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Self-assembling enzymes and the origins of the cytoskeleton

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Abstract

The bacterial cytoskeleton is composed of a complex and diverse group of proteins that self-assemble into linear filaments. These filaments support and organize cellular architecture and provide a dynamic network controlling transport and localization within the cell. Here, we review recent discoveries related to a newly appreciated class of self-assembling proteins that expand our view of the bacterial cytoskeleton and provide potential explanations for its evolutionary origins. Specifically, several types of metabolic enzymes can form structures similar to established cytoskeletal filaments and, in some cases, these structures have been repurposed for structural uses independent of their normal role. The behaviors of these enzymes suggest that some modern cytoskeletal proteins may have evolved from dual-role proteins with catalytic and structural functions.

Introduction and Overview of the Bacterial Cytoskeleton

Only in the past two decades has it become widely accepted that bacteria are internally organized and possess a complex multi-faceted cytoskeleton similar to eukaryotic cells. The proteins that make up the bacterial cytoskeleton can largely be grouped into families based on homology to the major types of eukaryotic cytoskeletal filaments: tubulin-like, actin-like, and intermediate filament-like. Additionally, bacteria are host to some filament systems that lack recognizable homology to eukaryotic cytoskeletons.

The first bacterial cytoskeletal element discovered was FtsZ, a tubulin homolog found in most bacteria that assembles into a ring-like complex at the site of cell division [1]. Like its eukaryotic relative, FtsZ polymerizes in a GTP-dependent manner, and the structure of the monomers bears significant similarity to α- and β-tubulins [2–5]. Proteins in the actin-like family include MreB and ParM, which maintain cell shape in non-spherical bacteria and carry out DNA partitioning, respectively [6–8]. These proteins are not strong amino acid sequence matches for actin, but do possess strong structural conservation with actin [9]. A recent bioinformatic study identified 35 different families of bacterial actin homologs, suggesting that there may be significant divergence in the sequences, structures, and functions of the members of the newly identified bacterial actin superfamily [10*]. Finally, a prokaryotic homolog of intermediate filaments, crescentin, was discovered in *Caulobacter crescentus*. In this species, crescentin helps maintain a slight curve in the overall cell shape [11], while other Coiled-Coil Rich Proteins (CCRPs) have been implicated as intermediate-filament-like proteins with diverse localizations and functions in other species [12]. More recently, several types of bacterial proteins unrelated to actin, tubulin, and intermediate

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filaments have been found to form filamentous structures. These newly appreciated filaments include bacterial-specific families such as the Walker A Cytoskeletal ATPases (WACA), which include the DNA segregation protein ParA and the division site placement protein MinD, and the bactofilin family of polymerizing proteins [13–15].

In addition to the "classical" cytoskeletal elements, there is a growing list of proteins that are conserved throughout bacteria, archaea, and eukaryotes and have previously characterized functions, but upon further investigation turn out to self-assemble in filamentous polymers. This new class primarily consists of enzymes that would not necessarily be expected to polymerize based on their cellular functions. Several recent reviews have focused on the non-enzymatic members of the bacterial cytoskeleton and the evolutionary relationships between bacterial and eukaryotic cytoskeletal elements [14–19]. Therefore, here we have decided to focus on the emerging class of filament-forming enzymes. We focus on the evidence that these proteins form filaments both *in vivo* and *in vitro*, the functional significance of polymerization for enzyme function, and the ability of cells to co-opt polymerizing enzymes for secondary functions. We also discuss how such dual-role enzymes may have served as the evolutionary precursors for the modern cytoskeleton.

