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Actin variant expression defines filament identity

Differential actin-binding protein interactions drive specific network formation

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Affidavit

I, undersigned, Micaela Boiero Sanders, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific direction of Alphée Michelot, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.

This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.

Fait à Marseille, le 19 janvier 2021.

Abstract

The actin cytoskeleton is a major player in many essential cellular processes, such as cell migration, cell division and endocytosis. A central question in the field is to explain the ability of the actin molecule to assemble into different structures and perform such a variety of functions. From careful genome analysis of eukaryotes, it appears that species might have evolved different strategies to achieve this objective. Some species, including animals and fungi, have few cytoplasmic actins but acquired a peculiar family of actin-binding proteins called tropomyosin. Tropomyosins can wrap around actin filaments, modify their biochemical properties, and could therefore differentiate all the filaments of the cell. Other species, such as plants, do not express tropomyosins but possess a high number of actin isoforms that arose through gene duplication. Actin isoforms are reported to segregate spatially within the cytoplasm of various eukaryotic cells. However, the physiological consequences of using different actin isoforms and the molecular mechanisms by which highly conserved actin isoforms are segregated into distinct networks, are poorly known. I was mainly focused on the molecular mechanisms that could lead to this segregation in different structures for specialized functions.

Because of the complexity of this question in higher eukaryotes, I have used a simple biological system, which is budding yeast, composed of two well-defined actin networks (branched and linear). This choice may seem surprising at first glance, as this system expresses a single actin and a limited set of actin-binding proteins. However, I will show that it is a powerful way to understand its reaction to actin genetic perturbations. I created a library of actin sequences that I replaced in yeast cells to observe the consequences in actin expression, cell viability and actin network organization. I analyzed the contribution of the actin intron, nucleotide sequence, amino acid sequence and of dual expression of actins in the same cell. To gain more insight in the molecular mechanisms at play, I reconstituted the assembly of these actin structures *in vitro* from the same purified proteins.

I demonstrated that few silent mutations in the actin gene are sufficient to modulate the level of actin expression, leading to cell growth defects below a certain threshold. At the amino acid level, I showed that small variations in the actin molecule are sufficient to induce a global reorganization of the actin cytoskeleton. While some actin orthologs assembled preferentially into branched networks, others assembled preferentially into linear networks. Biased assembly into a particular network is a consequence of defective interactions with actin-binding proteins, which inhibits assembly of the other pathway. Interestingly, expression of two heterologous actin variants, each specialized in assembling a different network, rescues cytoskeletal organization, proving the possibility to separate actin functions in yeast using two distinct actin isoforms. Strains expressing two actins are also more resistant to CK-666, an inhibitor of branched-network assembly. This observation suggests that while species using a unique actin have homeostatic actin networks, species using several actin isoforms assemble more independent actin networks.

These findings highlight the fact that despite a remarkably high conservation of actin proteins across species, they retain enough differences that cells expressing multiple isoforms could exploit to segregate them into diverse actin networks.

Résumé

Le cytosquelette d'actine est un acteur majeur dans de nombreux processus cellulaires essentiels, tels que la migration des cellules, la division cellulaire et l'endocytose. Une question centrale dans ce domaine est de comprendre comment la molécule d'actine s'assemble en différentes structures pour effectuer une telle variété de fonctions. L'analyse minutieuse des génomes eucaryotes indique que les espèces pourraient avoir élaboré différentes stratégies pour atteindre cet objectif. Certaines espèces, notamment les animaux et les champignons, expriment un nombre limité d'actines cytoplasmiques mais ont acquis une famille particulière de protéines de liaison à l'actine appelée tropomyosine. Les tropomyosines co-polymérisent autour des filaments d'actine, modifiant leurs propriétés biochimiques et différenciant ainsi les filaments de la cellule. D'autres espèces, par exemple les plantes, n'expriment pas les tropomyosines, mais possèdent en revanche un grand nombre d'isoformes d'actine issues de duplications de gènes. L'étude de ces isoformes a montré que celles-ci sont capables de s'assembler en réseaux spatialement distincts dans le cytoplasme de différentes cellules eucaryotes. Néanmoins, les conséquences physiologiques de l'utilisation de ces différentes isoformes d'actine, et les mécanismes moléculaires grâce auxquels ces isoformes d'actine, hautement conservées, peuvent être ségrégées spatialement sont peu connus. Au cours de ma thèse, je me suis principalement concentrée sur les mécanismes moléculaires qui pourraient conduire à cette ségrégation.

En raison de la complexité de cette question chez les eucaryotes supérieurs, j'ai utilisé un système biologique simple, à savoir la levure bourgeonnante, qui est composée seulement de deux réseaux d'actine bien définis (branchés et linéaires). Ce choix peut sembler surprenant à première vue, car ce système exprime une seule actine et un ensemble limité de protéines de liaison à l'actine. Cependant, je montrerai que c'est un outil puissant pour comprendre les effets de perturbations génétiques de l'actine. J'ai créé une bibliothèque de séquences d'actine que j'ai utilisé pour remplacer le gène d'actine sauvage dans des cellules de levure. Puis, j'ai observé les conséquences de ces perturbations sur l'expression de l'actine, la prolifération cellulaire et l'organisation du cytosquelette d'actine. J'ai analysé la contribution de l'intron d'actine, l'importance d'une conservation de la séquence nucléotidique, de l'expression d'actines hétérologues, et l'effet de l'expression de deux actines dans la même cellule. Pour mieux comprendre les mécanismes moléculaires en jeu, j'ai aussi reconstitué l'assemblage de ces structures d'actine in vitro à partir des protéines purifiées.

J'ai démontré qu'un faible nombre de mutations silencieuses dans le gène de l'actine est suffisant pour changer le niveau d'expression de celle-ci, entraînant des défauts de croissance cellulaire en dessous d'un certain seuil. En exprimant des actines hétérologues, j'ai démontré que de petites variations dans la molécule d'actine sont suffisantes pour induire une réorganisation globale du cytosquelette. Alors que certains orthologues d'actine s'assemblent préférentiellement en réseaux branchés, d'autres s'assemblent préférentiellement en réseaux linéaires. Ce biais pour un réseau particulier est la conséquence d'interactions défectueuses avec une ou plusieurs protéines de liaison à l'actine, ce qui inhibe l'assemblage par l'autre voie. Il est intéressant de noter que l'expression de deux variants d'actine hétérologues, chacun spécialisé dans l'assemblage d'un réseau différent, sauve l'organisation du cytosquelette, prouvant la possibilité de séparer les fonctions de l'actine dans la levure en utilisant deux isoformes d'actine distinctes. Les souches exprimant deux actines sont également plus résistantes à la drogue CK-666, un inhibiteur de l'assemblage des réseaux branchés. Cette observation suggère que si les espèces utilisant une actine unique ont des réseaux d'actine homéostatiques, les espèces utilisant plusieurs isoformes d'actine pourraient assembler des réseaux d'actine plus indépendants.

Ces résultats soulignent enfin le fait que, malgré une conservation remarquablement forte de l'actine chez les eucaryotes, ces isoformes conservent suffisamment de différences pour que des cellules en exprimant plusieurs puissent exploiter ces différences pour les séparer en réseaux d'actines distincts.

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PREFACE

Life requires movement, life is movement. A phrase coined by Aristotle in the 4th century BC and a slogan adopted countless times since. And indeed, for a lot of species this relationship is evident. While a rock cannot move by itself, animals can walk, run and exercise. Even plants, which seem to not move if we look at them for short periods of time, can grow and change shape over days/weeks. Although if we speak about plant moment, probably the first that comes to mind is the seismonastic movement of the *Mimosa pudica* leaves in response to touch. And for all species, even if we see them or we do not see them move, movement is occurring at the cellular scale as well. Cells can migrate by assembling different structures, e.g., a protrusion of the membrane or the creation of "cellular feet". Cells can also divide, a process which requires drastic changes in cell shape. And even in cells that are not actively migrating, if we look inside the cell, we will see movement. Some organelles such as mitochondria, the energy-producing organelle, can be transported across the cytoplasm. Vesicles and mRNA also get transported from here to there inside the cells. All these processes are very diverse and occur at completely different scales. All the mentioned processes, from muscle movement to vesicle transport, have a core element in common: The cytoskeleton. The cytoskeleton, the "skeleton of the cell", is composed of, among others, actin filaments. Actin has the capability to attach to itself (to polymerize) and form filaments. Even if it's called skeleton, these structures are not static at all. On the contrary, it is highly dynamic and undergoes constant remodeling. Actin or actin-like proteins are present in all known cells and are essential for their survival. Moreover, the protein sequence of actin is highly conserved, highlighting its vital role. How did actin evolve? What are the differences between the actin molecules of different species? And in between actins of the same species? How can actin perform so many functions? Can different actins specialize into different functions? These are some of the questions that I will address during the course of my thesis.

INTRODUCTION

1. Origins and evolution

Thinking about the creation of the Earth and the origins of life is enthralling. How and when was the Earth formed? When did life appear? The Earth formed between 4500 and 5000 million years ago (Fig. 1.1A) (Patterson, 1956). This number represents so many human generations that it seems impossible to fully understand how much time it means from our perspective. It is not much different for the beginning of life, which is thought to have happened around 4000 million years ago (Dodd *et al*., 2017). Today, the classification of all forms of life is structured in three domains: Archaea, Bacteria and Eukaryota (Fig. 1.1B). There is some discussion of when these domains separated. The current consensus in the field is that Bacteria and Archaea diverged first and then Eukaryotic life appeared, some say 2800 million years ago, others say 1500 million years ago (Knoll *et al*., 2006; Glansdorff *et al*., 2008). Regardless of when it happened, one thing is clear: it happened a long, long time ago. During all this time, cells belonging to all domains had time to evolve and diversify into the species that exist today. Genomic DNA was arranged during evolution and proteins changed their shape and gained specific tridimensional structures as part of the cells' adaptation to their surrounding environment. Proteins had time to mutate, specialize, change their function and even generate novel functions. It would then be normal to think that most of the proteins are specific to a certain group of species. However, some proteins are surprisingly conserved across very divergent domains. Such is the case for some of the cytoskeletal proteins, which can be found in all three domains.

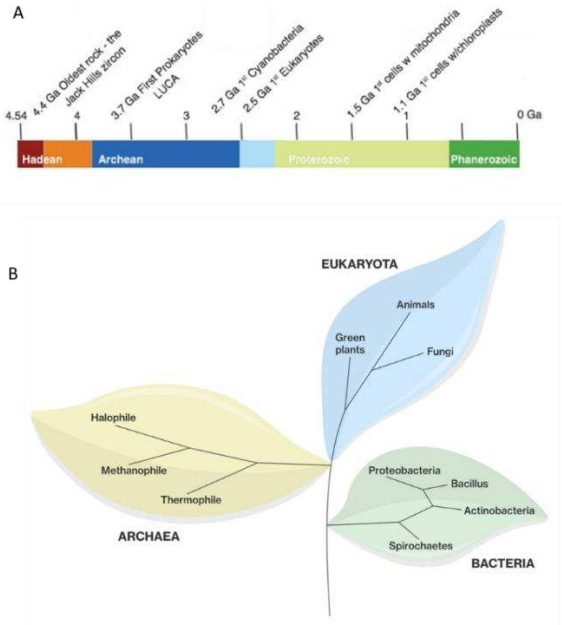


Fig. 1.1. Appearance of life.
(A) Timeline of the Earth. **(B)** The three domains of life, Eukaryota, Archaea and Bacteria.

2. The cytoskeleton

The cytoskeleton is found in the cell cytoplasm and it is composed of several proteins that form a rigid structure. Each element of the cytoskeleton is composed of protein subunits that are able to bind to other subunits, to polymerize, and create a chain or filament of different diameters and bending capacities (Fig. 2.1). Cytoskeleton means "skeleton of the cell" but contrary to the vertebrate skeleton that is composed of rigid bones that do not change much over short periods of time, the proteins that compose the cytoskeleton are highly dynamic. The cytoskeleton is involved in key cellular processes, mainly processes which involve movement, generation of forces, transport and/or structural support.

The eukaryote cytoskeleton is composed of three main elements: microtubules, intermediate filaments and actin microfilaments (Fig. 2.2A). Most of these elements are well conserved across all eukaryotes. Depending on the cell type, each of these components has a different relative contribution to the overall structure and functions. We can find related proteins in bacteria as well. For decades, it was believed that Bacteria and Archaea did not possess a cytoskeleton, although this view changed in the early 90s with the first discovery of a bacterial cytoskeletal protein (Bi and Lutkenhaus, 1991). Nowadays it is well accepted that bacteria and archaea have cytoskeletal proteins, mainly actin and tubulin homologs, although there is at least one case, the bacteria *Caulobacter crescentus*, that has a protein that resembles the intermediate filaments (Fig. 2.2B-C). The study of the bacterial cytoskeleton then gave insight on the evolution of eukaryotic life (Ettema *et al*., 2011; Izoré *et al*., 2016). In the next sections I will introduce these three components of the cytoskeleton as well as briefly discussing their non-eukaryotic homologs, which I will then supplement with a more in-depth description of the actin cytoskeleton.

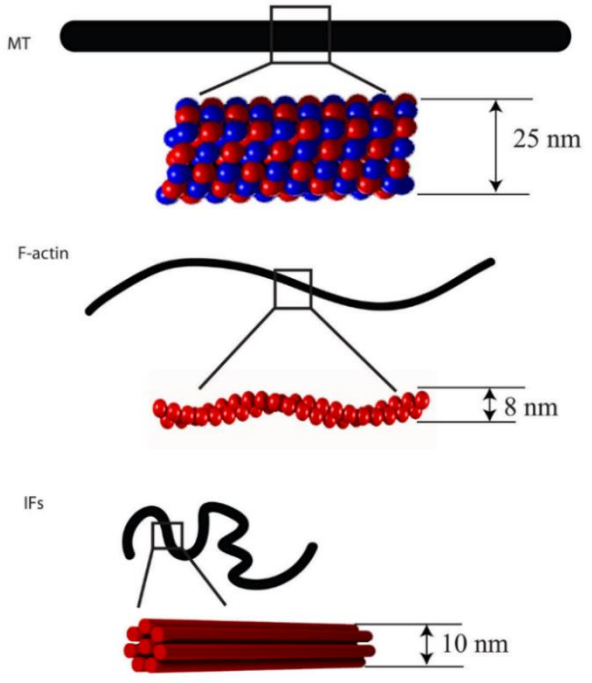


Fig. 2.1. Components of the cytoskeleton.
Comparison of the filament structure and persistence length for microtubules (top, MT), actin (middle, F-Actin) and intermediate filaments (bottom, IFs). Modified from (Wen and Janmey, 2011).

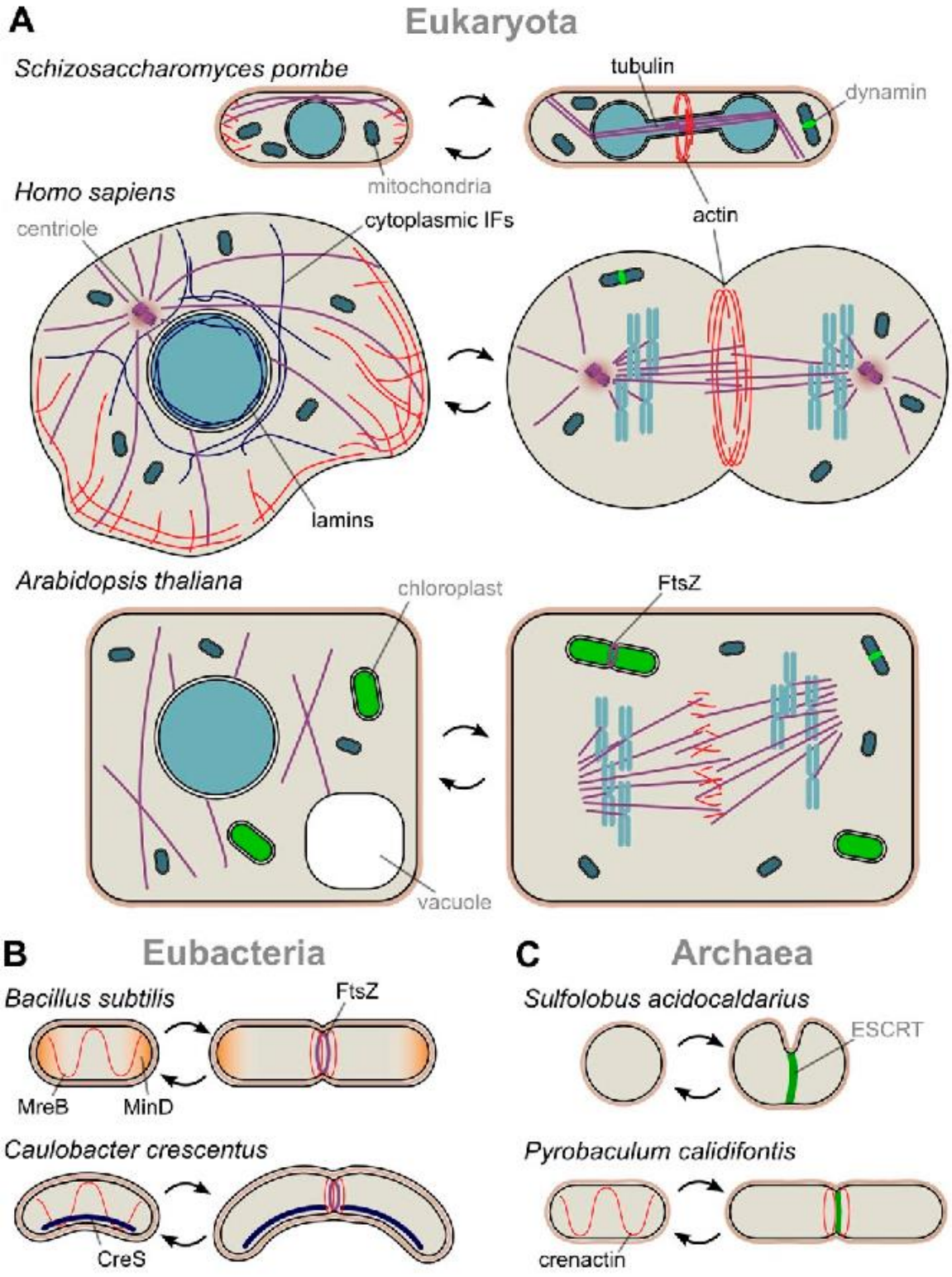


Fig. 2.2. Cytoskeletal structures in different cell types.
Schematic representations of the cytoskeleton for organisms of the different domains of life: Eukaryota **(A)**, Eubacteria **(B)** and Archaea **(C)**. For each cell type, a dividing and a non-dividing cell is shown. The actin cytoskeleton and the bacterial homologs of actin are depicted in red, tubulin and the bacterial homologs of tubulin are drawn in purple, and the intermediate filaments and the bacterial homolog CreS are drawn in blue. Modified from (Wickstead and Gull, 2011).

2.1 Microtubules

Microtubules are hollow cylinders with a diameter of 23-27 nm (Ledbetter and Porter, 1963) (Fig. 2.3A). They are stiffer than the other components of the cytoskeleton and their persistence length (the persistence length is a measurement of the stiffness of the polymer, it is related to how well the position of one section of a polymer is correlated to another section) is around 5 mm, which is higher than the persistence length of both actin and intermediate filaments (Fig. 2.11 A) (Pallavicini *et al*., 2014). This indicates that inside the cells, microtubules behave like rigid rods. Microtubules are dynamic structures, they can polymerize and also shrink. These polymers are composed of protofilaments formed by a dimer containing two subunits, α- and β-tubulin (Fig. 2.3A-C) (Nogales *et al*., 1998b). Up to thirteen protofilaments can interact laterally to give rise to the cylindrical structure of the microtubules. This cylinder is polarized, which means that the two ends are not equivalent. Indeed, due to the vertical orientation of the tubulins in the protofilament, one end of the microtubule possesses β-tubulin (plus end) and the other end possesses α-tubulin (minus end). Polymerization is significantly faster at the plus end, which leads to microtubule growth mainly from the plus end. Tubulins composing the dimer are bound to Guanosine-5'-triphosphate (GTP) which is a molecule composed of a nitrogenous base, a 5-carbon sugar and 3 phosphate groups bound to the sugar. The pyrophosphate bonds can be hydrolyzed in a catabolic reaction that releases energy to produce guanosine diphosphate (GDP) and inorganic phosphate (Pi). At some point after addition of a dimer in the microtubule, the GTP in the β-tubulin is hydrolyzed to GDP. The dimer composed of α-tubulin-GTP and β-tubulin-GDP is less stable and more prone to depolymerization. If the hydrolysis of the β-tubulin molecules reaches the beginning of the plus end the microtubule can rapidly depolymerize, a process called dynamic instability or catastrophe (Fig. 2.3B) (Mitchison and Kirschner, 1984; Cassimeris *et al*., 1987; Erickson and O'Brien, 1992). Tubulin is involved in many cellular processes, mainly in DNA segregation during cell division, cell movement such as cilia and flagella and vesicle transport (Mohri, 1976; Hirokawa, 1998; Petry, 2016).

There are several tubulin homologs in bacteria. The first discovered and most common one is FtsZ, which is involved in cell division and is also bound to GTP which can be hydrolyzed (de Boer *et al*., 1992; RayChaudhuri and Park, 1992; Mukherjee *et al*., 1993; Vaughan *et al*., 2004). Surprisingly, FtsZ and mammalian tubulin are very different in sequence, sharing only 10% identity. Nevertheless, they are very similar in terms of structure and even if it does not form microtubules, the protofilaments of FtsZ can present lateral interactions (fig 2.3 C) (Nogales *et al*., 1998a; Wickstead and Gull, 2011). This protein is involved in cell division and present in many bacteria and archaea, although it is not ubiquitous. Other families of tubulin-like proteins exist in bacteria, for example the families of TubZ and RepX, tubA/B and CetZ.

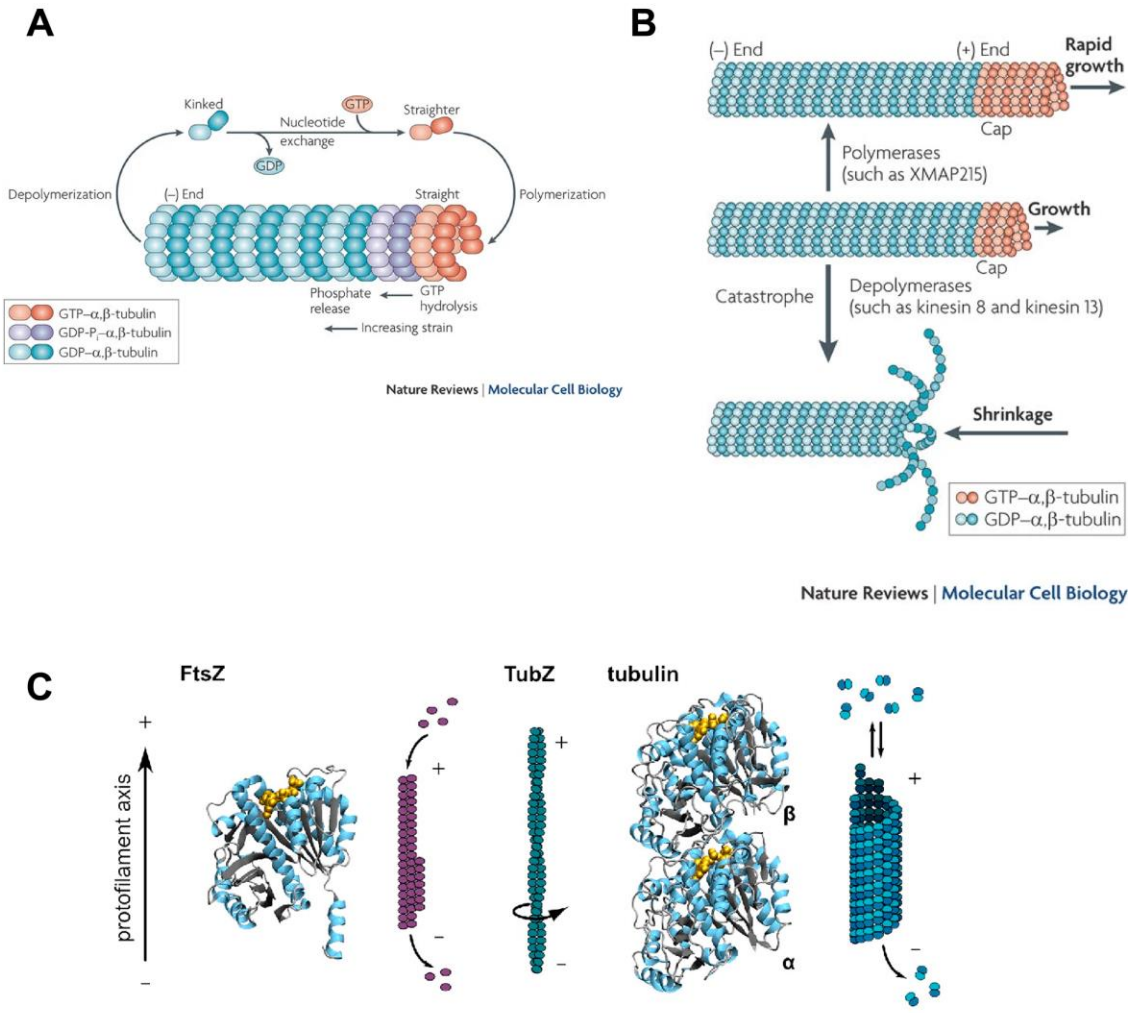


Fig. 2.3. Tubulin and its bacterial homologs, FtsZ and TubZ.
(A) Tubulin turnover cycle. GTP-tubulin subunits are incorporated in the microtubule. After GTP hydrolysis, GDP-tubulin is more prone to depolymerize because it is more strained. **(B)** GTP-tubulin at the growing end of the microtubule form a stabilizing cap (red subunits). If there is fast growth, the microtubule will elongate. If the growth is not fast enough or the cap is lost, the tubulin subunits in the cap will hydrolyze the nucleotide and this will produce a fast shrinkage of the microtubule. **(C)** Homology between eukaryotic tubulin and the bacterial homologs. They are similar in structure and folding, and they all form filaments. Modified from (Howard and Hyman, 2009; Wickstead and Gull, 2011).

2.2 Intermediate Filaments

Intermediate filaments are composed of eight parallel protofilaments that interact by hydrophobic and electrostatic interactions in between strands to make an unpolarized cable (Fig. 2.4) (Kreplak *et al*., 2004; Qin *et al*., 2009). They have an intermediate diameter in between microtubules and actin, of around 10 nm (Fig. 2.1) (Mücke *et al*., 2005). Contrary to other two components of the cytoskeleton, these filaments are less dynamic but much more flexible. Their persistence length is around 1 μm (Fig. 2.1) (Mücke *et al*., 2004). Their ability to withstand tensile and bending stress make them particularly efficient at providing structural support and bearing tension and mechanical stress

(Herrmann *et al*., 2007). They are structural components of the nuclear lamina and also play a cytoplasmic role such as anchoring organelles and forming cell-cell or cell-matrix junctions. Intermediate filaments are expressed in fewer organisms and cell types than the other cytoskeletal components. They are varied can be made out of different components.

Intermediate filaments are found in animals, but not in plants or fungi (Herrmann *et al*., 2009). They are not commonly found in bacteria. Only *Caulobacter crescentus* has a protein called CreS that is similar to the intermediate filament called Lamin A (Fig. 2.2B) (Ausmees *et al*., 2003). The structural similarity between CreS filaments and Lamin A is thought to be due to convergence and not because these filaments were present in the common ancestor (Wickstead and Gull, 2011). This indicates that the intermediate filaments, unlike microtubules or actin, were not found in the common ancestor between bacteria and eukaryotes. Probably they were not even present in the last eukaryotic common ancestor, since they are found only in holozoans out of all the eukaryotes.

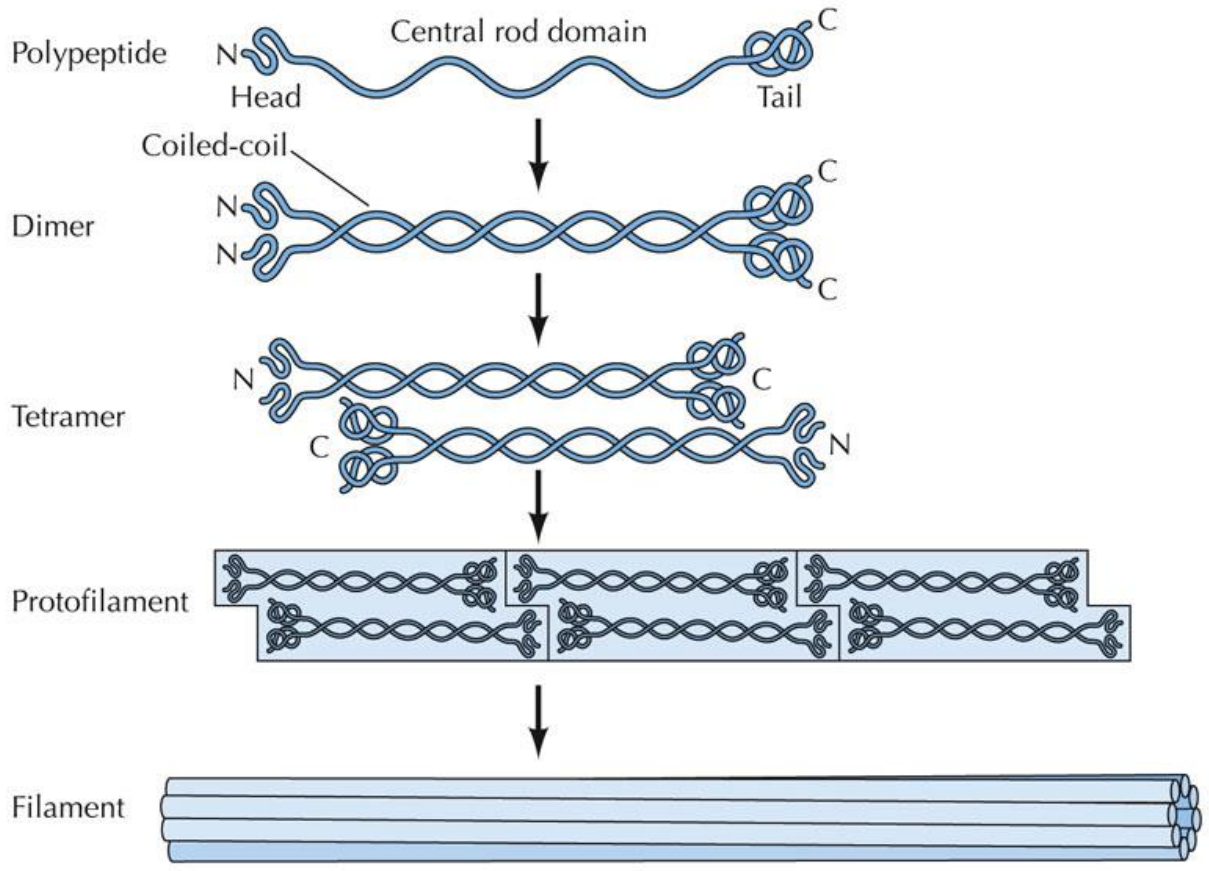


Fig. 2.4. Formation of an intermediate filament.
From (Alberts *et al*., 2002)

2.3 Actin filaments

The actin cytoskeleton, also called microfilaments, is composed of filaments that are formed by two protofilaments that interact to form a double-stranded helix (Fig. 2.5). These filaments have a diameter of 6-8 nm, which is smaller than both the microtubules and intermediate filaments (Fig. 2.1). The stiffness of actin filaments corresponds to an intermediate between the microtubules and the intermediate filaments, and its persistence length is approximately 10 μm (Fig. 2.1) (Liu and Pollack, 2002). This means that actin filaments much shorter than 10 μm can be considered to act as rigid rods while filaments much longer than 10 μm are more flexible and bend due to thermal fluctuations. Because filaments in cells are generally shorter than this value they should act as rigid rods. However, mechanical constrains may reduce the persistence length and bend filament at shorter scales (Harasim *et al*., 2013; Blanchoin *et al*., 2014). Actin assembly plays critical roles in a lot of cell processes, including cell division, migration, endocytosis, and establishment of cell polarity; functions for which it organizes in different structures (Fig. 2.5) (Chhabra and Higgs, 2007; Skau and Waterman, 2015). Actin also plays a central role in muscle contraction, where it is organized in a structure called sarcomere (Szent-Györgyi and Prior, 1966). Actin is able to carry out all these diverse functions thanks to its unique biochemical properties, binding partners and structures it can form.

Actin is the most abundant protein in eukaryotic cells. In line with to its role in many essential cellular processes, actin is found in all eukaryotic cells and its amino acid sequence is astonishingly conserved across all species. Species as different as *H. sapiens* (humans) and *S. cerevisiae* (baker's yeast) have actins which amino acid sequences share 89% of identity. Several types of actin-like molecules have been found in prokaryotic cells, further supporting its indispensable role during evolution. Actin homologs can also be found in bacteria and they comprise a large family of more than 35 families of actin-like proteins (Derman *et al*., 2009). The most common actin relative is called MreB. This protein is not so similar to actin in terms of amino acid sequence (they share only 15% of sequence identity), but it has a very similar structure (Fig. 2.6A). When it polymerizes, MreB can form a filament composed of two protofilaments, similarly to actin, but they lack the helical twist. Another example of a bacterial actin-like protein is ParM. ParM does not share a high identity in sequence with neither eukaryotic actin not MreB, but it can form twisted polymers similarly to actin (Fig. 2.6A). Expanding our search into other domains, crenactin is an archaeal actin which is a key cytoskeletal component of the archaeon *Pyrobaculum calidifontis*. It only shares around 20% of sequence identity with the eukaryotic counterpart, but it is believed to be closely related to actin and the origin of the eukaryotic cytoskeleton (Ettema *et al*., 2011; Merino and Raunser, 2016). Moreover, crenactin polymerizes into double helical filaments that are exceptionally similar to eukaryotic F-actin and the monomeric form has a structure that is very similar to actin monomers (Fig. 2.6B-C) (Izoré *et al*., 2016).

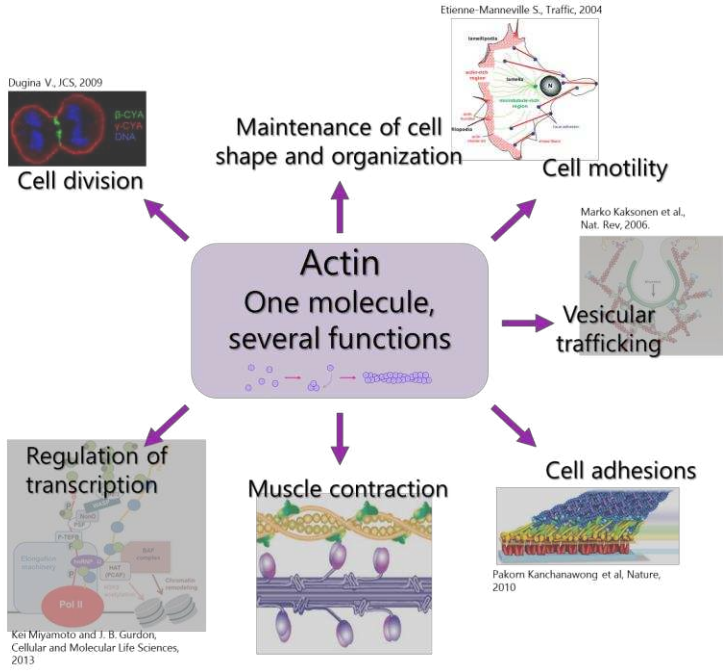
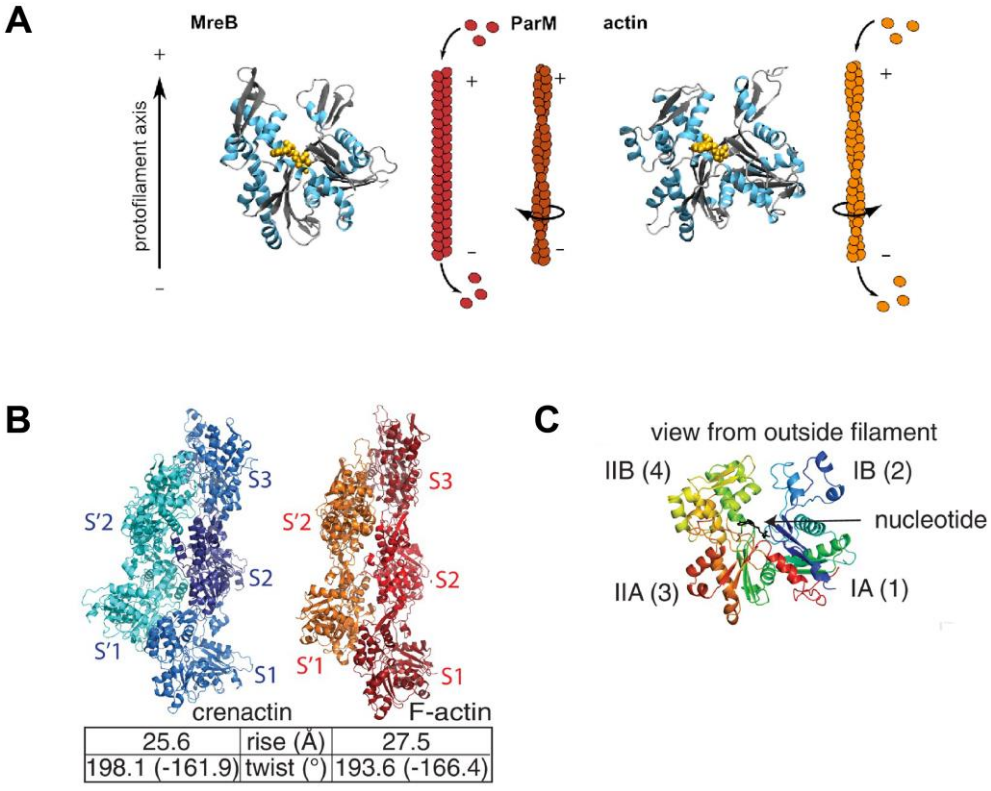


Fig. 2.5. The actin molecule is involved in many cellular functions.



25.6	rise (Å)	27.5
198.1 (-161.9)	twist (°)	193.6 (-166.4)

Fig. 2.6. The actin molecule and its homologs.

(A) Actin and its bacterial homologs, MreB and ParM, have a similar structure and can form filaments. **(B)** Comparison of the structures of crenactin filaments (blue and light blue) and actin filaments (red and orange). They are not only similar in structure but also in the helical parameters. **(C)** Structure of the crenactin subunit in the filament. It highly resembles the actin subunit. Modified from (Wickstead and Gull, 2011; Izoré *et al*., 2016; von der Ecken *et al*., 2015).

3. Structure and biochemical properties of actin

3.1 Actin Monomers

The actin molecule was first identified in 1942 by Straub F.B from rabbit skeletal muscle (Straub, 1942). It is a globular protein composed of approximately 375 amino acids. It has a molecular weight of 42 kDa and a diameter of 55 Å (Dominguez and Holmes, 2011). The structure of actin was first determined in 1990 by Kabsch *et al*. (Fig. 3.1A) (Kabsch *et al*., 1990). In this study, actin was crystalized bound to the bovine pancreatic deoxyribonuclease I (DNase I) which prevents the polymerization of actin. Since then, multiple studies have crystallized actin from different species coupled with other proteins, or bound to small molecules (Schutt *et al*., 1993; McLaughlin *et al*., 1993; Robinson *et al*., 1999). It is interesting to note that all these studies report a very similar structure.

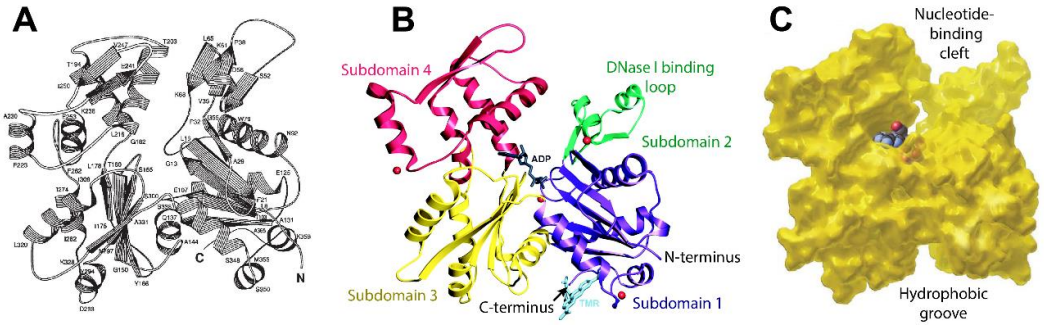


Fig. 3.1. Representations of the actin molecule.

(A) The first determination of the actin structure, done in 1990 by Kabsch *et al.* **(B)** Subdomains of the actin molecule are colored differently. The C- and N-terminal ends are found in subdomain 1 (blue) and the DNase I binding loop is found in subdomain 2 (green). **(C)** Surface representation of the actin molecular (yellow). The nucleotide can be easily seen in the middle of the molecule. Modified from (Kabsch *et al*., 1990; Otterbein *et al*., 2001; Pollard, 2016).

Actin is composed of one single amino acid chain that folds giving rise to 2 domains that are in turn subdivided in 2 other subdomains each (Fig. 3.1B) (Dominguez and Holmes, 2011). Both the N-terminal end and the C-terminal end of the protein are located in subdomain 1. The two main domains are connected by a linker helix that acts as a hinge. Two residues located in the hinge are critical for the proper folding of actin, which is assisted by the chaperonin containing TCP-1 (CCT) (McCormack *et al*., 2001; Willison, 2018). There is little contact surface between these domains, which results in the formation of a cleft on the upper side of the hinge and a groove on the lower side (Fig. 3.1C). The upper cleft is located between subdomains 2 and 4 and contains two binding sites: one for a nucleotide and one for a divalent cation. Adenosine triphosphate (ATP) binds this site, where it can be hydrolyzed to adenosine diphosphate (ADP). In cells, actin is normally bound to the divalent cation Mg^{2+}, although purified actin can also bind Ca^{2+}. The lower groove is located between subdomains 1 and 3 and is composed of hydrophobic residues.

This area is involved in the interaction with many proteins, including contacts between actin subunits in the filament and interaction with actin binding proteins (ABPs).

3.2 Actin Filaments

As all the elements of the cytoskeleton, actin is capable of forming polymers. In cells, actin can be found in either its monomeric (G-Actin) or filamentous form (F-Actin).

The atomic model of F-Actin was proposed at the time the first actin structure was published (Fig. 3.2A) and it corresponds to a double-stranded right-handed helix where each monomer is rotated by 166º (Depue Jr. and Rice, 1965; Holmes *et al*., 1990). In fact, the symmetry of the filament as a single unit is a single left-handed helix with a repetition of approximately 13 molecules every six turns in an axial distance of 35.9 nm (Dominguez and Holmes, 2011). Contrary to G-Actin, the filamentous form for actin cannot be grown in 3D crystals. For this reason, X-ray crystallography could not be used as a technique to provide an atomic description of the filament. The first models of the actin filament were based from low resolution X-ray fiber diffraction data (Holmes *et al*., 1990; Lorenz *et al*., 1993; Tirion *et al*., 1995; Holmes *et al*., 2003; Wu and Ma, 2004). Even though this technique has many limitations, it was possible to obtain some details about the structural changes of actin upon polymerization (Oda *et al*., 2009). Over the years, the use of electron cryomicroscopy (cyoEM) to obtain structural information highly increased, due to the improvement of the technique itself, the image analysis methods and mainly the sensitivity of the detectors. The first studies were carried out from low- or medium-resolution cryoEM maps, but the most recent studies achieved an incredibly high level of resolution, describing the actin filament and its interaction with ABPs at near-atomic resolution (Holmes *et al*., 1990; Fujii *et al*., 2010; Murakami *et al*., 2010; Galkin *et al*., 2010; Fujii *et al*., 2010; Merino *et al*., 2018; Pospich *et al*., 2020). This led to a description of the structure of F-actin with really high resolution (Fig. 3.2B).

The double-stranded actin filaments, as microtubules, are polarized, with the two ends having different properties (Fig. 3.2C). Indeed, actin monomers in a filament are always oriented in the same direction, with subdomains 1 and 3 found at the end which is called the barbed end, fast-growing end or plus end. Subdomains 2 and 4 are found at the other end, which is called pointed end, slow-growing end or minus end.

The filament is stabilized by intra-strand interactions (interactions between actin subunits on the same strand) and inter-strand interactions (interactions between actin subunits that are in different strands) (Fig. 3.2D) (Holmes *et al*., 1990; Oda *et al*., 2009). Each monomer in the filament can interact axially with two monomers of the same strand and laterally with two monomers from the other strand (Fujii *et al*., 2010). Intra-strand interactions are the strongest and consist of electrostatic and hydrophobic interactions. Axial or intra-strand interactions consist mainly of two interactions: 1) interactions between subdomain 3 of a monomer and subdomain 4 of the monomer below, and 2) the interaction of the D-

loop with domains 1 and 3 of the subunit above. Lateral contacts or intra-strand interactions include a plug-like insertion of the D-loop in subdomain 3 which stabilizes the helix, and another interaction between subdomain 4 of one monomer and subdomains 1 and 3 of a monomer in the other strand which is located just above the plug.

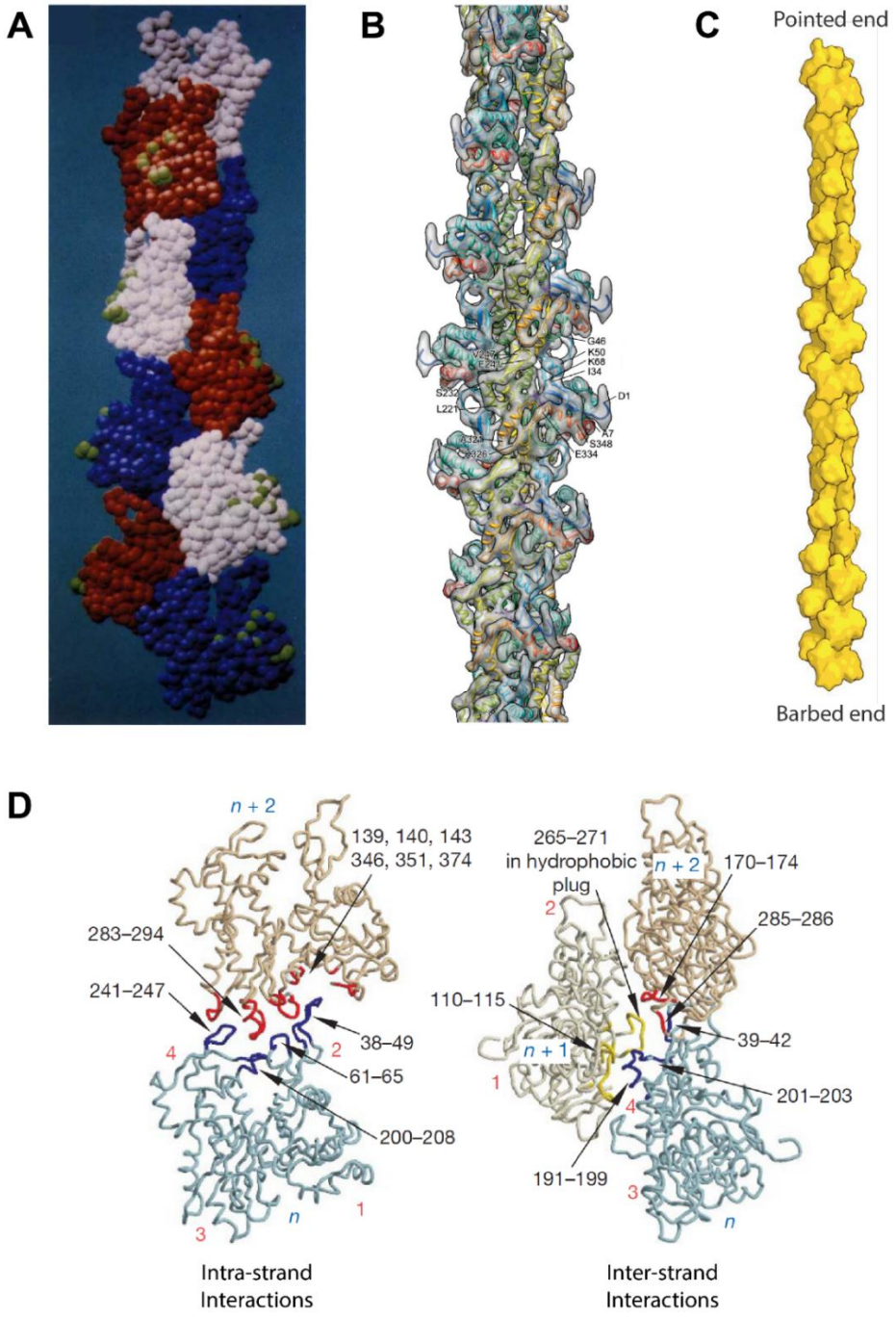


Fig. 3.2. Structure and contacts of the actin filament.

