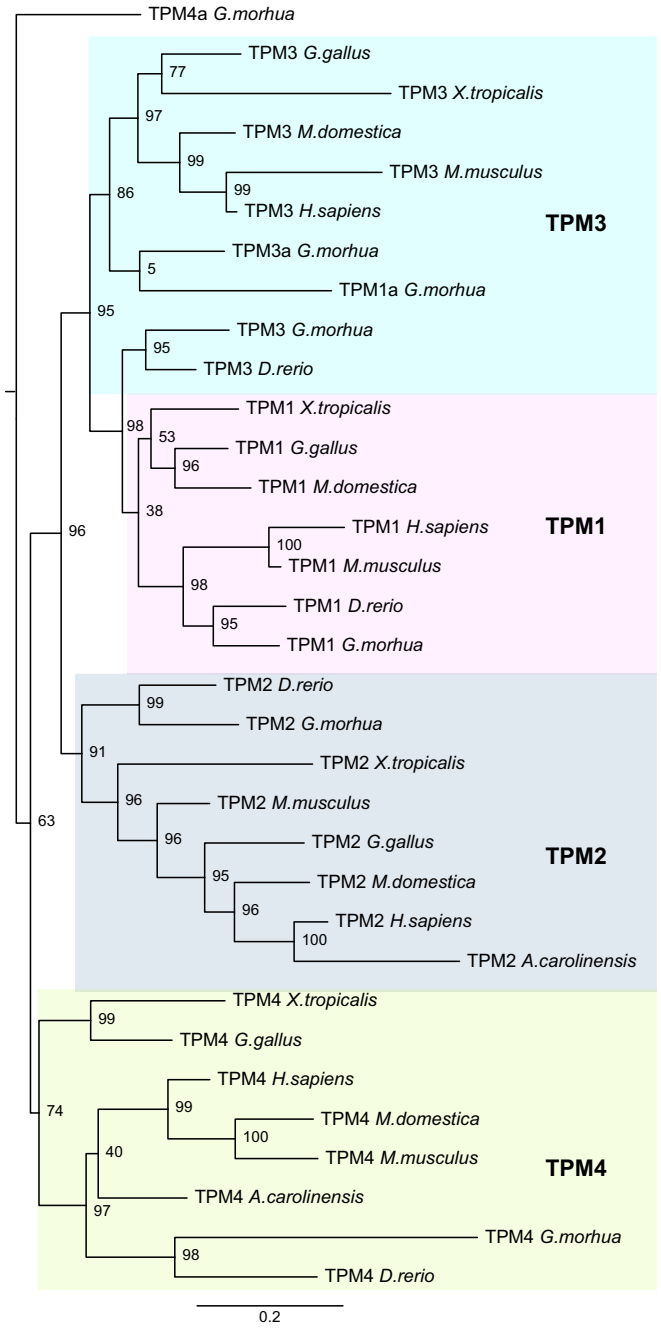


**Fig. 5. Phylogenetic relationships of selected tropomyosin genes between various metazoan species.** Tropomyosin isoforms are conserved between species. The relatedness of tropomyosin gene sequences is compared in a maximum likelihood phylogenetic tree. Here, gene sequences are compared, rather than protein sequences because of the difficulties in accurately aligning tropomyosin protein sequences owing to their repetitive coiled-coil sequence motifs of varying lengths. Shaded boxes indicate the clustering of tropomyosins TPM1, TPM2, TPM3 and TPM4. The tree-wide substitutions per base (SB) is 0.56, which compares with a SR of 0.40 in the equivalent protein phylogenetic tree (not shown).

differently to ADFs and cofilins (Bryce et al., 2003), and interact with specific tropomodulins and leiomodins, proteins that cap the pointed end of an actin-tropomyosin co-polymer and influence the filament dynamics and lifetimes (Yamashiro et al., 2012) and other actin-binding proteins (Creed et al., 2011; Kostyukova and Hitchcock-DeGregori, 2004; Sung and Lin, 1994; Watakabe et al., 1996).

Perhaps the most dramatic specialization of the actin filament is found in muscle. The ability to form contractile tissue came from specialization of the cytoskeleton. The principle of contractile force generated by the interaction of myosin II motors with actin–tropomyosin co-polymers is as ancient as the yeast contractile ring. The mechanism by which these compositionally distinct filaments, in terms of their tropomyosin content, are generated in fission yeast has recently been identified (Johnson et al., 2014). The two fission yeast formins generate actin filaments with different tropomyosin isoform compositions, and hence, with different functional properties. Manipulation of a formin to a new location in the cell led to the assembly of actin filaments complete with the formin-specific tropomyosin at the new site (Johnson et al., 2014).

Comparisons of the mammalian actin and tropomyosin sequences with those of tubulins and intermediate filaments are revealing. There are many more tubulin genes (24 in humans) than actin genes (Table 1) and they show a distribution in sequence identities that ranges from almost identical isotypes to those that show relatively more diversity in sequence than mammalian actins (Ludueña, 2013). Several of the tubulin isotypes have been implicated in discrete processes. Thus tubulins would appear to fit with the model that we have suggested for plants, whereby the number of genes have expanded and diversified in a background of negative selection pressure. That negative selection probably arises from a common set of interacting proteins. The sequence differences between tubulins allowed extension of function through the acquisition of sets of unique interactions. The mammalian intermediate filament gene family has also expanded, particularly among the keratins of which there are 54 in humans. The selection of keratins that are expressed changes with epithelial cell type, differentiation state and developmental stage (Chu and Weiss, 2002). This pattern has parallels with the prokaryotic one-filament-one-function model, whereby keratin genes have expanded to produce independent systems that have varying properties. Thus, a mammalian cell expresses the subset of keratins that meets its requirements.

We propose that the compositional diversity of actin filaments in terms of the actin and tropomyosin (in the case of fungi and metazoa) isoforms they contain, has provided an extraordinary diversity of function. Spatial and temporal segregation of both actin and tropomyosin isoforms provided the context in which to specialize. As cells became more specialized and architecturally complex, the actin, and subsequently tropomyosin, isoforms provided the opportunity to independently regulate a range of actin filament functions.

## Conclusions

Organisms, from bacteria to plants and man, use multiple actins to conduct the numerous functions demanded of this polymer system. In general, the actins appear to mostly form homopolymers, which provide fidelity of function to each polymer. The ability to include force and form into an expanding number of biological processes during eukaryotic evolution fashioned actin as a universal polymerization machine. Once created, we hypothesize that the central player actin was 'frozen' in evolutionary time due to negative selection imposed by its involvement in a multitude of functionally crucial processes. Thus, the inherent structural and dynamic characteristics of the actin filament system will have shaped its own evolution. The 'one-filament-one-function' system in bacteria was replaced by a 'universal-actin-pool' in eukaryotes which could be tapped into by many processes. This communal approach probably encountered challenges, particularly during the specialization of intracellular space in cells and in moving from single cellular to multicellular organisms, resulting in the expansion in the available variety of actin filaments. Different branches of eukaryotes appear to have evolved distinct methods to do this. Plants have an expanded number of actin genes allowing subtle variations in the actin produced, whereas tropomyosins have evolved in animals, which can differentially regulate the interactions with the filaments. In eukaryotes, post-translational modifications provide a further level in variety of actin filaments (Terman and Kashina, 2013). These adaptations have expanded the repertoire of actin function in the multicellular environment. However, the principle first established for bacteria of a specialized function for each type of compositionally distinct actin filament can be applied to understanding actin filaments across the kingdoms.

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#### Supplementary material

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