A diverse set of metabolic enzymes self-assemble into higher-order structures

While post-translational modifications and allostery have long been viewed as the major mechanisms of enzymatic regulation, in this review we will discuss increasing evidence that higher-order assembly represents an additional general mechanism for modulating enzyme activity. This higher-order assembly goes beyond assembly of individual protein monomers into functional oligomers, as it involves the conglomeration of functional units into large quanternary structures that build in two or three dimensions to form larger scale biological structures such as filaments. A subset of enzymes use higher-order assembly as a way to regulate activity either positively or negatively (we refer to these as filament-regulated enzymes). This assembly may either foster cooperativity between subunits or may hold enzymes in a conformation that is more or less susceptible to other regulatory mechanisms. Another group of enzymes assemble to form specialized structures for a role independent from their enzymatic activity (we refer to these as dual-role filaments). Filament-regulated enzymes and dual-role filaments may be related, as the two classes are not necessarily mutually exclusive and may have evolved from one another. The enzymes in these classes are involved in a wide range of cellular processes, but are usually at pathway points where tight control of activity would be desirable. In some cases enzyme polymerization is conserved between species, while in others it may not be. The examples to follow come from both eukaryotes and prokaryotes and have been organized not by the specific pathways of the enzymes of interest, but rather by the general principles of how polymerization affects enzymes and cytoskeletal evolution. These two groups provide evidence for the model we propose for the evolution of the cytoskeleton.

Higher-order assembly can regulate enzymatic activity

Perhaps the earliest-described and best-characterized relationship between higher-order assembly and enzyme activity occurs in the case of acetyl-CoA carboxylase (ACC). ACC catalyzes the first committed step of fatty acid synthesis, the carboxylation of acetyl-CoA to form malonyl-CoA. As the committed step of this energetically costly biosynthetic pathway, ACC is subject to tight regulation, such that it is not surprising that ACC is regulated by multiple distinct mechanisms including transcriptional regulation, phosphorylation, feedback inhibition, and allostery [20–23]. Initial biochemical studies of ACC showed that, upon incubation with high levels of citrate, high molecular weight ACC assemblies were observed

upon sedimentation [24,25]. Electron microscopy showed that these assemblies form long filaments [20]. ACC higher-order structure was first observed among mammalian protein samples and has also been described in yeast [20,26]. Later studies confirmed that polymerization occurs in response to allosteric activation by citrate [20,27]. Citrate activation ensures that fatty acid synthesis is coordinated with carbohydrate synthesis. Biochemical studies have demonstrated that the polymerized ACC population is more active than the unpolymerized (protomeric) population. These studies were facilitated by the use of avidin, a molecule that binds the biotinyl prosthetic group of ACC, thus inactivating the enzyme. ACC polymers are resistant to avidin binding, providing means to distinguish and quantify the relative amounts of protomeric and polymerized ACC [28,29]. The feedback inhibition of the enzyme also works through the modulation of its polymerization state. A high concentration of product, malonyl-CoA, rapidly dissociates polymers into smaller units. ACC transitions between active and inactive states in a cooperative non-linear manner [30]. Taken together, this suggests that polymerization into a higher-order structure stimulates ACC activity.

Recent work has shown that human and rat liver ACC allosteric activation by citrate can be increased and even bypassed by the addition of another polymerization-activator, the protein MIG12 [31**]. Thus, polymerization could be a mechanism for cooperatively stabilizing an enzyme's active conformation. Kim et al. showed that polymerization state changes with metabolic conditions such that when fatty acid biosynthesis is more active *in vivo*, a higher level of polymerization is observed. Additional evidence for higher-order assembly of ACC comes from *Trypansoma brucei*, where cytoplasmic foci which may represent ACC polymers are present [32*]. Two distinct mechanisms have been proposed to explain the increased activity of polymerized ACC. The first hypothesis is that the functional units of ACC bind cooperatively and that polymerization stabilizes a conformational form with increased activity [30]. Alternatively, the polymerized subunits may be protected from negative regulation by phosphorylation [33].