(A) First published atomic model of the actin filament. Actin monomers in the filament are in colors white, blue and red. **(B)** High-resolution atomic model. It corresponds to approximately fifteen actin subunits. **(C)** Cartoon of the filament showing the position of the pointed end and the barbed end. **(D)** Residues involved in the intra-strand (left) and inter-strand (right) contacts are specified. Figures modified from (Holmes *et al.*, 1990; Fujii *et al.*, 2010; Pollard, 2016; Oda *et al.*, 2009).

The two binding sites in the actin molecule, the nucleotide and the divalent cation binding sites, are also important for the properties of the actin filament. For example, the conformation of some loops in the actin subunit, such as the DNase binding loop, depend on the bound nucleotide and it can also modify the binding of other proteins to the filament (Fig. 3.3) (Merino *et al*., 2018). The bound cation can also affect the properties of the actin. For example, actin bound to Mg^{2+} polymerizes faster than Ca^{2+} actin and it also presents a higher ATP hydrolysis rate (Cooper *et al*., 1983; Frieden, 1983; Blanchoin and Pollard, 2002).

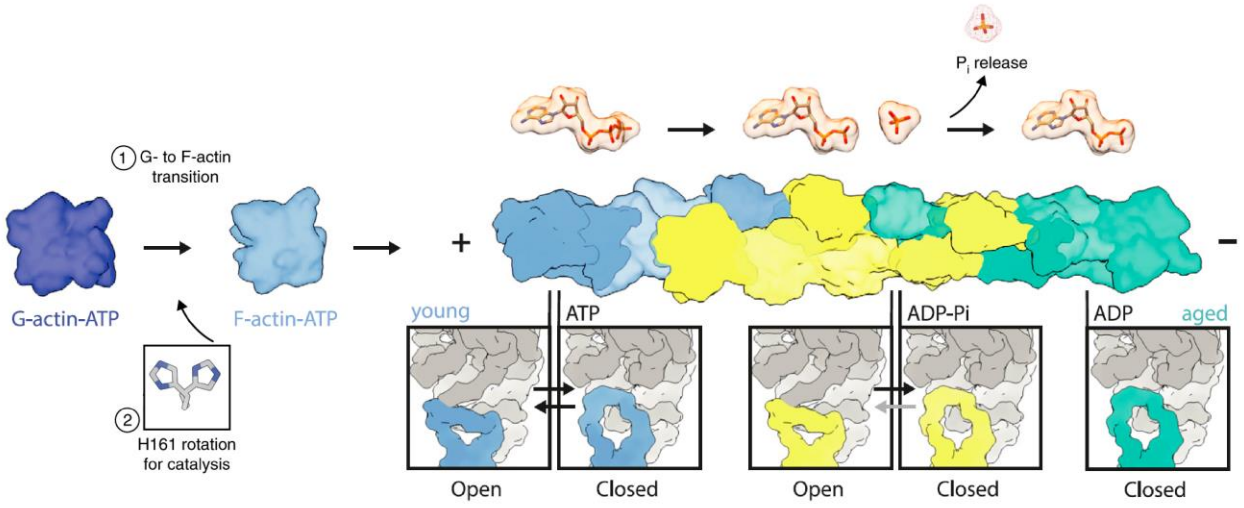


Fig. 3.3. Actin polymerization and ATP hydrolysis in the actin filament.
G-actin-ATP gets incorporated into the filament and undergoes a conformational change. ATP can be hydrolyzed, and after Pi release, the DNAse loop in the actin subunit is locked in the closed state. Modified from (Merino *et al*., 2018)

4. Actin dynamics

4.1 Actin polymerization *in vitro*

The formation of actin filaments *in vitro* is a dynamic process. The reaction is described in three phases: nucleation, elongation and a final steady state (Fig. 4.1A-B).

The nucleation is the limiting step in this reaction and it's a very slow process. First, actin monomers that are bound to ATP and Mg^{2+} undergo a conformational change that is essential for Mg^{2+}-induced polymerization (Frieden and Patane, 1985). This conformational change does not occur for ADP-monomers which nucleate even slower. Nucleation begins with the formation of a dimer of two actin subunits, which can then bind a third one to form a trimer. These steps are slow because even though the binding reactions to form dimers and trimers are fast, the products are very unstable and fall apart rapidly. This makes the formation of the trimer the limiting step in this reaction (Sept and McCammon, 2001). With the addition of the fourth monomer a lot of interactions between the subunits can be formed and the structure becomes stable.

The elongation step is the progressive addition of monomers to the filament after it has reached the trimer. The elongation of the actin filament is a well-studied process. As described in the previous section, the filament is polarized and has two ends: the barbed end and the pointed end. Actin subunits can be added at both of these ends and as well both ends can depolymerize, losing actin subunits (Fig. 4.1C). Since the biochemical properties of both ends are not the same, the dissociation and association constants of actin monomers for both ends are different. These constants also depend on the nucleotide state of the monomers/subunits and on the concentration of the different species (Wegner and Engel, 1975). Association of the ATP-Actin monomer is favored at the barbed end which elongates faster than the pointed end. The association rate of ATP-Actin to barbed end is limited by the diffusion of the monomer and has a value of 10 $\mu m^{-1}s^{-1}$, whereas the dissociation is very slow (1 s^{-1}).

As the filaments grow in length, the concentration of actin monomers decreases. At a certain point, the system will reach a steady state. At the steady state the concentration of monomeric actin remains stable and it is called critical concentration (C_c) which under standard in vitro conditions has a value of approximately 0.1 μM. When this point is reached, the polymerization at the barbed end is favored while at the pointed end the depolymerization is favored. This means that even though the filaments do not change their length, addition of monomers is constantly happening at one end while the other end is losing monomers at the same rate. (Fig. 4.1B). This phenomenon is called treadmilling of actin.

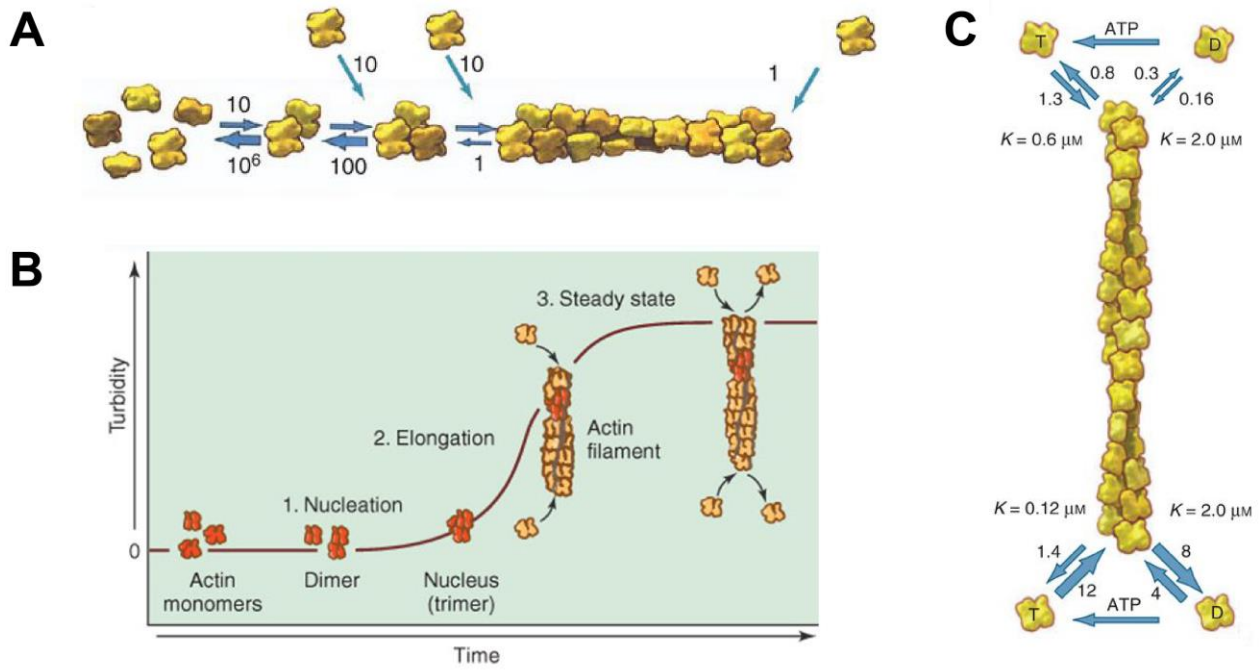


Fig. 4.1. Actin polymerization.

(A) Actin nucleation is not favorable until the trimer state. Once the trimer is formed, the filament can elongate. The numbers represent the estimates of rate constant for each step. Unit of association rate constants, μm^{-1} sec^{-1}; unit of dissociation rate constants, sec^{-1}. **(B)** The three stages of spontaneous actin polymerization: nucleation, elongation and steady state. **(C)** Dissociation and association constants for ATP- and ADP-monomers at both ends of the actin filament. Unit of association rate constants, μm^{-1} sec^{-1}; unit of dissociation rate constants, sec^{-1}. K corresponds to the critical concentration for each reaction. Modified from (Pollard, 2016) and Jones & Bartiett Learning.

4.2 Actin turnover

In physiological conditions, ATP-G-Actin is incorporated into the filaments at the barbed end. Few seconds after polymerization, the ATP is hydrolyzed in ADP and inorganic phosphate (Pi) (half-time of 2 seconds) (Pollard, 2016). Actin subunits can stay in their ADP+Pi form which has similar properties than those of the ATP-subunits. The Pi can later be released and the subunit will become an ADP-subunit. Once the actin molecule is incorporated in the filament, the nucleotide binding pocket remains inside the structure which means that ADP within the actin filament cannot exchange ADP for ATP (De La Cruz *et al*., 2000). This creates a gradient along the actin filament in which the newer subunits are found in the ATP-state whereas the older subunits are found in the ADP-state, showing somehow the "age" of the filament. The hydrolysis of ATP is believed to occur randomly across the filament which means that there are no discrete ATP, ADP+Pi and ADP areas in the filament (Jégou *et al*., 2011). The gradient is probably created by the fact that older subunits have a higher probability of hydrolyzing the ATP because of their lifetime inside the filament. Moreover, ADP-subunits in the filament are less stable and prone to disassemble. The state of the nucleotide bound to the subunit is related to conformational changes, for example the stabilization of the D-loop in the closed

conformation mentioned in section 3.2 (p. 10, Fig. 3.3) (Merino *et al*., 2018). The nucleotide binding pocket is accessible in G-actin, meaning that ADP-G-actin in solution can exchange the nucleotide back to ATP in a process that we call recycling. The ATP-monomers can get re-incorporated in the filament and this cycle is called "actin turnover" (Fig. 4.2). The nucleotide binding pocket is accessible in the G-Actin molecule, meaning that the nucleotide can be recycled in G-Actin.

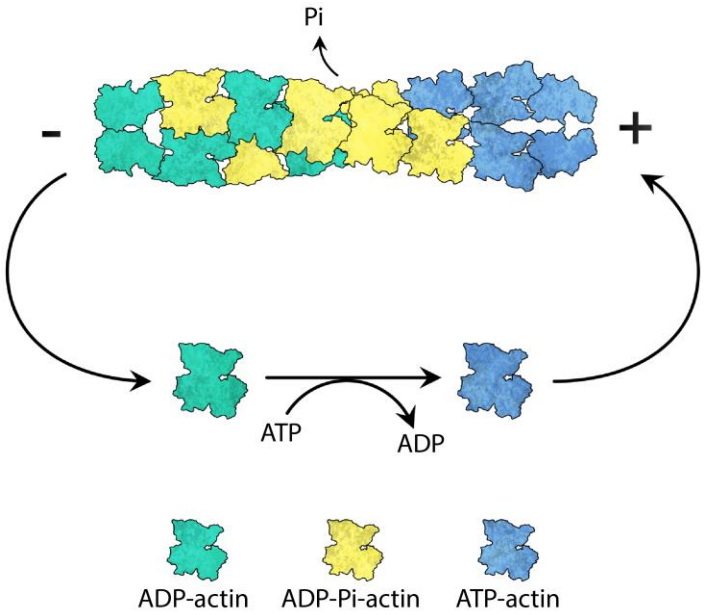


Fig. 4.2. Actin turnover.
ATP-actin can be added to the filament. ATP bound to actin subunits is hydrolyzed randomly. After hydrolysis, the release of the inorganic phosphate (Pi) is4 slow. ADP-subunits from the minus end can depolymerize. Once the nucleotide is exchange back to ATP, the monomer can be re-incorporated in the filament.

5. Actin organization in the cell

In cells, actin filaments can organize into different types of architectures or networks (Pollard and Cooper, 2009; Blanchoin *et al*., 2014; Skau and Waterman, 2015). The main types of organizations include (but are not limited to) branched and linear networks of actin filaments (Fig. 5.1).

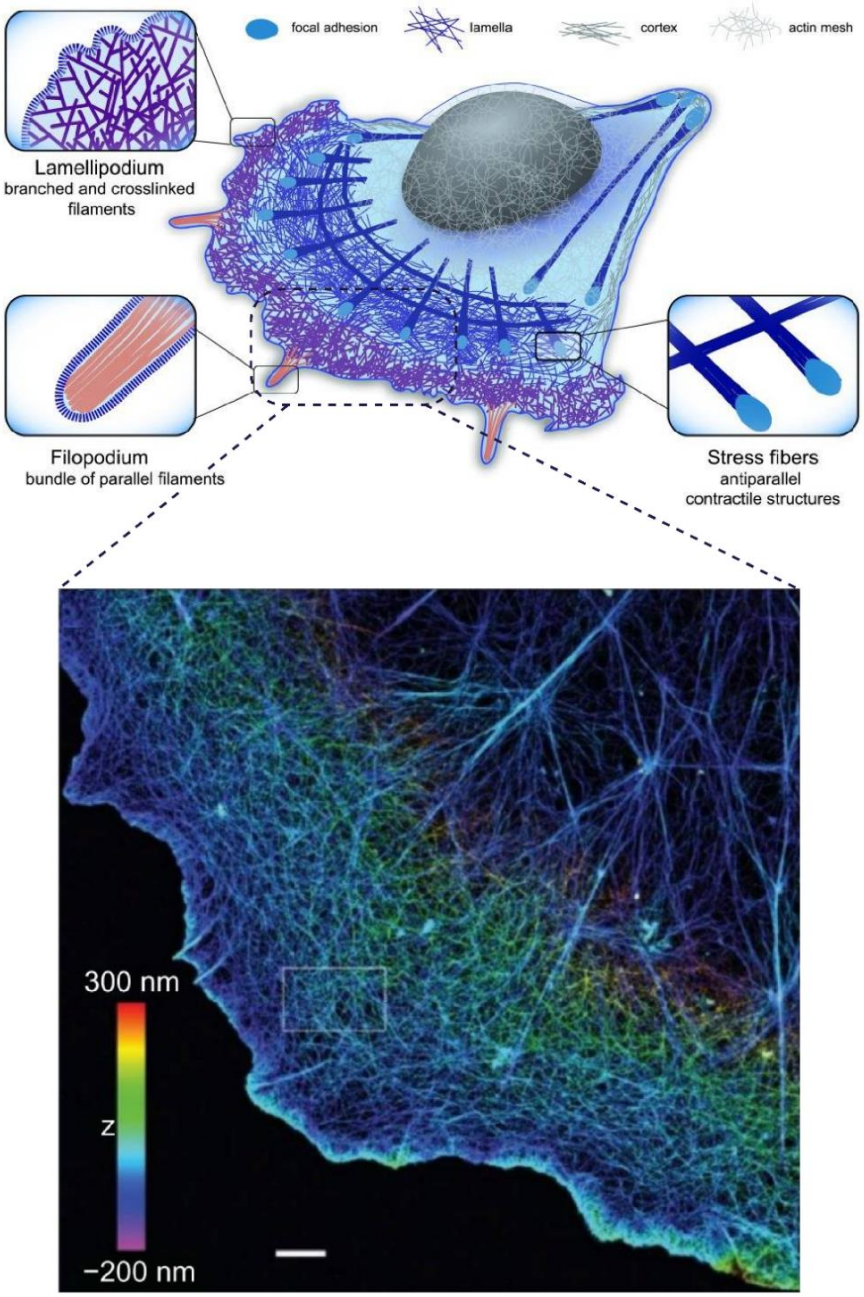


Fig. 5.1. Actin structures in cells.

(top) Schematic representation of the cellular organization of actin. Linear actin bundles can be found in the filopodium (parallel) and in the stress fibers (antiparallel) and the branched network can be found in the lamellipodia. **(bottom)** Super-resolution image of actin filaments in a COS-7 cell. Actin is labeled with Alexa 647-phalloidin and the image was taken using a dual-objective STORM microscope. Scale bar: 2 μm. Modified from (Letort *et al*., 2015; Montgomery and Leong-Hoi, 2015).

These structures have optimized dynamics and mechanical properties that allow them to perform specific functions. They are also highly organized and their spatial localization is tightly regulated by several factors. Although the two types of architectures can be spatially segregated in different areas of the cell, it is also possible to find both networks juxtaposed in small structures and one network can even grow from the other network. It is not evident how this high level of organization is achieved, since all the filaments composing the different networks are built from the same block: the actin molecule. Ongoing research is trying to uncover the cellular mechanisms that control the size and the assembly into the different networks. Moreover, the proteins and regulators that can bind to actin can also segregate in the different structures. In other words, although all these proteins coexist in the cell cytoplasm, the set of proteins that binds the branched network is not the same as the set of proteins that binds the linear network (Skau and Kovar, 2010; Michelot and Drubin, 2011; Kovar *et al*., 2011; Blanchoin *et al*., 2014). This observation is surprising since it would be logical to assume that all actin filaments in the cell represent equivalent substrates for ABPs. On the contrary, ABP segregation reveals the existence of complex mechanisms capable of rendering some actin filaments distinguishable from others. How this protein segregation originates is not fully understood and it is one of the main questions that motivated my work. I will discuss about the current hypotheses of the mechanisms that could drive this segregation in section 7 (p. 31), but before addressing that point I will explain the main properties of these networks and some of the proteins that play a role in their regulation.

5.1 Branched Networks

The branched network is composed of many branched actin filaments that create a meshwork. The branches in the network are done by an actin nucleator called the Arp2/3 complex. The branched network is mainly found near the cell membrane, mainly because the Arp2/3 complex is locally activated by the nucleating promoting factors which are localized at the membrane, although the branched network can also be found in other structures (Fig. 5.2A-B) (Rottner *et al*., 2010). Nucleated filaments can elongate pushing against the membrane, effectively biasing the direction of the movement while deforming the membrane. In 1993, Peskin *et al*. proposed the Brownian Ratchet as a mechanism of the protrusive force generation (Peskin *et al*., 1993). Briefly, rigid filaments that have their barbed ends against the membrane can add subunits because thermal fluctuations of the membrane leave a gap in between the tip of the filament and the membrane. Once an extra monomer is added to the filament, the membrane cannot go back to its initial position and therefore it gets pushed forward. This model has since been updated several times (Mogilner and Oster, 1996, 2003). Branched actin networks can generate forces of several pN, while the fastest crawling cells can move at rates as high as several micrometers per second (Prass *et al*., 2006; Barnhart *et al*., 2011; Milo and Phillips, 2016). Moreover, the actin filaments in these structures undergo a rapid turnover. The branched network can be found in many cellular structures, but the two most broadly studied

examples are the lamellipodia and endocytic sites. The lamellipodia is an actin meshwork found at the leading edge of the cell below the plasma membrane (Fig. 5.2A-C) (Small *et al*., 1995; Svitkina and Borisy, 1999). This structure is highly dynamic and the polymerization of branched actin occurs against the membrane during migration and pushes the cell forward (Wang, 1985). During endocytosis, the branched actin network produces the force necessary to invaginate the membrane to form a vesicle (Fig. 5.2B-D) (Skau and Waterman, 2015).

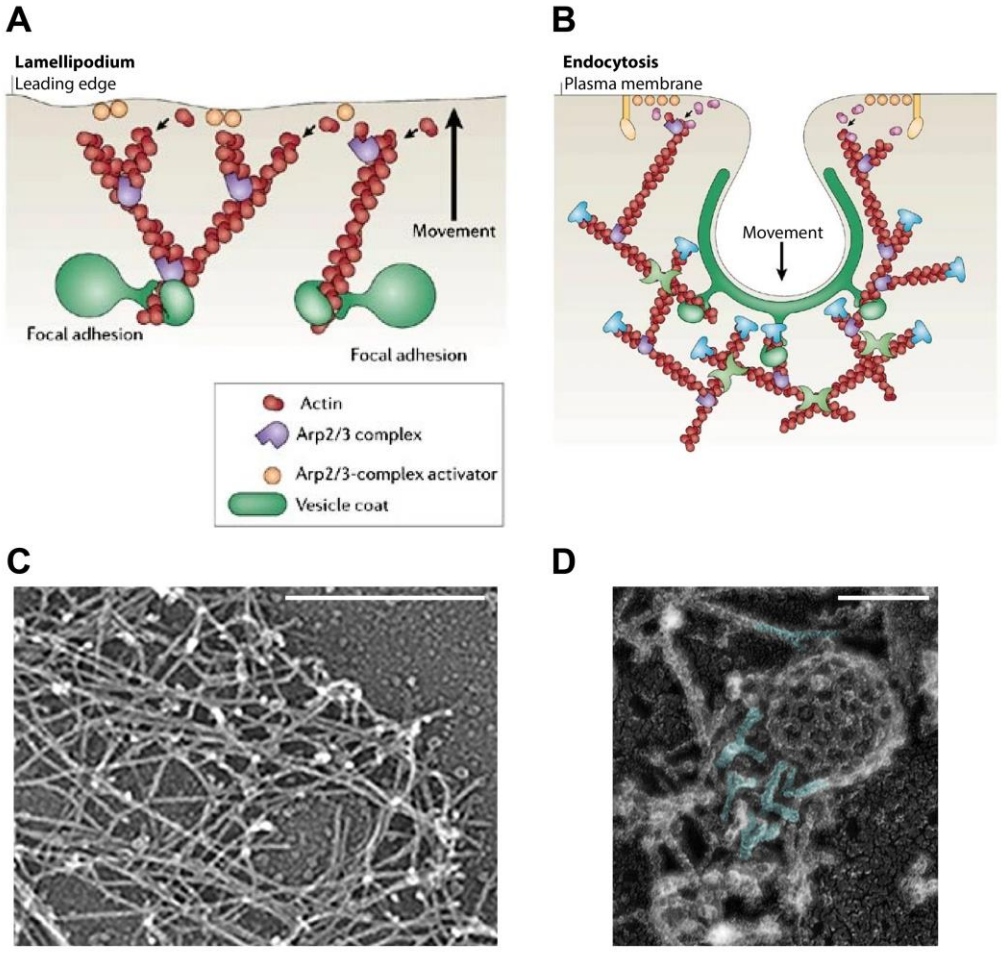


Fig. 5.2. Branched networks.

(A) Schematic representation of branched actin organization in the lamellipodia. Nucleation promoting factors are localized at the cell membrane where they act to activate the Arp2/3 complex. This promotes actin nucleation and pushes the membrane forward. **(B)** Schematic representation of the actin organization in an endocytic site in *Saccharomyces cerevisiae*. Branched actin assembly provides the necessary force to invaginate the membrane. Many other proteins are involved in this process. **(C)** Organization of actin filaments in the lamellipodia observed by Electron Microscopy in BG2 cells. Scale bar: 1 μm. **(D)** Branched filaments associated with clathrin-coated structures observed by Electron Microscopy. Scale bar: 100 nm. (Kaksonen *et al*., 2006; Biyasheva *et al*., 2004; Collins *et al*., 2011).

5.2 Linear Networks

Linear filaments can get together to form big bundles of filaments called actin cables (Fig. 5.3A-B). These bundles can be parallel if all the filaments are oriented in the same direction, or anti-parallel if the filaments are not in the same direction. Parallel cables can produce localized forces at the tip or act as a structural support and tethers. Bundles with mixed polarity can act as contractile units. The linear actin filaments composing cables are mainly nucleated and elongated by different formin isoforms, which are other type of actin nucleators. Some species have been found to express several formin isoforms and it's proposed that each of them can generate a certain linear structure. Linear structures are also dynamic and their turnover rate depends on the structure. Even in mature sarcomeres of muscle cells, which are one of the most stable structures, a population of the actin filaments in the structure can incorporate free actin within minutes (Wang *et al*., 2005; Bai *et al*., 2007, 2; Sanger *et al*., 2009). Actin cables can also be bundled by a specific type of actin binding proteins called "bundling proteins" or "crosslinkers". Bundling proteins are able to connect actin filaments because they either have more than one actin binding site or because they act in multimers (Otto, 1994). These proteins have different lengths so they can form bundles with different spacing between the filaments. Actin bundles can exert forces mainly when they are crosslinked by these proteins, otherwise they behave like single filaments (Mogilner and Rubinstein, 2005; Footer *et al*., 2007). Linear bundles can be found in many different structures and have varied roles (Faix and Grosse, 2006; Goode and Eck, 2007). Examples of actin linear bundles are filopodia, stress fibers and mitotic contractile rings. They are polar linear structures nucleated by a formin and bundled by a bundling protein (Fig. 5.3A-C) (Bornschlögl, 2013). This growing structure pushes the membrane forward, acts as a sensor of the surroundings of the cell and plays a role in migrating and initiating cell contacts (Galbraith *et al*., 2007). Stress fibers are contractile units which composed of bundles of ~10–30 actin filaments oriented in an antiparallel manner (Fig. 5.3B-D) (Tojkander *et al*., 2012). They can be generated by formins or by annealing of bundles (Hotulainen and Lappalainen, 2006). There are many categories of stress fibers and they are usually associated with focal adhesions (Tojkander *et al*., 2012). The contractile ring is composed of filaments that have different orientations and they are bound to myosin (Mangione and Gould, 2019). This ring is present in Amoebozoa and Opisthokonta (which includes fungi and animals) and it contracts the plasma membrane in the last stage of cell division to generate two daughter cells.

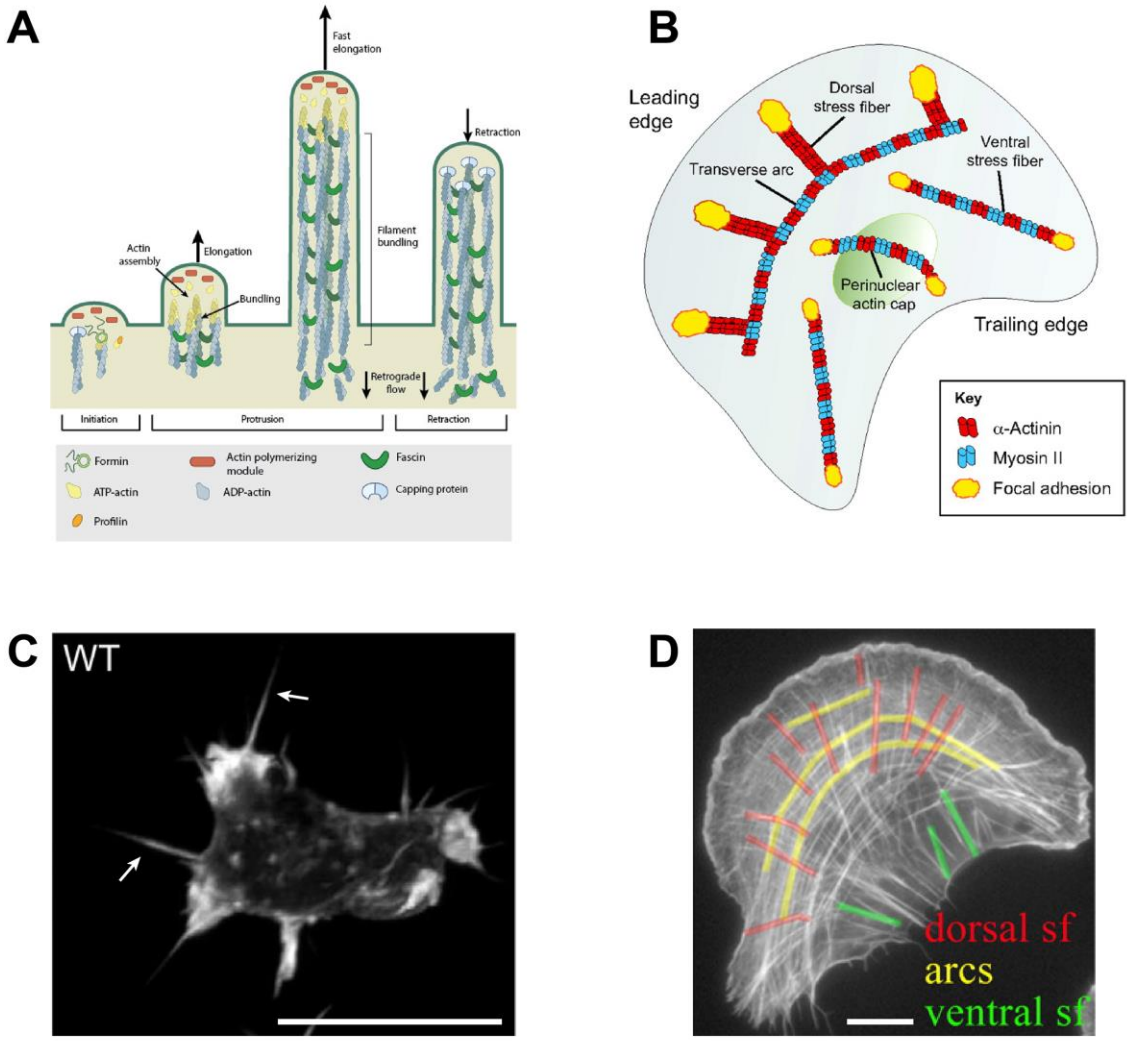


Fig. 5.3. Linear networks.

(A) Schematic representation of filopodia formation. Filopodia are composed of linear actin filaments with a parallel orientation. **(B)** Schematic representation of stress fibers in a mesenchymal cell. They can have different functions and protein compositions. **(C)** F-actin organization in *Dictyostelium* cells observed by confocal microscopy. Arrows point towards filopodia. Scale bar: 5 μm. **(D)** F-actin organization in U2OS cells (phalloidin staining). Actin linear networks are highlighted: dorsal stress fibers (red), transverse arcs (yellow) and ventral stress fibers (green). Scale bar: 10 μm. Modified from https://www.mechanobio.info/cytoskeleton-dynamics/what-are-filopodia/how-does-cross-linking-of-actin-filaments-aid-in-their-extension/ and (Tojkander *et al.*, 2012; Steffen *et al.*, 2006; Hotulainen and Lappalainen, 2006).

6. Actin Binding Proteins

As mentioned before, the actin molecule and actin structures inside cells are bound and regulated by a variety of proteins. These proteins are called "actin binding proteins" (ABPs). They have different functions and bind to different regions of actin monomers or filaments. Some of them can even respond to external stimuli. The main ABPs are found in most eukaryotes, meaning that they were probably present in the common ancestor. However, some organisms have more isoforms giving rise to large families of proteins. This makes the interactome of actin very complex but also very interesting.

Two very iconic ABPs are the main actin nucleators, which nucleate actin assembly into the different kinds of networks. The branched network is created by the nucleator Arp2/3 complex and the linear network mainly by formins (Fig. 6.1A) and their organization has been observed by electron microscopy and other techniques (Fig. 6.1B).

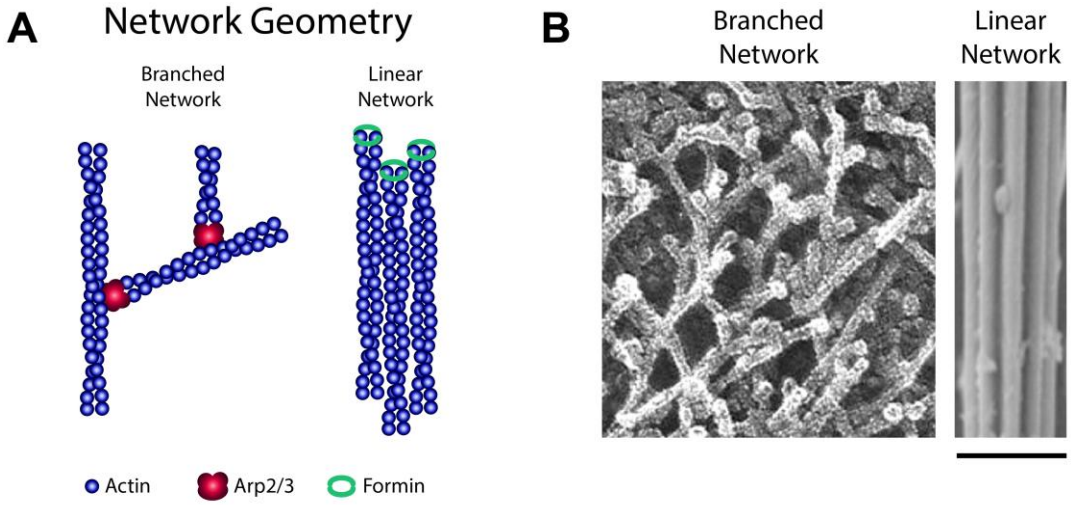


Fig. 6.1. Actin nucleators and network architecture.
(A) Schematic representation of the action of actin nucleators. The Arp2/3 complex branches actin filaments, whereas formins nucleate and elongate linear structures. **(B)** The architecture of both networks observed by electron microscopy. (left) Branched actin in the lamellipodia of a keratocyte. (right) Linear actin filaments in *Drosophila melanogaster* bristles. Scale bar: 0.1 μm. Modified from (Svitkina and Borisy, 1999; Hudson and Cooley, 2002).

The *in vitro* dynamics of purified actin are not compatible with the time scale of actin dynamics in cells. In cells, the dynamics of actin occur at a much faster scale. One of the reasons why actin can perform so many functions within the proper time scale is because it interacts with many proteins that regulate actin dynamics and function. These ABPs are involved in many different functions that can be seen in Fig. 6.2. Among all the ABPs I will describe some of them that regulate different stages of the actin turnover: nucleators, elongators, capping proteins, stabilizers, disassembling factors. The combination of the action of actin nucleators and all the other ABPs that regulate the different aspects of actin networks renders the formation and disassembly of the branched and the linear network a complex process with a lot of key players (Fig. 6.3).

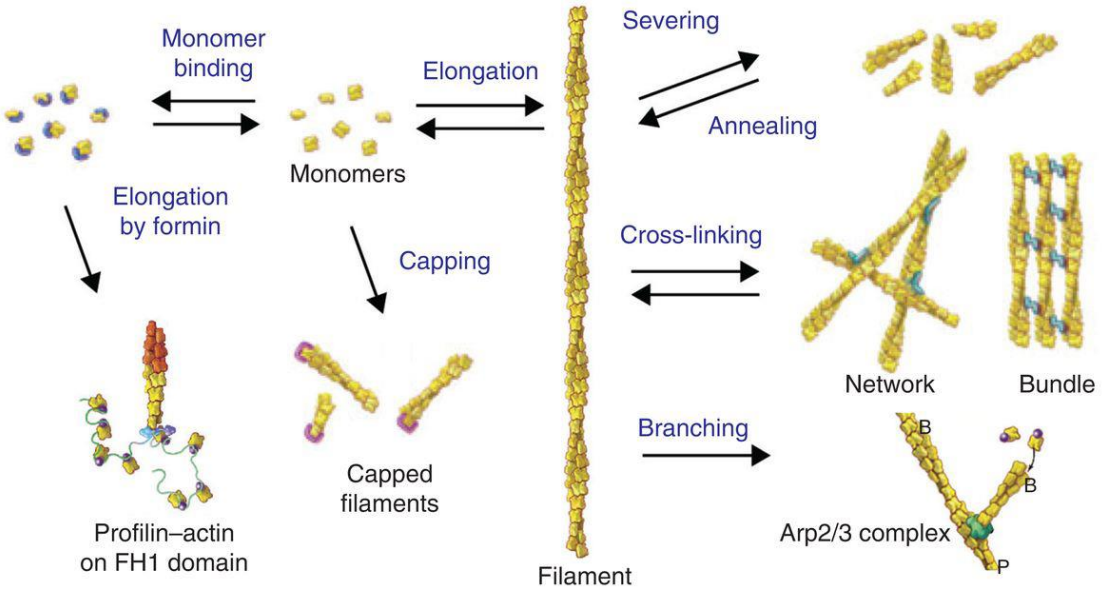


Fig. 6.2. Different functions of actin-binding proteins.

Numerous ABPs in cells regulate the dynamics of actin. There are many families of ABPs and they are specialized in different functions. This cartoon represents an overview of some of those functions. Modified from (Pollard, 2016).

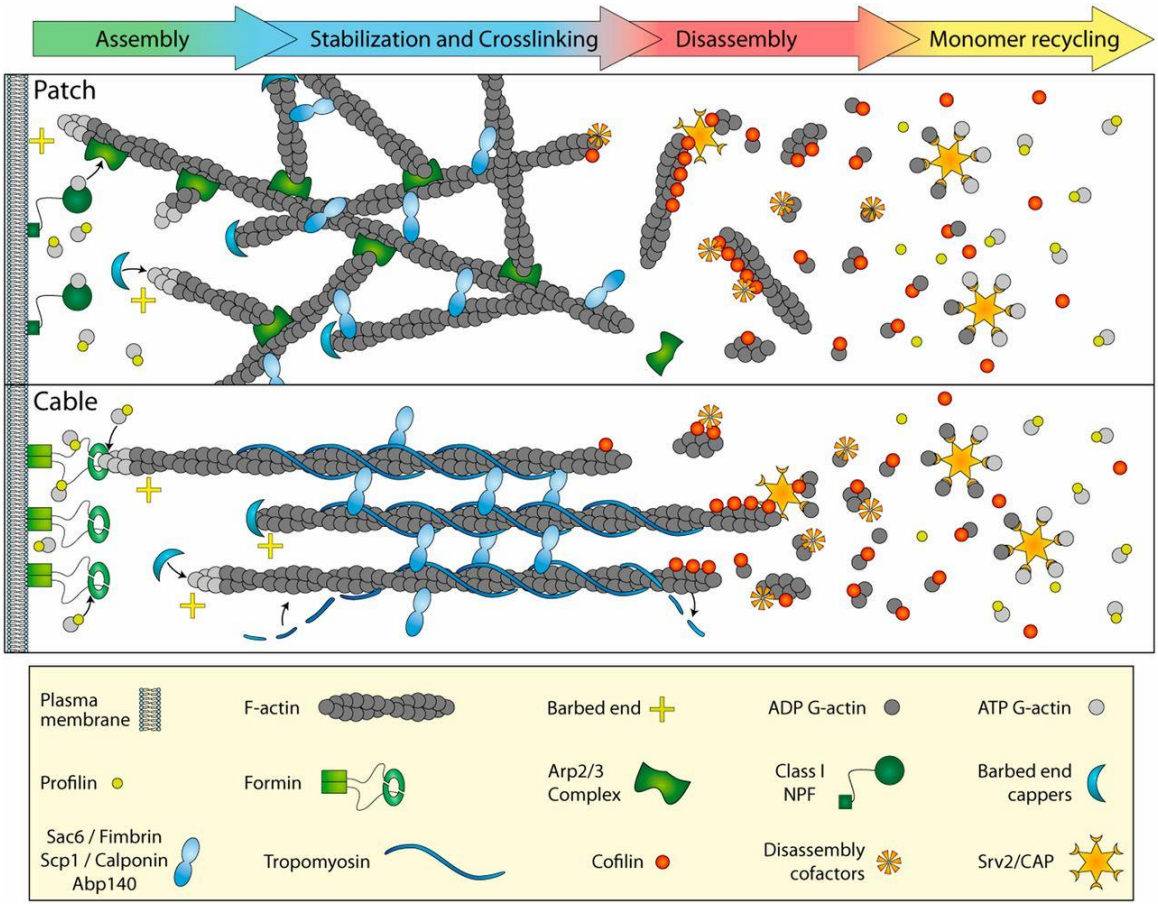


Fig. 6.3. Assembly, dissasembly and turnover of actin structures in cells.

Different steps in the formation, disassembly and turnover of actin patches (top) and actin cables (bottom) in budding yeast. Many ABPs with different functions are involved in each step. Adapted from (Goode *et al.*, 2015).

6.1 Nucleators

During actin polymerization, the process of actin nucleation, meaning the formation of the 3-subunit complex, is not a favorable process. In cells, this process is catalyzed by proteins that are called actin nucleators. Here, I will describe two of them, but they are not the only nucleators that we can find in cells.

6.1.1 Arp2/3 complex

The Arp2/3 complex is an actin nucleator capable of generating branches from pre-existing filaments (Fig. 6.4A). This complex is evolutionary conserved, an observation made at the time it was discovered in 1997 (Machesky *et al*., 1997; Welch *et al*., 1997, 3). It is widely spread among all eukaryotes, although it has been secondarily and independently lost in some branches of the phylogenetic tree, mainly in the Chromista (protists) and Archaeplastida (plants and algae) branches (Veltman and Insall, 2010).

The Arp2/3 complex is a 7 subunit complex which include 2 actin-like proteins, called Arp2 and Arp3 (Fig. 6.4B) (Robinson *et al*., 2001). The Arp2/3 complex can bind on the side of the actin filament mimicking the formation of the actin trimer, the limiting step in actin nucleation (Fig. 6.4C). This triggers the formation of a branch of actin (daughter filament) which grows from the mother filament at a 70º angle (Fig. 6.4A) (Blanchoin *et al*., 2000; Amann and Pollard, 2001). After nucleation the Arp2/3 complex remains bound to the pointed end of the daughter filament while the barbed end can elongate (Mullins *et al*., 1998, 3; Amann and Pollard, 2001).

Under physiological conditions the Arp2/3 complex is auto inhibited and its activity can be activated by different proteins, called nucleating promoting factors (NPFs) (Fig. 6.4A-first step) (Machesky *et al*., 1999; Rottner *et al*., 2010). Many of these factors are also inhibited themselves and are activated by Rho and Rac GTPases under certain conditions. Not only can branches can form, but branched filaments can be de-branched. This is mediated by another set of ABPs, for example ADF/cofilin (Chan *et al*., 2009).

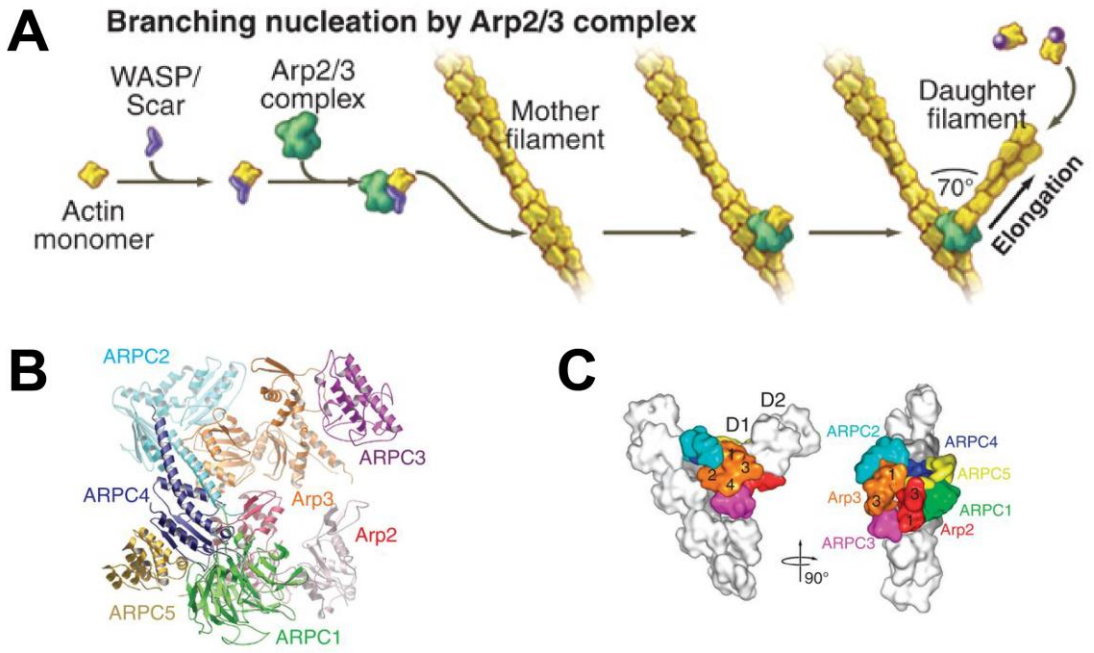


Fig. 6.4. Nucleation by the Arp2/3 complex.

(A) Scheme of Arp2/3-complex-mediated nucleation. An NPF binds actin and the Arp2/3 complex. The Arp2/3 complex can bind to the side of a pre-existing filament, nucleating the formation of a new branch. **(B)** Structure of the Arp2/3 complex solved by X-ray crystallography (PDB: 1K8K). **(C)** Model of the binding of the Arp2/3 complex to a filmanet. D1 and D2 represent the first two subunits from the daughter filament (branch). Modified from (Pollard and Cooper, 2009; Robinson *et al*., 2001; Rouiller *et al*., 2008)

6.1.2 Formin-Homology Proteins

Formins are a family of proteins found in all eukaryotes. Some species can have a lot of isoforms, such as mice which has 15 formins, and others can have only a few, which is the case of *S. cerevisiae* which has only two isoforms (Higgs and Peterson, 2005).

Formins form homodimers and they play a role in the nucleation and also the elongation of actin filaments (Fig. 6.5A). They bind to the barbed end of actin filaments to promote nucleation of free monomers. They act processively which means that they remain bound to the barbed end of the actin filaments and they mediate the addition of new monomers into the filaments (Kovar and Pollard, 2004; Higashida *et al*., 2004; Paul and Pollard, 2009). The affinity of formins for actin monomers is not very high, so the action of formin alone actually decreases the actin elongation rate. However, formin can nucleate very efficiently actin monomers that are bound to a small molecule called profilin (Fig. 6.5A). The elongation rate of filaments that elongate from formins and actin profilin is higher, but it depends on the formin isoform. For example, the elongation rate of mDia1 is 5 time faster than the elongation rate of free barbed ends (Kovar and Pollard, 2004; Kovar, 2006). Structurally, all formins have two formin homology domains (FH1 and FH2) (Fig. 6.5B-C). The FH1 domain is a proline rich domain which binds profilin. The FH2 domain is involved in the dimerization of formin and it nucleates and caps the barbed end of actin filaments (Fig. 6.5B). The dimer binds around the actin filament to stabilize the trimer and

regulating monomer addition at the end (Xu *et al*., 2004; Moseley *et al*., 2004, 1; Shimada *et al*., 2004, 2; Otomo *et al*., 2005b). Basically, actin monomers that bound to profilin can bind to the FH1 domain of formin. The FH1 domain is very flexible and by diffusion it can transfer the monomer to the barbed end of the filament. After the addition of the new monomer, the formin dimer rotates to remain bound to the barbed end (Kozlov and Bershadsky, 2004; Mizuno *et al*., 2011).

Formin activity can regulated though a diaphanous autoinhibitory domain (DAD) and its DAD-interacting domain (DID) if they are present in the isoform (Fig. 6.5C) (Alberts, 2001). This autoinhibition can be abolished by the binding of Rho-GTPases (Otomo *et al*., 2005a).