A growing body of evidence suggests that regulating enzyme activity by higher-order assembly is not unique to ACC. Glutamate dehydrogenase (GDH) catalyzes the conversion of glutamate to α-ketoglutarate and has a key role in regulating nitrogen flow within the cell. It is subject to allosteric activation by numerous molecules including purine nucleotide triphosphates [34,35]. At the high protein levels observed in the mitochondria, GDH can either aggregate or form ordered filaments in several types of mammalian cells [35–37]. GDH first oligomerizes into small complexes of at least four identical subunits, depending on the species [38]. These smaller complexes can then assemble into higher-order filaments. This process is reversible, as functional GDH oligomers can cycle between polymerized and dissociated forms [39,40]. GDH oligomers can also cycle between active and inactive forms. Polymerization has been suggested to modulate activity, though the connection between higher-order assembly and enzymatic activity remains the subject of debate [39,41–45]. Though less well characterized, a number of other enzymes have been found to form filamentous structures either *in vivo* or *in vitro*. These are enzymes whose assembly was identified biochemically, such as argininosuccinate lyase [46], enzymes whose assembly was identified in a bacterial protein localization screen, such as UDP-N-acetylmuramate--alanine ligase [47**], and enzymes whose assembly was identified in a yeast protein localization screen, such as glutamate synthase, guanosine diphosphate-mannose pyrophosphorylase, and the eIF2/2B translation factors [48**]. As detailed below, the enzyme CTP synthase was also independently found to form filaments in bacteria, yeast, flies, and rats.

In addition to the above examples of filament-forming enzymes, recent studies have identified many examples of proteins that change their subcellular localization in an activity-

dependent manner. For example, the enzymes involved in mammalian purine biosynthesis cluster when activated [49], and a yeast screen identified 180 proteins that form punctate foci in various metabolic states, including many metabolic enzymes whose localization appears to correlate with activity [50*]. It remains unclear whether these enzymes self-assemble or localize by interacting with other structures, and the resolution limits of light microscopy prevent the accurate assessment of whether they form disordered aggregates or ordered polymers. Nevertheless, these examples reinforce the idea that higher-order organization may be a general and widespread mechanism of regulating enzyme activity.

A prototype for the mechanism of enzyme regulation by polymerization: IRE1

There are at least three distinct mechanisms by which polymerization can regulate enzymatic activity: 1) polymerization may induce conformational changes that stabilize or destabilize the active form, 2) protein-protein interactions in the polymer may generate or occlude active sites, and 3) polymerization may promote or inhibit interactions with accessory factories that regulate enzyme activity (Figure 1). Another important question raised by the identification of polymerization-regulated enzymes is the physiological benefit or significance of using polymerization as a regulatory mechanism (as opposed to other regulatory mechanisms such as allostery or protein modification). While the mechanism and significance of polymerization-based regulation remain unclear for most metabolic enzymes, a combination of structural and enzymology approaches has led to significant insight for a regulatory enzyme, the inositol-requiring enzyme 1 (IRE1).

IRE1 is one of several unfolded protein response sensors that serve to tune endoplasmic reticulum function [51,52]. IRE1 is a transmembrane protein that consists of both a domain within the lumen of the endoplasmic reticulum that senses misfolded proteins and cytoplasmic domains with kinase and endoribonuclease function [53–56]. The RNase function stimulates the unfolded protein response through X-box binding protein 1 (XBP-1), upregulating the transcription of stress response genes. In both humans and yeast, this signaling through IRE1 is ultimately controlled by the assembly state of the protein. Higher-order assembly of oligomers arranges the various domains into conformations that promote higher RNase activity. As protein dimers bind one another, the kinase domains of separate units come into contact, creating a filamentous self-assembled network and facilitating or stabilizing an open kinase domain. This conformation increases the surface area available for IRE1-RNA substrate contact. Such extensive contacts are not possible in monomeric or dimeric forms. Regulation based on high order assembly is also evident from the non-Michaelis Menten kinetics of IRE1 catalysis. Specifically, IRE1 exhibits an extreme degree of cooperativity with a Hill coefficient approaching 8. Thus, polymerization-based regulation enables IRE1 to cooperatively transition extremely rapidly and sharply between active and inactive states, enabling a switch-like regulation of enzymatic function. Furthermore, appearance of foci *in vivo* coincides with higher enzyme activity as it results in more effective interaction between enzyme and substrate. As the stress response weakens and eventually ceases, the IRE1 protein structures disassemble and IRE1 deactivates [57**, 58*]. This well characterized mechanism provides a clear physical explanation of just how self-assembly affects enzyme function, and the extreme cooperativity provides an explanation for the benefit of using polymerization as a regulatory mechanism.

Self-assembling enzymes can be co-opted for independent structural roles

The newfound appreciation that many enzymes can form higher-order structures raises the intriguing possibility that cells can use these polymers for structural functions independent of their enzymatic origins.

Crystallins are metabolic enzymes that have been repurposed to form lenses

The most widely-appreciated class of dual-role enzymes are the crystallin proteins of the vertebrate eye. The lens of the vertebrate eye is composed of higher-order structures of crystallin protein that tightly packs to form a transparent lens (reviewed in [59,60]). Surprisingly, when the crystallin proteins were identified, many of them turned out to be metabolic enzymes or have strong homology to metabolic enzymes. Some of these enzymes have undergone gene duplication, allowing the crystallin protein and its metabolic counterpart to diverge, as is the case with argininosuccinate lyase and δ-crystallin of birds and lizards. In other organisms, the single argininosuccinate lyase coding region can produce a protein used as a crystallin in the eye but as a metabolic enzyme elsewhere in the body (reviewed in [61]). The extent to which the enzymes in the lens remain active is unclear, though the enzymes are found at such high concentrations that they are predicted to be largely inactive [59].

If the fact that the crystallins are metabolic enzymes with additional functions was the first big surprise in this field, the second big surprise was the discovery that different species use completely unrelated metabolic enzymes for their eye lenses. Thus, crystallins are related by their ability to form similar structures, but are unrelated at the sequence level. That such a wide diversity of metabolic enzymes can form crystallin structures is consistent with the concept that the propensity to form higher-order assemblies is a common feature in many enzymes.

CTP synthase can form cytoskeletal filaments that regulate bacterial cell shape

Crystallins clearly play important structural roles, but they do not form ordered linear polymers like cytoskeletal proteins. To date, the best example of co-opting a polymerizing enzyme for a cytoskeletal function is that of CTP synthase (CtpS) in *C. crescentus*. CtpS is a component of the pyrimidine biosynthesis pathway, catalyzing the conversion of UTP to CTP at the expense of ATP and glutamine. CtpS cooperatively self-associates to form active tetramers, and CtpS assembly can be regulated by the binding of its substrates, its product, and the allosteric effector GTP [62–66]. In 2010, three independent studies discovered that CtpS can form filamentous structures in two species of bacteria (*C. crescentus* and *Escherichia coli*), yeast (*Saccharomyces cerevisiae*), fly (*Drosophila melangaster*), and rat (*Rattus norvegicus*) [47**,48**,67**,68]. Purified *E. coli* CtpS can self-assemble into linear polymers *in vitro*, suggesting that the CtpS filaments observed *in vivo* are intrinsic to CtpS protein properties. In *C. crescentus,* addition of the CtpS inhibitor 6-Diazo-5-oxo-L-norleucine (DON) led to rapid filament disassembly, and in yeast, the addition of the CtpS product and enzymatic inhibitor, CTP, to the growth media shifted CtpS assembly from long filaments to short foci. Thus, CtpS polymerization and activity appear to be coupled, though exactly how remains unclear.

While CtpS filaments in most species appear randomly dispersed, their localization is highly stereotyped in the bacterium *C. crescentus*. *C. crescentus* cells are normally gently curved, and CtpS filaments specifically localize to the inner cell curvature [67**]. Consistent with the ability of CtpS to polymerize *in vitro,* linear polymers can be detected at the sites of CtpS localization by whole cell electron cryotomography [69]. The localization of CtpS to the inner cell curvature suggested that CtpS might play a role in regulating cell shape, and indeed CtpS overexpression led to dramatic cell straightening while CtpS depletion led to hyper-curved cells. Point mutations in CtpS that specifically abolish CtpS enzymatic activity without inhibiting polymerization were still capable of regulating *C. crescentus* curvature. Thus, CtpS appears to be a dual-role protein with two distinct functions; an enzymatic function that depends on the active site and may be regulated by polymerization, and a morphogenic function that depends on polymerization but not enzymatic activity.