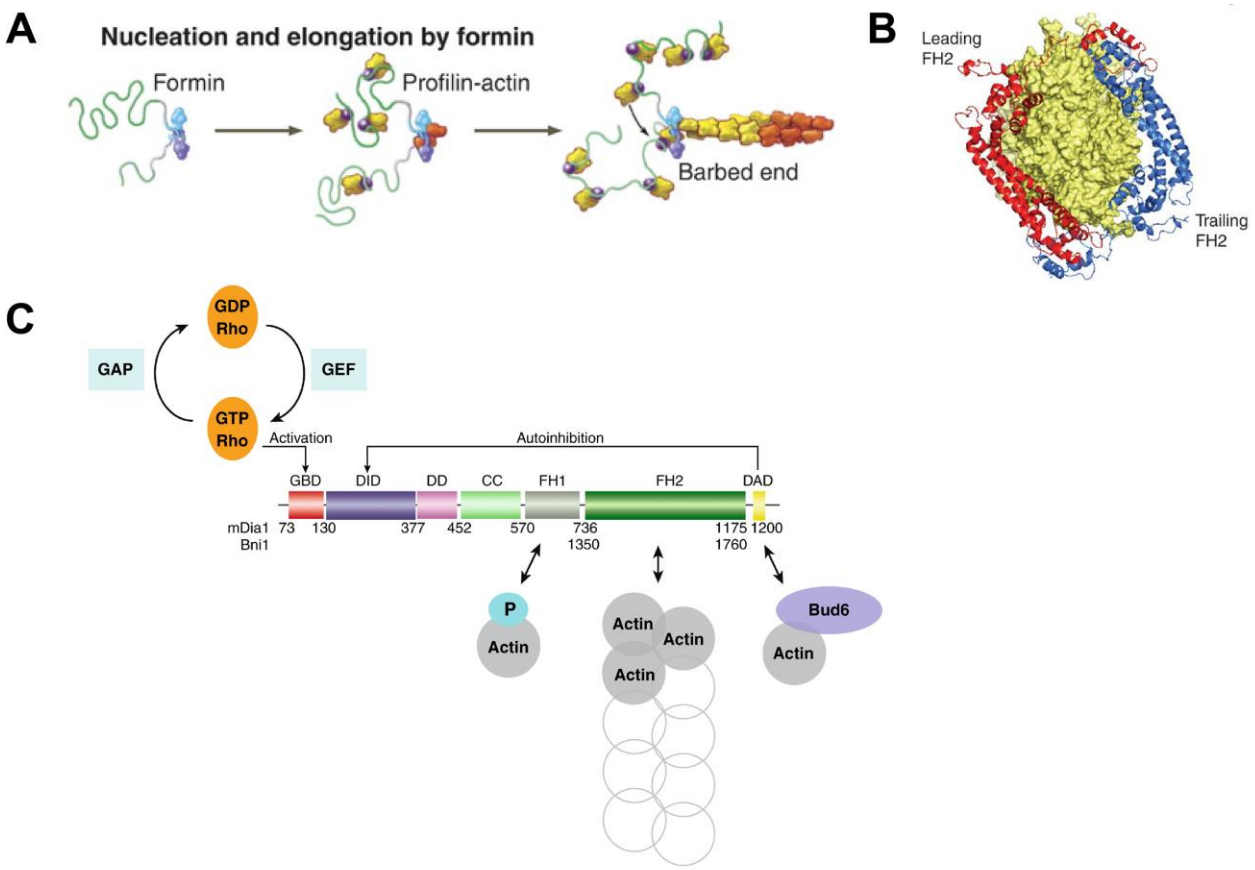


Fig. 6.5. Nucleation and elongation by formins.
(A) Scheme representation of the mechanism of formin nucleation and elongation. Formins initiate nucleation from profilin-actin and remain bound to the barbed end of the nascent filament to promote elongation. **(B)** Structure of a formin dimer (red and blue) bound to an actin subunit. **(C)** Domain structure of a diaphanous-type formin. These formins are autoinhibited by interaction of the DAD domain (yellow) and the DID domain (blue) and can be activated by Rho-GTPases. Domain names: CC, coiled-coil domain; DAD, diaphanous autoregulatory domain; DD, dimerization domain; DID, diaphanous inhibitory domain; FH1 and FH2, formin homology 1 and 2; GAP, G protein–activating protein; GBD, GTPase-binding domain; GEF, guanine nucleotide exchange factor. Modified from (Pollard and Cooper, 2009; Pollard, 2016; Goode and Eck, 2007).

6.2 Profilin

Profilin is a very small protein (14 kDa) that is found in high concentration (50-100 μM) in cells and binds to G-actin (Pollard, 2016). It is present in all eukaryotes, with the exception of parasitic organisms such as *Giardia duodenalis* (Rivero and Cvrcková, 2007).

Profilin binds actin monomers occupying the actin-actin binding site on subunits 1 and 3, therefore inhibiting spontaneous nucleation (Fig. 6.6) (Reichstein and Korn, 1979; Xue and Robinson, 2013). However, it helps polymerization by delivering the actin monomers to elongation factors with proline-rich sequences, such as formins dimers that are bound at the barbed end of the filament (Higashida *et al*., 2004; Romero *et al*., 2004; Kovar *et al*., 2006). After binding to the filament profilin is released because it's binding affinity for the ATP-F-actin is much lower than for the monomer (Jégou *et al*., 2011; Courtemanche and Pollard, 2013). Profilin can also bind ADP-G-actin, although with a 5-8-fold lower affinity than ATP-G-actin, and it accelerates the nucleotide exchange of ADP for ATP so that the ATP-G-actin can be recycled and re-added at the barbed end of the filaments (Vinson *et al*., 1998; Blanchoin and Pollard, 1998).

Profilin phosphorylation is induced in the presence of phosphatidylinositol bisphosphate (PIP2), which could play a regulatory mechanism to control the interaction of Profilin with proline-rich domains (Hansson *et al*., 1988; Singh *et al*., 1996). However, the precise role of this modification is not yet understood (Krishnan and Moens, 2009).

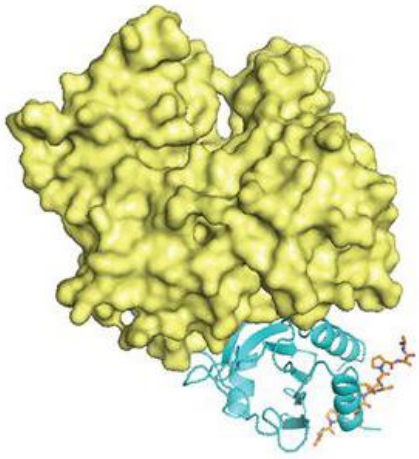


Fig. 6.6. Structure of profilin (light blue) bound to an actin monomer (yellow).
Modified from (Pollard, 2016).

6.3 Nucleation promoting factors

Nucleation promoting factors are proteins capable of activating Arp2/3-mediated nucleation. It is believed that all these proteins or complexes were present in the last eukaryotic common ancestor, although there is the possibility that WASH is more ancient than the others (Veltman and Insall, 2010).

Many proteins or complexes have been characterized as NPFs. Type 1 NPFs are complexes from the WASP/WAVE family and they can bind both the Arp2/3 complex and actin monomers. Those protein interactions are possible thanks to a conserved catalytic core which is composed by three independent regions: the VCA or WCA domain (Miki *et al*., 1998; Boczkowska *et al*., 2008). The verprolin homology domain or WASP homology domain 2 (WH2) binds G-actin (Paunola *et al*., 2002; Dominguez, 2007). The central domain has affinity for both actin and the Arp2/3 complex (Marchand *et al*., 2001; Kelly *et al*., 2006). The acidic region binds the Arp2/3 complex and is essential for its activation (Marchand *et al*., 2001). The proteins of this family have a variation in the N-terminal region that accounts for the differences in their regulation which reflect their biological activities (Disanza and Scita, 2008). NPFs that bind the Arp2/3 complex and the actin filament exist as well, and they are denominated type II NPFs (Ammer and Weed, 2008; Ren *et al*., 2009; Guo *et al*., 2018).

The activity of the NPFs generally has to be activated due to their catalytic domain being inaccessible. This is achieved by RhoGTPases at specific times and locations of the cell, generally in response to external cues (Stradal and Scita, 2006; Ladwein and Rottner, 2008).

6.4 Tropomyosin

Tropomyosin is a protein that forms dimers to create a double-stranded coiled coil structure (von der Ecken *et al*., 2015). It is found in many eukaryotes, but not all of them. They are present mainly in fungi and animals, while plants, amoebas and other groups do not have tropomyosin (Gunning *et al*., 2015).

Tropomyosin binds along the side of the actin filaments (Fig. 6.7A). It interacts with the actin filament mainly by electrostatic interactions: the negatively charged surface of tropomyosin interacts with a positively charged groove of actin (Fig. 6.7B) (von der Ecken *et al*., 2015). It binds cooperatively to actin (Fig. 6.7C). This means that when one dimer of tropomyosin is bound, the affinity for another tropomyosin dimer to bind is higher (Tobacman, 2008). Tropomyosin stabilizes actin filaments and protects them from severing by cofilin since they compete for the same binding site (Fig. 6.7C-D) (Christensen *et al*., 2017; Gateva *et al*., 2017; Jansen and Goode, 2019). As it will be discussed in the next sections, it promotes formin assembly while inhibiting Arp2/3-mediated assembly (Blanchoin *et al*., 2001; Wawro *et al*., 2007; Skau *et al*., 2009; Alioto *et al*., 2016).

Tropomyosin's affinity for actin filaments is regulated by a specific post-translational modification which is the N-terminal acetylation (Skoumpla *et al*., 2007). The acetylation of tropomyosin increases its affinity for actin by 10-fold (Urbancikova and Hitchcock-DeGregori, 1994).

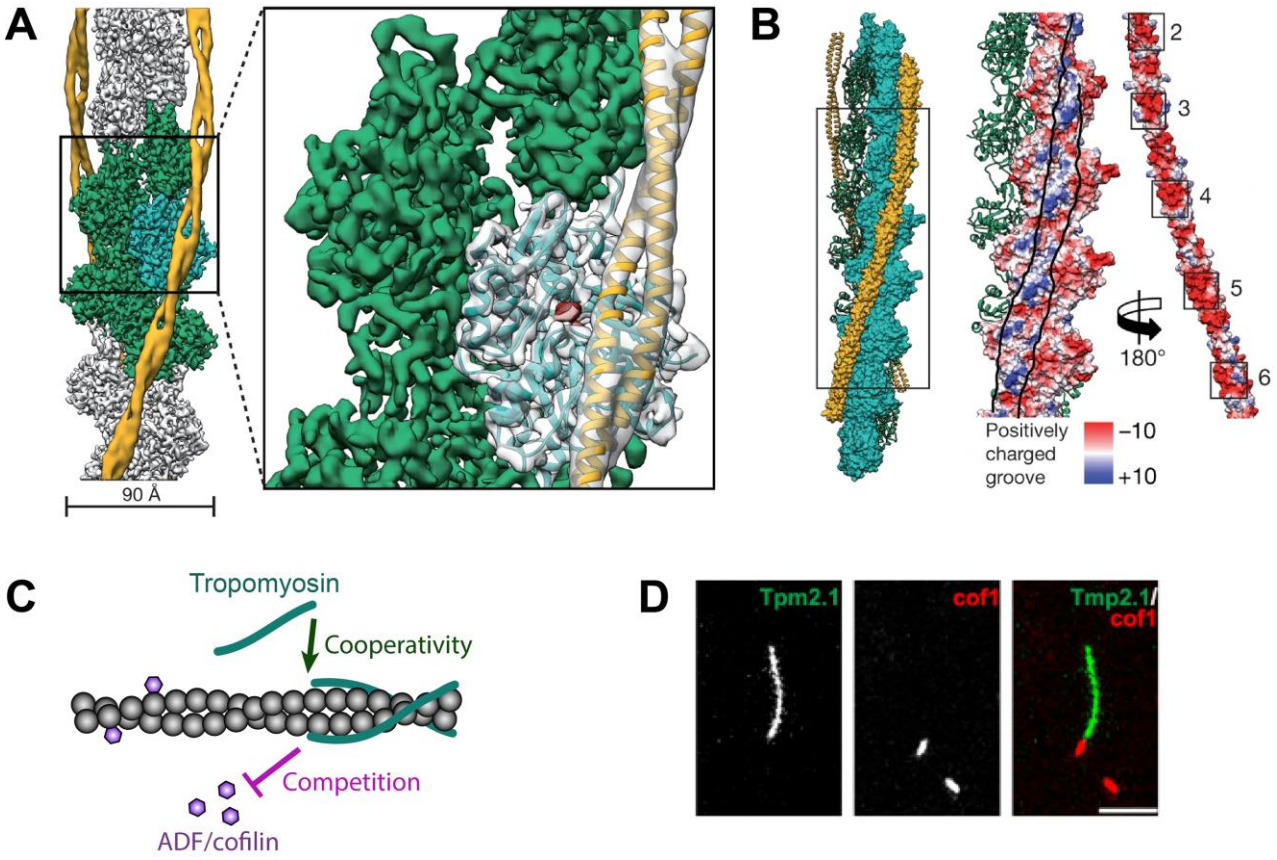


Fig. 6.7. Structure and function of tropomyosin.

(A) Structure of an actin filament (grey and green) decorated with tropomyosin (yellow), with a close up showing the molecular model for both proteins. The red dot corresponds to the ADP density. **(B)** Structure of the actin filament (green and teal) decorated with tropomyosin (yellow). The representation on the right shows the negatively charged surface of tropomyosin (red) that binds a positively charged groove in the actin filament (blue). **(C)** Scheme representation of the competition between tropomyosin and cofilin. **(D)** TIRF image of a single filament showing that tropomyosin (green) and cofilin (red) are not bound simultaneously to actin filaments. Scale bar: 5 μm. Modified from (von der Ecken *et al*., 2015; Boiero Sanders *et al*., 2020; Gateva *et al*., 2017).

6.5 ADF/cofilin

The actin depolymerizing factor (ADF) ADF/cofilin is a small protein (15-21 kDa) and it is found in all eukaryotes (Fig. 6.8A) (Maciver and Hussey, 2002). The amino acid sequence is poorly conserved but the tridimensional folding of the protein is maintained across species (Poukkula *et al*., 2011).

ADF/cofilin can bind actin filaments and sever them stochastically (Fig. 6.8B) (Michelot *et al*., 2007). It binds the actin filaments in a cooperative manner, meaning that binding of

one molecule of ADF/cofilin increases the affinity of other ADF/cofilin molecules next to where the original one bound (De La Cruz, 2005; Hayakawa *et al*., 2014). The decoration of actin filaments with ADF/cofilin stabilizes a state of the filament with a mean twist of 162°, compared to the twist of bare filaments which is 167° (McGough *et al*., 1997; Galkin *et al*., 2001). This rotation weakens lateral and longitudinal contacts in the actin filament making the filaments more flexible (Paavilainen *et al*., 2008; McCullough *et al*., 2008, 2011). The outcome is that the interphase between the decorated area of the filament and the undecorated area of the filament is very unstable and prone to sever. ADF/cofilin binds preferentially to ADP-F-Actin so it is more prone to sever aged actin filaments (Carlier *et al*., 1997). Bound ADF/cofilin also accelerates Pi release from the actin filament (Suarez *et al*., 2011). Severing is enhanced at low concentrations of ADF/cofilin (Andrianantoandro and Pollard, 2006). If the concentration of ADF/cofilin is very high ADF/cofilin decorates fully the actin filaments and stabilizes them (Andrianantoandro and Pollard, 2006). It can also bind ADP-G-actin and it inhibits nucleotide exchange which can be counteracted by profilin (Nishida, 1985; Blanchoin and Pollard, 1998; Paavilainen *et al*., 2008). Other proteins act in coordination with ADF/cofilin to promote severing. For example, ADF/cofilin severing activity is highly enhanced by Aip1, leading to a catastrophic disassembly of the actin filaments similar to microtubules (Fig. 6.8B) (Ono, 2003; Chen *et al*., 2015; Gressin *et al*., 2015).

ADF/cofilin activity can be negatively regulated by either binding to PIP2 or by phosphorylation (Mizuno, 2013). LIM kinase is the enzyme that catalyzes ADF/cofilin phosphorylation at the position Ser3 and there are phosphatases that can remove this phosphate group (Yang *et al*., 1998).

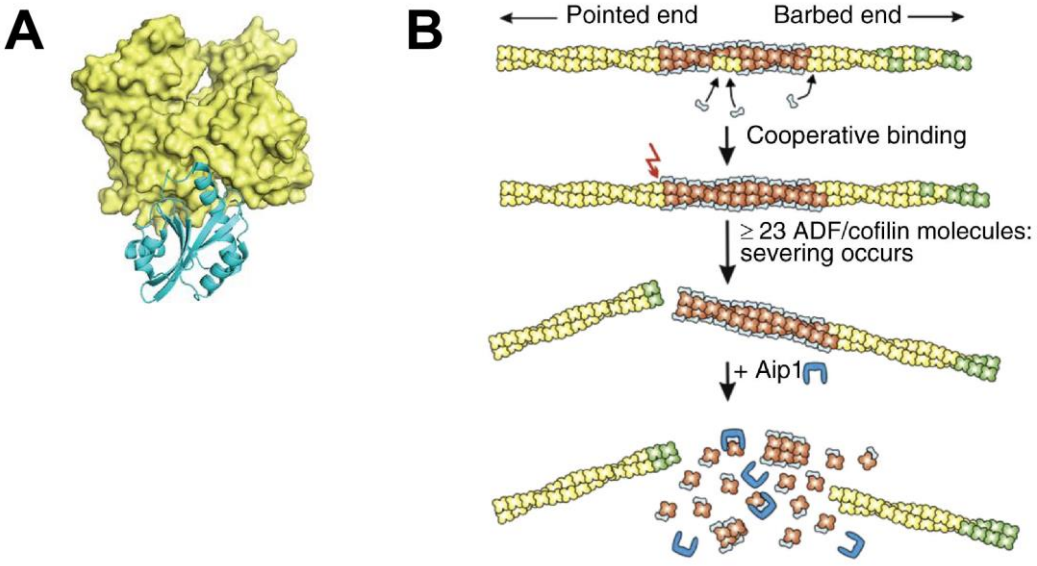


Fig. 6.8. ADF/cofilin structure and function.
(A) The carboxy-terminal cofilin domain from twinfilin (light blue) bound to an actin monomer (yellow) (PDB: 3DAW). **(B)** Mechanism of ADF/cofilin mediated severing. ADF/cofilin binds cooperatively to actin filaments and promote severing. Action of ADF/cofilin and Aip1 induces fast disassembly of the actin filaments. Modified from (Pollard, 2016; Winterhoff and Faix, 2015).

6.6 Capping Protein

Capping proteins are protein heterodimers and they are found in almost all eukaryotes with the exception of the parasitic diplomonads (Rivero and Cvrcková, 2007).

Capping proteins is a heterodimer of two subunits, α and β subunits, which have a similar size (30 kDa) (Fig. 6.9). They are different in sequence but their tertiary structure is similar. Capping protein binds to the barbed end of the actin filament with high affinity and remain bound, blocking polymerization and depolymerization at that end (Isenberg *et al*., 1980; Caldwell *et al*., 1989). By blocking most actin filament barbed-ends in cells, capping proteins help prevent the depletion of the G-Actin pool (Loisel *et al*., 1999). Capping protein plays also a role in increasing the density of branched networks (Achard *et al*., 2010). It does so by limiting the length of actin filaments after their nucleation, although it has also been proposed that it could promote Arp2/3 filament nucleation (Akin and Mullins, 2008). From the same pool of actin monomers, filaments will be short and numerous in the presence of capping protein, while they would be less numerous and long in its absence.

Capping protein activity is regulated by different mechanisms (Takeda *et al*., 2010; Edwards *et al*., 2014). Some proteins, such as formins, can bind to the barbed-ends like capping protein. Even though some studies claim that these two proteins compete for the same binding site, other studies have shown that they can bind simultaneously which results in the displacement of either the capping protein or the formin (Zigmond *et al*., 2003; Moseley *et al*., 2004; Kovar *et al*., 2005; Bartolini *et al*., 2012; Shekhar *et al*., 2015). Other molecules, like phospholipids directly block the interaction of capping protein by abolishing its binding (Heiss and Cooper, 1991; Schafer *et al*., 1996; Kim *et al*., 2007; Kuhn and Pollard, 2007). The activity of capping protein can also be negatively regulated by the multidomain proteins called CARMILs, which bind directly to capping protein to decrease its activity by inducing a conformational change (Yang *et al*., 2005; Uruno *et al*., 2006; Stark *et al*., 2017).

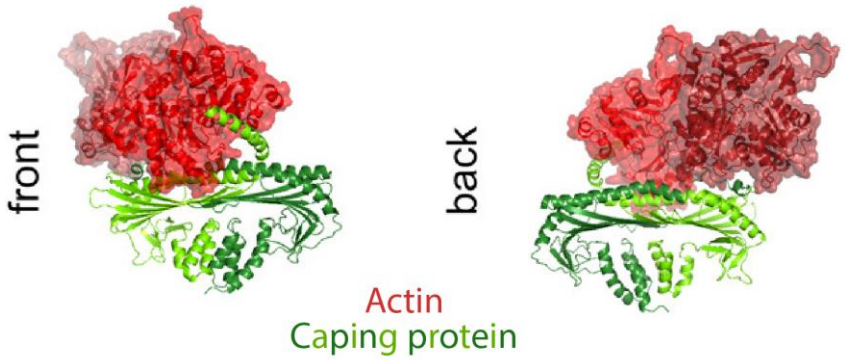


Fig. 6.9. Structure of capping protein (green) bound to an actin monomer (red).
Modified from (McConnell *et al*., 2020)

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7. Principles of actin organization

So far we have described a very complex regulation of the actin cytoskeleton. Actin can polymerize into different types of filaments that can organize into different networks. Each network binds a set of actin binding proteins. All the mechanisms and regulatory pathways that are involved in this regulation are under constant study. Of course, the final organization of actin networks in cells is a combination of many mechanisms, which act together to bring the final result. Studying simple organisms shed light on some of the mechanisms, but also comparative information about the different species uncover different mechanisms that have developed along the evolution of the modern species. In this section I will explain the current knowledge on these topics and then I will dedicate section 8 (p. 43) to describe in detail one of them.

7.1 Homeostasis of Actin Networks

In cells, the size and density of actin networks has to be finely tuned so that they are able to carry out their functions correctly. All the ABPs previously described are involved in the formation and regulation of the different networks. All these structures and ABPs coexist in the same cytoplasm, along with the building block for all the structures: the actin molecule. Originally, it was believed that networks assembled independently from a large pool of cytoplasmic actin. However, studies in the past years show that that polymerizable actin monomers are not in excess in the cytoplasm (Burke *et al*., 2014; Suarez and Kovar, 2016). As a consequence, branched and linear networks compete for a this limited pool of actin monomers (Burke *et al*., 2014). This means that the different architectures do not grow independently, but that their relative size is limited by the growth of the other competing networks. For example, if all branched networks are disassembled or disrupted, linear networks will grow more due to the higher availability of monomers, and vice versa. This balance of actin networks means that perturbation to any network will affect the whole actin cytoskeleton. This has been referred to as homeostasis of the actin cytoskeleton and also global treadmilling (Fig. 7.1) (Burke *et al*., 2014; Suarez and Kovar, 2016; Carlier and Shekhar, 2017). It is a key regulatory mechanism that contributes to the size and density changes of the different actin networks. In combination with many other regulatory mechanisms, it allows the proper assembly or disassembly of actin structures at the correct time and location which is crucial for the proper functioning of the actin cytoskeleton.

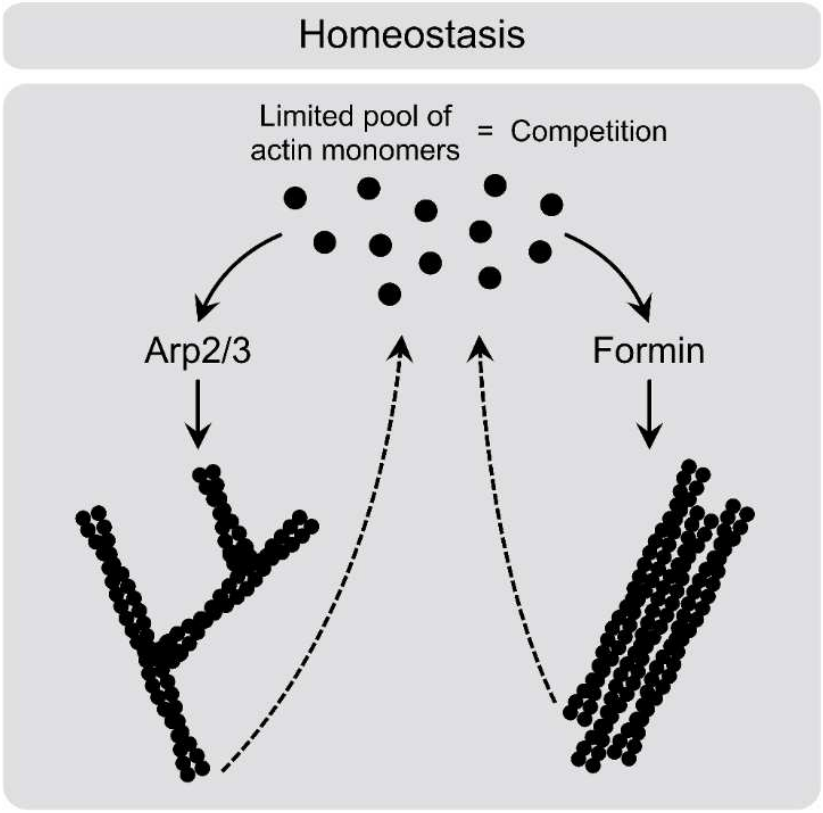


Fig. 7.1. Principles of actin homeostasis.
Actin networks compete for a limited pool of cytoplasmic actin monomers. These monomers can be incorporated in any of the networks, therefore increasing their size. Networks can also disassemble and those actin monomers can be recycled back to the cytoplasmic pool.

The homeostasis of the actin cytoskeleton has been observed in many cell types (Fig. 7.2). Fig. 7.2A illustrates the actin network homeostasis in representative graphs by showing the dependence of the decrease of a certain network, which is coupled with the increase of the size of the competing network For example, inhibiting branched network formation by Arp2/3 leads to the increase in the linear network assembled by formins (Fig. 7.2A). Actin assembly into the linear structure of the cytokinetic ring during cell division would require the reduction in size of the branched network (Fig. 7.2B). The main evidence for these models emerges from experiments where the active form of the nucleators, normally formins or the Arp2/3 complex, is inhibited. In fission yeast, Arp2/3 complex inactivation by addition of the drug CK-666 led to a 20-fold increase in actin assembled into linear structures (cables and contractile ring), whereas inhibition of formins led to a 50% increase of the amount of patches which are composed of branched network nucleated by the Arp2/3 complex (Fig. 7.2C). Similar results were obtained when using other cell types. The inhibition of the formation of the branched network by Arp2/3 depletion or inactivation leads to an increase of linear structures, such as filopodia, in both fibroblasts and *Drosophila melanogaster* S2 cells (Fig. 7.2D-E).

But formins and the Arp2/3 complex are not the only factors that can regulate the size of actin networks through homeostasis. Other actin binding proteins play a vital role in regulating this balance and shifting the equilibrium towards one network or the other. Profilin has been proven to be a key regulator of actin homeostasis by favoring formin over Arp2/3-mediated assembly (Rotty *et al*., 2015; Suarez *et al*., 2015). Moreover, other actin binding proteins have been shown to favor one or the other network not only by their direct action on the actin cytoskeleton but also because many actin binding proteins compete for the same binding site (Antkowiak *et al*., 2019; Christensen *et al*., 2017; Skau and Kovar, 2010; Billault-Chaumartin and Martin, 2019; Gateva *et al*., 2017). The full picture involves the combination of many mechanisms and it is complicated to understand.

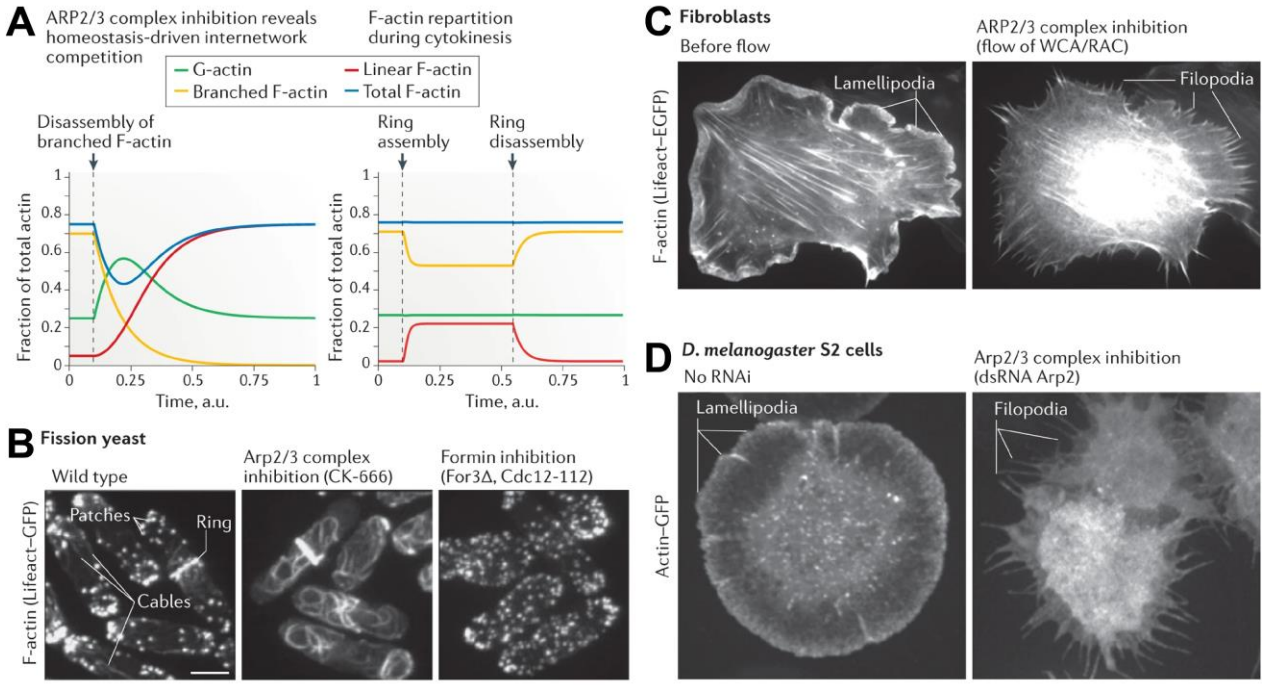


Fig. 7.2. Cellular evidence of actin homeostasis.

(A) Representative graphs illustrating the effects of actin homeostasis. When a network is disassembled, actin is reorganized in the other structures. **(B-D)** Examples of actin homeostasis in fission yeast (B), fibroblasts (C) and S2 cells (D). Whenever the assembly of a structure is somehow impaired, regardless of the mechanism used, the other networks will assemble.

7.2 Strategies to confer the actin filaments a molecular identity

Actin networks, even though they can be organized into different types of architectures, are all composed of actin filaments. However, the different networks can localize different sets of proteins. As it mentioned in section 5 (p. 16), not all ABPs present the same binding pattern, and some localize to only one type of network, while being excluded from others. Since the actin molecule is extremely conserved in sequence and it is the building block for all the filaments, it is not trivial to understand how the filaments or the network composed by these filaments can represent different substrates for ABPs. In this section will address strategies that could explain how ABPs are localized to the proper networks and the molecular mechanisms behind these strategies. This section is based on a review that I wrote alongside with Adrien Antkowiak and Alphée Michelot (Boiero Sanders *et al*., 2020), a copy of which you can find at the end of the manuscript. For more detailed information about what is written in this section, please refer to the review.

The branched and linear actin networks can in some cases be spatially separated in the cytosol. However, there are also cases when both networks can be very close to each other, grow from a pre-existing one, or intertwined (Fig. 7.3). This is the case, for example, of filopodia, which emerge through the elongation of actin filaments assembled in lamellipodia or the lamella which grows from the retrograde flow of branched actin towards the center of the cell (Fig. 7.3). In this case, ABPs could have contact with both networks nearly simultaneously. Anyhow, most ABPs can diffuse fast through the cytoplasm (typical diffusion rates measured for globular proteins of 10 to 100 kDa range from around 10 to 100 μm2 s−1) and thus can be in contact with both networks even when they are not proximal (Arrio-Dupont *et al*., 2000; Milo and Phillips, 2016). For these reasons, transport or local activation of ABPs cannot account for the protein segregation observed in cells. This observation indicates that actin networks themselves or even the actin filaments represent different substrates for downstream protein interactions. To date, two main hypotheses, not mutually exclusive, are guiding this field towards a better understanding of these principles. The first hypothesis is that the geometrical organization of filaments within actin networks is itself a sufficient characteristic to make these substrates distinct for ABPs (Fig. 7.4 left). The second hypothesis is that the actin filaments themselves within the actin networks could present different biochemical signatures, which could differentiate them for the different ABPs of the cell (Fig. 7.4 mid, right). These biochemical signatures include the use of different actin isoforms, the modification of actin monomers by post-translational modifications, competition between proteins and the binding of an ABP that could give a molecular identity to actin filaments. These mechanisms are often not distinct from each other, but interrelated. In the next sections I will introduce all of these concepts and then I will make a particular focus on the actin isoforms.

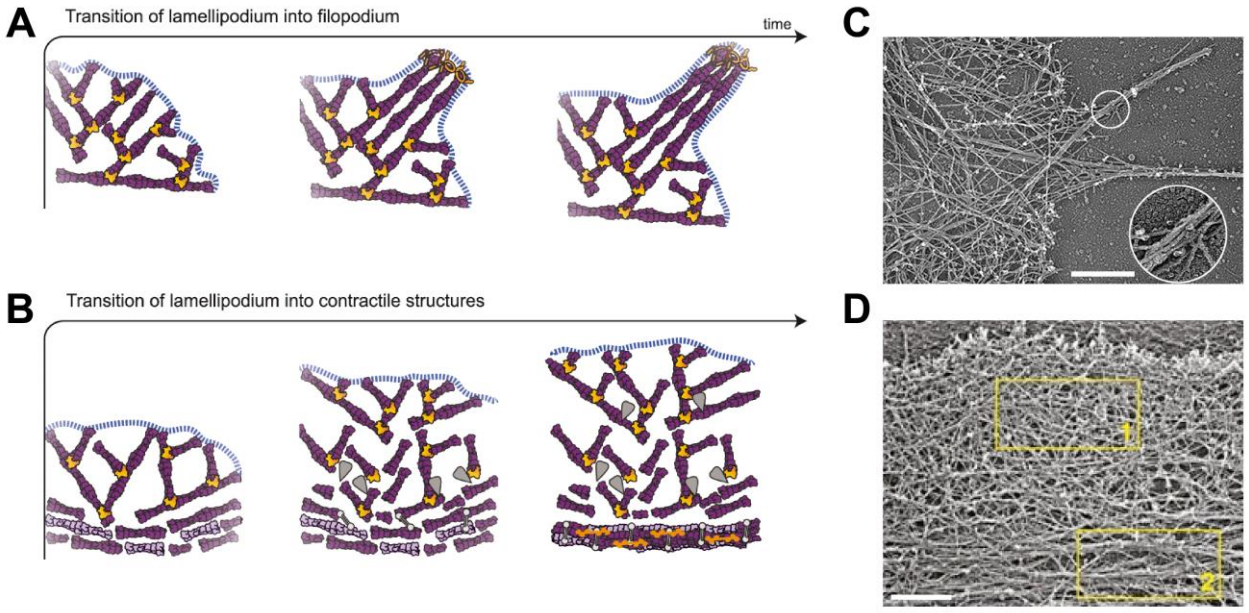


Fig. 7.3. Actin architecture transitions.

(A) Schematic representation of the growth of a filopodia from the branched network in the lamellipodia. **(B)** Schematic representation of the formation of actin cables in the lamella from the collapse of filaments in the branched network of the lamellipodia. **(C-D)** Electron microscopy pictures of these structures in cells. (C) shows a filopodia and the lamellipodia, (D) shows the lamellipodia and the lamella. Scale bars: 1 µm. Modified from (Letort *et al.*, 2015; Biyasheva *et al.*, 2004; Burnette *et al.*, 2011).

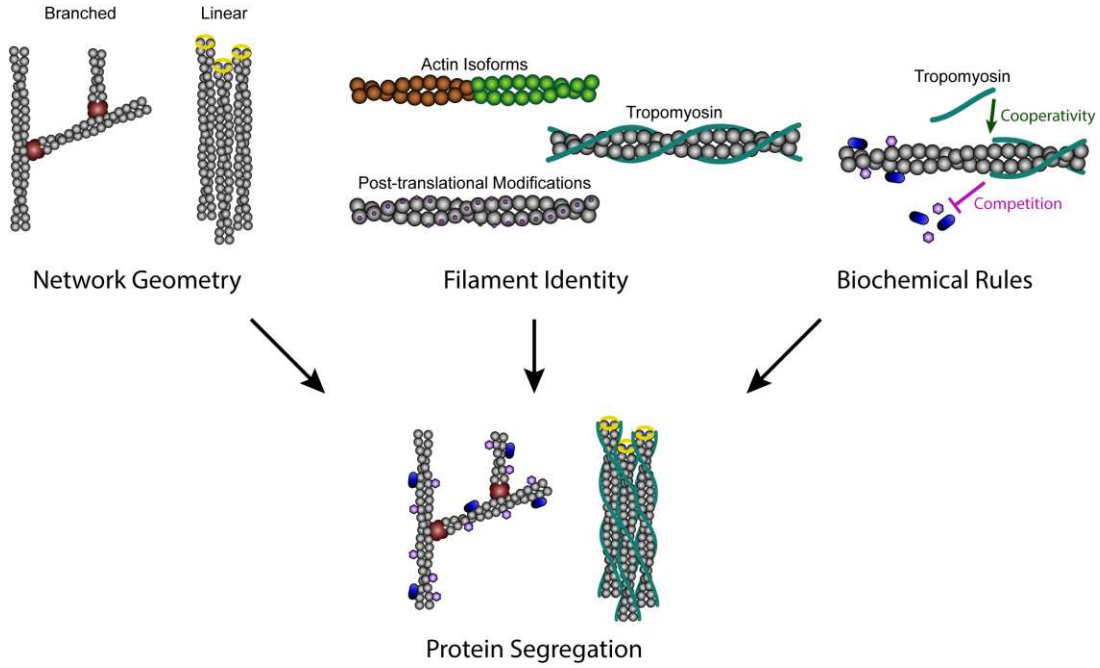


Fig. 7.4. Strategies for protein segregation in the actin networks.

Schematic representation of the three main mechanisms that account for the segregation of ABPs to different actin networks in cells. Differences in network geometry can direct the localization of some proteins to a particular network. Filaments can gain a particular molecular identity by many mechanisms: the use of different isoforms, the binding of tropomyosin to a particular network and post-translational modifications of the actin molecule. Cooperativity or competition between actin-binding proteins can also dictate which proteins can bind to a particular network. Figure from (Boiero Sanders *et al.*, 2020).

7.2.1 Actin network geometry as a feature for ABP segregation

The different actin networks are not only differentiated by how the filaments are organized (branched or linear) but also by the difference in filament length and orientation. For example, the branched actin networks of the lamellipodium are composed of short actin filaments of 7–18 actin subunits (Svitkina and Borisy, 1999), and the orientation of the filaments is forming a diagonal meshwork where the barbed ends of the filaments are directed towards the membrane of the cell (Verkhovsky *et al*., 2003; Risca *et al*., 2012). Branched filaments at endocytic sites appear to be longer than lamellipodial filaments, ranging between 18 and 68 actin subunits (Young *et al*., 2004; Rodal *et al*., 2005; Sirotkin *et al*., 2010; Akamatsu *et al*., 2020). Linear arrays, on the contrary, are often composed of longer filaments with up to 300 actin subunits which can be parallel to each other or have random orientations (Svitkina *et al*., 2003; Kamasaki *et al*., 2005).

This difference in geometrical organization, orientation and length of the filaments could affect the affinities of ABPs that are sensitive to these characteristics. This has been observed in contractile myosins with multiple motor domains, where their binding and activity has been shown to be affected by whether the actin networks are disorganized, branched by the Arp2/3 complex, parallel or antiparallel (Nagy *et al*., 2008; Nagy and Rock, 2010; Stachowiak *et al*., 2012; Reymann *et al*., 2012; Ennomani *et al*., 2016). *In vivo,* these myosins exhibit contractile activity in filaments with opposite polarity, whereas they play a role in trafficking on parallel bundles (Svitkina, 2018; Koenderink and Paluch, 2018). The sensitivity to filament orientations is not a feature exclusive of myosin. This has also been observed for some bundling proteins that bind preferentially when the spacing between two actin filaments is favorable (Winkelman *et al*., 2016). Moreover, other proteins such as ADF/cofilin, accumulate on linear networks of actin filaments, but are not efficiently recruited on branched actin networks in vitro (Fig. 7.5A) (Gressin *et al*., 2015). The observation of the in vitro pattern of ADF/cofilin binding is particularly interesting. In cells, ADF/cofilin is recruited to branched networks and hardly detectable on linear networks (Fig. 7.5B) (Lappalainen and Drubin, 1997; Dawe *et al*., 2003; Okreglak and Drubin, 2007). This indicates that even if the geometrical organization of actin network is a key parameter to modify the binding affinity and activity of some proteins, such as myosin, it is not sufficient to explain the localization pattern of other proteins, such as ADF/cofilin. These observations indicate that additional principles, beyond the actin filament network architecture, need to be taken into account to obtain a global picture of how ABPs are addressed in cells.

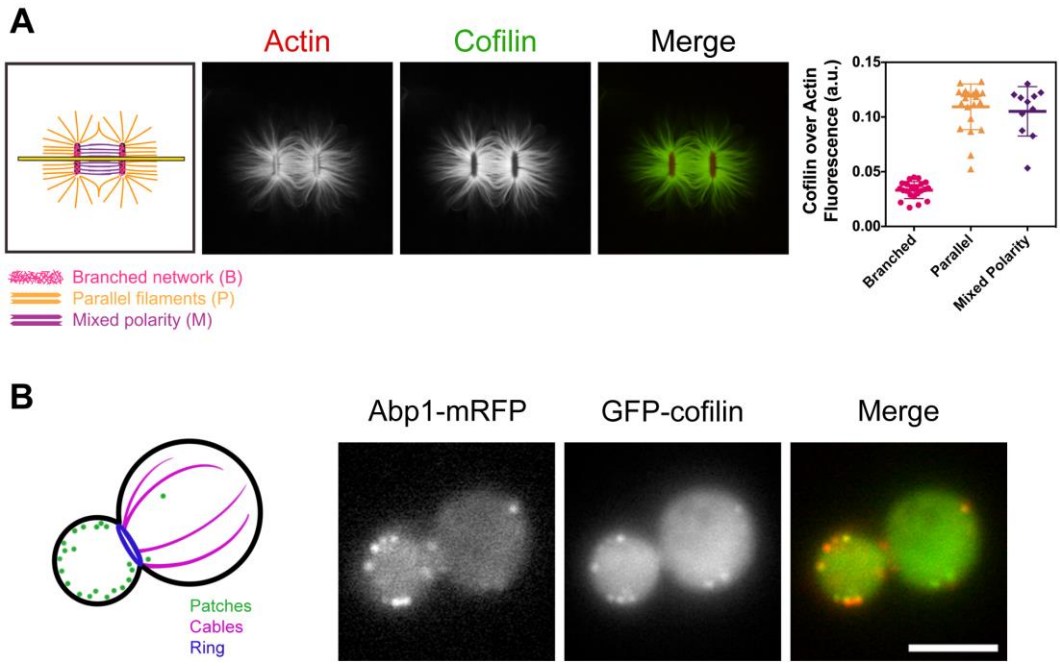


Fig. 7.5. Preference of ADF/cofilin for linear networks *in vitro* does not explain its *in vivo* localization.
(A) (left) Schematic representation of the geometry of actin networks in a reconstitution assay *in vitro*. (mid and right) Binding pattern of ADF/cofilin shows that it binds preferentially to linear networks *in vitro*. **(B)** (left) Schematic representation of actin structures in *Saccharomyces cerevisiae*. (right) ADF/cofilin colocalizes with Abp1, a protein that is found in the branched network in the actin patches.

7.2.2 Biochemical signatures of the actin molecule: Origins and characteristics

The first hypothesis, which states that the geometry of the networks is sufficient to address the ABPs to the different networks, originates from observations that some ABPs can indeed differentiate the networks. However, it is not the case for all ABPs. This is why geometry alone cannot account for the observed protein segregation. The necessity to discriminate different populations of actin filaments in cells must lead us to consider other possibilities, including that small differences in structure and surface properties of the actin filaments themselves could modulate their affinity for certain ABPs.

Different actins across all eukaryotes tend to be almost identical. It is believed that the reason why actin is so conserved in sequence is that once it became involved in more than one function, it had little chance to mutate without compromising any of these functions. Today, actin is involved in many key cellular processes and the pressure to remain conserved is even higher. The reason why this pressure is so high is because almost every actin residue is either buried in the actin core or plays a role for an important protein-protein interaction. This is why even between two distant eukaryotes such as budding yeast and human, which diverged more than a billion years ago, actin sequences still retain around 90% identity. In mammals, mutations in the cytoplasmic actins are generally rare, and usually lead in humans to serious diseases such as Baraitser–Winter syndrome (Rivière *et al*., 2012). If the actin molecule itself cannot change, how can we

consider differences in surface and structural properties as a possible mechanism to confer actin filaments a molecular identity? If we analyze the genomes of all eukaryotes, it seems that across evolution different strategies emerged to confer differences in the actin molecule without the need to change the original actin gene. The first one corresponds to the expansion of actin-related genes by genome duplication and the second one corresponds to the use of tropomyosin as a source of filament diversity (Gunning *et al*., 2015). The use of different actin isoforms, which corresponds to the first scenario, has been observed in many organisms. These isoforms can be either cell-specific or expressed simultaneously in the same cell types. An extreme example is plants, which express multiple actin isoforms, that originated from genome duplications (Fig. 7.6). The number of actins varies for each plant species and can reach, for example, 21 isoforms in Zea mays. Interestingly, some plant isoforms are more closely related to the isoforms of other species, rather than inside the same species (Gunning *et al*., 2015). This means that plant actins do not show the same level of conservation that mammalian actins have. An alternative strategy to differentiate actin filaments without the need to duplicate the actin gene to produce a different actin is through the use of specialized ABPs. Indeed, whereas some species, such as plants and amoebas, generally express dozens of different actin isoforms, other eukaryotes, for example, those from the kingdoms Animalia or Fungi, express only one or a very limited number of cytoplasmic actin isoforms (Fig. 7.6). Conversely, species expressing one or few actin isoforms express a multitude of tropomyosins, which are specific ABPs that wrap around actin filaments, whereas plants or amoebas do not express any (Fig. 7.6) (Gunning *et al*., 2015). This very strong anticorrelation is a signature that these two phenomena are probably related to each other. It suggests that while some species use multiple actin isoforms, representing different substrates for ABPs, in order to create different actin-related functions, other species that had gained tropomyosins in the course of evolution could use a limited number of actins, decorated by different tropomyosins, to generate functional diversity.

We expand these two scenarios by including all kinds of biochemical properties and interactions, taking into account post-translational modifications (PTMs) as another way to modify the surface properties of actin and protein competition and cooperative as an extra source of segregation. Combining all these possibilities, we end up with two options. One option is that specific ABPs could progressively decorate actin filaments to give them a specific identity, reinforcing or limiting the binding of other ABPs to the same filaments by competition, steric effects or by stabilizing particular conformations of the filaments (Michelot and Drubin, 2011). A second option is that cells could possibly use different actins, either through the expression of different actin isoforms or through post-translational modifications (PTMs) (Perrin and Ervasti, 2010; Šlajcherová *et al*., 2012). These two scenarios are not mutually exclusive, meaning that cells could also use several strategies simultaneously to create the greatest possible diversity of actin substrates.