The mechanism by which CtpS regulates cell shape involves cross-talk with another cytoskeletal element, the intermediate filament crescentin [67**]. Crescentin also localizes to the inner cell curvature and is responsible for recruiting CtpS to this site. CtpS in turn negatively regulates crescentin assembly, thereby limiting crescentin's impact on cell shape and keeping curvature in check. Despite the fact that *E. coli* cells are not curved and lack crescentin, Ingerson-Mahar et al. showed that the *E. coli* CtpS homolog can complement both the enzymatic and cell shape functions of *C. crescentus* CtpS. Thus, *C. crescentus* cells do not appear to have specially adapted CtpS, but rather simply co-opted the conserved property of CtpS polymerization for the secondary structural function of regulating cell shape.

The modern cytoskeleton may have evolved from polymerization-regulated enzymes

Together, the evidence presented above for the apparently widespread nature of polymerizing enzymes and the ability of cells to repurpose these enzymes for structural functions provide a possible mechanism for the evolution of ordered cellular structures like the cytoskeleton. In the ancestors of today's cells, ancient enzymes may have formed higher-order structures as a means of regulating metabolic activity. Such regulation may be mechanistically related to the cooperative regulation of IRE1 by polymerization and appears to have been retained in some modern enzymes such as acetyl-CoA carboxylase. Later, these structures may have been adapted as structural elements that formed the first rudimentary cytoskeleton, much as *C. crescentus* has more recently adapted CtpS filaments to regulate cell shape. Eventually, some of these structure-forming dual-role enzymes could have duplicated and diverged, with the major cytoskeletal elements losing their enzymatic activities.

The hypothesis that the modern cytoskeleton evolved from polymerizing enzymes is supported by the structural similarity of actin and MreB with sugar kinases such as hexokinase. These proteins do not have strong primary sequence homology, but retain significant structural alignment [9,70] and similar crystal structures [71]. Though these proteins have vastly divergent functions within the cell, their structural similarity points to a possible common ancestry. Though only a few proteins from a limited number of species have been examined, polymerization has not been observed to date for any sugar kinases. Nevertheless, perhaps the common ancestor of actin, MreB, and hexokinase was an enzyme that evolved the ability to assemble higher-order structures as a mechanism for regulating its activity depending on environmental conditions. Once filaments were present, cells may have exploited their structural properties for cell biological functions distinct from the enzymatic functions for which they were originally selected. Later, gene duplication and divergence could have allowed one copy of the gene to fine-tune its structural properties while losing its enzymatic activity, leading to the actin cytoskeleton. The other gene may have retained the enzymatic activity, perhaps eventually replacing polymerization with other regulatory mechanisms such as phosphorylation, which is common in sugar kinases. The similarity of tubulin to GTPases and WACA proteins to P-loop ATPases suggests that these cytoskeletons may also have evolved from enzymes.

Conclusions

Here we have summarized evidence that some enzymes form higher-order structures, that polymerization can regulate enzymatic activity, and that polymerizing enzymes can be repurposed for structural functions. What remains to be determined is just how widespread the phenomenon of enzyme polymerization is and through what mechanisms it can be used to control enzyme activity under various growth conditions. Understanding these aspects of

higher-order assembly will allow for better models of the formation and evolution of cellular structures like foci and filaments. These new perspectives on ancient proteins will also help us to understand the nature and evolutionary origins of the cytoskeleton as a whole.

It is also worth noting that the number of known cytoskeletal elements is rapidly growing, expanding our appreciation of the cytoskeleton beyond actin, tubulin, and intermediate filaments. As the cytoskeleton is responsible for successful cell division and also cell shape, it offered a strong advantage in genome integrity and survival over cells with less organized architectural networks. The rudimentary cytoskeleton may have started with just one or a few types of filament-forming proteins. This population of structures would have expanded in number and type over time as other proteins were incorporated. Additionally, these structural filaments would have then have faced selective pressure towards optimization as cytoskeletal, rather than metabolic proteins. Environmental factors like habitat pushed bacteria to diverge into different shapes, causing their cytoskeletal composition to differ based on which filaments adapted to forming more advantageous structures. Among dual-role enzymes, gene duplication events over time may have freed one population to be used for structural and allowed another to be retained for metabolic use until the two were recognizably different (Figure 2). This mechanism potentially explains the evolution of the growing list of bacterial cytoskeletal proteins observed today.

Highlights

- The list of proteins forming bacterial cytoskeletal structures is expanding.
- Some enzymes self-assemble into higher-order structures such as filaments.
- Assembly may regulate activity and be repurposed for structural roles.
- Dual-role filaments may have been the origin of the cytoskeleton.

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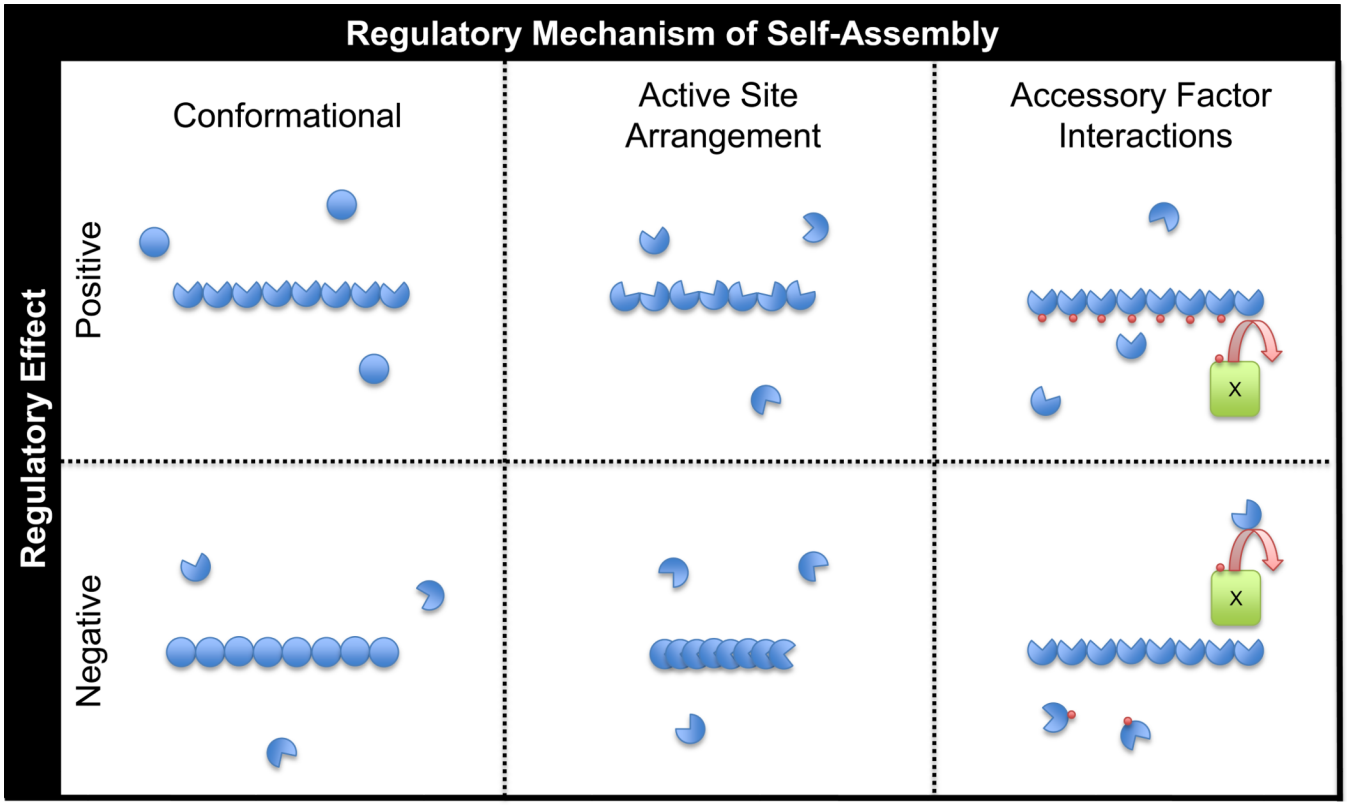

Figure 1.
A table of the generalized mechanisms through which higher-order assembly may control enzymatic activity. In the first situation, the conformation of enzymes in filaments may stabilize or destabilize the active form. Second, the filament may assemble active sites in relation to one another such that they promote or inhibit substrate binding. Finally, filaments may make enzymes more or less likely to interact with accessory factors that control activity such as kinases or phosphatases.