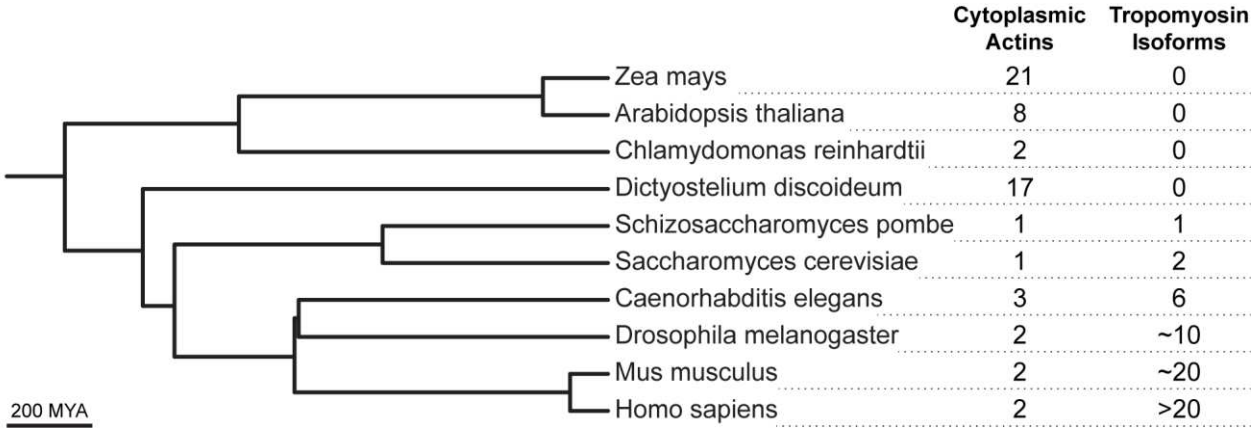


Fig. 7.6. Analysis of the number of cytoplasmic actins and tropomyosin isoforms across eukaryotes.
Anti-correlation of the number of cytoplasmic actin and tropomyosin isoforms. Plants express many actin isoforms but they lack tropomyosins. On the other hand, animals and fungi possess less actins but they can express a variety of tropomyosin isoforms.

7.2.2.1 Tropomyosins as a player in the identity of actin filaments

Sometimes the different networks can be composed of identical (or nearly identical) actin molecules. In this case, the actin molecules themselves cannot account for the differences in between the networks and the biochemical particularities of the ABPs composing each of the networks have to be taken into account. The branched and linear networks are nucleated by different ABPs, mainly the Arp2/3 complex and the formins, respectively. One could hypothesize that filaments acquire particular identities at the moment when they are generated by nucleation factors. However, formins and the Arp2/3 complex can be present in the same network and generate filaments that are being elongated by formins but are also branched (Block *et al*., 2012, 2). In cells, mixed actin networks can also be found (Fig7.1). These observations suggest that other regulators are needed to explain the protein segregation observed in between the branched and the linear networks. We have already seen in the previous paragraph that careful genomic analysis strongly suggests that proteins of the tropomyosin family are responsible for functional diversity of the actin cytoskeleton in higher eukaryotes (Gunning *et al*., 2015). We shall see that genetics, cell biology and biochemistry have also provided additional evidence for the importance of tropomyosins.

Tropomyosins are dimers of α-helices forming parallel coiled-coils that span several actin subunits (Gunning *et al*., 2005). Most cells express multiple tropomyosin isoforms and splicing variants, and those proteins have been proposed to provide actin filaments specific identities (Gunning *et al*., 2005; Michelot and Drubin, 2011; Vindin and Gunning, 2013; Gunning and Hardeman, 2017). There are many tropomyosin isoforms in metazoans and each of them has a specific binding pattern (Hardeman *et al*., 2019; Manstein *et al*., 2020). The different actin structures in cells are usually decorated by specific families of tropomyosins. Tropomyosin isoforms are recruited mainly with linear networks (filopodia, lamella, stress fibers) but do not bind branched networks (lamellipodia,

endocytic sites) (DesMarais *et al*., 2002; Vindin and Gunning, 2013; Gunning and Hardeman, 2017; Hardeman *et al*., 2019). An increase in the amount of intracellular tropomyosin clearly affects actin homeostasis by favoring the linear network over the branched network, while reducing tropomyosin levels favors the branched network (Gupton *et al*., 2005; Iwasa and Mullins, 2007; Antkowiak *et al*., 2019). The evident preference for tropomyosin for linear networks led researchers to study it in more depth if there was an interaction between these two proteins. Some tropomyosin isoforms have been shown to positively affect formin elongation and nucleation (Wawro *et al*., 2007; Skau *et al*., 2009; Alioto *et al*., 2016). On the same line, some formins were shown to dictate the localization of specific tropomyosins (Johnson *et al*., 2014). However, some formins have been shown to not affect tropomyosin localization (Meiring *et al*., 2019). Even when actin filaments are not generated de novo by formins, tropomyosin recruitment correlates also very well with filament debranching and the re-organization of actin filaments into new linear actin structures (Bugyi *et al*., 2010; Hsiao *et al*., 2015). All these results show that even if some tropomyosin isoforms can interact with specific formins, the localization pattern and function of tropomyosins is not purely dictated by formins.

The particular and unique binding pattern of tropomyosin is a good candidate to confer the actin filaments a specific network identity. Indeed, tropomyosin binding regulates to the recruitment of particular families of ABPs, and the dissociation of others. Tropomyosin impacts both the organization and the activity of myosin (Ostap, 2008; Clayton *et al*., 2010; Hu *et al*., 2019). Tropomyosin binding to actin filaments prevent the binding of other ABPs, such as fimbrin or ADF/cofilin, therefore preventing filaments from disassembly (Christensen *et al*., 2017; Gateva *et al*., 2017; Christensen *et al*., 2019; Jansen and Goode, 2019).

In summary, tropomyosins can differentiate different types of filaments and bind to them conferring a specific molecular identity. Filaments decorated by tropomyosin can regulate the binding of many families of ABPs, thereby leading to the segregation of these proteins to different actin networks.

7.2.2.2 Cooperative and competitive binding effects on the actin network protein composition

There are many studies that show that not only tropomyosins, but also most ABPs, display cooperative or competitive binding effects to actin filaments. These protein interactions cannot be ignored when trying to understand how the different ABPs are addressed to each network (Michelot and Drubin, 2011). A number of cellular biology studies have unambiguously demonstrated that the removal of a given ABP from one actin network may trigger a global relocation of ABPs from other actin networks (Skau and Kovar, 2010; Billault-Chaumartin and Martin, 2019). As a consequence, phenotypes observed in cells can arise not only due to the absence of a specific ABP, but also due to the mislocalization of other ABPs. Several hypotheses could explain this phenomenon.

First, it is possible that the absence of a protein in a given network may open a binding site for other proteins, or allow the binding of competing proteins. For instance, in yeast, removal of fimbrin from actin patches, which are Arp2/3-branched networks, causes an ectopic localization of tropomyosin to those networks (Skau and Kovar, 2010). Under another hypothetical scenario, it is possible that ectopic protein localization triggers the secondary recruitment of additional proteins. For instance, loss of CP from actin patches creates free actin filament barbed ends, where formins can bind, which in turn favors the ectopic binding of tropomyosin (Billault-Chaumartin and Martin, 2019). In a third scenario, the absence of an ABP could also have consequences on the geometry of the network, which would consequently impact its ABP composition. Overall, these results indicate that although tropomyosins are key regulators for addressing ABPs to appropriate networks, proper segregation of ABPs on specific actin networks in cells also relies on a global and complex biochemical equilibrium, involving many different families of ABPs. Addressing these questions further will require to integrate all these parameters into a comprehensive model.

7.2.2.3 Post-translational modifications of actin

Co- and PTMs are modifications that are done covalently to one or several amino acids of a protein by specific enzymes. These modifications can affect the interactions of the protein with its partners by changing its surface charge density, its structure, or by steric hindrance. For this reason, an actin modified with a PTM could have a different molecular identity than a non-modified actin.

The most abundant PTM for β- and γ-actin is N-terminal acetylation, which is the addition of an acetyl group (Drazic *et al*., 2018). In animals, this PTM occurs after cleavage of the first one or two amino acids, and most of the actin in the cell is modified by this PTM (Redman and Rubenstein, 1981; Rubenstein and Martin, 1982, 1983; Solomon and Rubenstein, 1985). N-terminal acetylation of actin is mediated by the acetyltransferase NAA80, which is specific to actin and acetylates preferentially the monomeric actin-profilin complex (Goris *et al*., 2018; Rebowski *et al*., 2020). Acetylation has been shown to affect cell motility and the cytoskeletal organization (Drazic *et al*., 2018). Acetylated actin has faster dynamics, both for assembly and disassembly (Drazic *et al*., 2018). Other actin residues can also be acetylated. A complex of lysine-acetylated actin and cyclase-associated protein (CAP) was shown to promote the inhibition of the formin INF2 (A *et al*., 2019).

Actin can also present another modification at the N-terminal end. It can be argynilated, which is the addition of an arginine residue. This modification is mediated by the arginyl-tRNA-protein transferase Ate1, which has been identified in several organisms, including mammals, plants and budding yeast (Kashina, 2014). In all observed cells, only β-actin seems to be arginylated. The reason behind this difference between β-and γ-actin is that the latter is specifically degraded when it is arginylated, so it does not accumulate inside

the cells (Karakozova *et al*., 2006; Zhang *et al*., 2010). Studies in Ate1 knock-out cells indicate that actin arginylation is responsible for both a decreased interaction with gelsolin and for an increased recruitment of capping protein (CP) and twinfilin (Saha *et al*., 2010).

Relatively few studies have compared the dynamics of non-modified versus modified actins. Here, we present evidence that PTMs can play a role in conferring actin a specific molecular identity. PTMs can not only regulate actin properties and its binding to other proteins, but also the activity of the other proteins themselves.

Arginylation and acetylation are two main PTMs of actin. Other PTMs, including phosphorylation and methylation, can also modify the chemistry of the actin molecule. For more details, we refer readers to a more detailed review (Terman and Kashina, 2013).

8. Actin isoforms

We saw that different mechanisms could explain the variety of functions that actin can achieve. These particularities can come from the linear and branched network themselves, from tropomyosin binding only to one type of filaments, from competition between the bound ABPs or from PTMs that modify the actin molecule. Yet, is it always necessary to have an external factor to the actin molecule to confer the actin filaments a molecular identity? We saw that plants usually possess more actin genes than animals and fungi. Do plants use different actins for different functions? And what about the other groups? In addition, we saw that actins are exceptionally conserved in amino acid sequence in the same organism and also across different species. They also have nearly identical tridimensional structures. Are those tiny differences enough to confer a molecular identity to the actin filaments? Or do all actin molecules behave the same? What is the mechanism by which highly similar actins could generate such a difference in localization or function? These are some of the questions that I will address in this section but also during the course of my PhD.

Protein isoforms or variants are proteins that are functionally similar and are produced from the same gene or family of genes. It normally refers to proteins that are produced from the same gene via alternative splicing, as is the case for the tropomyosin isoforms. However, in the case of actin isoforms they are proteins produced from the same family of genes.

In the next sections I will discuss whether very similar eukaryotic actins from different species are able (1) to assemble separately within a common cytoplasm and (2) to form filaments of sufficiently specific molecular identity to interact differently with ABPs and carry specific cellular functions. The answer to this question is far from being clear today and is the subject of intense research. This is the reason why I will end up by discussing the conflicting results and topics we have yet to understand.

8.1 β- and γ-actin localization and functions

For a period longer than 30 years after its discovery in 1942, actin was thought to be a single, highly conserved protein in all cell types (Straub, 1942; Bray, 1973). It was during the mid-70s that actin isoforms were discovered (Gruenstein and Rich, 1975; Garrels and Gibson, 1976; Whalen *et al*., 1976; Vandekerckhove and Weber, 1978). Mammalian organisms have six different actin isoforms: four muscle actins and two cytoplasmic actins. The latter, called β- and γ-actins, differ only in four amino acids at the N-terminal end and are simultaneously and ubiquitously expressed in cells.

Shortly after the discovery of actin isoforms, the scientific community was highly interested in the possible advantage of having several actins. Many studies were done during the 80s to better understand the localization and functions of these isoforms (Lubit

and Schwartz, 1980; Otey *et al*., 1986, 1988; Casale *et al*., 1988; DeNofrio *et al*., 1989; Hoock *et al*., 1991). The differential sorting of muscle actins vs non muscle actins was the first to be observed (Lubit and Schwartz, 1980; Otey *et al*., 1988; DeNofrio *et al*., 1989). During these first studies, β-actin and its mRNA were shown to localize preferentially in the lamellipodia beneath the plasma membrane (Hoock *et al*., 1991; North *et al*., 1994). This matter was not addressed for many years, and later and newer studies show a different localization pattern. β-actin was found mainly in actin bundles of basal stress fibers, filopodia, at cell–cell contacts and in contractile rings (mainly linear network), whereas γ-actin is present mainly in lamellar and dorsal cell regions (mainly branched network) (Fig. 8.1A) (Dugina *et al*., 2009; Chen *et al*., 2017). This difference in β-actin localization could be explained by a difference in the staining conditions (Dugina *et al*., 2009). In podosomes, which are actin-rich adhesive structures involved in migration and invasion, actin filaments consist of a β-actin core which is surrounded by a γ-actin envelope, composed of linear actin (Fig. 8.1B) (van den Dries *et al*., 2019).

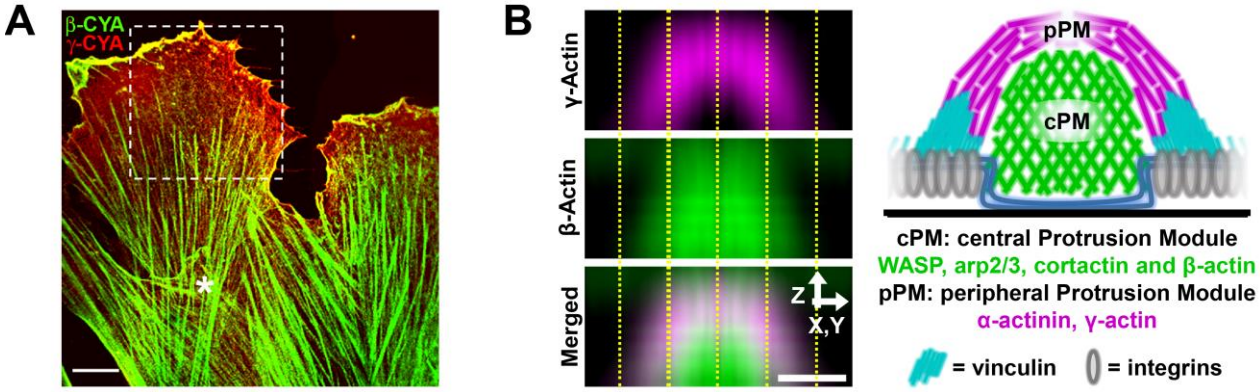


Fig. 8.1. Localization of β- and γ-actins into different networks.

(A) Example of the differential localization of β- (green) and γ-(red) actins at the cell scale, in migrating human subcutaneous fibroblasts. Scale bar: 10 μm. **(B)** Differential localization of β- (green) and γ-actins (magenta) in podosomes. Scale bar: 0.5 μm. Adapted from (Dugina *et al*., 2009; van den Dries *et al*., 2019).

These differences in localization suggest that these two isoforms, despite being so similar, could be associated with different cellular functions. Numerous isoform-specific knock down studies have shown the implication of β-and γ-actin in several processes. B-actin has been shown to play a role in cell motility (Hoock *et al*., 1991; Bunnell *et al*., 2011; Joseph *et al*., 2014). Loss of γ-actin has been shown to be decrease migration in some cases (Dugina *et al*., 2009; Shum *et al*., 2011), but it can also induce migration in another model (Lechuga *et al*., 2014). In agreement with β-actin localization in the cytokinetic ring, β-actin has been shown to be an important factor for cell division, although γ-actin seems to also play a role in early stages of this process (Bunnell *et al*., 2011; Joseph *et al*., 2014; Patrinostro *et al*., 2017; Dugina *et al*., 2018). In conclusion, these experiments are not sufficient to distinguish the roles of β-and γ-actin, since both isoforms appear to be implicated in both processes.

8.2 Role of the nucleotide sequence

The understanding of β-and γ-actin in the generation of different functions has been further complicated by the analysis of the nucleotide sequence contribution. Despite their amino acid sequences being so similar, the nucleotide sequences of β-and γ-actin genes possess silent mutations that affect 40% of the codons (Fig. 8.2A). These silent mutations have been proven to be an important factor of actin regulation. For example, the translation of β-actin is produced in bursts and it accumulates much faster than γ-actin (Zhang *et al*., 2010; Buxbaum *et al*., 2014). This difference in translation exists because γ-actin mRNA induces a stalling event during translation which is not observed for β-actin and leads to their differential arginylation (Zhang *et al*., 2010). In addition to the effect of the coding sequence, the untranslated regions (UTR) of the actin gene are also involved in actin regulation. These regions include elements that control different aspects of mRNA regulation, for example, mRNA secondary structure, transport and localization (Andreassi *et al*., 2018). In the particular case of the cytoplasmic actins, the most studied is the 3' UTR of β-actin which includes an element that is called "zipcode" and is involved in the localization and local translation of the transcript (Condeelis and Singer, 2005).

In 2017, Vedula *et al.* tested the hypothesis of the importance of the nucleotide sequence at the functional level using mice and mouse embryonic fibroblasts (Vedula *et al*., 2017). Knock-out mice for both cytoplasmic actins have been done in the past. B-actin mice are reported to be embryonic lethal, whereas γ-actin knock-out have a milder phenotype (Shawlot *et al*., 1998; Shmerling *et al*., 2005; Belyantseva *et al*., 2009; Bunnell and Ervasti, 2010). This observation led the scientific community to believe that β-and γ-actins were not functionally equivalent, as γ-actin could not replace the lack of β-actin and mice could not develop. Interestingly, Vedula *et al.* were able to generate viable mice that are not expressing any β-actin (Fig. 8.2B) (Vedula *et al*., 2017). They achieved so by using a gene that expresses γ-actin but is carrying β-actin's silent mutations. These mice have the γ-actin gene and they also have another gene, which is similar to the β-actin gene, but that was point mutated so that it produces γ-actin. This gene has only five point mutations compared to the β-actin one. In addition, actin structures in cells lacking β-actin (but expressing γ-actin from this "β-actin coded" gene) were normal (Fig. 8.2C), as well as their ability to migrate. They conclude the study by suggesting that the *in vivo* functions of β-and γ-actins are defined by their nucleotide sequence, rather than their amino acid sequence. It appears that this difference in nucleotide sequence lead to a higher density of ribosomes in β-actin mRNA than γ-actin mRNA. This means that cells can produce β-actin in bursts, while this would not be possible for γ-actin (Fig. 8.2D).

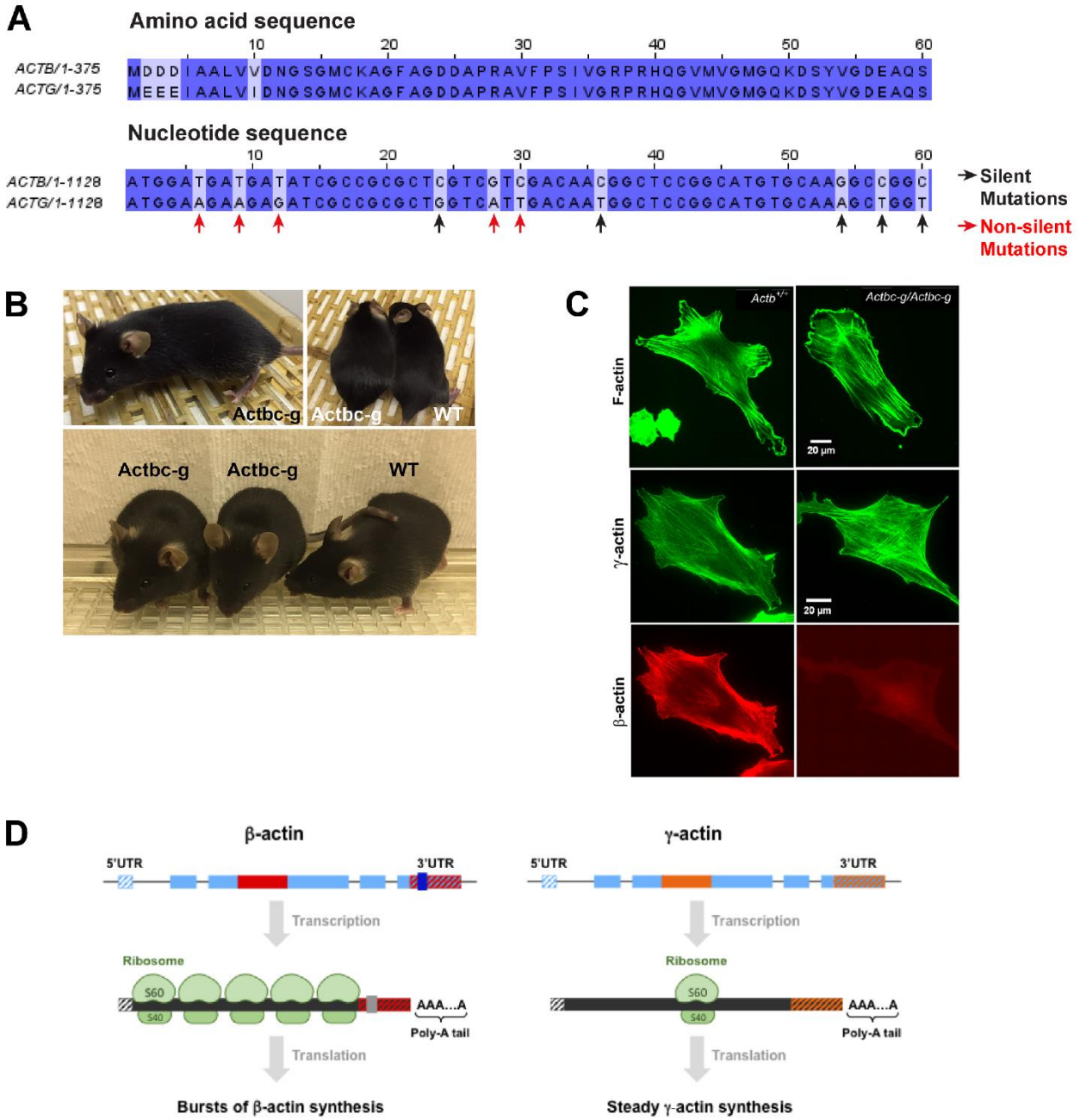


Fig. 8.2. Role of the nucleotide sequence in the actin gene.

(A) First sixty positions of the amino acid and nucleotide sequences of β- and γ-actins. These proteins only differ in four amino acids located at the N-terminal end, although their nucleotide sequences have a much higher number of silent mutations (e.g. black arrows). **(B)** Mice lacking β-actin but carrying a β-coded gene to express γ-actin (Actbc-g) are healthy and reach adulthood similar to wild type mice (WT). **(C)** Mouse embryonic fibroblast derived from mice lacking β-actin (Actbc-g/ Actbc-g) have a normal cytoskeletal organization. **(D)** Differences in different areas of the the β- and γ-actin nucleotide sequence cause, among other consequences, that both actins are not translated in the same manner. While β-actin has a high ribosomal density and is produced in rapid bursts, γ-actin is produced steadily at a low rate. Modified from (Boiero Sanders *et al*., 2020; Vedula *et al*., 2017; Gunning and Hardeman, 2018).

8.3 Plant actins localization and functions

Other organisms that express several actin isoforms are plants. Moreover, plants do not have genes encoding tropomyosin. For this reason, it has been hypothesized that the variety of actin-related functions in plants is generated by the use of different actin isoforms. *Arabidopsis thaliana*, the most famous plant model organism, has 10 actin genes that are grouped in two classes according to their sequence similarities and their tissue-specific expressions: vegetative and reproductive (Šlajcherová *et al*., 2012). Vegetative and reproductive actins are involved in different cellular processes and have different patterns of expression (McDowell *et al*., 1996). However, having a tissue pattern of expression is not the only way that plant actins can form separate structures. Plant actin isoforms that are expressed in the same tissue can also be spatially segregated. For example, the main vegetative isoforms ACT2 and ACT7 had a separated distribution in leaf epidermal and mesophyll sponge cells (Kijima *et al*., 2018). ACT2 assembled into thinner and longer filaments, whereas ACT7 formed thick bundles (fig). Besides their ability to assemble into different structures, plant isoforms are not functionally equivalent. Expression of the reproductive actin ACT1 in vegetative tissues causes dwarfing and altered morphology in most organs, showing that expression of ACT1 in these tissues is affects the dynamics of actin and its regulators (Kandasamy *et al*., 2002).

Speaking about actin isoforms, a curious case is the one of the green algae *Chlamydomonas reinhardtii*, which carries two actin genes. The main isoform IDA5 is a conventional actin that is expressed in normal conditions. The second actin, called NAP1 (for Novel Actin-like Protein 1), is highly divergent as it shares only 65% sequence identity with IDA5 (Lee *et al*., 1997). These two actins are expressed in different conditions, for example, when IDA5 is absent or after addition of the actin monomer sequestering drug latrunculin B (Kato-Minoura *et al*., 1998; Onishi *et al*., 2016). This drug can prevent IDA5 polymerization, but surprisingly NAP1 generates latrunculin B-resistant structures. Despite being so different, essential actin functions can be performed by either of these actins, and cells lacking any of the actin genes can grow and divide normally (Onishi *et al*., 2016) (fig). and have been shown to overlap in the main functions but yet have isoform-specific functions as well (Detmers *et al*., 1983, 1985; Kato-Minoura *et al*., 1997; Hirono *et al*., 2003).

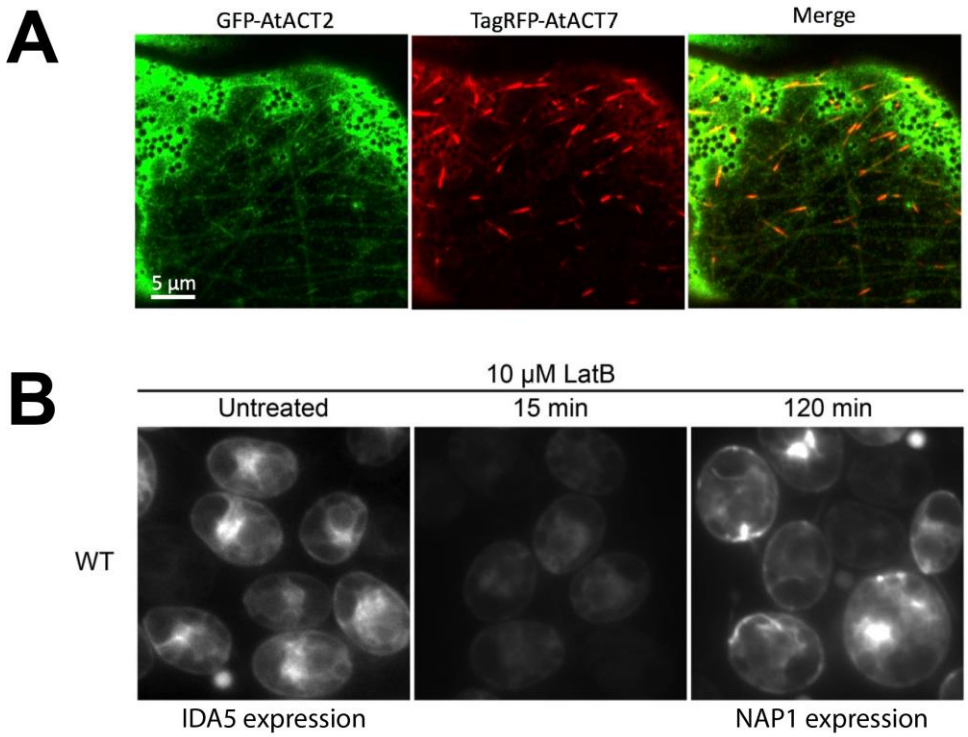


Fig. 8.3. Actin isoform localization in *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*.
(A) Localization of the actin isoforms ACT2 (green) and ACT7 (red) in *N. benthamiana* leaf epidermal cells. ACT2 assembled into thinner and longer filaments, whereas ACT7 formed thick bundles. **(B)** Actin structures in in *Chlamydomonas reinhardtii* cells. Untreated cells express the conventional actin IDA5, which is disassembled by LatB. 120 minutes after LatB treatment, drug-resistant actin structures appear. Modified from (Kijima *et al*., 2018; Onishi *et al*., 2016).

8.4 Biochemical similarities and differences between actins

Actin isoforms in various organisms are spatially segregated and appear to be involved in specific functions. This suggests that actin isoforms, even though they are very similar, may have different biochemical properties.

Since the purifications of actins coming from rabbit muscle and yeast are well established, most of the studies have been done on these actins. These two actins present 87% of identity, which indicates that these two actins are quite different comparatively to all actins expressed in eukaryotes. Even so, budding yeast and rabbit muscle actins can copolymerize (McKane *et al*., 2006). Surprisingly, this is less clear for β-and γ-actin, despite being 99% identical. While these two isoforms were shown to copolymerize in some studies, other studies reported their ability to assemble into independent filaments (Bergeron *et al*., 2010; Müller *et al*., 2013; Chen *et al*., 2017). Some studies show the differences in the biophysical and biochemical properties of some actins. Yeast and rabbit muscle actins show differences in flexibility (Orlova *et al*., 2001; McCullough *et al*., 2011) and yeast actin polymerizes faster than muscle actin (Kim *et al*., 1996; Buzan and Frieden, 1996; Takaine and Mabuchi, 2007; Ti and Pollard, 2011). This difference in rates of

polymerization is also observed in plants' isoforms (Kijima *et al*., 2016). Nucleotide hydrolysis, nucleotide exchange and Pi release are also faster for yeast actin compared to muscle actin filaments (Eads *et al*., 1998; Yao and Rubenstein, 2001; Bryan and Rubenstein, 2005; Takaine and Mabuchi, 2007; Ti and Pollard, 2011). In summary, we can hypothesize from few well-characterized actins, that the combination of these biochemical and biophysical subtleties might overall account for important functional differences in cells.

If the combination of these differences in the biochemical properties may render actin isoforms sufficiently different to generate a molecular identity, the affinity or activity of ABPs could also change depending on the actin isoform. The simplest explanation for one particular actin to be enriched in a specific structure would be that the actin nucleators are isoform-specific. There are only a few studies that investigate the preference of actin nucleators for a specific isoform. One study that I would like to highlight is the one published by Chen *et al.* in 2017 (Chen *et al*., 2017), where the authors investigated the regulation of cytokinesis. Cytokinesis, the last step of cell division where the mother cell divides into two daughter cells, requires a linear actin structure called the cytokinetic ring. Using immunofluorescence to visualize β- and γ-actin it has been observed in different studies that the cytokinetic ring is composed of β-actin (Fig. 8.4A). Chen *et al.* showed that the nucleation of β-actin at the cytokinetic furrow depends on a formin called DIAPH3 and its ability to interact with the scaffolding protein Anilin. The authors performed *in vitro* experiments using purified actin from platelet and gizzard. The purified actins are a mix of both β-and γ-actin, but their proportions are different. Platelet actin is enriched in β-actin while gizzard actin is enriched in γ-actin. These experiments show that the formin DIAPH3 preferentially nucleates β-actin when both β-and γ-actins are available, suggesting that actin cables assembled from DIAPH3 could be enriched with β-actin (Fig. 8.4B). Another study has investigated the implication of both cytoplasmic actins during the spread of vaccinia virus (Marzook *et al*., 2017). This virus infects the cells and induces branched actin nucleation and this process depends mainly on β-actin. However, the VCA domain of N-WASP, an activator of the Arp2/3 complex, does not show specificity for β- or γ-actin (Marzook *et al*., 2017). These findings suggest that the specific nucleation of a certain actin isoform might occur at the level of the Arp2/3 complex, rather than with its activator. However, this hypothesis has not been tested.

Other studies have investigated the possibility of different actins to have different interactions with the ABPs. Some of these studies have been performed with proteins from the same organism and other explore the differential interactions when using actins or ABPs from other organisms, mainly comparisons between yeast proteins and human proteins. In *Arabidopsis thaliana*, profilins seem to have different binding affinities depending on the actin isoform (Kijima *et al*., 2016). Moreover, to achieve different actin functions, it appears that that many actins may have co-evolved with their binding partners, rendering proteins from the same species optimized (Nefsky and Bretscher,

1992; Kandasamy *et al*., 2007; Takaine and Mabuchi, 2007; Ezezika *et al*., 2009; Ti and Pollard, 2011; McCullough *et al*., 2011; Kang *et al*., 2014).

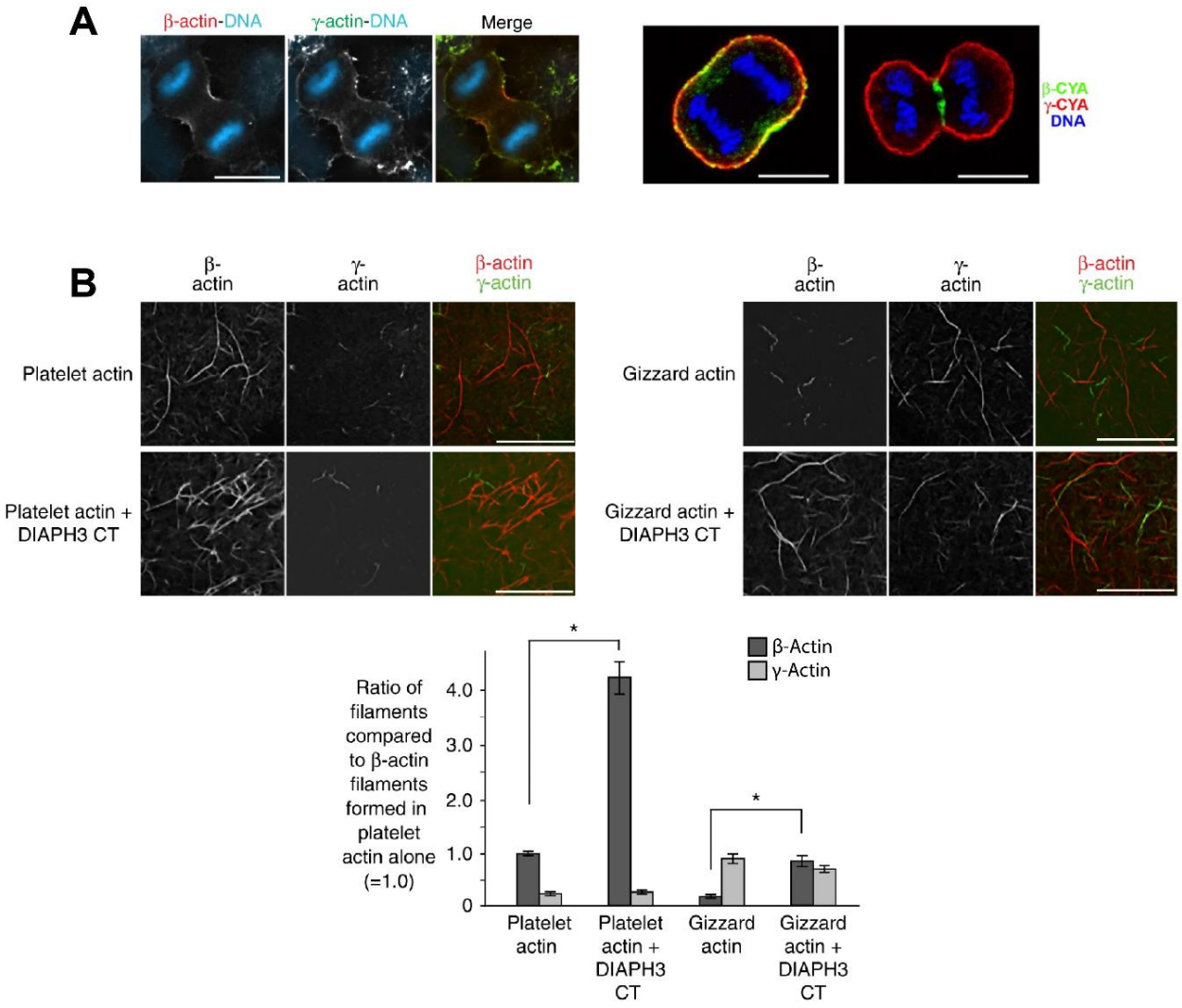


Fig. 8.4. Preference of the actin nucleator DIAPH3 for β-actin.

(A) Antibody staining showing the localization of β- and γ-actin during cytokinesis in HeLa cells (left) and HaCaT cells (right). β-actin localizes to the cytokinetic ring. Scale bar: 10 μm **(B)** Single filaments from actin isolated from platelet (left) or chicken gizzard (right). Filaments were stained with antibodies against β-actin (red) and γ-actin (green). In both cases, after addition of the formin DIAPH3 there is an increase of nucleated β-actin compared to γ-actin.

9. Tools to study the basic mechanisms that regulate the actin cytoskeleton

All the mechanisms and strategies described before probably act in concert to regulate the actin cytoskeleton. Understanding the base of these mechanisms, especially when using complex models such a mice or mammalian cells, is extremely difficult. Mammalian systems in particular possess large families of ABPs which can very often compensate for each other's function which makes the interpretation of the molecular mechanisms particularly challenging. For this reason, I would like to investigate this topic using a one-actin system with a smaller set of ABPs. I will use this system to measure the consequences of expressing a heterologous actin at the cellular level and cytoskeletal structures. I decided to use *Saccharomyces cerevisiae*, known as well as budding yeast, as a model system. There are many benefits to using this model which I will detail in the next section. To uncover the molecular principles of a possible segregation, I will use a reconstituted *in vitro* assay. Both methods by itself would not be sufficient but combined they provide a better understanding of the results at different levels: the cellular level and the molecular level. In this section I will describe both models and the advantages of using them.

9.1 An *in vivo* model: Budding yeast

9.1.1 General characteristics

Saccharomyces cerevisiae, also called budding yeast or baker's yeast is a unicellular organism that belongs to the Fungi Kingdom and the Ascomycota Division. It has been established as a model organism to study biological processes due to many reasons, which I will discuss in the next sections.

First of all, *S. cerevisiae* shares many cellular mechanisms and pathways with human cells, even if both diverged around 1100 MYA. This makes it a great model to study and to draw a parallel with *Homo sapiens* and other eukaryotic cells. The advantage of using budding yeast to better comprehend mammalian cells was already highlighted in 1997 where Botstein *et al.* reported that nearly 31% of the potential protein-encoding genes of *S. cerevisiae* has a statistically robust mammalian homolog (Botstein *et al*., 1997). Moreover, the mechanisms and machineries that govern the assembly of different actin structures are highly conserved among the eukaryotes (Moseley and Goode, 2006).

The life cycle of *S. cerevisiae* is very fast, and cells have a doubling time of around 2 hours at 25ºC (Fig. 9.1) compared to human cells which divide once every 24 hours at 37ºC. Yeast cells are stable in the diploid and haploid states and divide through budding (Fig. 9.2 - 1). Budding is a type of mitosis (cell division) in which a small swelling starts growing from a specific location in the mother cell. This outgrowth is called bud and it will give rise to the daughter cell after cytokinesis. Diploid cells have two copies of each of the 16

chromosomes and therefore they carry two copies of each gene. In adversary conditions, for example lack of nutrients, yeast cells can undergo sporulation. The result of this process is the creation of a spore, a resistant structure carrying four quiescent cells (Fig. 9.2 - 2). The four cells inside the spore are haploid and are produced by meiosis. In favorable conditions, haploid cells can proliferate. Haploid cells from different mating types (a or α) can mate (Fig. 9.2 - 3) and generate a diploid cell. In the wild, haploid cells in the spores start growing again if the conditions become favorable and haploid cells instantly mate with the opposite mating type cells that are in the vicinity. For this reason, yeast cells in nature are found mainly in the diploid state. In lab conditions, however, we can isolate haploid cells of a certain mating type so that they remain in that state.

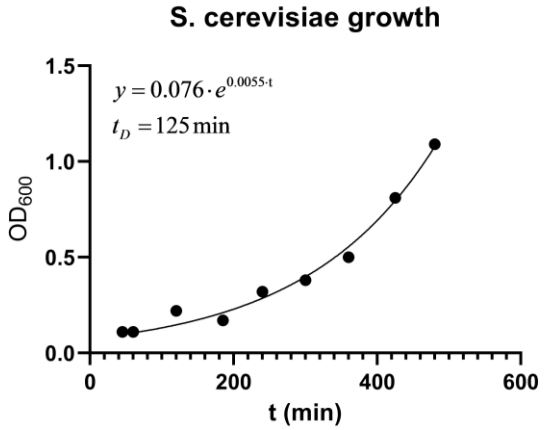


Fig. 9.1. *Saccharomyces cerevisiae* growth.

Growth of *Saccharomyces cerevisiae* cells depicted as increase in the optical density measured at 600 nm over time at 25ºC. The doubling time (t_D) of the culture is approximately 2 hours.

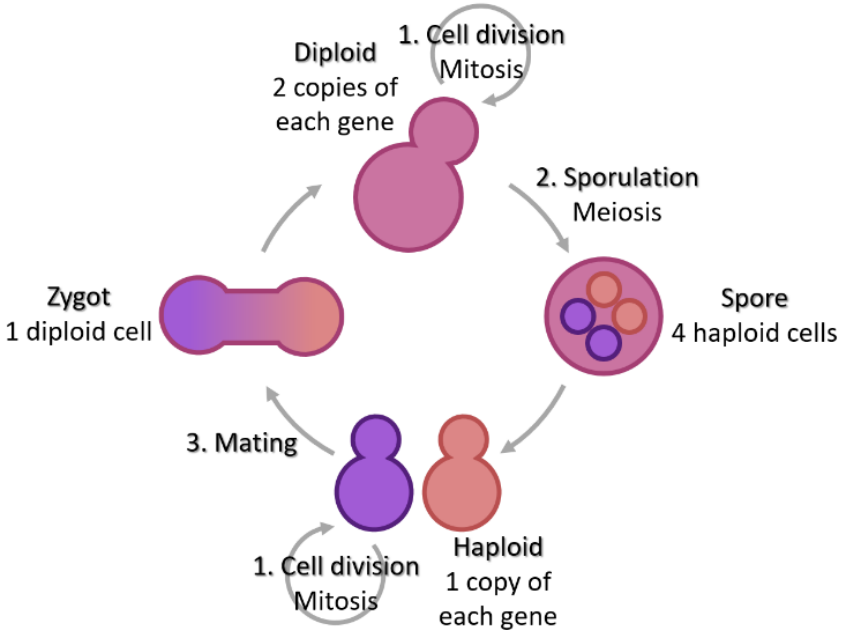


Fig. 9.2. The life cycle of *Saccharomyces cerevisiae*.

1. Diploid and haploid cells can proliferate by diving through mitosis. 2. In adverse conditions, diploid cells can sporulate generating a spore through meiosis. The haploid cells in the spore can proliferate if the conditions are beneficial. 3. Haploid cells can mate and generate a diploid cell.

In 1996, *S. cerevisiae* was the first eukaryotic organism to have its genome fully sequenced (Goffeau *et al*., 1996, 600). The sequences of all genes and proteins, along with their position in the genome, can be found at Saccharomyces Genome Database (https://www.yeastgenome.org/). Besides being fully annotated, the genome of *S. cerevisiae* can be easily modified by homologous recombination. Homologous recombination is a DNA repair pathway that occurs regularly in cells when there is DNA damage, such as double or single strand breaks. Experimentally, a researcher can take advantage of this process to modify a target sequence of the cell genome using a designed DNA construct. For this project in particular, another interest is that this yeast has only one actin locus, which we can mutate and or replace at will by homologous recombination (Gallwitz and Seidel, 1980; Ng and Abelson, 1980). Taking advantage of the yeast life cycle, it is possible to study a mutation when cells are haploids or diploids. Haploid cells carry only one copy of the gene and therefore we can study the direct effect of the mutation. Diploid cells will have one copy of the gene carrying the mutation of interest and another copy of the gene, that could be wild type or mutated as well.

9.1.2 Actin structures in budding yeast

Budding yeast is a great model to study actin structures. When studying the linear and the branched networks, it presents an invaluable advantage compared to some other model organisms: actin structures in budding yeast are well defined and spatially segregated along the cell cycle. In dividing cells, three main structures can be easily seen: patches, cables and a ring (Fig. 9.3).

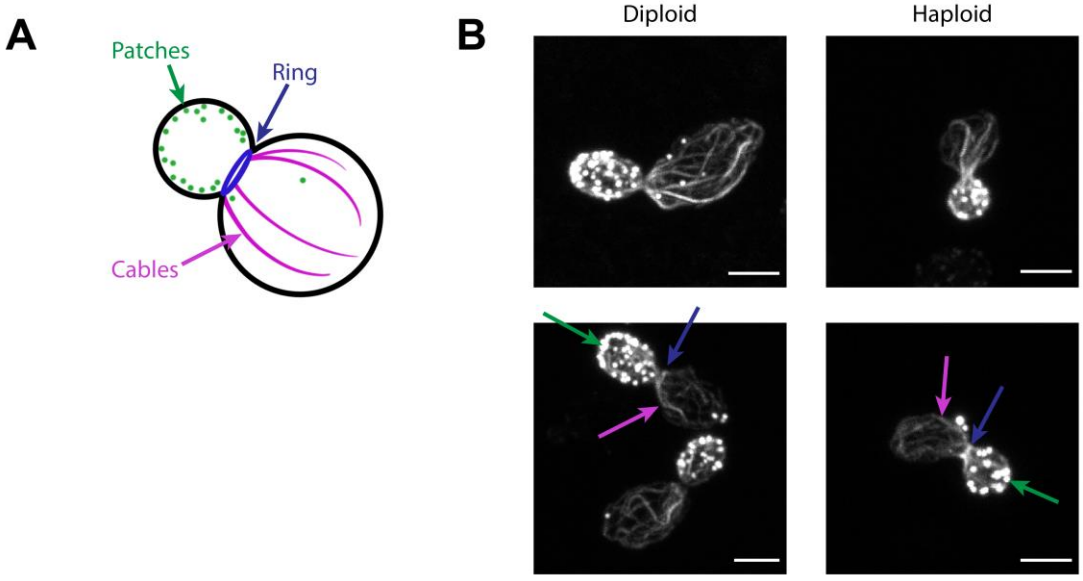


Fig. 9.3. Actin structures in *Saccharomyces cerevisiae*.
(A) Schematic representation of the main actin structures in a dividing yeast cell. Branched actin network can be found in the patches (green) and linear actin networks can be found in the cables (magenta) and cytokinetic ring (blue). **(B)** Actin organization in diploid and haploid yeast cells. Arrows point towards the actin structures schematized in (A). Cells were stained with fluorescent phalloidin and pictures were taken with a confocal microscope. Scale bar: 3 μm.

9.1.2.1 Actin patches & Endocytosis

The actin network in the patches is composed of branched actin and as such bind all the regulators of the branched actin network, including the most iconic one, the Arp2/3 complex. These structures play a role in clathrin-mediated endocytosis; they are highly dynamic with a turnover rate of 10 to 20 seconds (Moseley and Goode, 2006). Endocytosis is a process in which the cellular membrane is invaginated and substances from the media are brought into the cell. This process helps as well to regulate the composition of the cellular membrane by internalization of transmembrane proteins. During their lifetime, endocytic patches undergo three different phases: (1) a non-motile phase, which corresponds to the initial recruitment of early patch components, (2) a slow-moving phase, which corresponds to the recruitment and activation of the actin nucleating machinery, which in turn leads to membrane invagination driven by actin polymerization and finally (3) a fast-moving phase, which corresponds to membrane scission and rapid transportation of the vesicle along actin cables, following actin depolymerization (Fig. 9.4) (Goode *et al*., 2015). The internalized vesicles then fuse with the endosome so that the components can be degraded or recycled. This process is highly regulated and dozens proteins are recruited sequentially to each of these 3 stages.

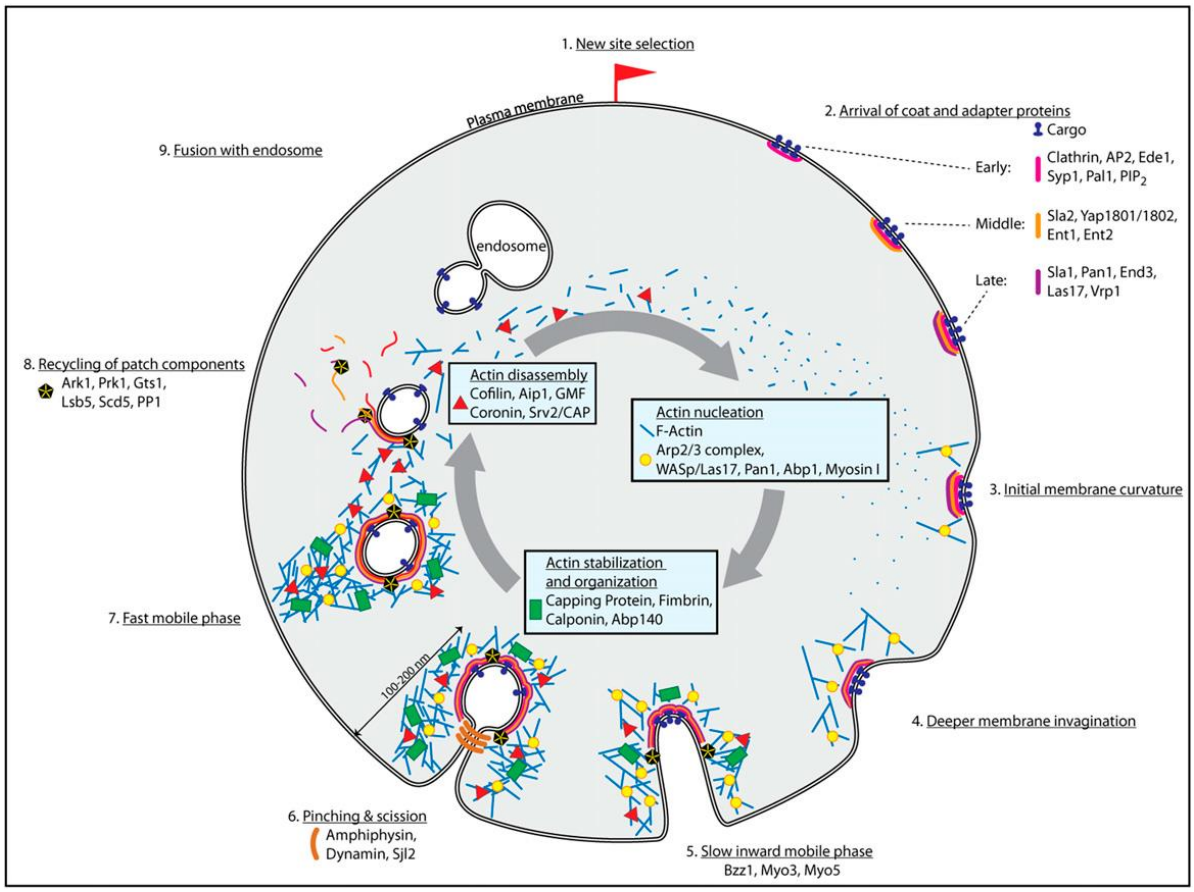


Fig. 9.4. Initiation, maturation and turnover of endocytic patches.

Representation of the different endocytic phases: The non-motile phase (1 to 3), the slow-moving phase (4-6) and the fast moving phase (7 to 10). Some of the many proteins that are involved in this process are detailed in the image. Modified from (Goode *et al*., 2015).

9.1.2.2 Actin cables & transport/polarity

Actin cables in budding yeast are linear structures, composed of short filaments that are oriented in the same direction, creating a polarized actin cable. Actin cables act as the rails that are necessary to transport of secretory vesicles, organelles and other cargos to direct polarized cell growth. These cargos are transported mainly by type V myosin motors (Myo2 and Myo4). Actin filaments composing cables are nucleated by the budding yeast formins: Bni1 and Bnr1. Bni1 is located at the bud tip and Bnr1 is located at the bud neck (Fig. 9.5) (Garabedian *et al*., 2018). These two forms have different dynamics (Buttery *et al*., 2007). Bni1 is highly dynamic when it is free and displays a retrograde movement when associated with an actin filament. On the contrary, Bnr1 is relatively static and it does not exchange with a cytoplasmic pool. Most actin cables are decorated with yeast tropomyosins, Tpm1 and Tpm2, which stabilize the filaments and occupy the Cofilin binding site (Bernstein and Bamburg, 1982; Ono and Ono, 2002; Gateva *et al*., 2017). Cables that are not decorated by tropomyosin are rapidly disassembled by the action of Cofilin and Aip1 (Gressin *et al*., 2015).

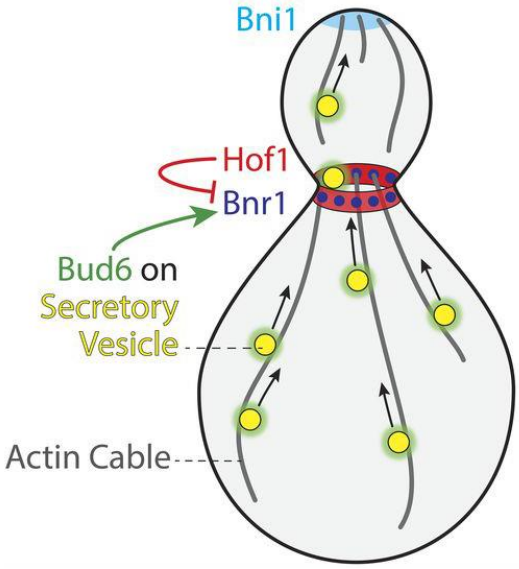


Fig. 9.5. Myosin-mediated transport on actin cables.
Secretory vesicles are transported through actin cables. These cables are nucleated by two formins: Bni1 which locates at the bud tip, and Bnr1 which locates at the bud neck. Modified from (Garabedian *et al*., 2018).

9.1.2.3 Actin ring & cytokinesis

The actin cytokinetic ring is a contractile structure located at the bud neck (Fig. 9.6). It is composed of actin and a type II myosin (Myo1) alongside other regulatory proteins (Lippincott and Li, 1998). Similar to cable assembly, the ring formation in budding yeast requires formins, profilin and tropomyosin (Tolliday *et al*., 2002). This structure is anchored to the cell membrane and it is formed during the last step of the cell division process, cytokinesis (Bi *et al*., 1998). This actomyosin ring constricts the bud neck creating a cleavage furrow which is followed by the formation of a septum, process that culminates in the separation of one cell into two cells (Moseley and Goode, 2006).

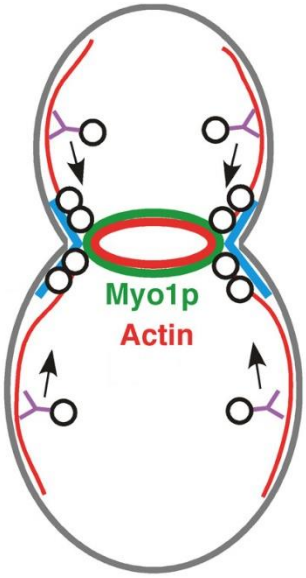


Fig. 9.6. The actomyosin ring.
The actomyosin ring constricts the bud neck during cytokinesis. Modified from (Luo *et al*., 2004).

9.1.2.4 Other actin structures

Other actin structures also exist in yeast. Some of them are less visible while others exist outside of normal growing conditions. For example, in quiescent cells we can observe the formation of a non-dynamic structure composed of actin and some ABPs called actin bodies (Sagot *et al*., 2006). This structure is thought to be an actin reservoir that can be reused for cable and patch production upon reentry into a proliferation state. Less visible actin structures could play a role in vacuolar fusion, endoplasmic reticulum cortical dynamics, and chromatin remodeling, as actin was shown to be important in all those processes (Prinz *et al*., 2000; Eitzen *et al*., 2002; Blessing *et al*., 2004).

9.1.3 Imaging of the actin cytoskeleton in *S. cerevisiae*

There are many ways to observe the actin cytoskeleton in budding yeast cells. Each method is useful to obtain different information. I will describe two of the imaging methods that I have used during my PhD.

9.1.3.1 Actin cytoskeleton in fixed cells

There are many ways to observe the actin cytoskeleton in cells. A widely used technique for F-actin visualization in fixed cells is by labelling it with fluorescent phalloidin. Phalloidin is a toxin that is found in the deadly mushroom *Amanita phalloides* that binds and stabilizes actin filaments. By coupling is with a fluorophore it is possible to use as a probe to specifically targ*et al*l the actin filaments in the cells. Phalloidin is commercially available coupled with many fluorophores and this allows to label actin with different colors, which is useful when the objective is to do dual-color microscopy (Fig. 9.7). The variability of possible fluorophores also offers the possibility to use different types of microscopes. This technique is not used to see the dynamics of the cytoskeleton, as the cells are not alive anymore, but to see the structures themselves.

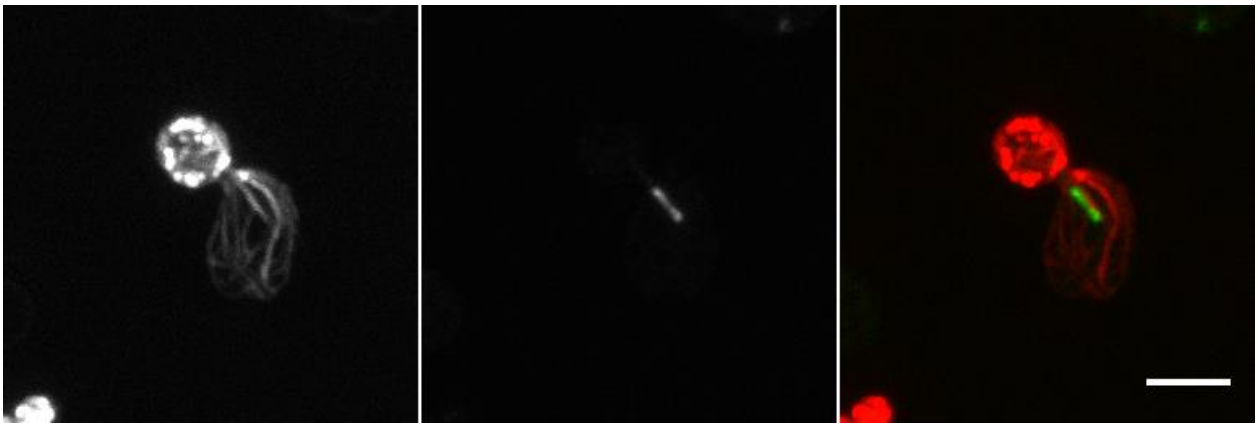


Fig. 9.7. Phalloidin labelling of a *Saccharomyces cerevisiae* strain expressing GFP-tubulin.
Z-projection of a confocal image of a yeast cell showing actin (red) and tubulin (green). Scale bar: 3 μm.

The actin cytoskeleton in fixed cells can be observed using different kinds of microscopes. The easiest way is to observe it using a regular epifluorescence widefield microscope (Fig. 9.8A, left). As the cells are fixed and the dynamics cannot be observed, it is possible to use microscopes which take more time to take the picture, such as a confocal microscope (Fig. 9.8B, left). A confocal microscope is a microscope designed to not collect the light coming from out-of-focus planes using a pinhole. This allows for resolution lower signal-to-noise ratio compared to a fluorescence microscope, but the acquisition of the images take more time and the fluorophores have to be more resistant to photo bleaching due to the higher exposure to the light. For this reason, it is advantageous that phalloidin can be coupled with fluorescent dyes that have a higher photostability, such as the Alexa or ATTO-dyes.

This variety of possible dyes grants the possibility to do super resolution microscopy. One example out of many, is Structured illumination microscopy (SIM) (Fig. 9.8A-B, right). This technique relies on the mathematical deconvolution of the interference patterns of images acquired using a known spatially structured pattern of light to acquire is highly resolved image. Unfortunately, I was not able to use this technique because some of the samples, which had a dimmer signal, were bleaching.

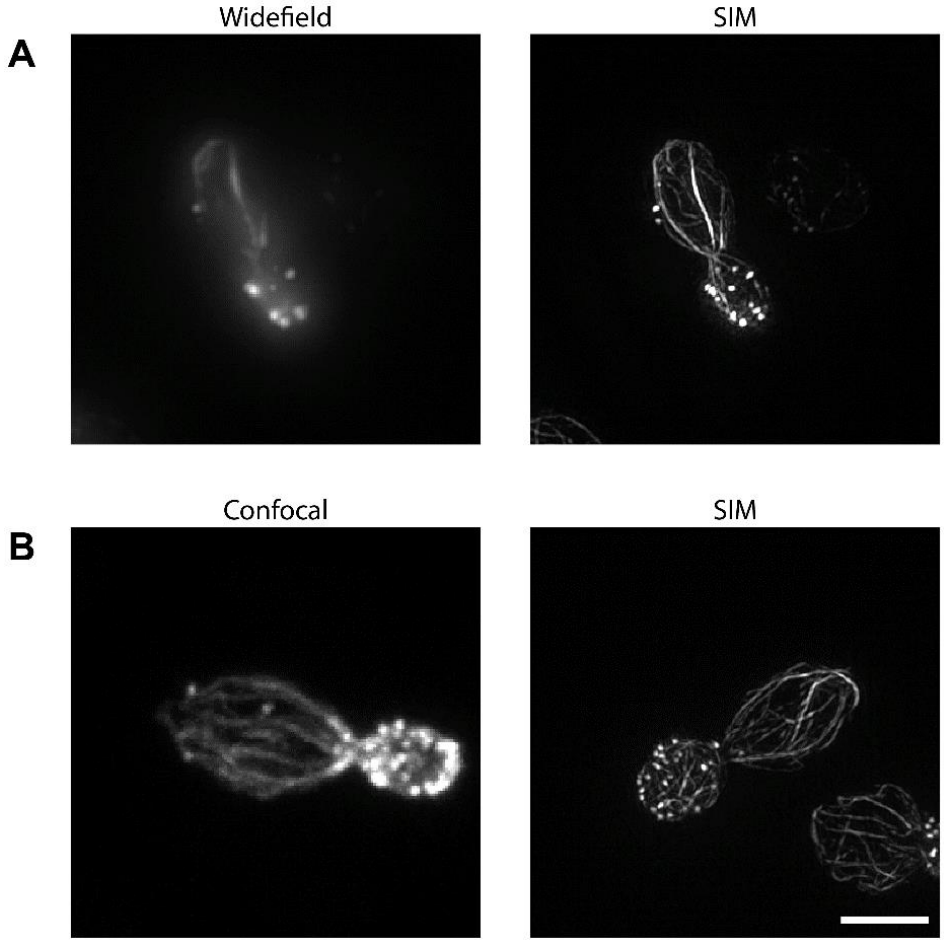


Fig. 9.8. Imaging the actin cytoskeleton in fixed cells.
S. cerevisiae cells fixed and labeled with fluorescent phalloidin. **(A)** Comparison of the same cell imaged with widefield microscopy and SIM microscopy. **(B)** Comparison between confocal microscopy and SIM microscopy (different cells). Scale bar: 3 μm.

9.1.3.2 Live imaging of the actin cytoskeleton

The live observation of the actin cytoskeleton in *S. cerevisiae* consists of using yeast strains which contain a fluorescently-tagged protein that localizes to a cytoskeletal structure. This technique is used to observe and measure different aspects of the dynamics of the actin cytoskeleton, for example, the appearance/disappearance of different structures, the order in which different proteins are recruited to those structures, etc. For example, one typical yeast strain to observe the movement of the actin patches is Abp1-GFP (Fig. 9.9, left). Abp1 is a protein that binds actin filaments and regulates Arp2/3 complex-mediated actin assembly. The human homolog of this protein is calledHIP-55. Other strains are also used to observe the movement of the patches. A typical strain used to observe both actin patches and cables in vivo is Abp140-3GFP (Fig. 9.9, middle). This strain is the only one where actin cables were successfully labeled by the tagging of an endogenous protein. For this reason, another probe was developed to label the actin cytoskeleton in vivo using the first 17 amino acids of ABP140 fused to GFP, which is called Lifeact-GFP (Riedl *et al*., 2008). It is also possible to observe the actomyosin ring with a Myo1-GFP strain (Fig. 9.9, right). It is important to note that when trying to see the dynamics of the actin cytoskeleton in a particular mutant, the strain should also have the GFP-tagged protein, making it a double mutant. Making this experiment then includes not only creating the strain, but also to verify that there are no synthetic defects in the strain.

Similar to fixed cells, the actin cytoskeleton in fluorescent strains can be observed in many kinds of microscopes, although there are some limitations. For example, if the objective of the experiment would be to analyze the dynamic behavior of actin patches, the speed of the acquisition is a very important parameter. Taking a picture of a yeast cell with a good signal-to-noise ratio using a confocal microscope can take around 30 seconds, but the lifetime of an actin patch is around 15 seconds. For this reason, faster microscopes are needed to observe the dynamics. With the development of the Airyscan, it is now possible to take confocal images at a faster scale. However, it is still important to check for the bleaching of GFP-tagged proteins when using these microscopes.

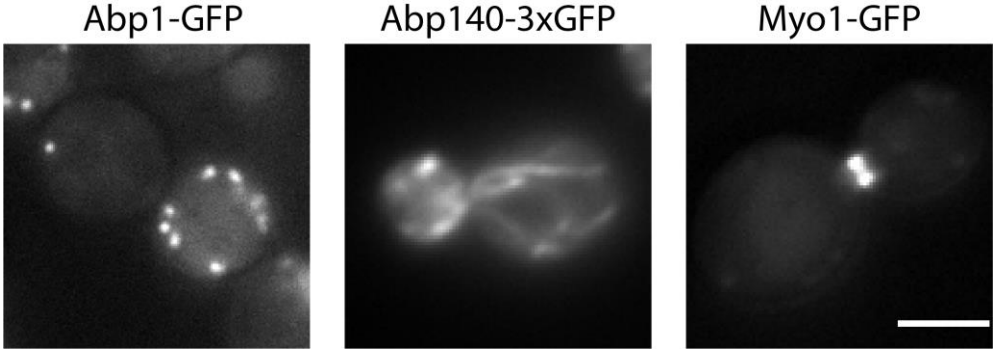


Fig. 9.9. Live-imaging of the actin cytoskeleton.
Observation of the actin cytoskeleton using strains with GFP-tagged proteins. Aip1-GFP labels the actin patches, ABP140-3xGFP labels both patches and cables and Myo1-GFP labels the actomyosin ring. Scale bar: 3 μm.

9.2 In vitro assays

9.2.1 General characteristics

As much as it is useful to study the actin regulation in a cellular context, it is hard to dissect the basic mechanisms when all the possible interactions are present, even when using a simple organism such as budding yeast. To get more information about protein-protein interactions and the mechanistic details, in vivo observations need to be complemented with the usage of simplified systems. There are two ways to approach in vitro reconstitution: the top-down approach and the bottom-up approach. In the top-down approach, cellular extracts are used so that the reconstituted system will have all the possible factors and proteins needed for the system to function properly. Using this approach, it is possible to produce cellular extracts from knock-out strains for a certain protein, therefore completely removing only one protein from the mix, to see how this would affect the system. Bottom-up approaches consist of using purified proteins to generate a minimal system. In this approach, purified proteins can be added one by one to identify the minimal set of proteins needed to reconstitute a certain structure. This way it is possible to test the role of a molecule in the presence of a limited number of partners while controlling the concentration of all components in the mixture. Over the years, different groups have made effort to reconstitute actin networks in vitro developing different techniques. These include the usage of functionalized beads, generating the networks in micro patterns and the usage of liposome-like structures where actin can be polymerized in the interior or the exterior of the liposome. Reconstituted systems have been a key factor to dissect how cellular systems work since they permit us to simplify mechanisms that would otherwise be too complex to understand. These systems provided insight in many cellular processes, like signaling processes, membrane remodeling, signal transduction and of course, cytoskeleton organization and dynamics. This is illustrated in Fig. 9.10 and the discoveries that have been achieved thanks to reconstituted systems are reviewed in (Ganzinger and Schwille, 2019).

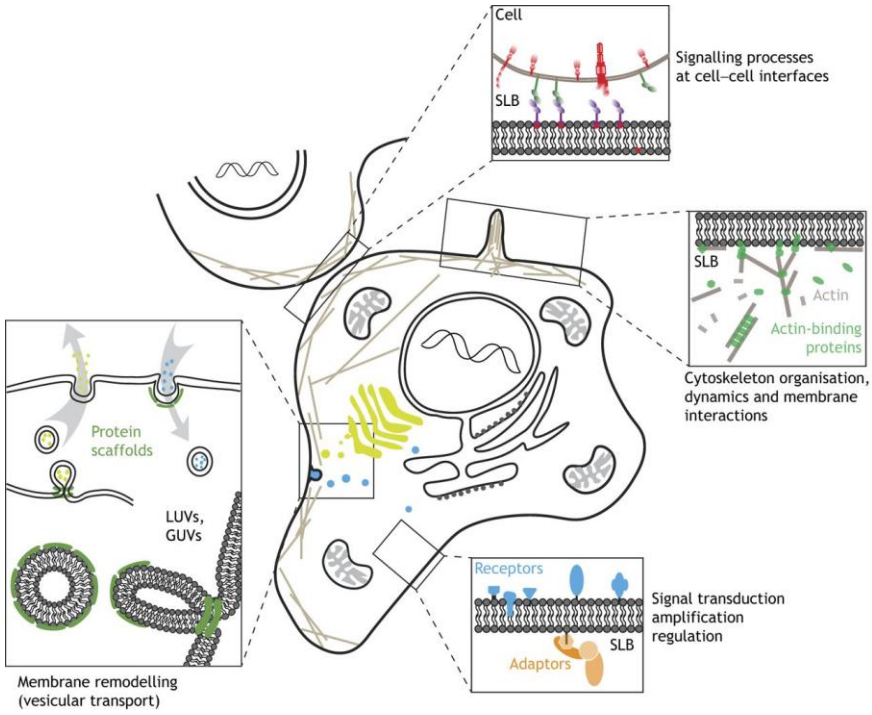


Fig. 9.10. Questions that can be addressed by using reconstituted systems.
Synthetic reconstitution of membranes led to discoveries in the fields of membrane remodeling, membrane-associated cytoskeletal rearrangements and signalling processes. From (Ganzinger and Schwille, 2019).

9.2.2 Reconstituting actin networks using functionalized beads

One powerful approach for the reconstitution of actin networks is through the usage of functionalized beads.

The idea to use functionalized beads to reconstitute the actin branched network in vitro comes from *Listeria monocytogenes*, a bacterial parasite that infects cells and uses the host actin machinery to propel itself inside the cell (Fig. 9.11A-B). This bacterium presents a polar distribution on its surface of ActA, a transmembrane protein that activates the Arp2/3 complex. This actination of the Arp2/3 complexinduces branched actin polymerization from the bacterium and this produced force is what propels the bacterium inside the host cell. Using this model as an inspiration, beads functionalized with ActA were incubated with cellular extract and the same kind of actin comet was formed (Fig. 9.11C) (Cameron *et al*., 1999). The first successful attempt to reconstitute an actin network not from cellular extract but from purified proteins *in vitro* was done the same year using Listeria cells (Fig. 9.11D) (Loisel *et al*., 1999).

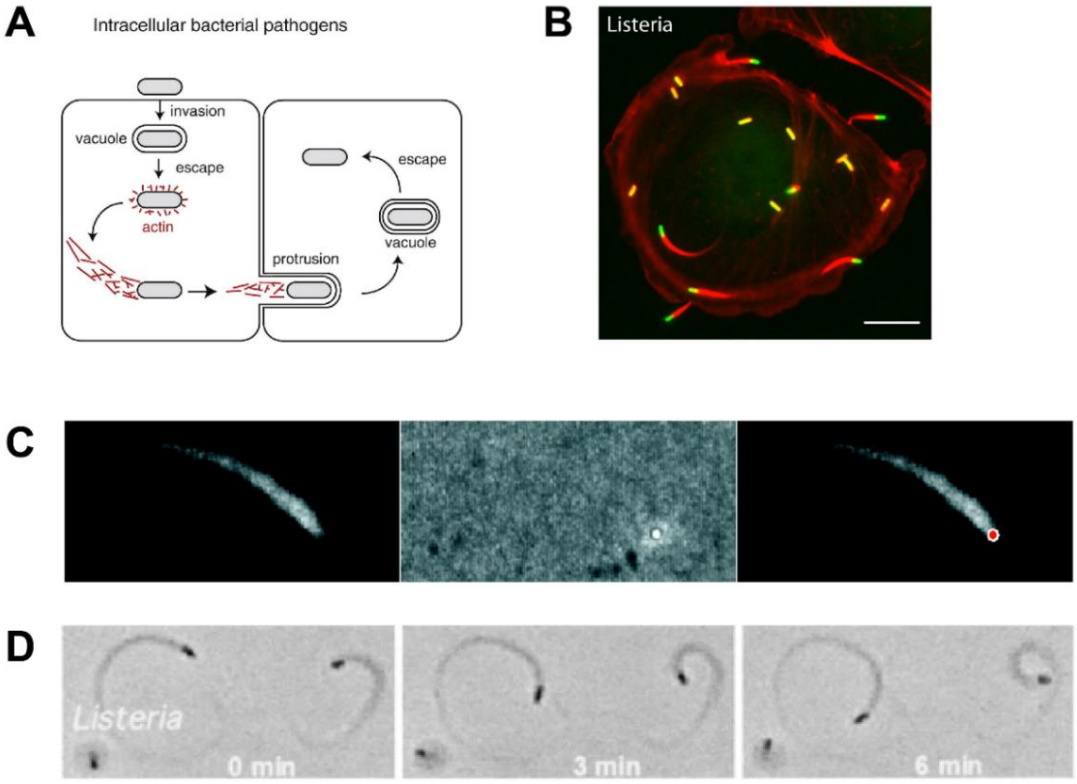


Fig. 9.11. *Listeria monocytogenes* as an inspiration to reconstitute branched network assembly.
(A) Schematic representation of the Arp2/3-complex-mediated polymerization from the surface of the parasite *Listeria monocytogenes*. **(B)** Actin comets (red) polymerizing from the surface of *Listeria monocytogenes* (green). Scale bar: 10 μm. **(C)** Branched actin polymerization from beads coated with ActA and incubated with cellular extract. **(D)** Branched actin polymerization from the surface of *Listeria monocytogenes* incubated with purified proteins. Modified from (Welch and Way, 2013; Cameron *et al*., 1999; Loisel *et al*., 1999).

It is possible to reconstitute both branched and linear networks by the use of functionalized beads (Fig. 9.12A). In the first case, the bead is covered by an Arp2/3 complex activator (NPFs), that will locally activate the Arp2/3 complex which will initiate branched network assembly from the bead. In the second case, the bead is covered by formins which nucleate linear actin filaments.

Functionalized beads with NPFs have a homogeneous distribution of the Arp2/3 complex activator all along the surface of the bead, whereas Listeria cells have a polar distribution of this activator. This means that actin is polymerized only from one side of the cell when Listeria infects a host cell. However, actin comets are observed even when using functionalized beads, where actin polymerization is not directed to only one side of the bead. Indeed, actin branched network can polymerize from the whole surface of the functionalized bead. At a certain point the pressure generated from the constant actin polymerization on the surface of the bead reaches a threshold and it leads to the breakage of the structure, a process called symmetry breaking (Achard *et al*., 2010). After this happens, actin starts to polymerize faster on one side of the bead, pushing the bead

forward in the process and therefore producing the actin comet that we can see in the images. (Fig. 9.12B).

Not only branched network can be reconstituted in vitro by the usage of functionalized beads. It is also possible to generate a linear network by functionalizing the beads with formins. In general, beads are functionalized with the FH1-FH2 domains of formin, which are the catalytic domains (Romero *et al*., 2004; Michelot *et al*., 2007). By not including the regulatory domains, the FH1-FH2 construct is constitutively active. In this system, linear filaments grow from the beads nucleated and elongated by formins (Fig. 9.12C). The linear network can be observed by microscopy and it has the shape of a "sun". The "rays of the sun" are actin bundles of linear actin that polymerize from the surface of the bead.

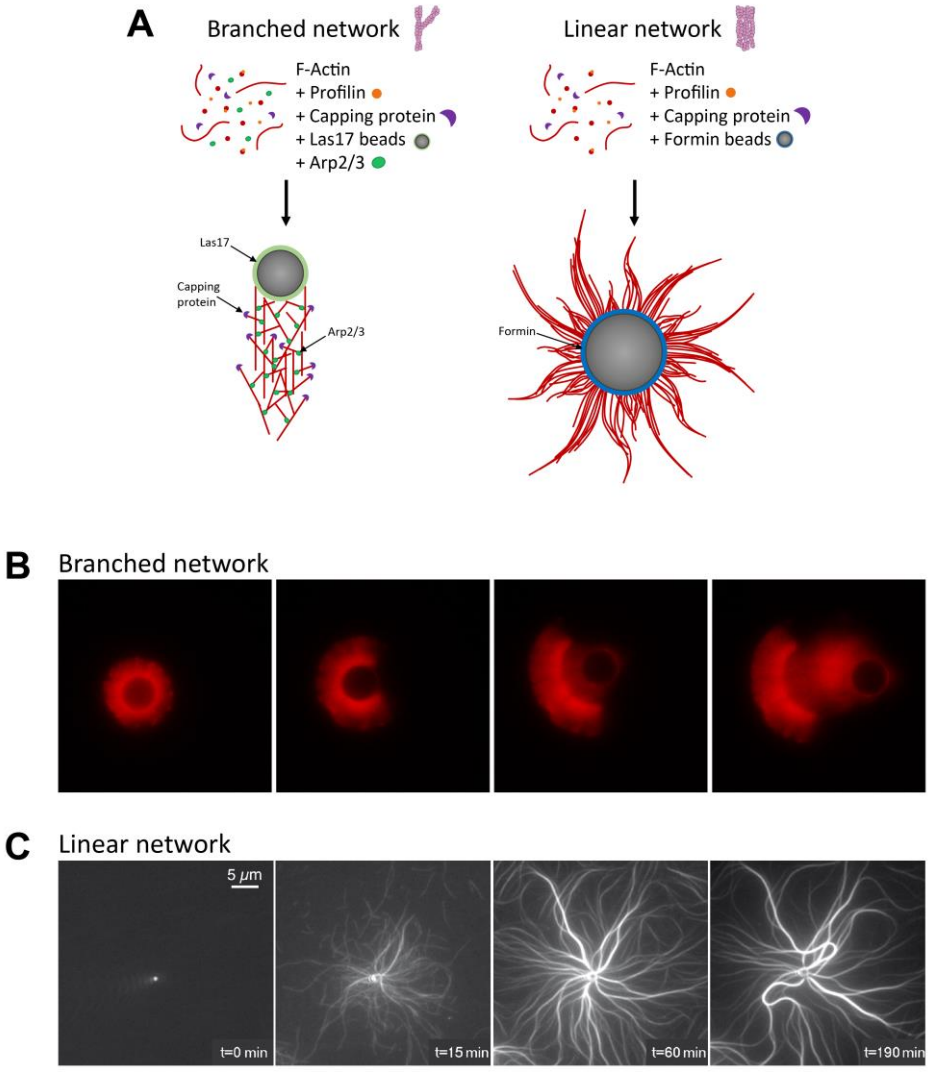


Fig. 9.12. Reconstitution of branched- and linear-networks using functionalized beads.
(A) Strategy to reconstitute actin networks *in vitro*. Las17-coated beads and formin-coated beads are incubated with a minimal set of purified proteins to reconstitute the branched- and the linear-network, respectively. **(B)** Time series of branched actin assembling from a ActA-coated bead. We can observe the symmetry breaking in the second panel. **(C)** Time-lapse of the linear actin polymerization from a mDia1-coated bead in the presence of ADF/cofilin. Modified from (Dayel *et al*., 2009; Michelot *et al*., 2007).

OPEN QUESTIONS AND OBJECTIVES

As we have seen across the introduction, there are many hypotheses that aim to explain how is one single molecule able to perform so many functions. There are a large number binding partners of actin and also several mechanisms that are involved in the actin regulation. My goal was to understand whether and how cells can use different actin orthologs to assemble different networks and perform different cellular functions. As a consequence, the molecular mechanisms that could lead to this segregation in structure and functions have to be investigated. In particular, I was interested in studying the effect that a small variation in the actin protein can have in the cytoskeletal organization and the binding with other proteins. Although many efforts have been done in the last years to understand more this subject, there is still uncertainty and questions to answer.

As it was described in the previous sections, there are several studies that observed the segregation of different yet very similar actin isoforms in different cell types. However, sometimes it is not evident to combine the knowledge gathered in all these studies since results can be contradictory. For example, cellular localization of β-and γ-actins does not suggest any particular preference for a certain type of architecture. In particular, β-actin has been observed to be incorporated in both the branched network in the podosomes and beneath the plasma membrane but it also localizes to linear structures such as the stress fibers and the contractile ring (Fig. 8.1A-B) (Dugina *et al*., 2009; van den Dries *et al*., 2019). This lack of a general obvious rule complicates our understanding of the molecular mechanisms implicated in the assembly of these two actin isoforms into distinct networks.

An interesting hypothesis would be that a PTM that affects only one actin could account for their differences. To this date, the only PTM that has been shown to modify differentially γ-and β-actin is N-terminal arginylation. This makes arginylation a likely candidate to explain the differences in localization and function of the cytoplasmic actins. Only arginylated β-actin is found in cells, since arginylated γ-actin is specifically degraded (Zhang *et al*., 2010; Karakozova *et al*., 2006). The positive charges that arginylation brings to the surface of β-actin could be driving a new molecular identity specifically for this isoform and it has been linked to its function on cell migration at the leading edge of the cell (Karakozova *et al*., 2006; Pavlyk *et al*., 2018; Chen and Kashina, 2019). Whereas arginylation is an important factor to make a distinction between both actins, only around 1% of all the expressed β-actin is arginylated (Chen and Kashina, 2019). This means that even if arginylation plays an important role in the regulation of cytoplasmic actins, it is not sufficient to explain the observed spatial segregation in cells.

All these observations are further complicated by the studies that argue that the importance is not on the amino acid sequence, but on the nucleotide sequence. This raises several questions which remain open since the mechanisms by which nucleotide sequence is involved are not understood. On one side, the nucleotide sequence is related to the rate of translation, which can lead to many regulatory differences, including the differential

arginylation mentioned in the previous paragraph. On another side, the nucleotide sequence, at least in the case of the cytoplasmic actins, generates a difference in the ribosome density of both actins (Vedula *et al*., 2017), which leads to both actins being translated with different kinetics. The translation in bursts produced by the β-actin gene is required for the proper development of mice, but not β-actin itself. However, these mechanisms do not explain how the actin isoforms are segregated in cells. The nucleotide sequence though, can play a role in protein localization. The mRNA of β-actin possess a sequence called "zipcode" which localizes the mRNA to the leading edge of fibroblasts (Kislauskis *et al*., 1994). However, β-actin is not only localized at the leading edge of the cell, but also at the stress fibers. These observations reinforce the idea that not only one mechanism is needed for the proper distribution of the functions of the different actins, but a combination of several mechanisms.

An appealing hypothesis to explain how actin isoforms can generate different functions is through the tuning of the interactions of each isoform with the ABPs involved in the regulation of the different structures. Indeed, since actin isoforms were discovered, scientists wonder if these isoforms, being so similar, are functionally equivalent or if the small differences can account for the functional difference and their segregated localization. This question is explored by Chen *et al.* who show that a formin has a preference for a specific actin isoform (Fig. 8.4B) (Chen *et al*., 2017). Their observation is appealing and it can very well explain the enrichment of β-actin in the cytokinetic ring *in vivo*. However, something interesting to note is that actin isoforms in their assay are fully segregated even before the addition of the formin (Fig. 8.4B). This result is surprising as both isoforms used have 99% of identity and they have also been shown to have the ability to co-polymerize (Bergeron *et al*., 2010; Müller *et al*., 2013). In this case, the formin is not the source of segregation but the source of enrichment in a particular isoform. How this initial segregation occurs is still unknown.

All the points highlighted above address the necessity for a better understanding on how almost identical actins can segregate and generate different functions. The combination and interconnection of all these pathways and mechanisms make actin regulation and function extremely complex to study in depth. Specially in mammalian systems, where ABPs have many isoforms composing big families of proteins. This means that the disruption of a protein can probably be compensated by another isoform or pathway, which would result in no effect, a minor one or a multifactorial result. Moreover, actin networks in these cells can depend on other networks to be able to generate, so the perturbation in one network will have an effect on other networks as well, not only through homeostasis but also through direct interaction. In addition, modifications are more complex to bring about compared to more simple organisms, and purification of a specific actin from these cells is complicated due to the simultaneous expression of more than one actin. It is also possible that each actin isoform is optimized to work with a particular ABP isoform. If that would be the case, purifying all the proteins and finding

the right combination of actin and ABPs for the *in vitro* experiment becomes extremely complicated.

For this, I decided to implement a different approach, using a one-actin system to observe the consequences of making modifications in the actin gene. For this purpose, *Saccharomyces cerevisiae* (budding yeast) is an ideal model to carry out my project. It is a one-actin-system where we can easily edit the genomic DNA sequence. It has defined actin structures that we can observe by microscopy. Budding yeast is also a great tool to purify proteins that we can then use to perform biochemical assays. Performing these essays is extremely useful. By combining the *in vivo* observations and the results obtained *in vitro*, we can get more detailed information about the process that we are interested into. Also, biochemical assays are extremely useful when trying to understand the molecular interactions.

The big question that I will address during my PhD is if the use of different actins can account for the generation of different functions and what are the molecular mechanisms that could allow such segregation.

It is evident from the data collected in the last years of research about the actin cytoskeleton that the actin molecule is able to perform to many different functions via more than one mechanism (which were detailed in section 7, p. 31). These mechanisms are equally important and a deep understanding of all of them are needed to get the full picture of how actin is regulated. During these 4 years of work my aim was to bring new information about actin regulation to the scientific community.

1/ My main project will address the actin isoform mechanism. This project will be divided in three parts.

- The first step is the generation of a library of actin sequences. These sequences have to produce functional actins so the method to generate the library has to be chosen carefully. A subset of the sequences will be used to transform *S. cerevisiae* cells to create strains carrying different actin genes.
- The generation of yeast strains carrying different actin genes will enable us to test the consequence of swapping the actin gene for heterologous actins in cells. The yeast strains are generated so that we can study many situations: removing the intron of the actin gene in yeast, introducing a gene with silent mutations that still produces the endogenous actin protein, introducing a gene that will change the amino acid sequence of the protein and analyze strains where 2 different actins are expressed (Fig. 0.1). More specifically, this approach will allow me to address the following questions:
    - How does changing the nucleotide sequence affect cell viability, actin expression and structures? (including silent mutations and removal of the intron).

- Is it possible to express any heterologous actins in yeast? If they are expressed, how does that affect cell viability, actin expression and structures? And more specifically, are they able to form both the branched network and the linear network *in vivo*?
- For cells expressing two different actins, is this dual expression beneficial or detrimental for the cells?

- The last stage of my project involves the purification of candidate actins to do *in vitro* experiments. As we saw in section 9.2 (p. 60), we can reconstitute the branched- and the linear-network using a minimal set of proteins in the mix and adding fluorescent proteins we can see their addition on the networks.
  - Can these actins assemble into linear and branched network *in vitro*? How do the results obtained correlate with the phenotypes observed in cells?
  - Do the different actins present defective interactions with an ABP *in vitro*? If so, can this be explained by the differences in the amino acid sequence?

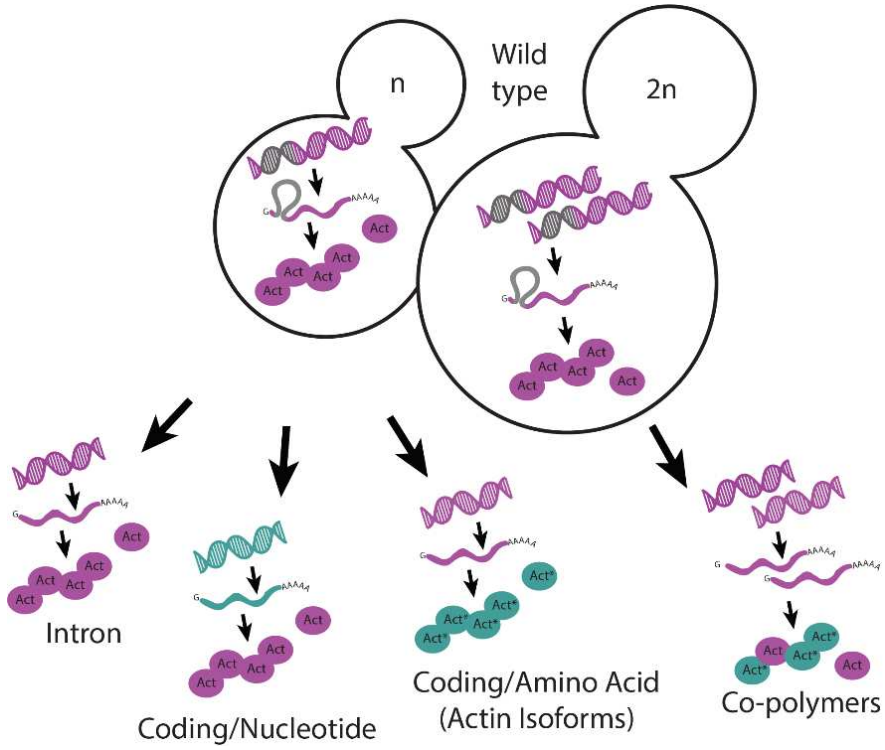


Fig. 0.1. Questions addressed during this PhD.
Schematic showing the mutagenesis strategies applied in this study, enabling to question respectively the importance of actin's intron, the nucleotide sequence, the amino acid sequence, and the effect of expressing copolymers. Green color shows at which level the modification is brought. It can be either in the coding sequence but maintaining a wild-type (pink) protein, or it can be a gene maintaining wild-type coding that produces a protein with another amino acid sequence (green).

During these years I also helped with the development of other projects from the lab to understand the other mechanisms of actin regulation and function. These projects were carried out primarily by Dr. Adrien Antkowiak (2/) and Dr. Jessica Planade and Dr. Reda Belbahri (3/). For those projects I was involved in the generation of *S. cerevisiae* strains and the visualization of the actin cytoskeleton in them, both in live imaging and fixed samples.

2/ How the sizes of actin linear and branched networks are controlled/affected by the different ABPs.

3/ How actin cross linkers affect the physical properties of actin networks and the efficiency of endocytosis.

RESULTS

10. Defective interactions with regulatory factors trigger spatial segregation of actin variants

10.1 How to choose mutants?

With the objective of finding actins that have different affinity for certain ABPs, I proceeded to design a robust method to introduce mutations in the actin sequence. Some studies have analyzed the effect of mutations by random mutagenesis. This technique is a powerful tool to change the properties of an enzyme. It can be very useful because it allows for the generation of a really big library of mutants containing different versions of the gene. After transformation in cells, it is possible to screen this library to find the desired properties, for example a temperature-sensitive variation of the protein. However, this method requires careful analysis to make sure that the mutations do not disrupt actin activity, prevent proper folding, etc. For this reason, we thought to design the mutant variants according to other possible strategies: using structural data to identify regions of interest, using actin sequences from other organisms and finally, using mutations found in patients that present actin-related diseases.

We decided to use S. cerevisae as a model, due to the wide array of genetic tools available. In all cases, to screen for interesting mutants, cells were transformed in diploids, sporulated and spores were dissected (Fig. 10.1). This first dissection allows us to spot if the mutants present growth defects, since we can separate the haploid cells that have the wild type gene and the ones that have the mutant gene. At the bottom of Fig. 10.1 you can see two examples, the first one of a mutant that presents no growth defect and the second one of a mutant that does present a growth defect. Since actin is involved in cell growth, cell division and other important mechanisms, we can assume that if cells grow slowly it means that at least one of those functions of actin is affected.

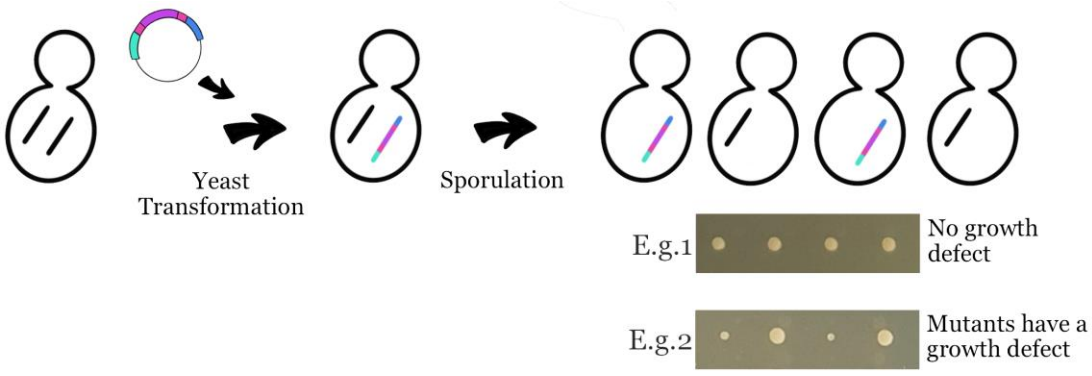


Fig. 10.1. Workflow to create a haploid yeast strain.

Saccharomyces cerevisiae cells carrying wild-type actin genes (black) can be transformed with a plasmid containing a different actin gene and selection markers. The result after sporulation and dissection is four haploid cells per spore, two of them carrying the wild-type gene and two of them carrying the mutated gene. If the mutation is detrimental for cell viability, the mutant strains will show a growth defect.

10.1.1 Structural Approach

In actin, there are 42 residues that change between human and budding yeast. According to structural data and the chemical properties of the amino acids, we can build predictions whether swapping for "human residues" in yeast will affect binding of the actin molecule to ABPs. For all the 42 residues that differ in between yeast and human, an analysis was done by the group of Robert Robinson, grouping each residue in 4 possible groups according to how much this change was predicted to affect actin function (see Tables A1 to A4 in annex.). Groups ranged from 1 to 4. Substitutions from group 1 are conservative substitutions that are predicted not to induce major changes in the actin molecule. Group 2 consists of surface residues that are located in between the 2 strands. This interaction is very weak and is dominated mainly by the shape of the protofilaments and not by strong protein-protein interactions. Group 3 includes residues that are involved in intra-strand contacts. Some of them can also affect the affinities of the actin with profilin and cofilin. Lastly, group 4 substitutions are those that have a higher chance of affecting the binding of other ABPs. From this analysis, we chose 4 residues to mutate in yeast actin. Two mutations were chosen from groups 1 and 2 as controls, with the expectation that cells carrying any of these substitutions in the actin molecule would not present any phenotype. The other two mutations were chosen from group 4 and they were predicted to interfere with actin structure and function. The mutants chosen were R68K (group 2), Q228A (group 4), V327I (group 1) and T350S (group 4). These mutations were inserted into the working plasmid and transformed in *S. cerevisiae* cells. Unfortunately, none of the 4 mutants showed growth defects. We then decided to move forward with another approach.

10.1.2 Disease-based predictions

Another possibility is to study mutations found in actin-related diseases. This is a very interesting approach, because since actin is an essential protein, we know these mutations cause defects in some of the actin functions, but not necessarily all of them. Moreover, it can help us understand and dissect the mechanisms that underlie the phenotypic manifestations of these diseases. More than 60 mutations have been identified in muscle actins and they are the reason behind many myopathies. On the other hand, not much is known about disease-related mutations in cytoplasmic actins. They are point mutations related to rare diseases such as Baraitser-Winter syndrome, which affects the development of the body, particularly the face and the brain. These abnormalities are related to impaired neuronal migration. The mutations that have been described are Arg183Trp, Arg196His in β-actin and Ser155Phe in γ-actin (FigDisease). One drawback of this approach is that disease-related mutations in cytoplasmic actins are very rare, while one of our objectives was to generate a big library of actins. For this reason, we thought of another approach to generate a big library of actin sequences.