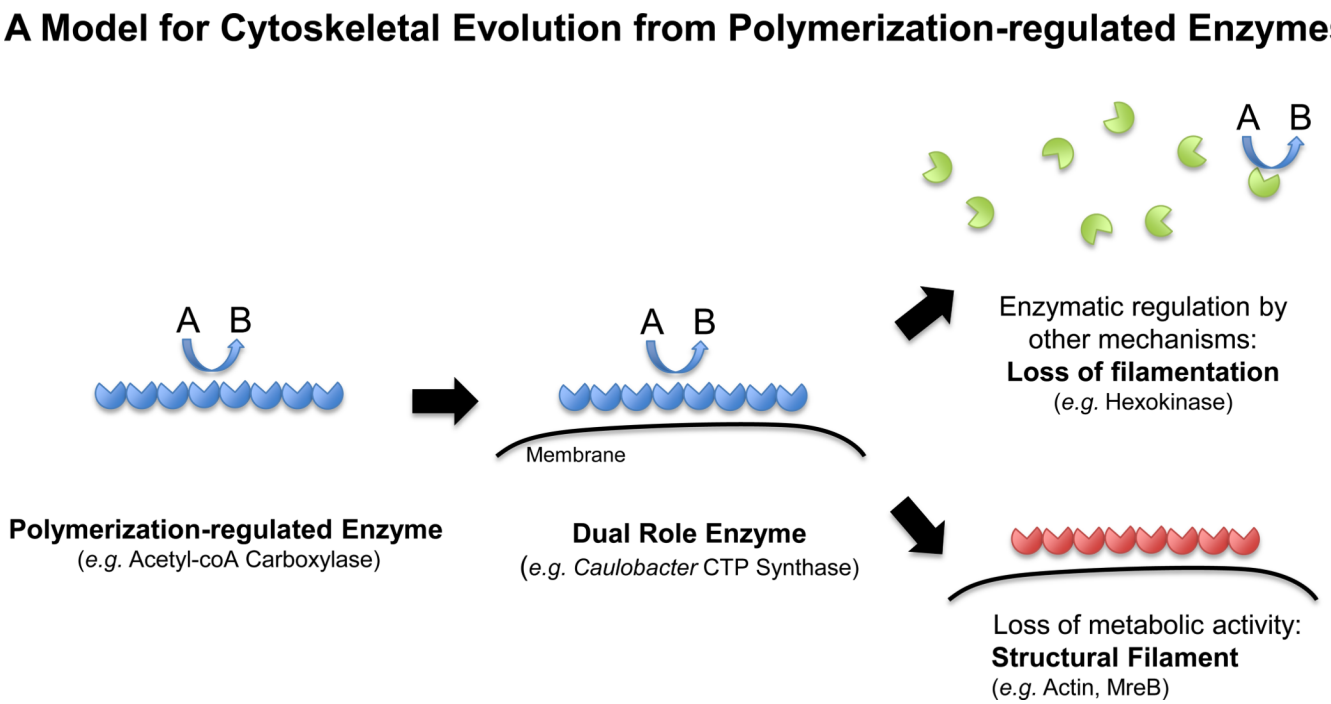

Figure 2.
A model of cytoskeletal evolution with modern examples of each step. The first event in this process is the evolution of self-assembly as an enzymatic regulatory mechanism. Polymerizing filaments can then be repurposed as dual-role enzymes with both enzymatic and structural functions (such as membrane association, shown here). Eventually dual-role enzymes may have diverged into cytoskeletal filaments that lost catalytic function (bottom) or active enzymes whose regulation by higher-order assembly was replaced by other regulatory mechanisms (top).