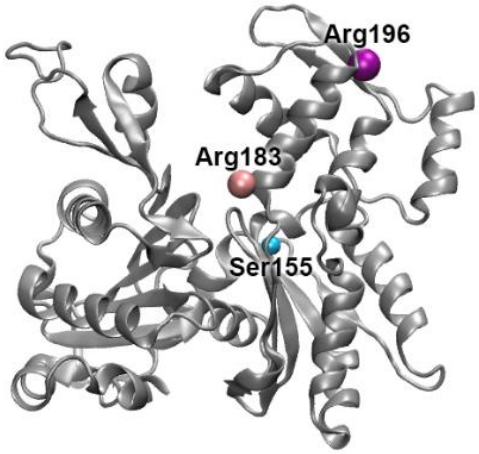


Fig. 10.2. Disease-related mutations in cytoplasmic actins.
This representation of the actin molecule shows the position of the three point mutations in the cytoplasmic actins that cause rare diseases.

10.1.3 Evolutionary Approach

The evolutionary approach consists of generating a library of actin sequences out of existing actins from different organisms or hypothetical actins coming from species that are predicted to have existed in ancient species. There are several benefits to choosing this approach. First, there is a wide range of sequences available and we can choose sequences that present different levels of sequence similarity to the *S. cerevisiae* actin. Our hypothesis is that the strength of phenotypes in cells should correlate somehow with the level of identity between the mutated actin and wild type actin. In other words, that the more different the actin is, the stronger the phenotype should be. For this reason, we chose to study sequences that range from very similar to budding yeast actin (around 99% of sequence identity) to very different (around 84% of sequence identity). Sequence identities between actins also correlate to a large extent with the evolutionary distance between species, so species that are in the same phylogenetic group of *S. cerevisiae* contain actins that are more similar than species from other groups. Second, besides being different at the sequence level, we also want to analyze actins which mutations are located at different regions of the protein. We reasoned that this will allow us to analyze a range of defects since the affected interactions with ABPs will be different for each actin variant. Last but not least, selected actins would need to be properly folded and functional actins. It is important to know that the chosen actins keep their main properties such as polymerization, depolymerization, nucleotide binding or hydrolysis. Otherwise, the observed phenotype could be related to a defect in the actin itself. Choosing actins from other species ensure that they are fully functional.

10.1.3.1 Ancestral reconstruction

Ancestral reconstruction is a technique that predicts a characteristic that was present in a common ancestor based on species that exist today. It consists of making an extrapolation back in time from said characteristics of several species to their common ancestors. This can be used to reconstruct the DNA or amino acid sequence of the hypothetical ancestors, for example. This analysis is coupled with the evolutionary relationship between contemporary species, and it builds relationships between their characteristics.

In my case, I was looking to generate ancestral reconstructions on the actin amino acid sequence to increase the number of actins in my library. First, I collected actin sequences from multiple species and then generated a phylogenetic tree including all species. I collected all actin sequences from UniProtKB/Swiss-Prot, which are manually annotated, reviewed and curated. From this list, only one actin sequence per species was chosen (always the most similar to *S. cerevisiae* actin), resulting in a list of 122 actins. The phylogenetic tree of all species was created based on the NCBI taxonomy using the phyloT website (https://phylot.biobyte.de/) The alignment of the 122 actin sequences and the phylogenetic tree of the 122 species were used as inputs for ancestral reconstruction using an algorithm called FastML (Ashkenazy *et al*., 2012). This algorithm returns, among other results, the most probable sequence for each node in the input tree. The result was a list of 101 ancestral reconstructed species belonging to each node, and each of them represents a hypothetical ancestor of the species branching from that node. Because the sequence of the actin protein is highly conserved across species, ancestral sequence reconstructions scored with high confidence. 99% of the amino acids in the ancestral sequences are predicted with an accuracy >95% and uncertain residues correspond to conservative substitutions (Grantham score <100, (Grantham, 1974)). The posterior probabilities for each residue for all the nodes used in this study can be found in the annex (Figure A1). Combining these reconstructed sequences with the UniProtKB/Swiss-Prot sequences, we generated a library of 223 actin sequences. After having established the database, I selected a total of 15 actins (Table 1, Fig. 10.3). These amino acid sequences were chosen to cover a wide variety of sequence identities compared to *S. cerevisiae* actin (Act_Sc). Another characteristic is that the mutations are distributed across all domains of the actin fold (Fig. 10.4). Species that are closely related to *S. cerevisiae* in the phylogenetic tree possess actins that are more similar, usually from 95% to 100% of sequence similarity. More distant species can have actins as different as 82%, as is the case for *Arabidopsis thaliana* (Act_At).

I chose these 15 actins to introduce them in *S. cerevisiae* cells. To achieve this, I designed the genes, the nucleotide sequences, that will carry the information to produce these actin proteins inside the cells. Genes were based of the *S. cerevisiae* coding sequence (the actin gene without the intron) and I manually curated the sequence to include the least possible number of changes. I generated a nucleotide sequence for each of the 15 actins, while the base gene is as similar to the *S. cerevisiae* actin as possible. Each of these genes can then

produce one of the 15 actin amino acid sequences that were chosen (Table 1). In addition to these sequences, I was also interested in investigating the role of the nucleotide sequence, since it has been shown to play an important role as well (see section 8.2, p. 45). For this purpose, I designed four more actin nucleotide sequences but in this case the four sequences produce the same actin protein, *S. cerevisiae* actin. To generate these sequences, I used the actin coding sequence from four species: *Homo sapiens*, *Arabidopsis thaliana*, *Schizosaccharomyces pombe* and *Candida albicans*. I manually modified these coding sequences so that they express *S. cerevisiae* actin by introducing single nucleotide synonymous mutations. This resulted in four very different actin genes that code for the same actin (Table 2). All these nucleotide sequences that I designed were then commercially synthesized.

Table 1. Details of the actin variants.

Name	Species	Coded by	Protein ref	Amino acid identity	Positive	Nucleotide identity
Sc	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	P60010	100.0	100.0	100
N1	Ancestral reconstruction	*Saccharomyces cerevisiae*	-	98.4	99.5	99.29
Kl	*Kluyveromyces lactis*	*Saccharomyces cerevisiae*	P17128	97.3	99.2	98.49
N2	Ancestral reconstruction	*Saccharomyces cerevisiae*	-	96.0	98.7	97.87
Op	*Ogataea parapolymorpha*	*Saccharomyces cerevisiae*	O74258	95.2	98.7	97.96
Ca	*Candida albicans*	*Saccharomyces cerevisiae*	P14235	94.6	98.1	96.9
N3	Ancestral reconstruction	*Saccharomyces cerevisiae*	-	93.1	98.4	96.81
N4	Ancestral reconstruction	*Saccharomyces cerevisiae*	-	92.5	97.9	96.45
Nc	*Neurospora crassa*	*Saccharomyces cerevisiae*	P78711	92.0	97.9	96.01
N5	Ancestral reconstruction	*Saccharomyces cerevisiae*	-	91.2	97.6	95.83
Yl	*Yarrowia lipolytica*	*Saccharomyces cerevisiae*	Q9UVF3	89.6	96.8	95.39
Sp	*Schizosaccharomyces pombe*	*Saccharomyces cerevisiae*	P10989	89.6	96.3	95.04
Hs	*Homo sapiens*	*Saccharomyces cerevisiae*	P60709	88.8	96.3	95.04
Sco	*Schizophyllum commune*	*Saccharomyces cerevisiae*	Q9Y702	88.0	96.3	94.41
At	*Arabidopsis thaliana*	*Saccharomyces cerevisiae*	Q96293	84.7	94.9	91.31

Table 2. Details of the nucleotide sequence variants.

Name	Species	Coded by	Protein ref	Amino acid identity	Positive	Nucleotide identity
Sc	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae*	P60010	100.0	100.0	100
Sc[Ca]	*Saccharomyces cerevisiae*	*Candida albicans*	P60010	100.0	100.0	89.8
Sc[Sp]	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	P60010	100.0	100.0	82.18
Sc[At]	*Saccharomyces cerevisiae*	*Arabidopsis thaliana*	P60010	100.0	100.0	77.93
Sc[Hs]	*Saccharomyces cerevisiae*	*Homo sapiens*	P60010	100.0	100.0	75.98

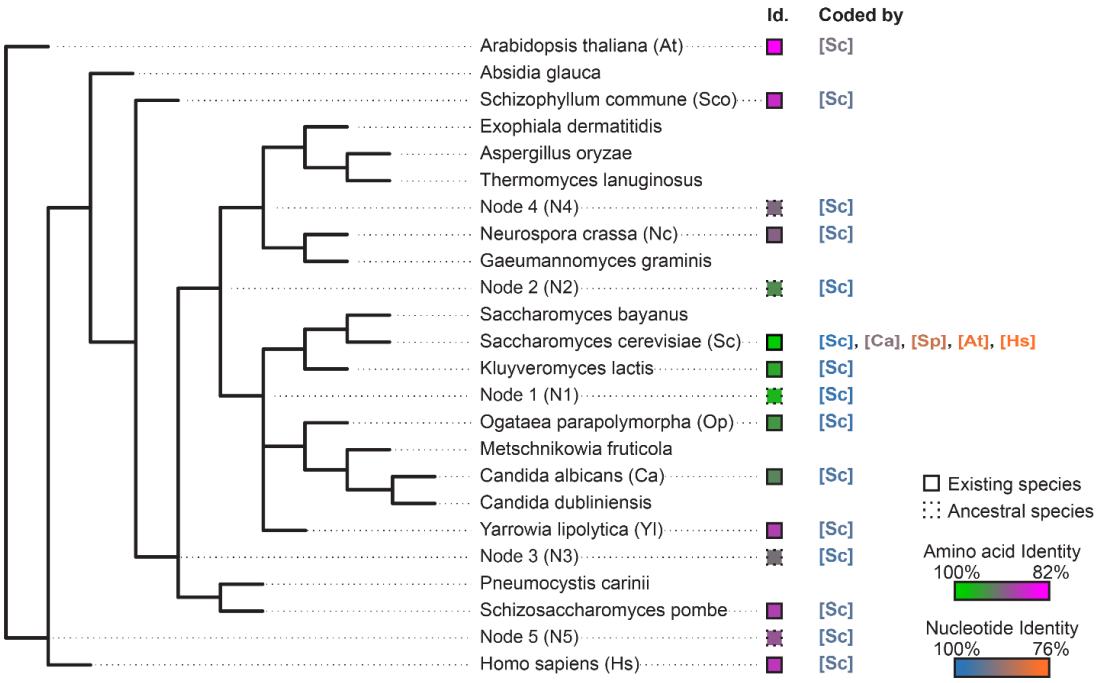


Fig. 10.3. Variety of actin selected for this study and their evolutionary relationship.

Simplified phylogenetic tree showing mainly the Dikarya subkingdom and including the external branches *Homo sapiens* (Hs) and *Arabidopsis thaliana* (At). The Id. column indicates amino acid sequences percentage identities, ranging from 100% (green) to 84% (magenta) identity to *S. cerevisiae*'s actin. Squares outlines are solid or dotted for sequences deriving from existing species or ancestral reconstruction, respectively. The "coded by" column indicates which coding sequences were originally used to code genes of interest. Nucleotide sequence identities are ranging from 100% (blue) to 76% (orange) compared to *S. cerevisiae*'s actin coding sequence.

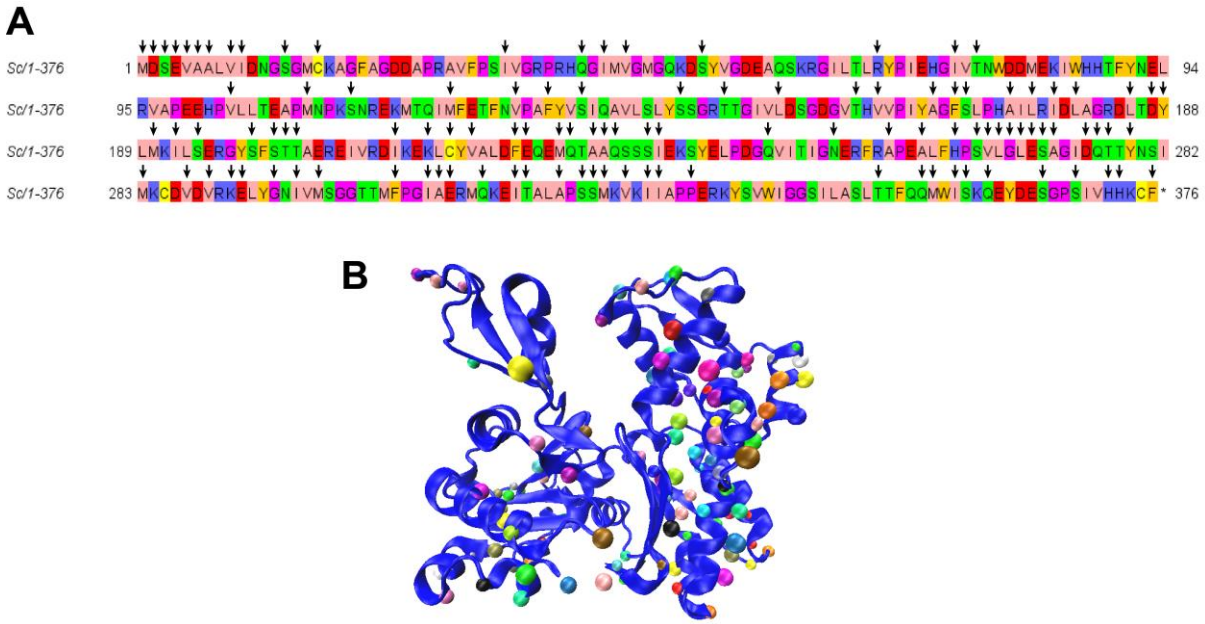


Fig. 10.4. Positions of the combined mutations of all actin variants.

(A) Amino acid sequence of *Saccharomyces cerevisiae* actin. Arrows denote all the positions that are mutated in at least one of the actin variants tested in this study. **(B)** Schematic representation of *S. cerevisiae* actin 3D structure (1YAG (Vorobiev *et al.*, 2003)), showing that mutations cover all regions of the protein. Dots indicate where mutations are located, using a different color code for all actins studied here.

With the objective of replacing the endogenous actin gene of *S. cerevisiae* cells for the other actin genes, I generated a plasmid that I designed for fast and robust gene replacement. The plasmid includes an insert that can be used to replace the actin gene by homologous recombination (Fig. 10.5). This insert is composed of the actin gene, including promoter and terminator flanked by two different markers, which are in turn flanked by the recombination sites. Selecting for the two markers allowed me to test for clones in which the whole actin gene has been replaced, and using constructs with different markers (for example, one with LEU/URA and another one with HIS/KAN) allowed me to generate strains carrying two mutant actins.

The genes were transformed in *S. cerevisiae* cells replacing one copy of the endogenous actin (Fig. 10.6, transformation). This method allowed me to generate diploid yeast strains carrying two actins, one copy of the wild type gene and one copy that was replaced for one of the 19 chosen sequences. Moreover, it was possible to generate haploid yeast strains carrying only the mutated gene where the wild type gene is not present at all (Fig. 10.6, sporulation). It is possible to go even further, and generate diploid yeast strains carrying two mutant actin genes (Fig. 10.6, mating). I will explore all these possibilities in the following sections. The objective of generating so many yeast strains was to screen the resulting strains for growth defects, measure actin expression and observe the actin cytoskeleton. This will allow me to see what is the effect of changing the endogenous actin for another actin gene in cell viability and how this change will affect actin structures. This strategy will allow me to investigate these aspects of actin regulation: the effect of changing the actin amino acid sequence, the effect of changing the nucleotide sequence, the effect of removing the intron and the effect of expressing two different actin variants at the same time (Fig. 0.1). Last but not least, these strains can be used to purify mutant actins for *in vitro* assays.

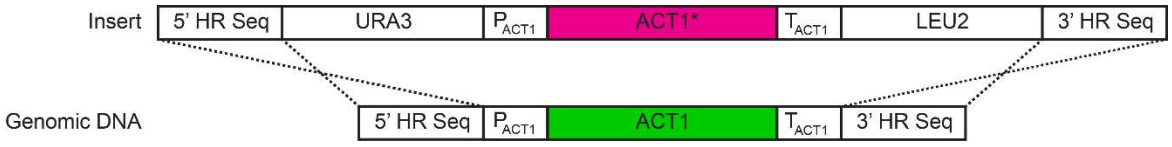


Fig. 10.5. Composition of the construct used for homologous recombination.
Schematic representation of mutagenesis strategy by homologous recombination used in this study.

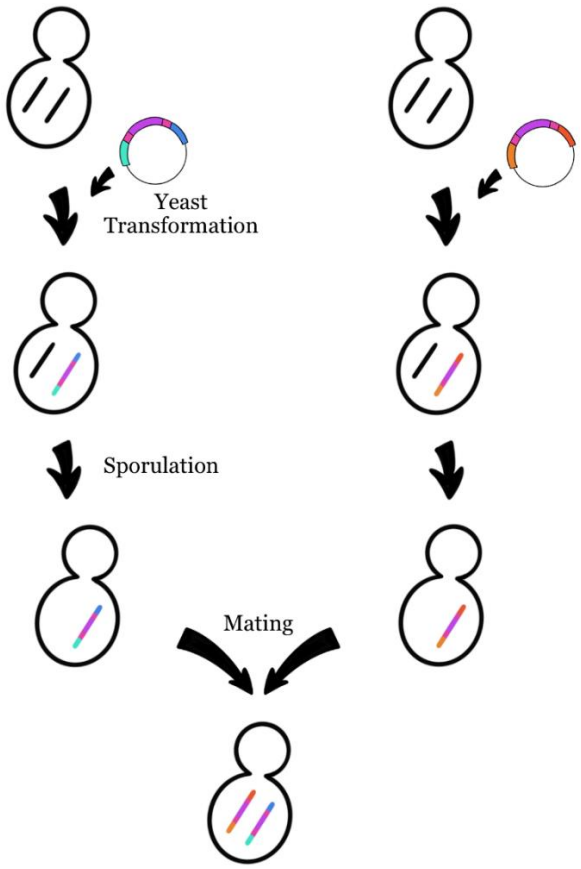


Fig. 10.6. Workflow to create a double-mutant diploid strain.
After yeast transforming yeast with a another actin gene, sporulating and dissecting, we obtain a haploid strain carrying a particular mutation. Haploid cells can carry one of two possible alleles of the mating types locus MAT, MATa or MATα. Haploid cells of opposite mating type can mate to generate a diploid cell. To be able to select this diploid cell, it is necessary that both haploid strains carry different selection markers.

10.1.3.2 Nomenclature

S. cerevisiae strains will be called according to the column "Name" in Table 1 and Table 2.

For the amino acid sequence changes, *S. cerevisiae* strains are called according to the provenance of the expressed actin according to the names in Table 1. The actin molecule that each strain codes for will be called Act_Name. For example, wild-type *S. cerevisiae* cells will be called Sc and the actin expressed in these cells is Act_Sc. *S. cerevisiae* cells transformed to express the actin protein coming from *Candida albicans* will be called Ca, and the actin molecule will be called Act_Ca.

For the nucleotide sequence analysis, all strains express wild-type *S. cerevisiae* actin (Act_Sc), but the genes are different because they have synonymous substitutions. Strains will be called to the names according in Table 2, meaning Sc[coding]. All strains which do not specify the provenance of the coding sequence, are coded according to the *S. cerevisiae* gene without the intron. So Sc cells are cells that express wild-type actin from a *S. cerevisiae* gene. Sc[Ca] cells are *S. cerevisiae* cells that express wild-type actin from a gene carrying synonymous mutations according to the *Candida albicans* gene.

10.2 Role of the intron in the actin gene of *S. cerevisiae*

Genes in *S. cerevisiae* can have introns, although only 4-5% of the genes do (Ng *et al*., 1985; Parenteau *et al*., 2008). The actin gene is one of the genes that carries an intron, which has been shown to not be essential for actin expression and functions (Ng *et al*., 1985). In any case, the first step for my work was to confirm this observation and explore the importance of the intron in actin expression, cell viability and actin structures. The reasoning behind this is that if we can confirm that the intron does not play an important role in those aspects, all the genes could be designed to not include the intron. It is also important to verify that the full construct that we are including in the genome does not affect any of the mentioned aspects neither.

For this reason, we compared wild-type cells (Sc) with two mutants: one carrying the full construct with a wild type gene, meaning actin and the intron (ScI) and another one carrying the construct with actin without the intron (ScNI).

Actin expression was measured by Western Blot and comparing the actin band intensity against a loading control. Neither the presence of the construct nor the presence of the intron seem to significantly affect actin expression (Fig. 10.7A-B).

Growth was measured by performing spot assays and measuring colony area of individual colonies after 2 days of growth. Cell growth was as fast as Sc cells in all tested strains (Fig. 10.7C-D).

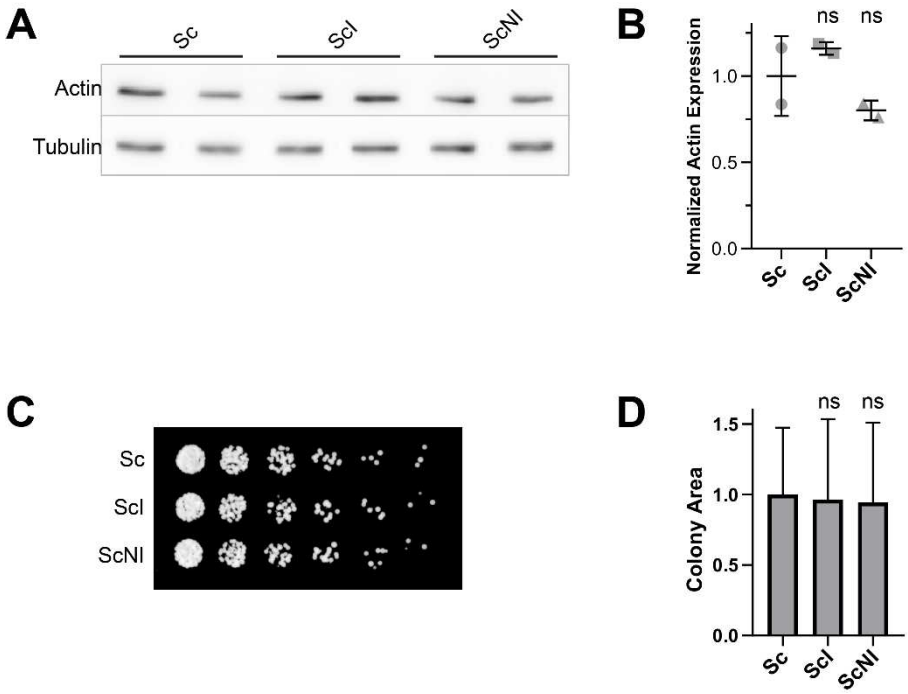


Fig. 10.7. Neither the presence of the construct or the absence of the intron affect actin expression levels or growth.

(A) Actin expression levels shown by western blotting, with tubulin (Tub1p) as a loading control. **(B)** Quantification of actin expression levels. **(C)** 3-fold serial dilutions of different yeast strains cultures grown at 25 ºC for 2 days on a YPD plate. **(D)** Quantification of (C) by measurement of colony area.

To observe the actin structures in these cells I fixed them and then stained for actin using phalloidin staining. Using a confocal microscope, I took pictures of medium budded cells were we can see the two main actin structures: cables (linear network) and patches (branched network) (see section 9.1.2, p. 53). To quantify how actin structures are affected, I developed two indexes: the *in vivo* actin network deviation index and the polarity index.

The *in vivo* actin network deviation index consists of a quantification of how the branched-to-linear balance is perturbed. The index is defined like this:

$$In\ vivo \text{ Actin Network Deviation Index} = \frac{\frac{I_{patch}}{\bar{I}_{patch,wild-type}} - \frac{I_{cable}}{\bar{I}_{cable,wild-type}}}{\frac{I_{patch}}{\bar{I}_{patch,wild-type}} + \frac{I_{cable}}{\bar{I}_{cable,wild-type}}} \quad (1),$$

where I_{patch} is the total patch fluorescence intensity of the cell of interest, and $\bar{I}_{patch,Sc}$ is the mean total intensity of actin patches in wild type Sc cells. I_{cable} and $\bar{I}_{cable,Sc}$ correspond to the same measurements but performed on cables instead of patches. Basically, if the value of this index is 0, the strain presents a ratio of linear and branched network that equals the one of wild-type cells (Sc). If the value is positive, the strain has more patches and/or less cables compared to wild type, and the value can reach a maximum of 1 if the strain has only patches and no cables at all. On the other hand, if the value is negative, the strain has more cables and/or less patches compared to wild type.

The polarity index is a measurement of where the patches are located. It is defined like this:

$$\text{Polarity index} = \frac{Patches_{bud} - Patches_{mother}}{Patches_{bud} + Patches_{mother}} \quad (2),$$

Where $Patches_{bud}$ corresponds to the number of visible patches in the bud and $Patches_{mother}$ corresponds to the number of patches in the mother cell. During division, Sc cells present most of their patches in the bud, whereas few patches are in the mother cell. Therefore, the polarity index of Sc cells has a value which is close to 1. A value of 0 would mean that the strain has an equal amount or patches in the bud and the mother cell, meaning that it is depolarized. A polarity index of -1 would mean that all the patches are located in the mother cell, meaning that these cells are wrongly polarized.

As expected, branched and linear organization, as well as polarity were not affected in any of these strains (Fig. 10.8). Actin structures in all cases were similar to Sc cells and all cells were properly polarized during division (Fig. 10.8). It is important to note that the full construct itself does not seem to affect any observed structures (comparing Sc with ScI). This is important because to exchange the actin gene we need to introduce a construct that does not only have the actin gene, but also auxotrophy markers or resistances. Even though these markers are right before the promoter and right after the terminator of actin, they do not affect the expression of the protein. In addition, these results further confirm

that the intron in the actin gene of *S. cerevisiae* does not play a role in actin expression, actin organization, polarity and cell growth (comparing Sc with ScNI).

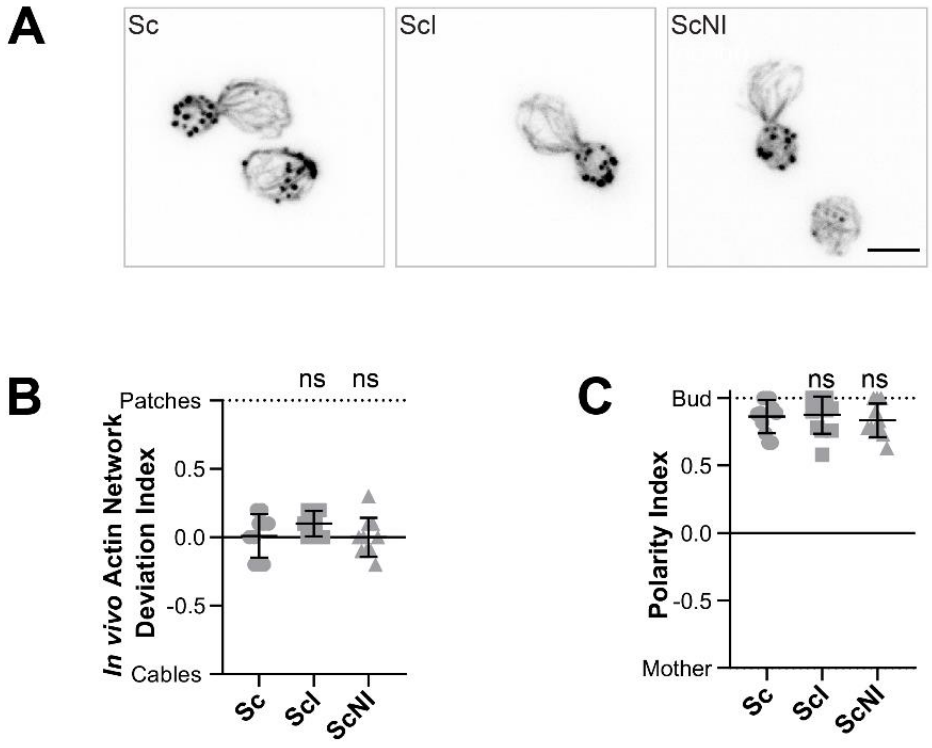


Fig. 10.8. Balance of actin structures and polarity in strains carrying the construct or lacking the intron. **(A)** Phallodin stain depicting F-actin organization. Images are maximum intensity projections of 3D stacks. Scale bar: 3 μm. **(B)** *In vivo* actin network deviation indexes, defined to evaluate the patch-cable balance compared to *S. cerevisiae* haploid cells (Sc) (value is 0 in Sc cells, 1 when cells contain only actin patches and -1 when cells contain only cables). **(C)** Polarity indexes, defined to assess whether cell polarity is normal or affected (value is 1 when all patches of medium to large budded cells are present in the bud, and -1 refers when all patches are in the mother cell.

10.3 Effect of silent mutations in the actin gene

Nucleotide sequence has been shown to be an important layer of regulation of actin biology. Therefore, I set out to check if variation in the nucleotide sequence could affect actin expression or function in my system. For this reason, I checked the strains that I generated which express wild-type Sc actin but that have silent mutations in their nucleotide sequence, based on the genes of other species (Table 2, p. 73).

Changes in nucleotide sequence affected actin expression (Fig. 10.9A-B). More specifically, the more the nucleotide sequence differed from the *S. cerevisiae* sequence, the lower actin expression was; these two parameters showed a linear correlation (Fig. 10.9C). This result is not surprising, since codon usage has been shown to control protein expression (Hoekema *et al*., 1987; Zhou *et al*., 2016). Each species has their own codon usage bias, meaning the codons that appear more frequently. This value is usually related to the amount of each transfer RNA available in the cells, that recognize the codons in the mRNA to transport the correct amino acid to the nascent protein during translation. We could predict that yeast cells would have difficulty in expressing a protein from a gene that is optimized for expression in a distant species. The fact that actin expression correlates with the nucleotide sequence identity is very interesting. This result could be explained purely by the difference in nucleotide sequence, but it could also be explained by evolutionary distance, since the most different sequences come from less related species. Moreover, changing the nucleotide sequence could be used as a tool to finely control the expression of a protein, to investigate the effect of expression in a precise manner.

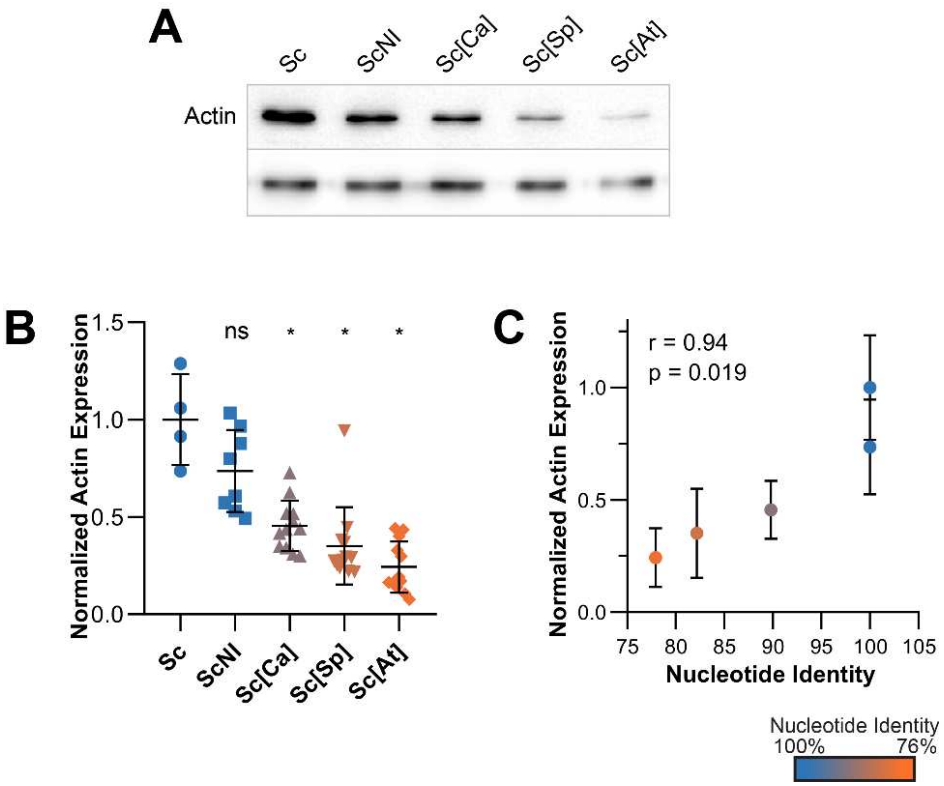


Fig. 10.9. Silent mutations in the actin gene reduce expression levels.
(A) Actin expression levels shown by western blotting for strains expressing *S. cerevisiae*'s actin protein from various coding sequences, with tubulin (Tub1p) as a loading control. **(B)** Quantification of actin expression levels, showing a decrease when more silent mutations are present. **(C)** Actin expression levels as a function of nucleotide conservation, showing that increased number of silent mutations lowers actin expression. Color code: nucleotide sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (blue) to 76% (orange).

Growth was also affected by silent mutations in the actin gene. More specifically, growth seemed not to be affected for Sc[Ca] and Sc[Sp] strains, which both have nucleotide sequences with 90% and 82% identity compared to Sc, respectively (Fig. 10.10A-B). However, growth was greatly impaired in Sc[At] (78% identity compared to Sc) and Sc[Hs] cells did not grow at all (76% identity compared to Sc) (Fig. 10.10A-B). Actin expression and colony area did not correlate (Fig. 10.10C). Interestingly, there seems to be a threshold of actin expression above which cell growth is not affected, and below which cells display severe growth defects or even lethality. This threshold seems to be located between 25% and 35% of expression. These results indicate that our system is not sensitive to mild drops in actin expression.

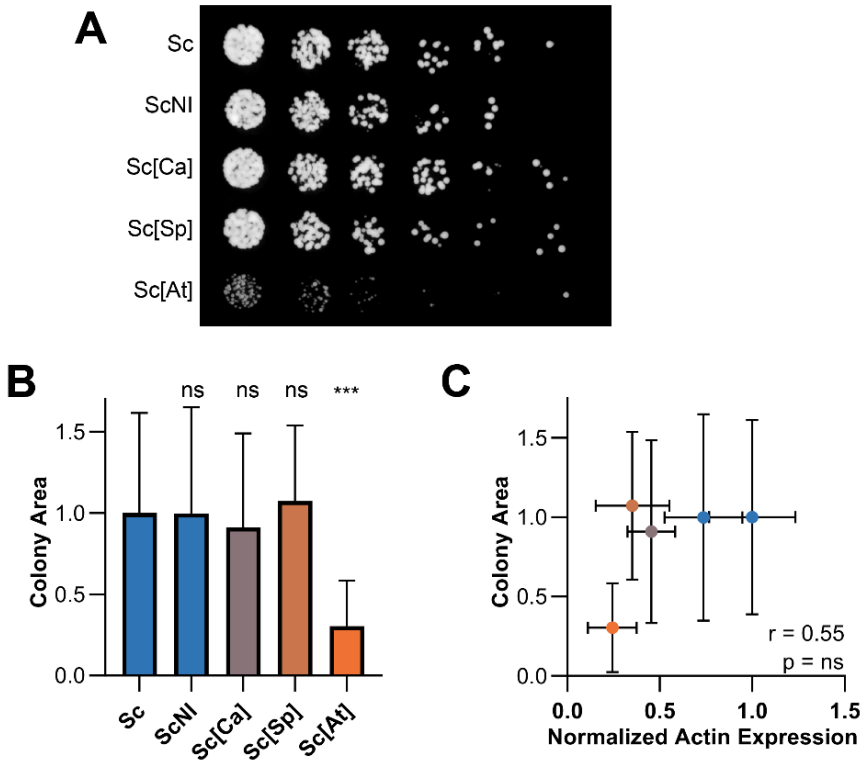


Fig. 10.10. Growth is not affected when actin expression is above a treshhold.
(A) 3-fold serial dilutions of yeast strains cultures, grown at 25 ºC for 2 days on a YPD plate. **(B)** Quantification of (A) by measurement of colony area. **(C)** Level of actin expression as a function of colony area does not show any clear correlation. Rather, there is an apparent level of actin expression (0.25 < expression < 0.35) below which the growth rate is drastically reduced. Color code: nucleotide sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (blue) to 76% (orange).

Phalloidin staining of these strains show that actin structures were affected at different levels. For strains above the threshold, Sc[Ca] and Sc[Sp], the patch-to-cable balance was maintained compared to Sc (Fig. 10.11A-B). On the other hand, actin structures were greatly affected in Sc[At], which is below the threshold (Fig. 10.11A-B). The *in vivo* actin network deviation index was positive, meaning that this strain has more branched-network compared to Sc cells. In agreement with these observations, *in vivo* actin network deviation indexes as a function of actin expression levels does not show any clear correlation. Rather, we observe a threshold of actin expression levels (0.25 < expression < 0.35) below which actin cytoskeleton organization is affected. Polarity index in strains above the threshold was only affected for Sc[Sp] (Fig. 10.11D). Sc[At] did not only have an abnormal amount of actin patches, but also cells were highly depolarized, meaning that they have a similar amount of patches in the mother cell and the growing bud (Fig. 10.11D). These results indicate that our system is not sensitive to mild drops in actin expression, and that only loss of over 60% of wild-type actin expression is deleterious for *S cerevisae* growth.

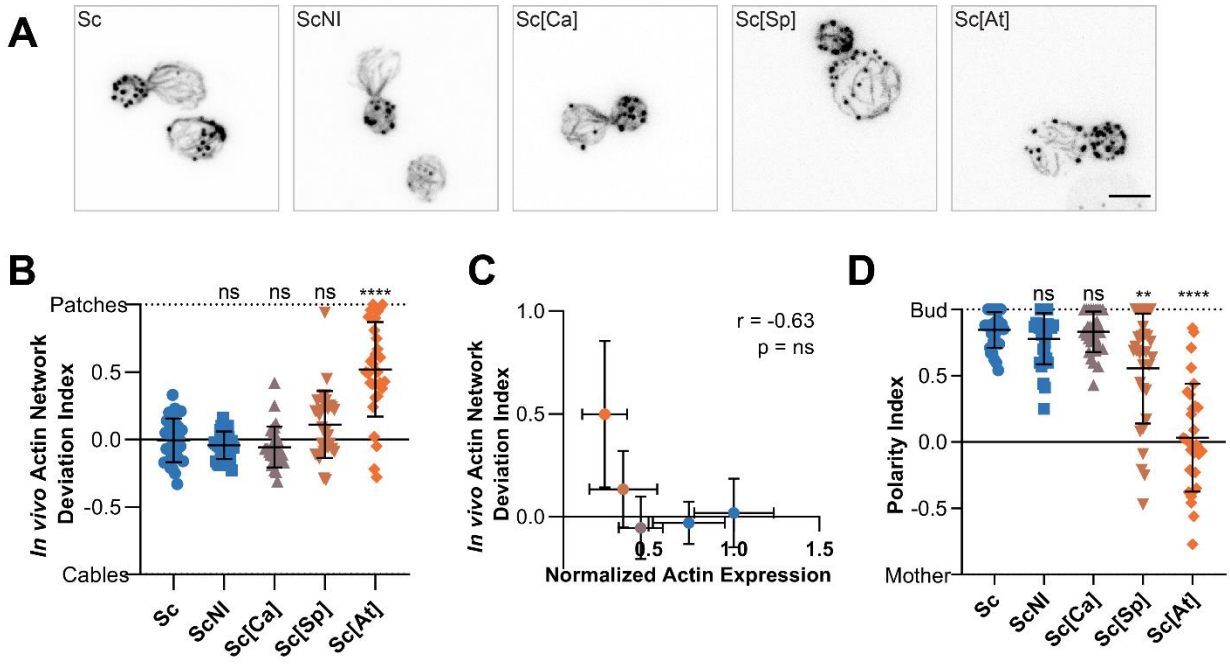


Fig. 10.11. Actin organization and polarity are not affected when actin expression is above a treshhold. **(A)** Phalloidin staining depicting F-actin organization. Images are maximum intensity projections of 3D stacks. Scale bar: 3 µm. **(B)** *In vivo* actin network deviation indexes for all strains. **(C)** *In vivo* actin network deviation indexes as a function of actin expression levels does not show any clear correlation. Rather, we observe a threshold of actin expression levels (0.25 < expression < 0.35) below which actin cytoskeleton organization is affected. **(D)** Polarity indexes for all strains. Color code: nucleotide sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (blue) to 76% (orange).

10.4 Effects of the amino acid sequence in cell viability and organization of the actin cytoskeleton

After investigating the effect of the nucleotide sequence, I was interested in assessing how small changes in the actin amino acid sequence could affect cell viability and actin regulation. For this part, I analyzed the yeast strains that are expressing actins from other species (Table 1, p. 73). It is important to note that yeast cells cannot survive only expressing some of the most different actins in the list, therefore they will not appear in the results. The genes for these actins were designed to maintain the nucleotide sequence as similar as possible to the ScNI nucleotide sequence. This means that all the nucleotide sequences used in this part have more than 90% identity with the ScNI nucleotide sequence.

The first step was to verify actin expression in the strains expressing different actins. One complication for that is that since the actin amino acid sequence is different for these strains, the primary antibody that recognizes actin could interact differently with each actin. For this reason, I chose to use the C4 anti-actin antibody that recognizes a highly conserved region in actin located between amino acid positions 50 and 70 and which sequence you can see in Fig. 10.12A (C4_Epitope). However, in the actins I used there is a one amino acid difference in this sequence, with the exception of Act_Hs (see Mutated_Epitope in Fig. 10.12). For this reason, the reactivity for this antibody against both epitopes was assessed by using purified proteins carrying one or the other epitope (Fig. 10.12B-C), and the value of Act_Hs was corrected accordingly in the actin expression measurements in vivo.

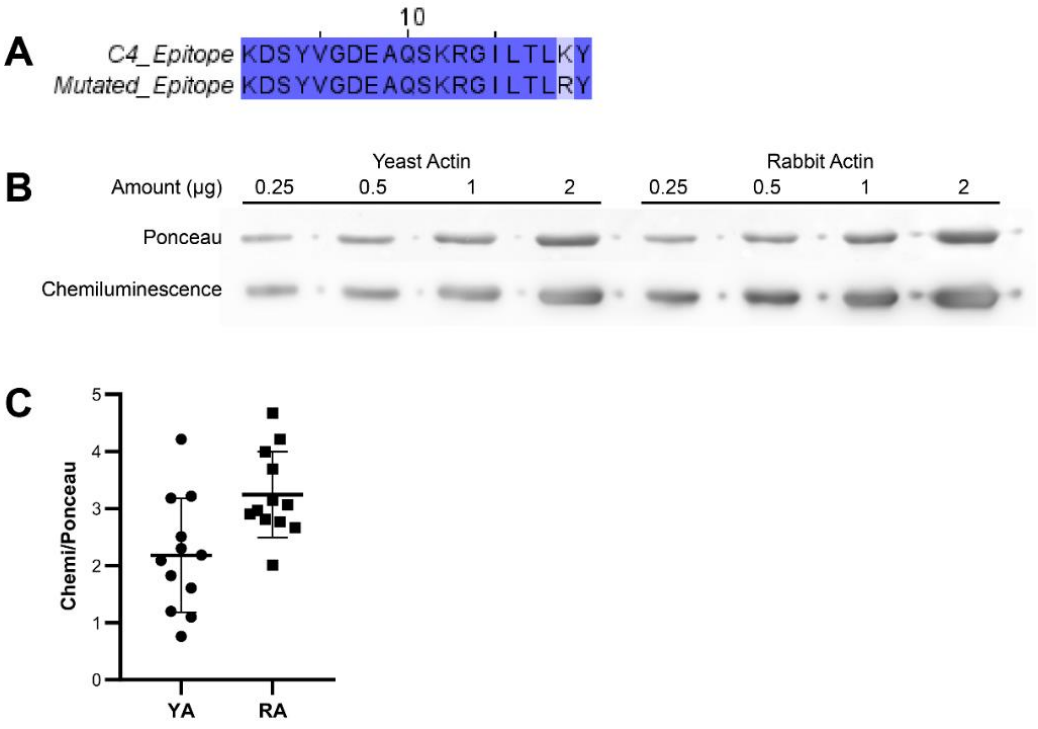


Fig. 10.12. C4 actin antibody has a higher affinity for rabbit muscle actin than for *S. cerevisiae* actin.
(A) The binding site of the C4 antibody, indicated as "C4_Epitope", is found on Act_Hs and on rabbit muscle actin. In all other actin variants used in this study, the sequence varies of one amino acid (called here "Mutated_Epitope") but is recognized by C4 antibody. **(B)** Western blot with equivalent amounts of purified yeast actin and rabbit actin. The amount of protein was revealed by two methods: Ponceau staining and chemiluminescence. The chemiluminescence signal corresponds to the one produced by the secondary antibody after incubation with a primary antibody anti-actin C4 and a secondary antibody conjugated with HRP. **(C)** Quantification of (B) indicates that immunolabeling of rabbit muscle actin with C4 antibody leads to a 1.48-fold more intense signal than immunolabeling of *S. cerevisiae* actin.

Actin expression was verified by Western Blot for all the haploid strains. The expression level of actin varied in each strain, and appeared to be random and not correlated with evolutionary relationship (Fig. 10.13A-B). For example, Act_N1, which has a 98.4% identity to Act_Sc, is only expressed at 39%. This result is surprising since the actins are highly similar but for some reason the expression levels drop down drastically. However, the expression of Act_N1 is above the expression threshold of 35% defined in the previous paragraph, so it was reasonable to think that actin was sufficiently expressed to not affect cell viability nor actin structures. Indeed, we will see in the next paragraph that this strain behaves like a wild type strain. I did not have sufficient time to explore what is the mechanism that controls actin expression in this strain, but addressing this question could maybe lead to an actin expression regulation pathway that is defective in these cells. Moving forward, we can see the actins which have an identity compared to Sc_Act between 97.% and 94.6% (Act_Kl, Act_N2, Act_Op and Act_Ca). The expression of these actins in budding yeast cells does not differ significantly from wild-type cells, although if we look closely each strain seems to have a different tendency. More different actins, Act_Nc and Act_Yl, have a significantly lower expression than Act_Sc, of 40% and 35% respectively. These two actins are expressed at a very low level but they are on the limit or over the threshold defined in the previous paragraph. Act_Hs corresponds to the expression of mammalian β-actin, and after correction of the signal we can see that it is expressed at normal levels.

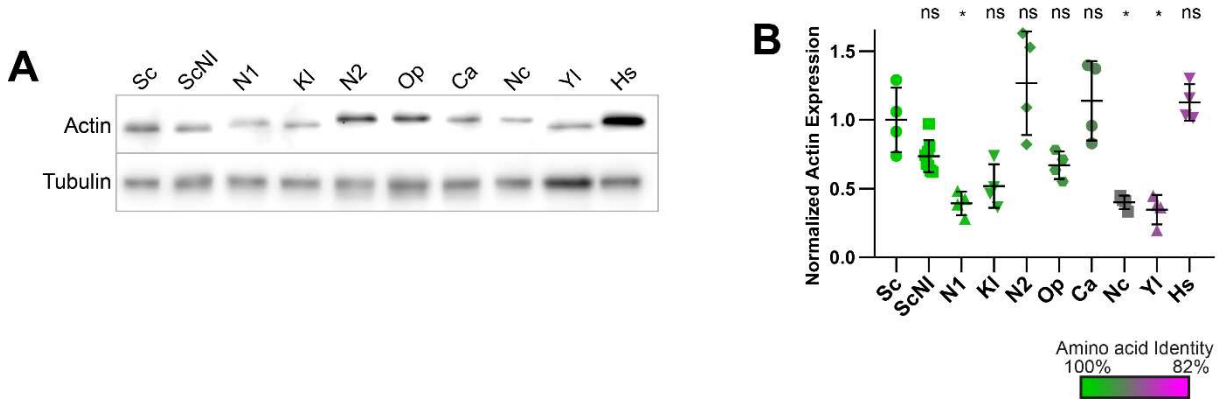


Fig. 10.13. Actin expression levels appear not to be correlated with evolutionary relationship.
(A) Actin expression levels shown by western blotting for strains expressing Act_Sc or other actins, with tubulin (Tub1p) as a loading control. **(B)** Quantification of actin expression levels showing varying levels of expression that do not correlate with evolutionary relationship. Color code: amino acid sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (green) to 82% (magenta).

Actin expression was not the only thing that varied in these cells. The growth of each strain was also different. More specifically, the more different the actin amino acid sequence is, the slower the cells grow (Fig. 10.14A-B). Indeed, the sequence identity and the colony area showed a linear correlation (Fig. 10.14C). Looking at the results, we can distinguish 3 groups. A first group formed of N1 and Kl, which are strains expressing actins with an identity of 97% or higher with Act_Sc, grew well and at a similar rate to Sc cells. A second group composed of N2, Op and Ca, presented impaired growth and a colony size around 40-50% of Sc cells. The second group expresses actins that have between 96% and 94% of sequence identity compared to Sc. The third group has very different actins, with less than 93% of identity to Sc actin, and the growth is heavily impaired or they cannot survive.

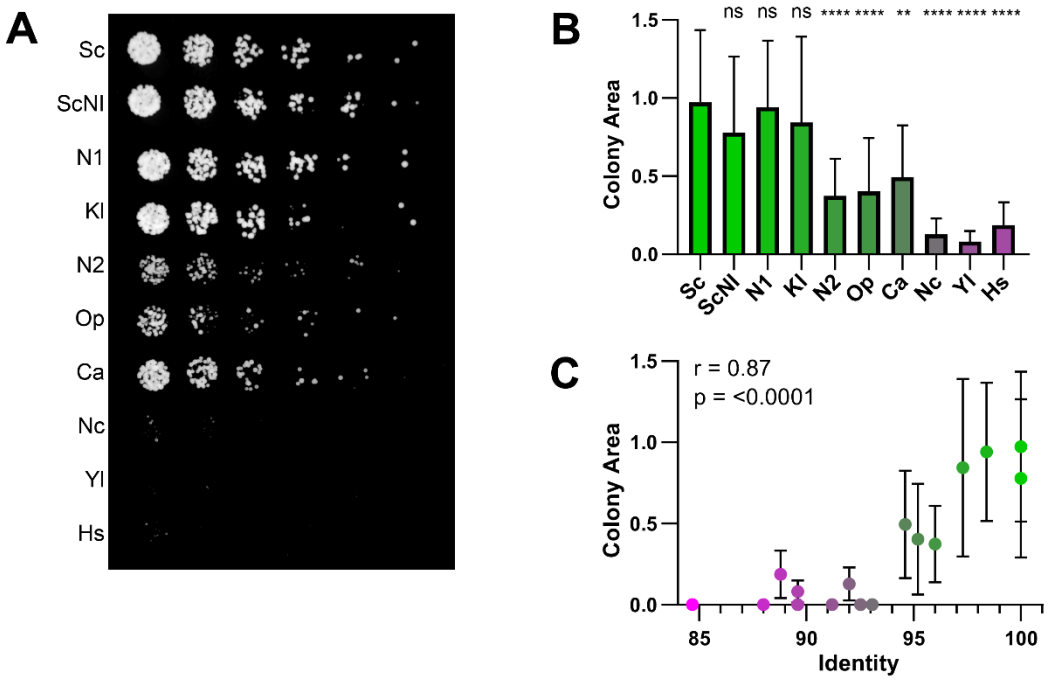


Fig. 10.14. The growth of strains expressing heterologous actins correlate with the evolutionary relationship.

(A) 3-fold serial dilutions of different yeast strain cultures grown at 25 ºC for 2 days on a YPD plate. **(B)** Quantification of (A) by measurement of the colony area. **(C)** Colony area as a function of percentage identity of the actin variant, showing a clear correlation. Color code: amino acid sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (green) to 82% (magenta).

Phalloidin staining of yeast strains carrying different actins was used to see how these small changes in the actins would affect the linear- and branched-networks. For these strains where the amino acid sequence of actin was modified, the actin structures presented some particularities. Whereas for the strains analyzed in the previous sections the intensities of the patches and cables were similar in all strains, it was not the case for these strains. For example, patches in N2 and Hs strains were much brighter. On the contrary, patches in Op and Ca strains were less intense. It is for this reason that the *in*

vivo actin network deviation index was calculated measuring the integrated intensities of each structure. Counting the number of patches and the number of cables, although faster to measure, would not have provided correct information about the relative size of both networks in each strain.

Interestingly, consequences on the organization of the actin cytoskeleton was not the same for all mutants. Some strains, mainly the ones of the first group mentioned before (N1, and Kl), presented a normal distribution of the actin cytoskeleton. The amount of linear and branched network in N1 and Kl cells was similar to Sc cells (Fig. 10.15A-B) and the cells were properly polarized, presenting most of the patches in the bud and not in the mother cell (Fig. 10.15C). The rest of the strains presented a different organization of the actin cytoskeleton. Some strains (Op and Ca) had a higher amount of linear network compared to Sc cells, reflected by the fact that their *in vivo* actin network deviation index has a mean negative value (Fig. 10.15A-B). On the contrary, N2 presents a really high number of patches (Fig. 10.15A-B). Hs cells have an abnormal size and cytoskeletal organization, with cells usually having a higher number of patches compared to Sc. However, the branched-to-linear balance is not significantly different to the one of Sc cells (Fig. 10.15A-B). All these strains (N2, Op, Ca and Hs) were completely depolarized (Fig. 10.15C). According to the homeostasis of the actin cytoskeleton, both the branched- and the linear-networks compete for the same G-actin pool. For the strains where the balance is shifted towards a particular network, we hypothesized that the cells cannot efficiently produce the other network. If we consider the actin homeostasis, the consequence of not assembling one network efficiently would be that more G-actin is available to polymerize into the other network. For example, for N2, we hypothesize that cells cannot efficiently generate or maintain the linear network and therefore there is an increase in patch number. Another possibility is that patch or cable assembly is more efficient with the use of a particular actin, although this seems less likely. In this case, one network could assemble more efficiently, using the actin monomers from the monomer pool before the other network can assemble. These results show that actin orthologs expressed in budding yeast cells, even though they are highly similar, can assemble predominantly in one particular network. In other words, the differences that these actins carry are sufficient to segregate them into the different networks.

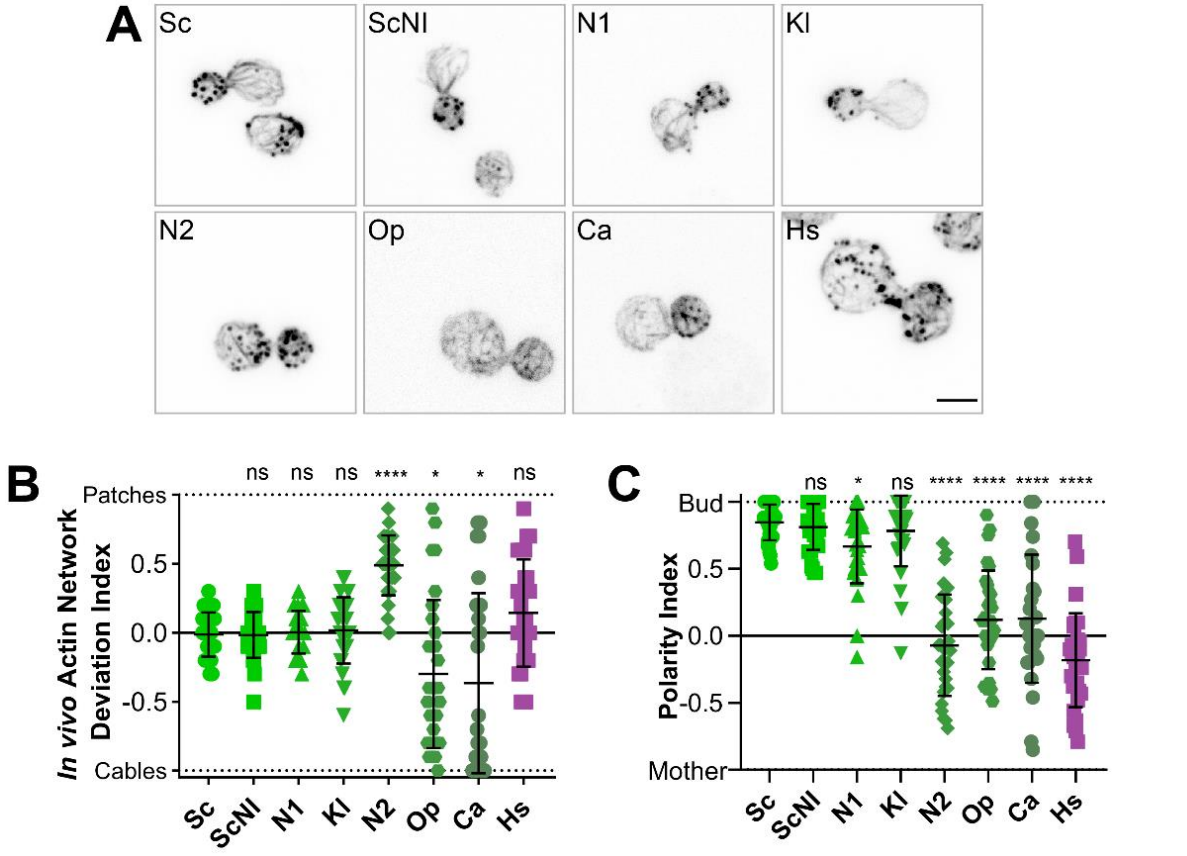


Fig. 10.15. Actin amino acid sequence variation imbalance the linear-to-branched actin network ratio. **(A)** Phalloidin staining of F-actin organization. Images are maximum intensity projections of 3D stacks. Scale bar: 3 μm. **(B)** *In vivo* actin network deviation indexes. **(C)** Polarity indexes. Color code: amino acid sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (green) to 82% (magenta).

It is interesting to note that the phalloidin staining did not have the same intensity for all strains. The staining in N2 and Hs cells was particularly higher than the staining in Sc cells, whereas the staining in Ca and Op cells were particularly low. This could be to a change in the polymerized actin in those cells, but it could also be that phalloidin has different affinities for each of these strains. Indeed, some of the mutations in these actins are located at the phalloidin binding site if the compared Act_Hs (for a detail on the actin amino acids that interact with phalloidin see Fig. 10.16A-B). Even though all of the mutations are conservative substitutions, still Act_Ca is the one that has the most mutations in the phalloidin binding site (T77S, L110M, T194S and V287M), followed by Act_Op that has 3 mutations, then Act_Sc that has 2 mutations and Act_N2 that has only one mutation. This could affect the interaction of the actins with phalloidin, which would explain the intensity pattern. The actins that have the least mutations in the phalloidin binding site would be the most intense in the microscopy pictures (Hs and N2) and the ones with the most mutations would be the less intense (Op and Ca). In any case, since we are calculating a ratio between patch and cable intensity for each cell, differences in intensities for the different strains do not affect our conclusions.

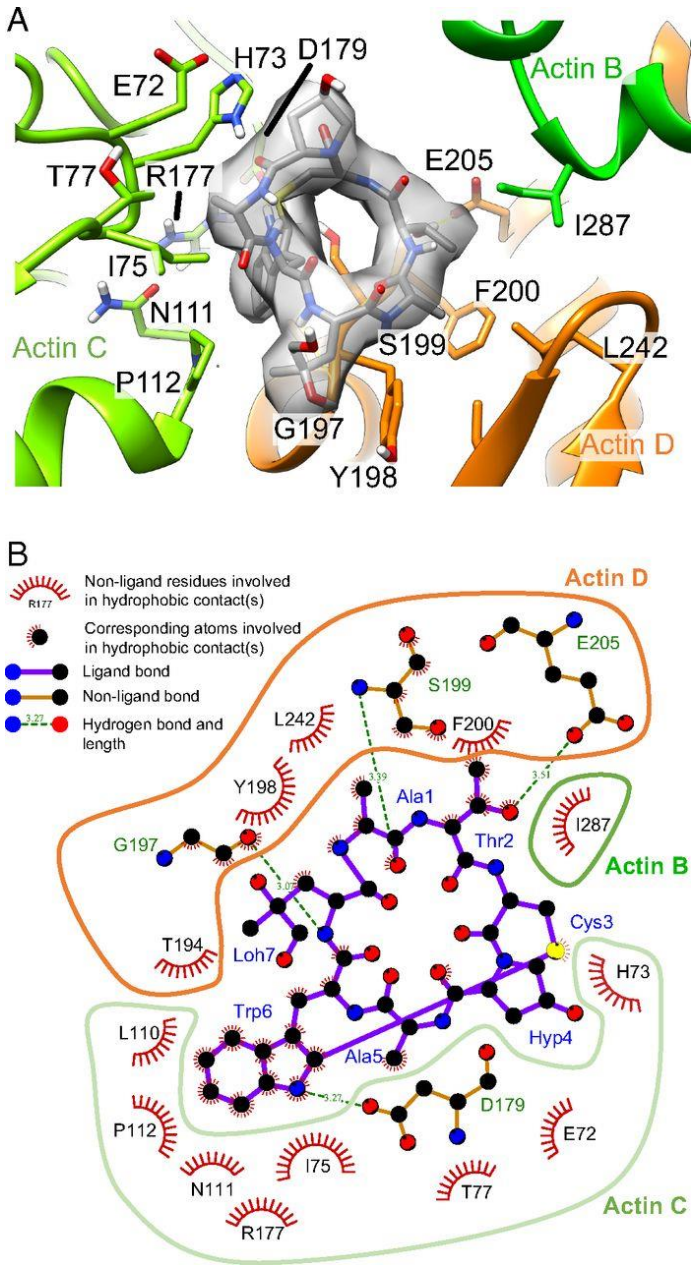


Fig. 10.16. Detail of the actin amino acids that interact with phalloidin.
(A) Phalloidin (gray) interacts with three actin subunits (green, light green and orange). This structure was modeled after a cryo-EM density. **(B)** Detail of the actin residues from the three subunits that interact with phalloidin. Adapted from (Mentes *et al.*, 2018).

The previous results show that some strains assemble an increased number of patches compared to cables and other strains do the opposite. One way to perturb the balance of the actin cytoskeleton is by the addition of drugs. Ck-666 is a small molecule that inhibits the Arp2/3 complex activity by stabilizing an inactive state of the complex, therefore inhibiting branched-network formation (Hetrick *et al.*, 2013). After the addition of CK-666, I fixed the cells and did a phalloidin staining to look at the actin structures. It is possible to see how many cells in a big field still present branched networks (patches) inside and

how many (Fig. 10.17A-B). For example, in Sc cells, 56% of the cells had no patches, and only 2% of the cells have 10 or more patches. N2 cells, where in normal conditions the balance is biased towards producing more patches, were less sensitive to the addition of CK-666. For this strain, only 28% of the cells have no patches and on the contrary, 14% of the cells have 10 patches or more. Strains that normally produce more linear network than branched network were more sensitive to Ck-666. This is the case of Ca and Op cells, where the percentage of cells without any patch is 71% and 87%, respectively. Hs cells had a similar distribution to Sc cells, although they presented a higher percentage of cells with 10 or more patches (10%). These results indicate that strains with increased branched network are buffered against Arp2/3 complex perturbations.

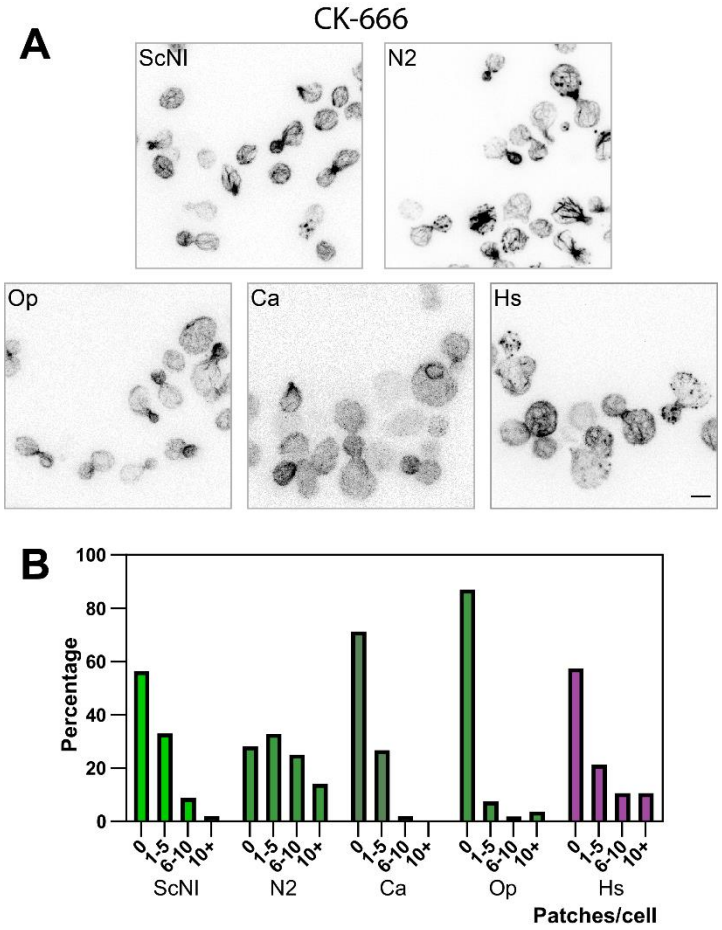


Fig. 10.17. Strains with increased branched-network are buffered against CK-666 perturbations.
(A) Effect of CK-666 (150 μM) on the organization of the actin cytoskeleton. Cells were stained with phalloidin after 30 min incubation with CK-666. Images are maximum intensity projections of 3D stacks. **(B)** Quantification of actin patch resistance to CK-666 treatment. Bar graphs represent the percentage of cells with a given number of visible actin patches after CK-666 treatment. Color code: amino acid sequences percentage identities compared to *S. cerevisiae* actin (Act_Sc), ranging from 100% (green) to 82% (magenta).

10.5 Reconstituted branched- and linear-networks using different actins

After obtaining this result, I was particularly interested in the fact that the changes in the amino acid sequence did not lead to the same phenotype: some actins seemed to favor the branched-network, while others seem to favor the linear-network. Because of this, I aimed to understand the molecular mechanisms behind this apparent preference. Our hypothesis was that heterologous actins may present defective interactions with some of the ABPs. It would be interesting to see if these defective interactions are with the nucleators themselves or with other proteins, since now we know that most of the ABPs can regulate the homeostasis of the actin cytoskeleton (Suarez *et al*., 2015; Antkowiak *et al*., 2019).

To answer this question, I moved from cells to an in vitro system where it is possible to reconstitute the linear- and the branched-network from a limited set of proteins. This approach is relevant to address this question because we can choose which proteins to include in the assay so we can directly see the effect of adding or removing them, but also because we can use fluorescently tagged proteins to see where they locate.

To test if some of these actins bind defectively to ABPs, I purified Act_Sc, Act_Ca and Act_N2. Act_Ca produces more linear-network *in vivo* and Act_N2 produces more branched-network. The system was reconstituted with either of these actins and a subset of proteins that are necessary for the assembly and stabilization of both networks in vivo. Beyond formins and the Arp2/3 complex, these proteins include: an NPF, which activates the Arp2/3 complex, profilin, a small globular protein that favors formin assembly, capping protein, a heterodimer that binds to barbed ends, ADF/cofilin, a small protein that promotes the disassembly of actin filaments, and tropomyosin, a helical coiled-coil protein that binds and stabilizes linear-actin filaments nucleated by formins (Moseley and Goode, 2006; Pollard, 2016). These proteins were included in a system with functionalized beads to generate the branched- and linear-networks (see section 9.2.2, p. 61). Beads functionalized with Las17 (the activator of the Arp2/3 complex, WASp homolog) are used to generate the branched-network, and beads functionalized with the FH1-FH2 domains of Bni1 (a budding yeast formin) are used to generate the linear-network. Both the branched- and linear-networks were reconstituted simultaneously in the same experimental environment (Antkowiak *et al*., 2019).

The first step was to assess the capability of the purified actins to assemble both networks using a minimal set of ABPs. As expected, Act_Sc can grow from both types of beads (Fig. 10.18A-B). Act_Sc polymerizes from the surface of Las17-coated beads, generating a branched network gel that grows uniformly until the point of symmetry breaking, where the network starts growing from one direction to generate an actin comet that pushes the bead forward (Fig. 10.18A-B and section 9.2.2, p. 61). Act_Sc polymerized from Bni-coated beads forming linear filaments that can bundle together to form cables that grow from the

bead, looking like rays of the sun (Fig. 10.18A-B and section 9.2.2, p. 61). Surprisingly, Act_N2 is able to grow both networks as well (Fig. 10.18A-B). Actin comets from Act_N2 where shorter but more intense than Act_Sc comets. As previously said, this actin polymerizes mainly into the branched-network *in vivo*, so this experiment cannot explain the observed phenotype in cells. Act_Ca assembled beautifully into linear-networks but did not assemble in branched networks, providing explanation for the inability of this actin to assemble actin patches in cells (Fig. 10.18A-C).

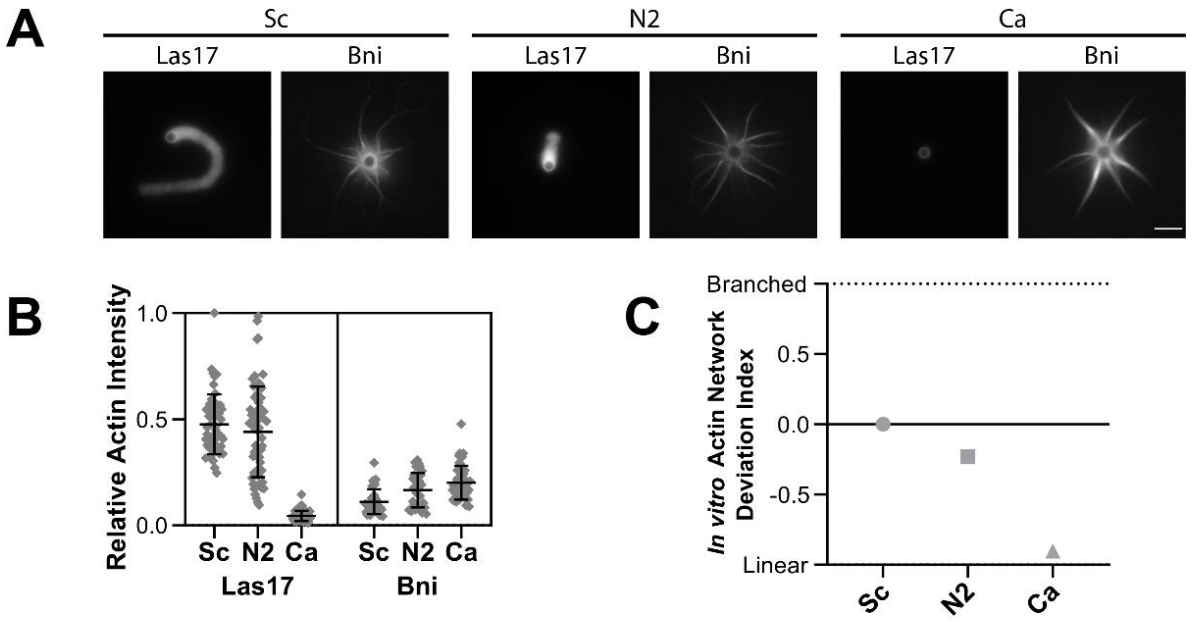


Fig. 10.18. *In vitro* reconstitution of branched- and linear-networks from purified actin variants.
Standard conditions include Las17- (branched) and Bni1- (linear) coated beads, 8 μM F-actin (1% Alexa-568-labeled), 15 μM profilin, 1 μM capping protein, 500 nM Arp2/3 complex and 600 nM ADF/cofilin. Snapshots of representative actin networks were taken after 30 min. Scale bar: 6 μm. **(A)** Snapshots of actin networks assembled from three different actins sources: Act1, Act_N2 and Act_Ca. **(B)** Quantification of actin fluorescence on beads. **(C)** *In vitro* actin network deviation indexes.

With the objective of observing the binding pattern of some ABPs in both networks, I used fluorescently tagged proteins to visualize their localization. I reproduced the experiment above and I either exchanged unlabeled or tropomyosin for their fluorescent counterparts. We labeled first ADF/cofilin, which is known to promote branched-network disassembly by inducing Arp2/3 debranching while stabilizing linear-networks (Michelot *et al*., 2007; Chan *et al*., 2009). By looking at Fig. 10.19A, we can see that ADF/cofilin is able to bind all networks from all actins even though it seems to bind with a slightly lower affinity to the mutated actins. ADF/cofilin bound to linear-actin networks with higher affinity than to the branched-actin network, as previously reported (Fig. 10.19A-B) (Gressin *et al*., 2015). The addition of fluorescent tropomyosin on the mix showed a different pattern. First, tropomyosin bound more efficiently to the linear-network than the branched-network

(Fig. 10.19C-D). Most importantly, tropomyosin did not bind to Act_N2, while it can bind to both Act_Sc and Act_Ca (Fig. 10.19C-D). Tropomyosin inhibits branched-network assembly and promotes linear-network stabilization (Blanchoin *et al*., 2001; Bernstein and Bamburg, 1982; DesMarais *et al*., 2002; Antkowiak *et al*., 2019). The inability of Act_N2 to bind tropomyosin could explain why the branched-network is favored in N2 cells. A possible explanation for this phenotype is that Act_N2 can assemble into both networks, but that in vivo tropomyosin cannot bind the actin cables and therefore they get disassembled very rapidly.

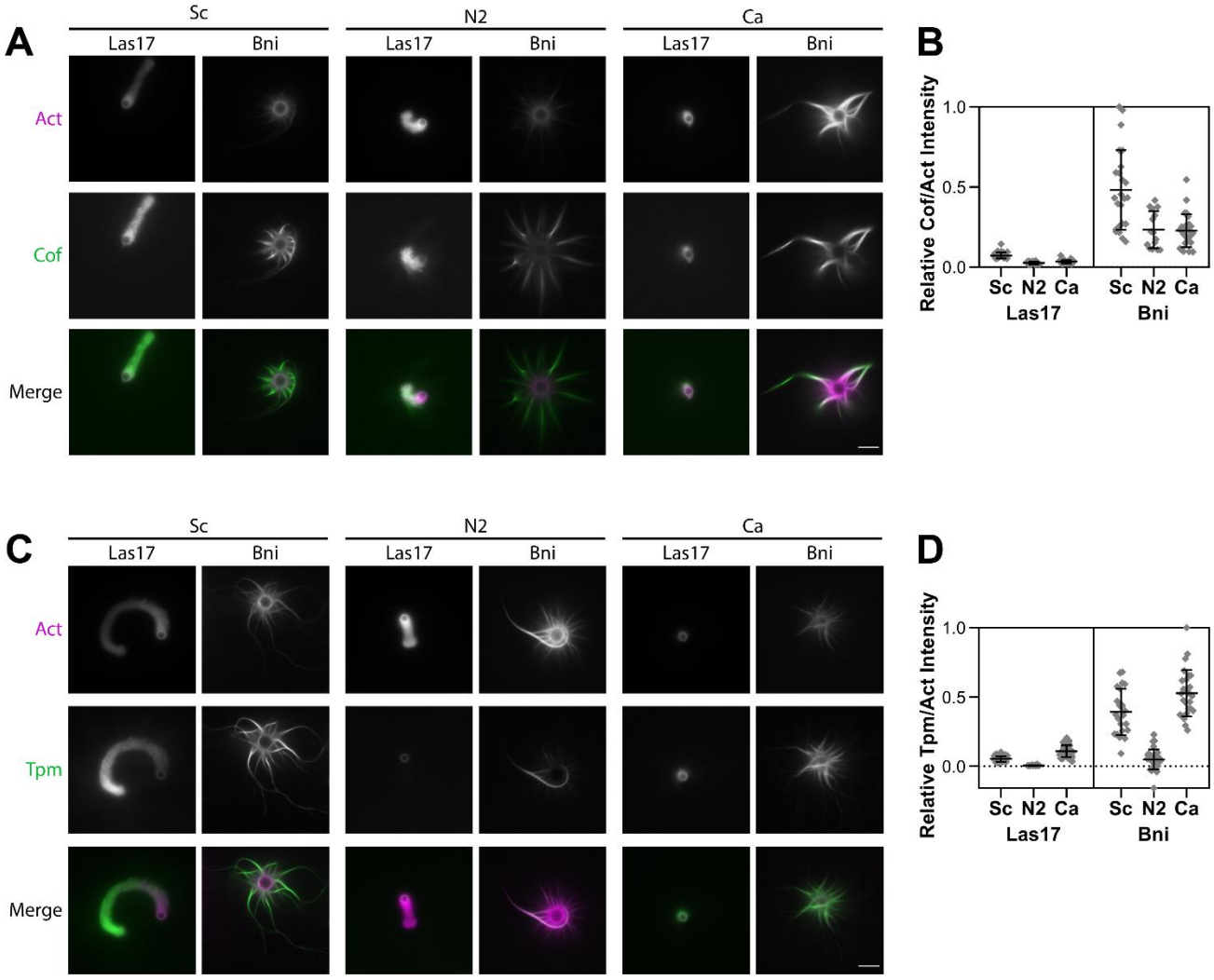


Fig. 10.19. Binding of fluorescently-tagged actin regulators to networks assembled from different actin variants.

(A) Snapshots of representative actin networks assembled in the presence of 600 nM Alexa-488-labeled ADF/cofilin (replacement of unlabeled ADF/cofilin). **(B)** Quantification of the amount of ADF/cofilin relative to the amount of polymerized actin. **(C)** Snapshots of representative actin networks assembled in the presence 1 µM Alexa 488-tropomyosin. **(D)** Quantification of the amount of tropomyosin relative to the amount of polymerized actin. Act_N2 does not recruit tropomyosin to actin networks. Contrast was adapted for comets and formin beads independently because their intensities were not comparable. Scale bars: 6 µm.

10.6 Structural analysis of the differences between the different actins

We then aimed for a structural understanding of why Act_N2 and Act_Ca do not interact properly with specific ABPs of *S. cerevisiae*. Based on structural information available of these interactions, we identified actin residues that are within 5 Å of at the actin-actin interface, or at the interface between F-actin and the ABPs used in our biomimetic assay (Winn *et al*., 2011), with the exception of the Arp2/3 mother filament which were within 10 Å since the coordinates have not been released (Fäßler *et al*., 2020) (Fig. 10.20A).

At protomer:protomer interfaces, wild-type actin differed by one residue (Val287Met) and two residues (Ala167Glu and Ser170Ala) relative to Act_Ca and Act_N2, respectively (Fig. 10.20B). In particular, the Ala167Glu substitution has been shown to effect actin filament stiffness (Hocky *et al*., 2016; Kang *et al*., 2012; Scipion *et al*., 2018). Furthermore, four differences were observed in inter-strand contacts relative to Sc (Ser194Thr and Glu270Asp for Act_N2) and (Ser201Thr and Thr203Ser for Act_Ca) (Fig. 10.20B). Together, these substitutions may subtly alter the relative filament plasticity, which in turn may have an influence on the association or activity of filament binding and filament nucleating proteins (McCullough *et al*., 2011; von der Ecken *et al*., 2015). In addition, we identified in total 15 non-conserved residues of Act_N2 or Act_Ca that are surface exposed on the actin protomer structures and contact a binding partner (Table 2, p. 73). Tropomyosin is likely to be particularly susceptible to small changes in the actin filaments, since it loosely associates with the actin filament surface via shape and charge complementarity (Popp and Robinson, 2012; von der Ecken *et al*., 2016). Particularly, Act_N2 filament Asp311 potentially places the negative charge at ~1.5 Å closer to the actin, relative to the Sc and Ca filaments, which can be inappropriate for tropomyosin binding. Act_N2 has substitutions in interfaces with all the proteins used in the in vitro assays, including Arp2/3 complex and formin interfaces, which could have impaired the activities of these filament nucleating complexes. Act_Ca has fewer substitutions in the actin regulating proteins, with the notable exception of the Arp2/3 complex. In particular, substitutions in the actin interfaces with Arp2/3 subunits in the daughter filament may indicate that the nucleation process of the daughter filament is impaired for Act_Ca with *S. cerevisiae*'s Arp2/3 complex.

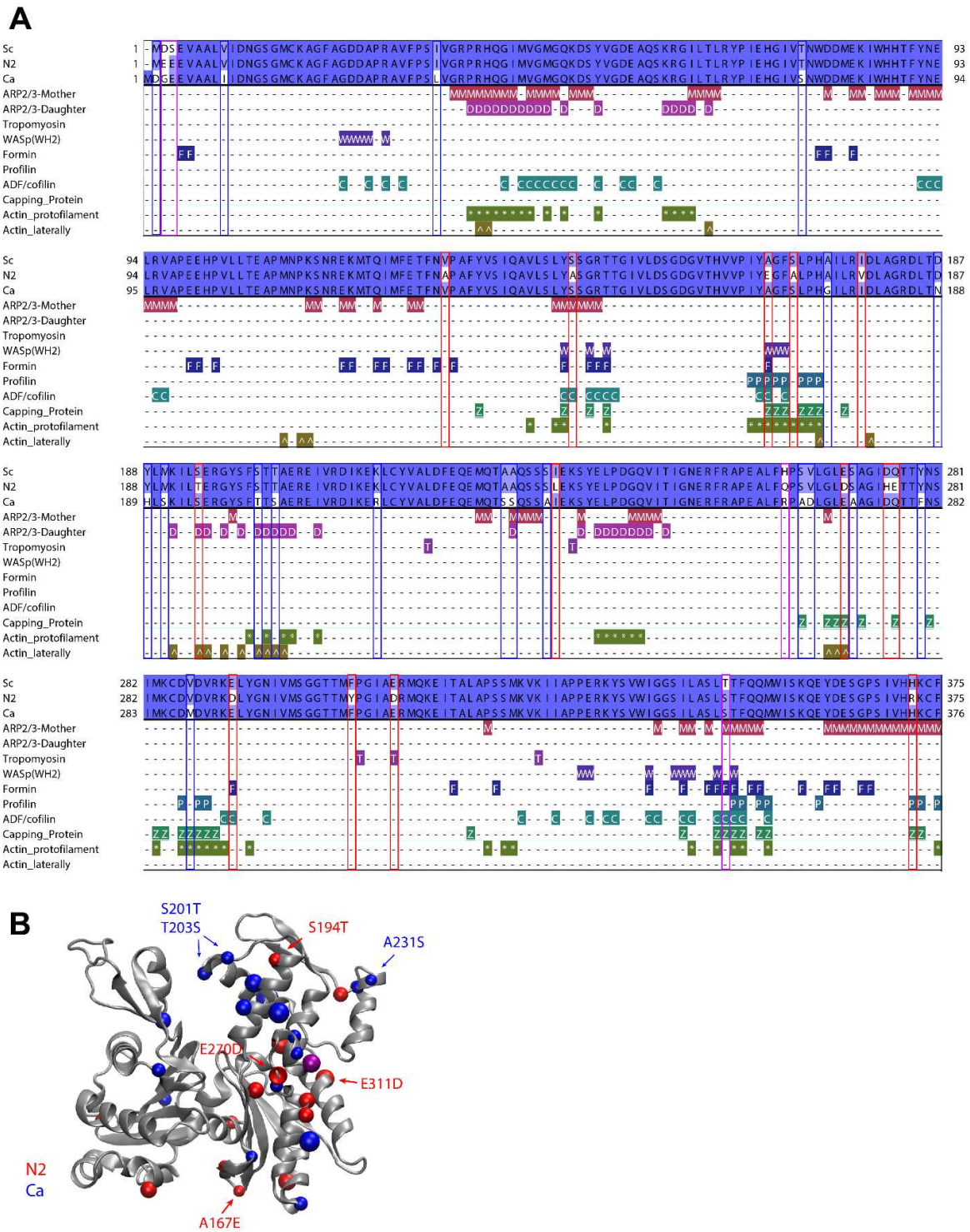


Fig. 10.20. Interaction interfaces of actin with actin-binding proteins.

(A) Sequence alignment of three actins (Act_Sc, Act_N2 and Act_Ca; deep blue indicates conserved residues, light blue and white indicates non-conserved), indicating contacts between proteins used in the biomimetic assay (with Arp2/3 complex at the mother filament interface (M), with Arp2/3 complex at the daughter filaments interface (D), with tropomyosin (T), with WASP's WH2 (W), with formin (F), with profilin (P), with ADF/cofilin (C), with capping protein (Z), at the protofilament interface (*) and laterally (^). **(B)** Schematic representation of actin 3D structure (1YAG (Vorobiev *et al.*, 2003)). Red (resp. blue) color dots correspond to positions where Act_Sc and Act_N2 (resp. Act_Ca) have different residues. Purple dots correspond to positions where both Act_N2 and Act_Ca have different residues compared to Act_Sc.

10.7 The effect of co-expressing a patch and a cable-favoring actin in diploid cells

S. cerevisiae cells are stable in both their haploid and diploid states. This is very useful because when using diploid cells, which have 2 actin alleles in the genome, we can investigate the effect of having two different actins inside the same cell. Considering that we found actins that assemble mainly into different networks *in vivo*, I wondered if co-expressing a "branched-network actin" and a "linear-network actin" would restore a normal cytoskeleton (

Fig. 10.21). If this is true, it could mean that one actin can compensate for the defect the other actin has and viceversa. With the objective to test this hypothesis, I generated several diploid strains. The control stains carry only one actin, Sc/Sc, Ca/Ca and N2/N2, where I expect to see the same phenotype as their haploid counterparts. N2/Ca and Ca/N2 strains carry two different actins, Act_N2 and Act_Ca. The difference between these two strains is only that I inverted the markers for the transformation.

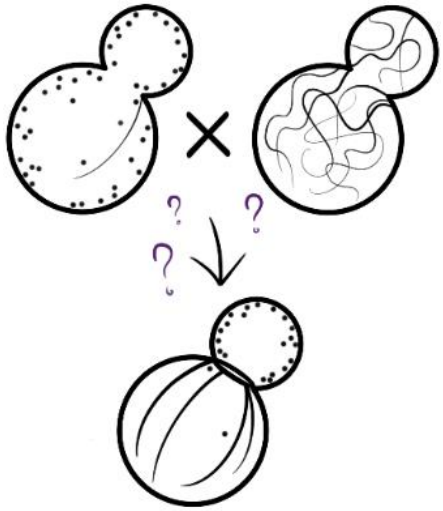


Fig. 10.21. Is it possible to recreate a normal actin cytoskeleton by co-expressing a patch-favoring actin and a cable-favoring actin?

First, I looked at the growth phenotype of the diploid strains. Sc/Sc cells have a normal growth rate, whereas N2/N2 grow slower, similar to the haploid N2 cells (Fig. 10.22A-B). Ca/Ca cells display a tendency to grow slower, but this slight difference is not significant (Fig. 10.22A-B). Interestingly, both strains expressing the two actins (N2/Ca and Ca/N2) can divide at the same rate as Sc/Sc cells, restoring the growth defect (Fig. 10.22A-B). This result give us a first indication that having two actins, each specialized in a different network, is beneficial compared to having only one unspecialized actin.

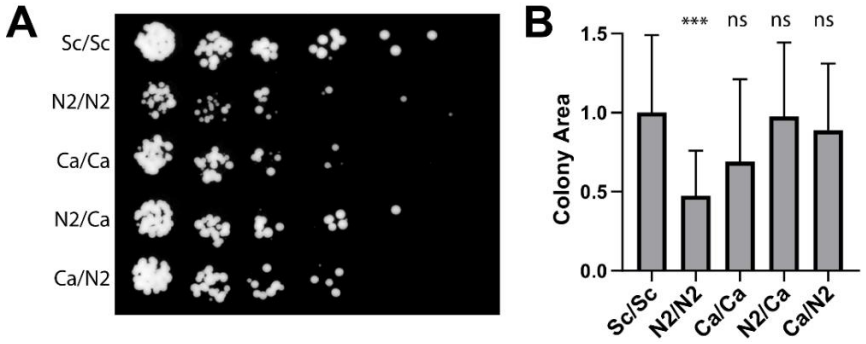


Fig. 10.22. Co-expression of a patch-favoring actin and a cable-favoring actin rescues cell growth.
(A) 3-fold serial dilutions of diploid yeast strains cultures, grown at 25 ºC for 2 days on a YPD plate. Act_N2/Act_Ca and Act_Ca/Act_N2 express the same actins but markers used for selection are exchanged. **(B)** Quantification of (A) by measurement of colony area.

The next step was to visualize the actin structures in medium-budded diploid cells. Fixation and phalloidin staining showed that Sc/Sc, N2/N2 and Ca/Ca present a similar distribution as their haploid counterparts (Fig. 10.23A). More specifically, Sc/Sc has a normal distribution of the actin structures where both networks are present: patches are located mainly in the bud, and cables are located mainly in the mother cell. N2/N2 has predominantly patches (branched-network) and Ca/Ca has predominantly cables (linear-network). This is reflected by their *in vivo* actin network deviation index which is negative for N2/N2 and positive for Ca/Ca (Fig. 10.23B-C). N2/Ca and Ca/N2 both showed a normal distribution of the cytoskeleton, confirming the proposed hypothesis (

Fig. 10.21 and Fig. 10.23A-C). It is tempting to speculate that in strains that carry both Act_N2 and Act_Ca, the first one would be found mainly in the branched-network and the second one mainly in the linear-network. Unfortunately, since we lack the tools to be able to stain both actins independently, such as specific antibodies, we cannot confirm this speculation. In any case, the results demonstrate that cells expressing two defective actins simultaneously can have a normal growth and cytoskeleton.

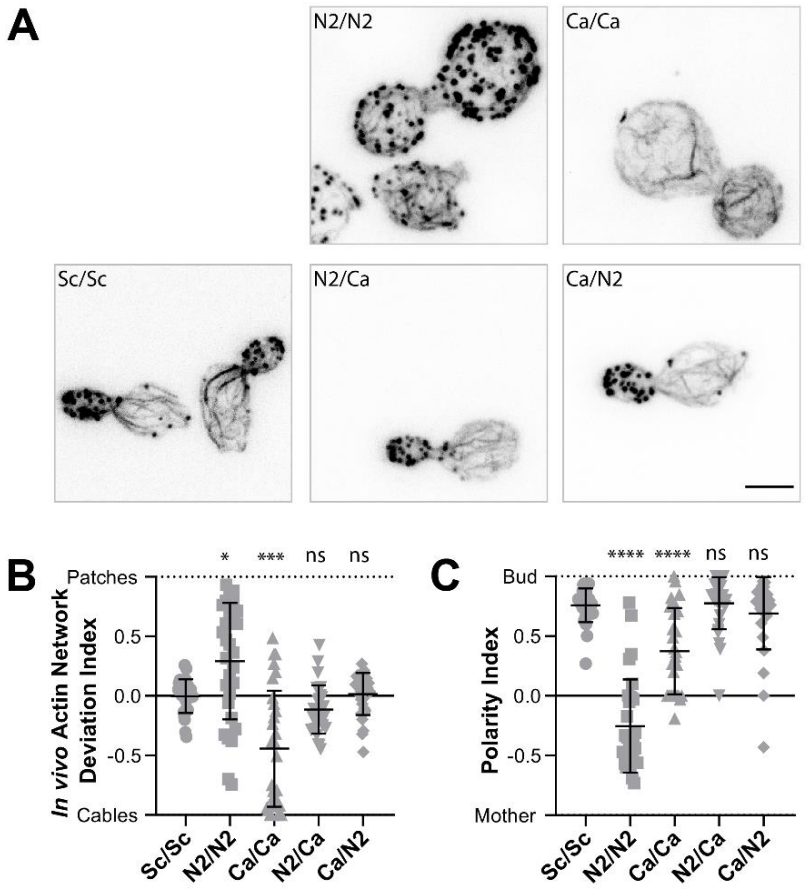


Fig. 10.23. Co-expression of a patch-favoring actin and a cable-favoring actin rescues cytoskeletal organization.

(A) Phalloidin staining depicting F-actin organization. Images are maximum intensity projections of 3D stacks. **(B)** *In vivo* actin network deviation indexes. **(C)** Polarity indexes. Scale bar: 3 μm

Generation of yeast strains with partially separated actin functions enabled us to question some differences between species sharing a single actin for multiple cellular functions, and species using different actins. We were especially curious to know what the physiological consequences would be on actin network homeostasis for wild type diploid cells and Act_N2/Act_Ca cells, which share the same ratio of branched and linear network but possess different actin orthologs. As expected, addition of CK-666 in wild-type cells resulted in the disappearance of actin patches and an increase of actin cables (Fig. 10.24A-B). On the contrary, addition of CK-666 to Act_N2/Act_Ca cells had a weaker effect, as a large number of actin patches could still be observed (Fig. 10.24A-B). Altogether, these results show that while F-actin network homeostasis is preserved in a yeast strain using a single actin to assemble several actin networks, homeostasis is less effective in the context of a yeast strain which expresses two different actins.

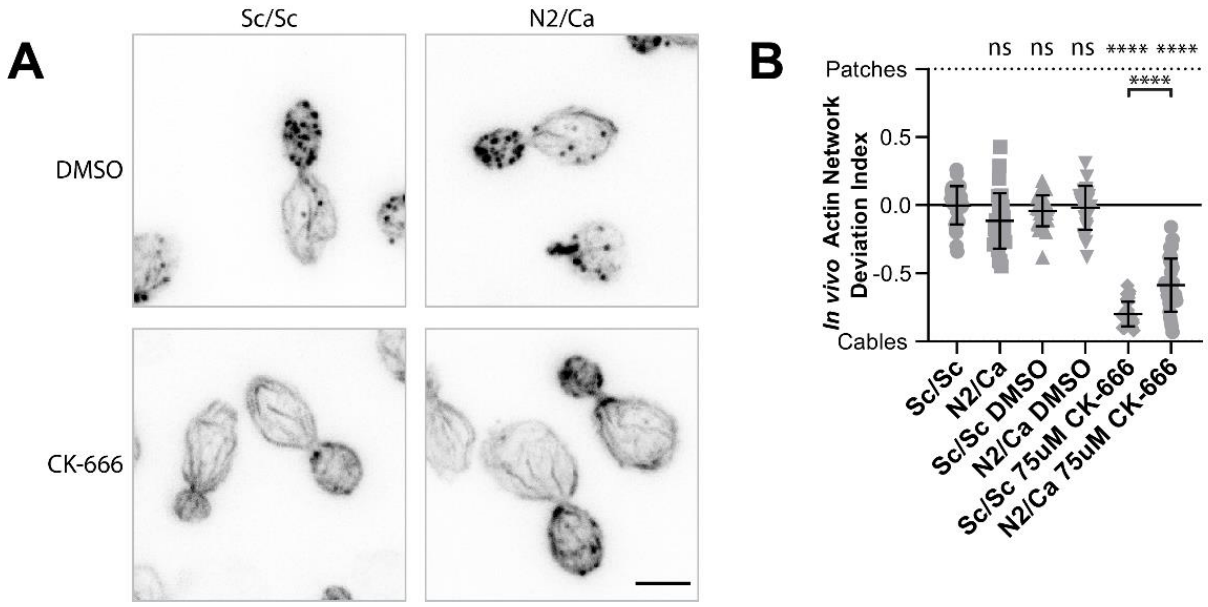


Fig. 10.24. Effect of dual expression of actin variants against external perturbations.
(A) Effect of CK-666 (75 μM) on the organization of the actin cytoskeleton. Cells were stained with phalloidin after 30 min incubation with CK-666. Images are maximum intensity projections of 3D stacks. Scale bar: 3 μm. **(B)** *In vivo* actin network deviation indexes of cells treated with DMSO or CK-666.

10.8 Consequences of expressing an actin ortholog alongside wild-type actin in diploid cells

I next sought to analyze the effect of having a mutant actin alongside wild type *S. cerevisiae* actin (Act_Sc). The objective of this section is to assess whether cell viability or actin structures are affected when expressing a heterologous actin alongside Act_Sc. Since all these strains carry one copy of the wild type gene, my hypothesis is that the defects, if any, will be milder. However, it is possible that the expression of another actin ortholog affects positively or negatively cell viability and actin networks.

For these experiments, the full spectrum of actins was considered; from the most similar actin to Act_Sc, which is Act_N1 with 98% of shared identity, to the most different, which is *Arabidopsis thaliana* actin (Act_At) which shares 85% of identity with Act_Sc. Strains will be called Sc/X, where X will be replaced for the initials of the species to which the mutated actin belongs (see "Name" in Table 1, p. 73). For example, the strain called Sc/Sc is a wild type strain, since it only carries wild type actin (Act_Sc). As another example, the strain Sc/N2 carries one copy of Act_Sc and one copy of Act_N2 in the other allele. In addition, I also generated a diploid strain that is lacking one actin gene, which will be called Sc/KO (*S. cerevisiae*/Knock-out).

Actin expression in all these strains was similar, with the exception of Sc/KO that expresses actin only at 75% (Fig. 10.25A-B). It is interesting to note that after removal of one of the two actin alleles, actin expression did not drop to 50%. This means that diploid yeast cells having one actin gene only can partially compensate for the expression from the missing allele, in the case that both alleles would contribute equally to actin expression in Sc/Sc cells.

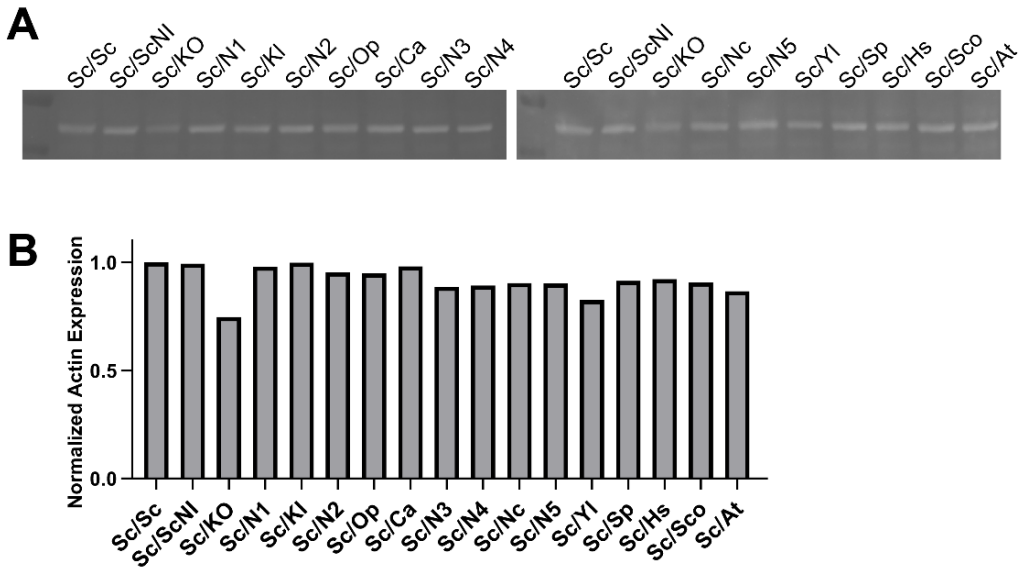


Fig. 10.25. Actin expression in diploid cells carrying only one mutant actin.
(A) Actin expression levels shown by western blotting for strains co-expressing Act_Sc and another actin variant. **(B)** Quantification of actin expression levels shown in (A).

Growth assays denote that most of the diploid strains that carry one copy of Act_Sc and one copy of an actin ortholog can grow well (Fig. 10.26A-B). Interestingly, Sc/KO cells presented a big defect in growth, which was around 40% compared to Sc/Sc cells. The actin expression threshold for haploid cells was around 35% of actin expression. This threshold has not been calculated for diploid cells. However, an expression of 75% is way above the threshold for haploid cells, and one could speculate that the growth defect in Sc/KO cells should not be related to the drop in actin expression. There must be then another reason why this strain is sick. Another interesting observation is that the strains Sc/Sp and Sc/At also grow slow, but they grow even slower than Sc/KO. Sc/Sp cells grew 34% compared to Sc/Sc cells, and Sc/At cells grew only 22% compared to Sc/Sc cells. This could mean that for these two strains, expressing a heterologous actin is more detrimental than having only one actin gene in a diploid cell. Act_Sp and Act_At share 87% and 85% of sequence identity with Act_Sc, respectively. Even though they are some of the more distant actins used in this study, there are others that share less than 90% of identity (for example, Act_Yl and Act_Hs) that grow well. This phenomenon was not investigated by me, but it could be interesting to understand why when these particular actins (Act_Sp and Act_At) are expressed in cells, their growth is highly affected. One option is that it could be related to their ability or inability to make co-polymers with Act_Sc. Another likely possibility would be that the different actin orthologs can co-polymerize with wild-type Act_Sc, but that these filaments composed of different actins have highly perturbed binding of some ABPs.

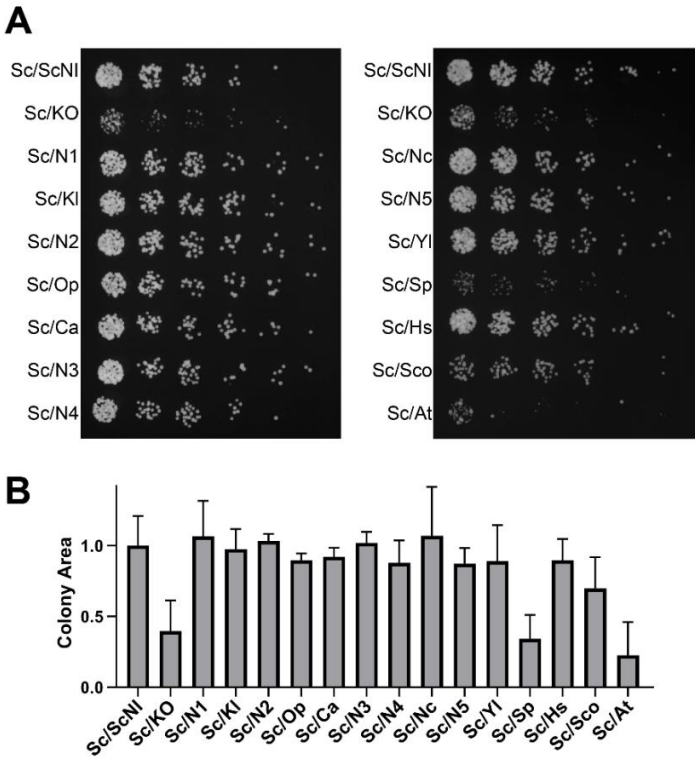


Fig. 10.26. Growth in diploid cells carrying only one mutant actin.
(A) 3-fold serial dilutions of diploid yeast strains cultures, grown at 25 ºC for 2 days on a YPD plate. **(B)** Quantification of (A) by measurement of colony area.

Actin structures in most of these strains were normal or only slightly perturbed (Fig. 10.27A-B). The only exception was Sc/At, strain which clearly presented more branched than linear network. By looking at the microscope image we can tell that cells from this strain are also depolarized.

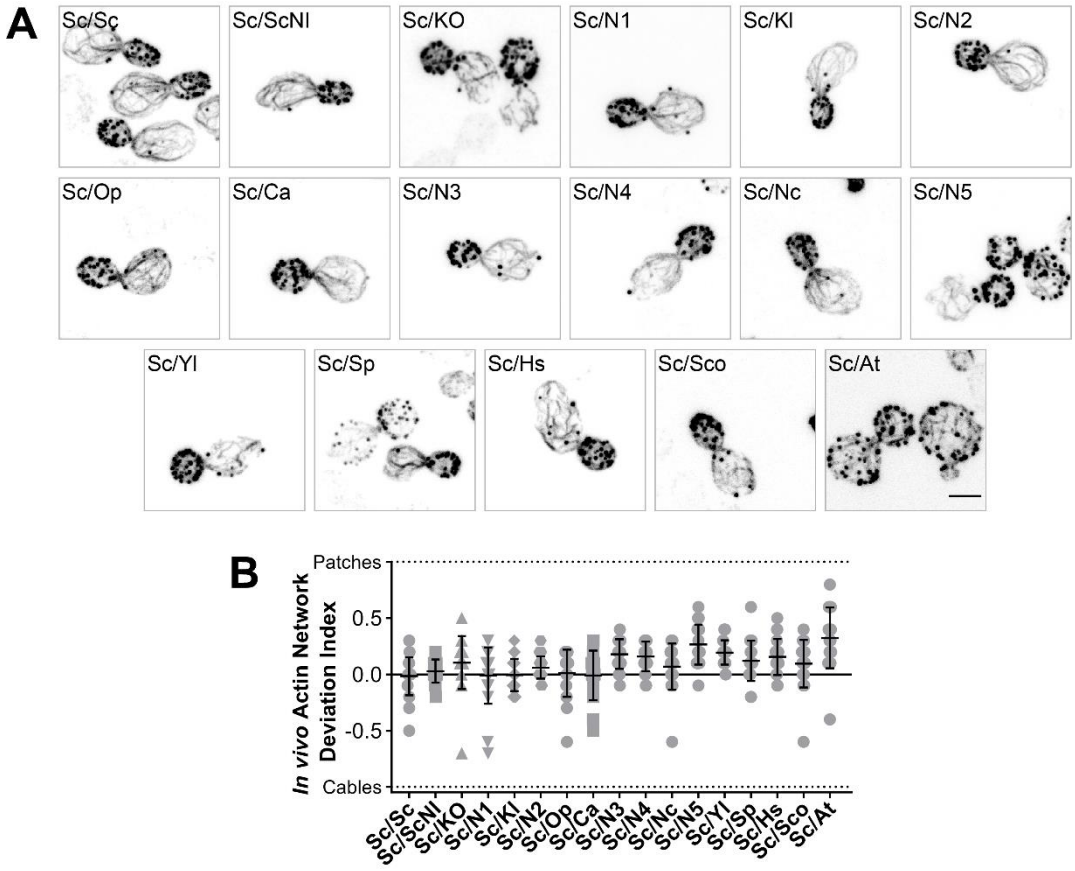


Fig. 10.27. Cytoskeletal organization in diploid cells carrying only one mutant actin.
(A) Phalloidin staining depicting F-actin organization. Images are maximum intensity projections of 3D stacks. Scale bar: 3 μm **(B)** *In vivo* actin network deviation indexes.

DISCUSSION AND PERSPECTIVES

During the course of my PhD, my main objective was to gain insight into how a cell can modulate the organization of its actin cytoskeleton from the expression of different actin isoforms. To simplify the complexity of this regulation and understand the basal mechanisms that are involved, I used a relatively simple organism, *Saccharomyces cerevisiae*, which expresses only one actin, and an *in vitro* system with purified proteins. I investigated actin regulation at different levels. First, I tested the contribution of the intronic sequence in *S. cerevisiae* cells. Then, I studied the contribution of the nucleotide sequence and separately the contribution of the amino acid sequence. Last, I analyzed the effect of expressing two different actins in the same system.

Models

Saccharomyces cerevisiae proved to be a very powerful model system for this study. I generated 18 heterozygous diploid strains, four carrying silent mutations and 14 carrying heterologous actins. Many of these diploids strains were also viable in the haploid state, allowing for their study in the presence of only one mutant actin. Using "*S. cerevisiae* coded" genes to express the heterologous actins proved to be very useful. For example, a yeast strain expressing the coding sequence from human β-actin is lethal in the haploid state, but a yeast strain expressing β-actin from a yeast coded gene is viable. Keeping the coding sequence as similar as possible to the one of budding yeast allowed us to study the effect of heterologous actins that otherwise would not have been possible. Moreover, it allowed me to generate stable yeast strains from which to purify different actins. This is extremely useful as, until recently, it has been impossible to separate actins that are co-expressed in the same cells for purification. This is the case for β- and γ-actins, where it was not possible to purify separately because of their similarity and co-expression in all cell types. In recent years, new protocols have been developed for the purification of actin isoforms in the yeast *Pichia pastoris* (Hatano *et al*., 2018, 2020). Experiments using purified proteins and functionalized beads can reconstitute effectively the branched and linear networks. Moreover, by using fluorescent proteins we could study their localization on the different networks but also when using different actins. These experiments allowed us to describe possible mechanisms underlying the *in vivo* phenotypes.

Intron

The actin intron in budding yeast has already been shown to not have an observable function and this study confirms that observation (Ng *et al*., 1985). However, this study focused on mRNA levels (measured by northern blot), which do not change. We can now expand this to the protein expression levels, that do not change significantly when removing the actin intron in budding yeast. This does not rule out the possibility that the

intron has an effect that is too subtle to observe, or that it plays a role in a function that has not been analyzed yet.

Nucleotide sequence

Variations in the nucleotide sequence of a gene that do not affect the final amino acid sequence of the protein are called silent mutations. In our system, silent mutations had an effect on actin expression levels. This result was somewhat expected, since each organism has its own codon usage bias according to what tRNAs are most expressed in cells. This means that cells carrying genes with many silent mutations could have difficulties to produce the actin protein from the mRNA. Interestingly, we found that the drop on actin expression did not affect cell growth and actin structures if the drop was above 35%. Cells with actin expression below this 35% threshold had difficulties to grow or did not grow at all. Actin structures in strains with low actin levels presented a bias towards the branched network. This result was surprising as in fission yeast actin underexpression was reported to reduce Arp2/3-mediated actin patches and an increase in the linear network in the cytokinetic ring (Burke *et al*., 2014). In this study, the authors hypothesized that the reason why the balance was shifted towards the linear network in fission yeast cells underexpressing actin is because of an increase in the actin-profilin ratio that would address the available actin to the formins. Even though that was only a hypothesis that was not directly tested in the study, it would seem that in budding yeast the mechanism is different. The variations in the nucleotide sequence can play various roles, some of them that have been not explored along this work. For example, other than directly affecting expression levels, it can affect the stability or tertiary structure of the mRNA and modify the translation speed. Translation speed is an important regulator for expression, alternative splicing and differential PTMs. As mentioned in section 8.2 (p. 45), the nucleotide sequence in actin has been shown to play a major role in actin regulation between β-and γ-actins (Vedula *et al*., 2017). In addition, arginylation does not affect β- and γ-actin in the same way because γ-actin mRNA has a slower translation that after a series of events drives the degradation of arginylated γ-actin (Zhang *et al*., 2010). As mentioned earlier, the aginylation of exclusively β-actin cannot account for all the differences observed in between both cytoplasmic actins, since the amount of aginylated β-actin equals only the 1% of the total β-actin in cells (Chen and Kashina, 2019).

Amino acid sequence

Variations in the amino acid sequence led to a different result. Cells expressing heterologous actins presented a massive reorganization of the actin cytoskeleton. Interestingly, not all actins showed the same tendency. Some actins assembled more efficiently into the branched network whereas other actins assembled more efficiently into the linear network. It is important to highlight that most of these actins share around 95% of sequence identity with *S. cerevisiae* actin. This proves that actins that are very similar

can have a preference to assemble into a specific architecture. This result means that the observation of ACT2 and ACT7 in different structures in *Arabidopsis thaliana* could be due to their differences (Kijima *et al*., 2018). These two actins share around 93% of sequence identity. However, actins like β- and γ-actin share 99% of sequence identity. In my system, yeast cells expressing an actin (Act_Kl) that shares 97% of sequence identity with *S. cerevisiae* actin (Act_Sc) have a normal cytoskeletal organization. This means that it remains to be proven if actins that share more than 97% of sequence identity have enough differences to segregate into different structures. It is also important to take into account that not only the percentage of identity is important, but also which is the amino acid that is different and where it is located. Therefore, it is possible that a single major point mutation could be sufficient to lead to cytoskeletal reorganization as described in this study. When exposed to the Arp2/3 complex inhibiting drug, CK-666, strains that had a higher number of patches presented some resistance. These cells would require a higher concentration of CK-666 to fully disassemble all the patches, implying that not only the number of patches but also their properties are different.

Molecular mechanisms

This biased assembly into a particular network is triggered by defective interactions with ABPs. The easiest way to explain this would be by the differential binding or activity of a nucleating protein with a particular actin. Our results demonstrate that other ABPs could play a part in this role, such as tropomyosin. This result highlights that the segregation of actin isoforms can be influenced after filament nucleation. Although actin nucleators tend to be in the spotlight, it must be stressed that most ABPs have different effects on branched- and linear-actin networks and their influence needs to be taken into account (Rotty *et al*., 2015; Suarez and Kovar, 2016; Antkowiak *et al*., 2019). Proteins like tropomyosin and ADF/cofilin stabilize linear networks of actin filaments, while enhancing disassembly of branched networks. Any actin variant with defective binding to ADF/cofilin or tropomyosin will naturally be more present within branched networks, while absent from linear ones. In this context, it is rational to postulate that the inability of an actin variant to assemble efficiently in a given actin network, leads to an expansion of the other actin networks, provided that those can use this actin normally.

Overall, the principles outlined above should be valid regardless of the mechanism by which variation is brought to the specific actin, whether it is through changes in the peptide sequence or through post-translational modifications. Also, from an evolutionary perspective, the proposed mechanism appears to be efficient in allowing the emergence of new actin isoforms associated with discrete actin functions. Our model implies that a simple actin gene duplication, followed by minimal mutation in one actin copy, which impairs an essential interaction with an ABP, could be sufficient to trigger a global reorganization of the actin cytoskeleton, whereby each actin network becomes enriched in one actin isoform or the other.

We now have precise structural information on how actin interacts with many ABPs (Fedorov *et al*., 1997; Eads *et al*., 1998; Otomo *et al*., 2005b; Baek *et al*., 2008; Thompson *et al*., 2013; Urnavicius *et al*., 2015; von der Ecken *et al*., 2015; Pollard, 2016; Tanaka *et al*., 2018; Shaaban *et al*., 2020). Careful analysis of actin-actin and actin-ABPs interactions can be a powerful tool to predict which ABPs affect the roles of specific actin isoforms in discrete actin networks. For example, such analysis indicates that most Act_Ca substitutions affect its interface with the Arp2/3 complex, providing potential explanation for defective assembly into branched-actin networks. In parallel, our knowledge of the molecular principles involved in the assembly of the different actin networks of the cell allows us to anticipate the consequences of varying the affinity between actin and an ABP.

Co-expressing two actins

Our biochemical results show that ABPs in general and not only nucleators can play a role in the regulation of the size of the branched and linear networks. However, in this context the conclusion is slightly different than in studies using one actin where it was shown that the different ABPs can shift the branched-to-linear balance. Differential interaction of ABPs with the different actins could control the predominance of one actin in the branched network and another actin in the linear network for organisms that express several actins. In agreement with this line of thought, we observed that co-expressing two actins inside the same diploid cell, one that assembles predominantly in the branched-network and another one that assembles predominantly in the linear-network restores normal growth and actin structures in the cells. This reinforces the possibility that actins could compensate for the other actin's lack of efficiency to form a certain structure. However, these diploid cells still show some resistance to CK-666. This shows that in these diploid cells carrying two actins, even though they present a wild-type like actin cytoskeleton, the homeostasis is shifted. This leads me to hypothesize that one benefit of carrying several actins is the possibility of having a higher adaptability, and if one of the actins assembles more efficiently into a certain type of network, then it is possible that making perturbations in that network requires more effort. A clear example in nature is the unicellular green algae *Chlamydomonas reinhardtii*. This organism carries two actin genes, one of them which is an unconventional actin that shares only 65% of identity with the first one (Lee *et al*., 1997). The expression of the unconventional actin is induced upon Latrunculin B exposure, since this actin is resistant to the drug (Kato-Minoura *et al*., 1998; Onishi *et al*., 2016). In the case of *C. reinhardtii* the actins are so different that it is expected to have an effect. But this may also happen even when actins are very similar. I believe that this extreme example, combined with my results, show that higher adaptability could be a possible benefit of having more than one actin gene. Reciprocally, using only one actin for several functions allows the organism for fast and efficient re-allocation of actin monomers when physiological conditions change. From this point of view, both strategies have their own advantages/disadvantages.

Co-expressing more than 1 actin in the same diploid cell can have various effects. Besides the beneficial effect discussed in the previous paragraph, expressing very different actins can be detrimental for the cells as well. For example, expressing a very different actin, such as *Arabidopsis thaliana* actin (Act_At), alongside wild-type *S. cerevisiae* actin (Act_Sc) leads to severe growth defects and an abnormal cytoskeleton. Interestingly, the growth defects are even stronger than the actin knock-out strain, which has only one wild-type copy of the actin gene. This means that the addition of Act_At adds an extra difficulty for the actin cytoskeleton to assemble correctly. We did not inquire into why these cells have growth defects, so we can only speculate. Maybe these actins cannot co-polymerize, leading to a deficiency in the total amount of available actin for the cell. Or maybe Act_At polymerizes but cannot be severed by the yeast disassembly factors, leading to an over production of F-Actin that is not dynamic enough to function properly. This is an open question that could be studied further. In any case, studying these possibilities would have been complicated since the haploid strain carrying only Act_At is not viable. This reinforces the idea that yeast cells cannot utilize properly this actin. It is interesting to note that the same trend is also observed when using the actin coming from *Schizosaccharomyces pombe* (Act_Sp), which is another yeast. This may sound surprising since both species are part of the Ascomycota Division. However, it is necessary to remember that even if these unicellular organisms may look very similar, they actually diverged 590 million years ago and their actins are as different as the comparison between *S. cerevisiae* and *H. sapiens*.

Model

With all these observations, I was able to propose a model (model). Actin monomers can either assemble in branched or linear networks, depending on if they can pass through the "filter" (represented by the color of the tap) or not. These filters include not only formins or the Arp2/3 complex but all the proteins involved in the regulation of a certain network. In a wild-type one-actin system (gray, left), the actin monomers can assemble into both the linear and the branched networks. Adding CK-666 to this system will shift the balance towards more cables than patches. Defective one-actin systems (pink and blue, middle) are those that carry one mutant actin that is unable to assemble efficiently into one of the networks. By combining the two mutants that are defective (two-actin system, right) it is possible to restore a normal cytoskeleton, with a normal amount of branched and linear networks. However, this system is more resistant to the addition of CK-666.

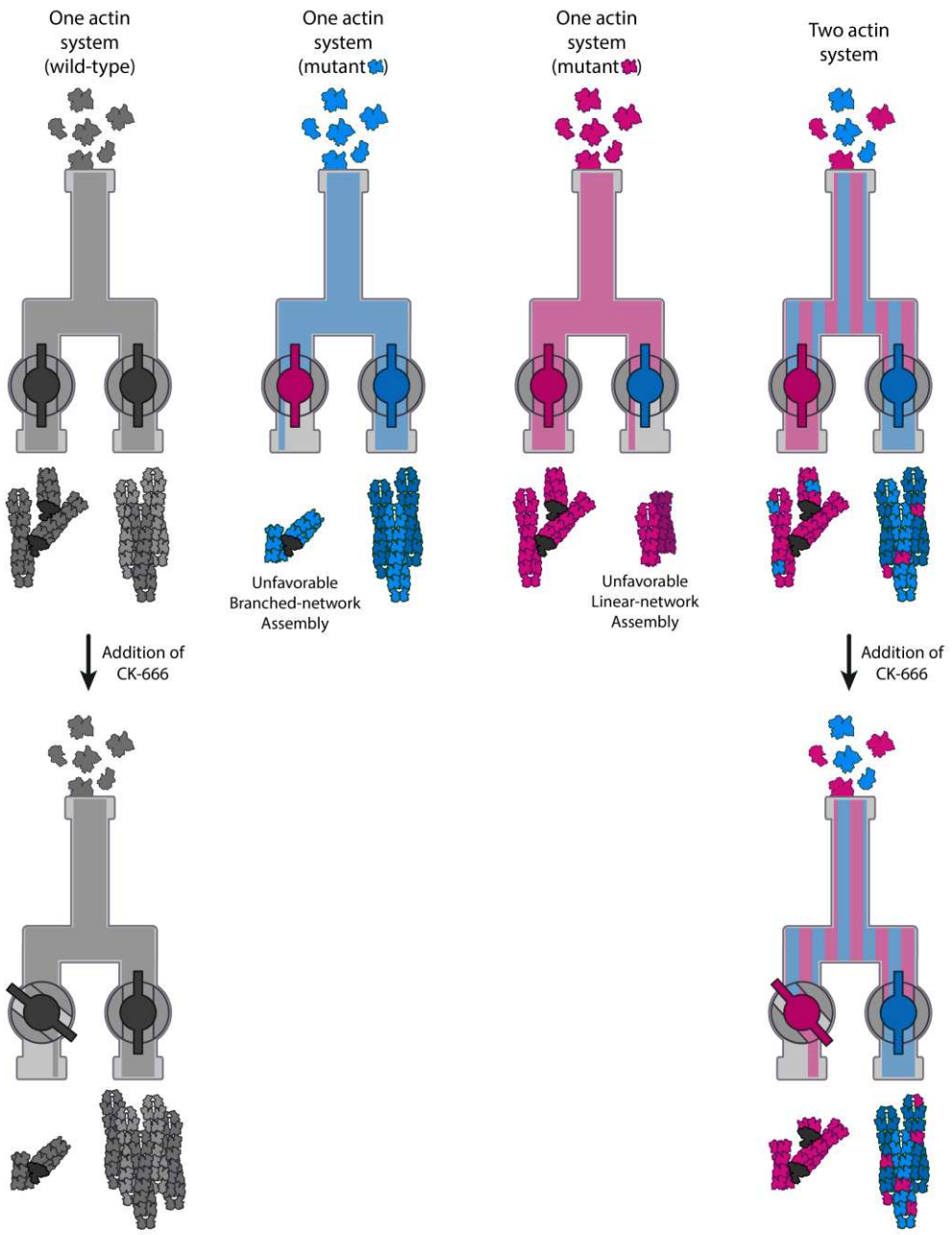


Fig. 0.1. Schematic model of the differences between a cell expressing one or two actins to perform two cellular functions.

(Top) A model of the molecular mechanisms by which two actin isoforms may segregate to different actin networks. On the left, a system carrying wild-type actin is able to generate both the branched- and linear-networks. On the two central panels, defective interactions of an actin isoform with one or several ABPs, affect branched- or linear-network assembly. On the right, combining these two actin variants in one cell should trigger a natural segregation of actins and rescues the wild type actin organization. **(Bottom)** Effect of perturbing an actin assembly pathway for cells using one or two actin variants. On the left, when one actin is shared for two actin functions, the inhibition of one actin assembly pathway (for example branched-networks with CK-666) leads to a reinforcement of the other actin assembly pathway. On the right, when two actin variants are used for two different actin functions, this effect is limited as both actin networks assemble more independently. In other words, having a system with two actin variants can buffer against the addition of the drug.

Open questions

There are several open questions concerning the results obtained by using different actins. Concerning Act_Ca, we were able to show that this actin has a bias towards cables *in vivo* and cannot efficiently polymerize branched network *in vitro*. However, we were not able to pinpoint specifically which ABP interaction is affected. The easiest possible explanation would be that this actin presents a defect with the Arp2/3 complex or its activator but it could be as well another interaction. Another possibility, although less likely, would be that some ABPs could have a higher activity with this actin, for example for cofilin or tropomyosin, that would reinforce cable assembly over branched network assembly (Antkowiak *et al*., 2019). Another unanswered question is whether there is actual segregation of actins in the diploid strain carrying two actins, biased towards different networks (Act_N2 for the branched network, Act_Ca for the linear network). The lack of specific antibodies against each of these actins complicates the direct observation of their localization, which would be crucial to verify whether partial segregation has directly occurred and to what extent. Another option would be the usage of fluorescently tagged-actins. However, fusion proteins of actin and fluorescent proteins such as GPF polymerize inefficiently and rely on the co-polymerization with untagged actin (Yamada *et al*., 2005; Riedl *et al*., 2008). In theory, we would expect the relative integration of an actin within a particular actin network to depend not only on the ability of this actin to assemble in such network, but being part of a complex equation of the relative ability of each actins to integrate within branched- and linear- actin networks. This hypothesis is purely speculative at this stage and needs to be formally tested. Nevertheless, our observation strongly suggests that we were successful in performing a partial separation of actin function, and transformed yeast from an organism that uses a single actin into an organism using two actin isoforms to perform several actin-based functions.

This work-flow that I have developed during my thesis can be adapted to study a variety of different questions. From a technical perspective, I have streamlined transforming actin in yeast cells, being able to purify actin and then addressing questions *in vitro* to pinpoint the affected interactions.

In humans, point mutations in cytoplasmic actins can lead to rare diseases. As it has been explained in section 10.1.2 (p. 70), not many mutations in the cytoplasmic actins have been described, implying that most mutations are probably lethal. It would be interesting to use my system to make those point mutations in yeast cells, observe actin structures and then purify the proteins to observe the *in vitro* interactions of these actins with other ABPs. In this case, the results could not be compared directly with what happens in mammalian cells if the experiments are done with yeast proteins. Nevertheless, if a certain defect is observed in this yeast-based system, it would help guide work in mammalian cells which have large families of ABPs. This could would be quite informative on the molecular mechanisms that give rise to the severe phenotypes.

These systems can also be used to address evolutionary questions. Budding yeast cells have a high mutation rate and can rapidly evolve and we can take advantage of those characteristics to design and perform evolutionary experiments. This technique has been used to address issues of multicellularity and aneuploidy (Rancati *et al*., 2008; Ratcliff *et al*., 2012). It would be interesting to generate a yeast strain with an actin of interest that presents a growth defect, and let it evolve over several generations. If we can isolate a variety that has recovered growth rates, it would be possible to do whole genome sequencing to understand which were the changes that led to this growth increase.

In a similar train of thought, this system could be used to ask co-evolution questions. To achieve different actin functions, it appears that actins may have co-evolved with their binding partners and this has been observed in several organisms (Nefsky and Bretscher, 1992; Kandasamy *et al*., 2007; Takaine and Mabuchi, 2007; Ezezika *et al*., 2009; McCullough *et al*., 2011; Kang *et al*., 2014). In plants, for example, some components of the cytoskeleton have been shown to co-evolve to be tissue-specific. Specifically, one reproductive actin (ACT1) appears to have co-evolved with the reproductive profilin (PRF4) and cofilin (ADF7) (Kandasamy *et al*., 2007). Evidence for coevolution of actin with ABPs can be also found by combining proteins from different species. For example, in both yeasts *S. cerevisiae* and S. pombe, profilin inhibits endogenous actin polymerization but has little effect on rabbit muscle actin polymerization (Nefsky and Bretscher, 1992; Takaine and Mabuchi, 2007; Ezezika *et al*., 2009). Another example is vertebrate cofilin, which can bind to *S. cerevisiae* actin but does not increase their flexibility nor promote severing (McCullough *et al*., 2011; Kang *et al*., 2014). *S. pombe* Arp2/3 complex is reported to be a better nucleator of *S. pombe* actin than rabbit muscle actin (Ti and Pollard, 2011). In the system I developed during my thesis, it would be possible to address these questions in a cellular context. For example, we have seen that N2 cells present more branched network and we also know that Act_N2 cannot interact properly with *S. cerevisiae* tropomyosin. Would transforming tropomyosin from N2 restore a normal cytoskeleton in these cells? This question is not easy to answer, as the actin sequence of N2 was predicted by ancestral reconstruction and does not correspond to any living species. It would be possible to do an ancestral reconstruction analysis of tropomyosin to find the theoretical tropomyosin in this node, although this would be complicated since tropomyosin varies in between different species much more than actin. Besides the specific complications of using an ancestral reconstructed actin as an example, the same logic could be applied to address co-evolution questions about actins from other species and their ABPs. If expressing a heterologous actin from a certain species in *S. cerevisiae* cells leads to a particular defect in the cytoskeletal organization, it is possible to co-express candidate ABPs in the same cells to observe if we can obtain at least a partial rescue of the any of the observed defects.

During the course of this study, I could not address the question of PTMs. It is clear that PTMs are another way to confer actin molecules small differences that could trigger a function specialization, as it has been proposed for arginylation and β-actin.

All in all, my results shed light on the consequences of perturbing a single-actin system at the cellular and molecular level. I demonstrated that in *S. cerevisiae*, silent mutations in the actin gene control the levels of actin expression. I also showed that small variations in the actin molecule are sufficient to induce a global reorganization of the actin cytoskeleton. This finding highlights the fact that despite a remarkably high conservation of actin proteins across species, actins retain enough differences that cells expressing multiple actins could exploit to segregate them into diverse actin networks.

ANNEX: Additional tables and figures

Structural analysis done by Robert Robinson.

Group 1

The first group is the residues buried with the actin fold. These are very conservative substitutions. Some residues are coupled with others. The prediction here would be that all of the yeast residues can be replaced by the human residues and the actin will be functional. My only worry is whether the mutant actins will fold properly which is the worry for all of this work. But I think that this is a small risk in general.

AA	Yeast	Human	G-actin	Comment
10	Ile	Val	buried	No predicted effect
129	Val	Thr	buried	No predicted effect Coupled with 132
132	Phe	Met	buried	No predicted effect Coupled with 129
135	Ser	Ala	buried	No predicted effect
144	Ser	Ala	buried	No predicted effect
153	Leu	Met	buried	No predicted effect Coupled 297/299
162	Val	Thr	buried	No predicted effect Coupled 178
170	Ser	Ala	buried	May effect polymerization
178	Ile	Leu	buried	No predicted effect Coupled 162
236	Ile	Leu	buried	No predicted effect
257	Ala	Cys	buried	No predicted effect Coupled 306
297*	Ile	Thr	buried	No predicted effect Coupled 299/153
299	Met	Leu	buried	No predicted effect Coupled 297/153
306	Phe	Tyr	buried	No predicted effect Coupled 257
327	Val	Ile	buried	No predicted effect

* This residue may be bad for folding

Table A1

Group 2

The second group are the residues on the surface of actin but pointing between the two strands. This is a very weak interaction dominated by the shape of the protofilaments and not by strong protein:protein interactions. I would guess that the yeast residues can be swapped to human without too much problem.

AA	Yeast	Human	F-actin	Comment
68	Arg	Lys	points between strands	No direct interaction
110	Met	Leu	Between strands	May effect polymerization Coupled 194
114	Ser	Ala	points between strands	No direct interaction
194	Ser	Thr	Between strands	May effect polymerization Coupled 110
201	Ser	Thr	Between strands	May effect polymerization
263	His	Gln	Between strands	No predicted effect Coupled 266
266	Val	Phe	Between strands	No predicted effect Coupled 263
269	Leu	Met	Between strands plug	May effect polymerization
272	Ala	Cys	Between strands plug	Plug Coupled 276, 275
275	Asp	His	Between strands plug	Plug Coupled 276, 272
276	Gln	Glu	Between strands plug	Plug Coupled 272, 275
279	Tyr	Phe	Between strands	No predicted effect

Table A2

Group 3

The third class is likely to be sensitive between yeast and human. These residues form the contacts along a single strand. Some of these residues double in binding to G-actin binding proteins such as profilin. My guess would be that changing them all would allow the actin to polymerize, but this may mess up the affinity for profilin and cofilin. We could possibly think about introducing mutated binding partners to overcome this problem.

AA	Yeast	Human	F-actin	Contact	Comment
43	Ile	Val	protofilament		May effect polymerization Coupled with 170
167	Ala	Glu	protofilament	Gelsolin profilin	May effect polymerization
169	Phe	Tyr	protofilament	Gelsolin profilin	May effect polymerization
289	Val	Ile	protofilament		No predicted effect
324	Ser	Thr	protofilament		May effect polymerization
372	His	Arg	protofilament	Gelsolin	May effect polymerization

Table A3

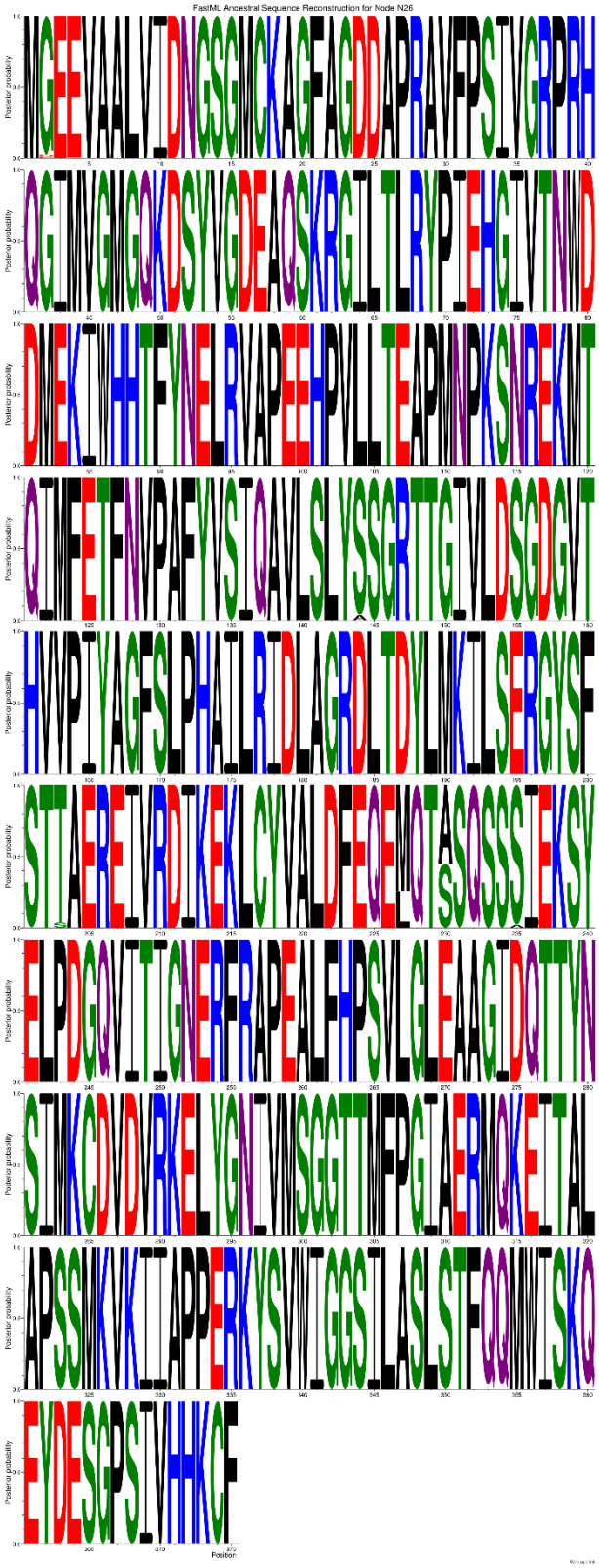
Group 4

The final set are those residues hanging out on the outside of the actin filament. These residues are possibly will effect binding to formins and side binding proteins.

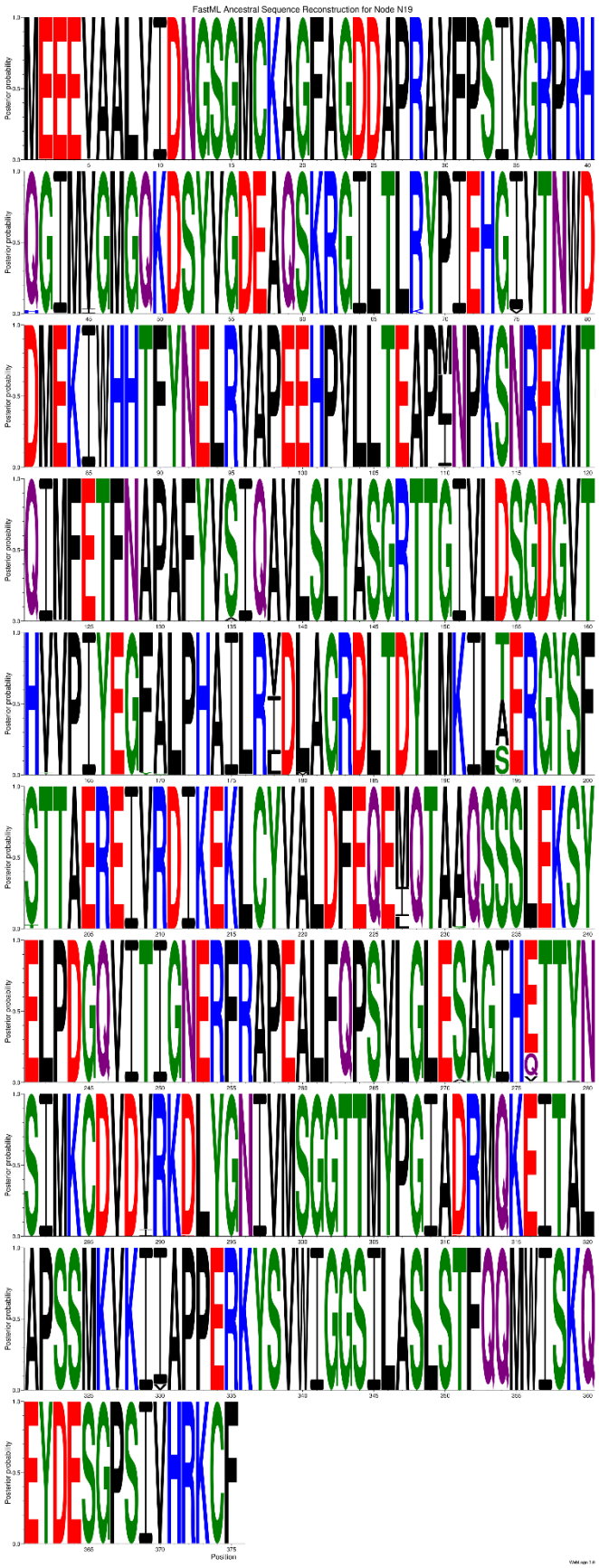
AA	Yeast	Human	G-actin	F-actin	Contact	Comment
3	Ser	Asp			formin?	Surface
4	Glu	Asp			formin?	Surface
5	Val	Ile			formin?	Surface
228	Gln	Ala			formin?	Surface
232	Gln	Ser			formin?	Surface
292	Glu	Asp			gelsolin	Surface
295	Gly	Ala			formin?	Surface
311	Glu	Asp			formin?	Surface
350	Thr	Ser			Profilin formin?	Surface

Table A4

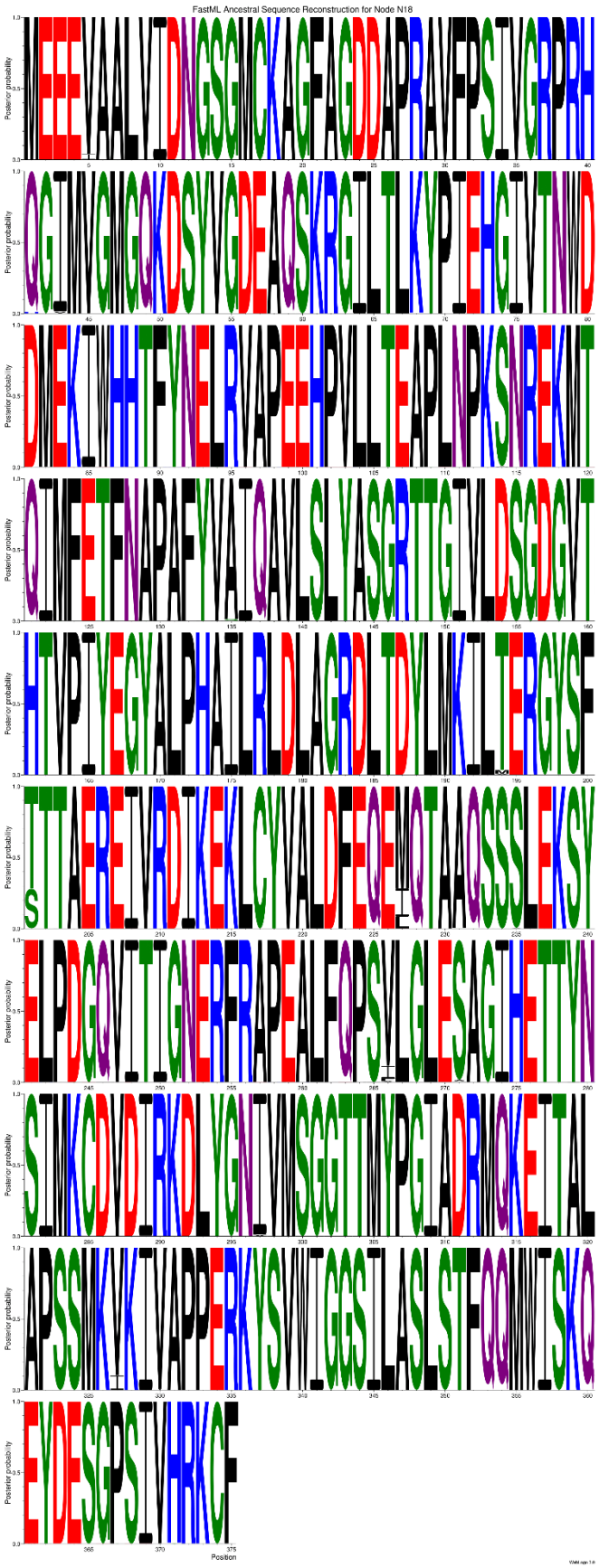
Figure A1. Posterior probabilities for all nodes used in this study: N1, N2, N3, N4 and N5.



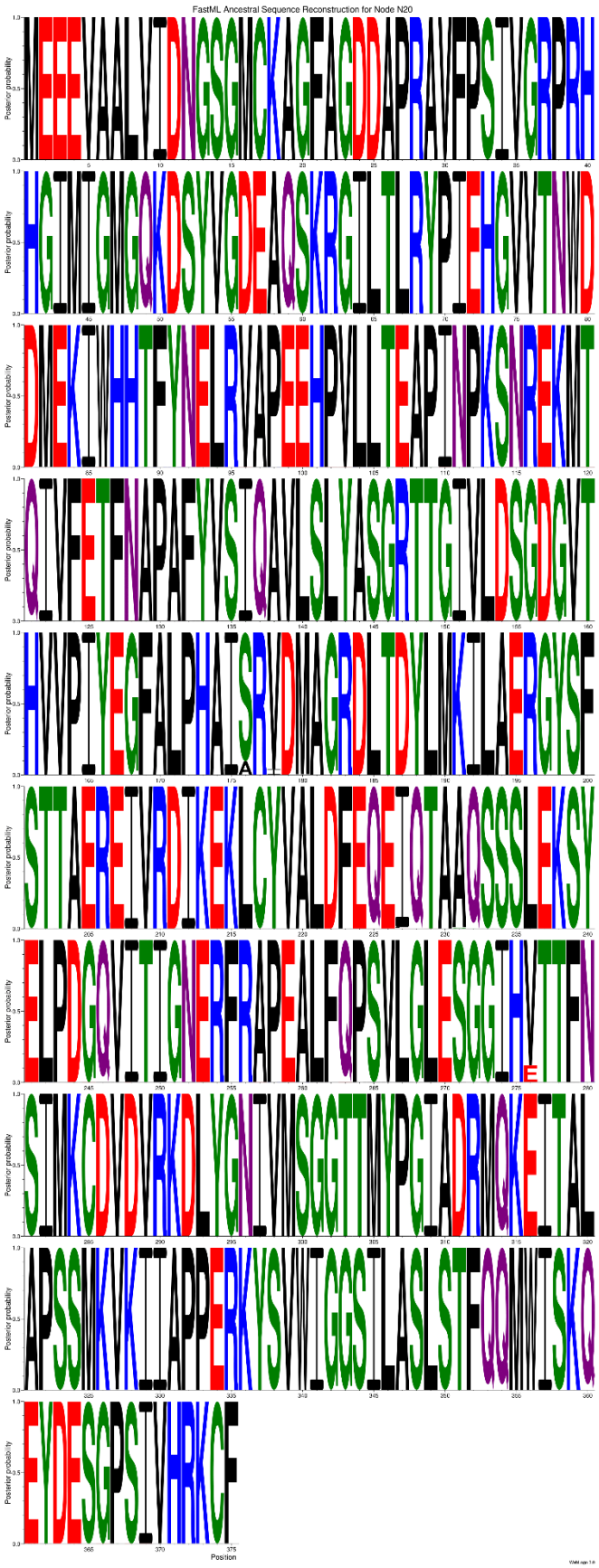
Node 1


FastML Ancestral Sequence Reconstruction for Node N19
Posterior probability
Position

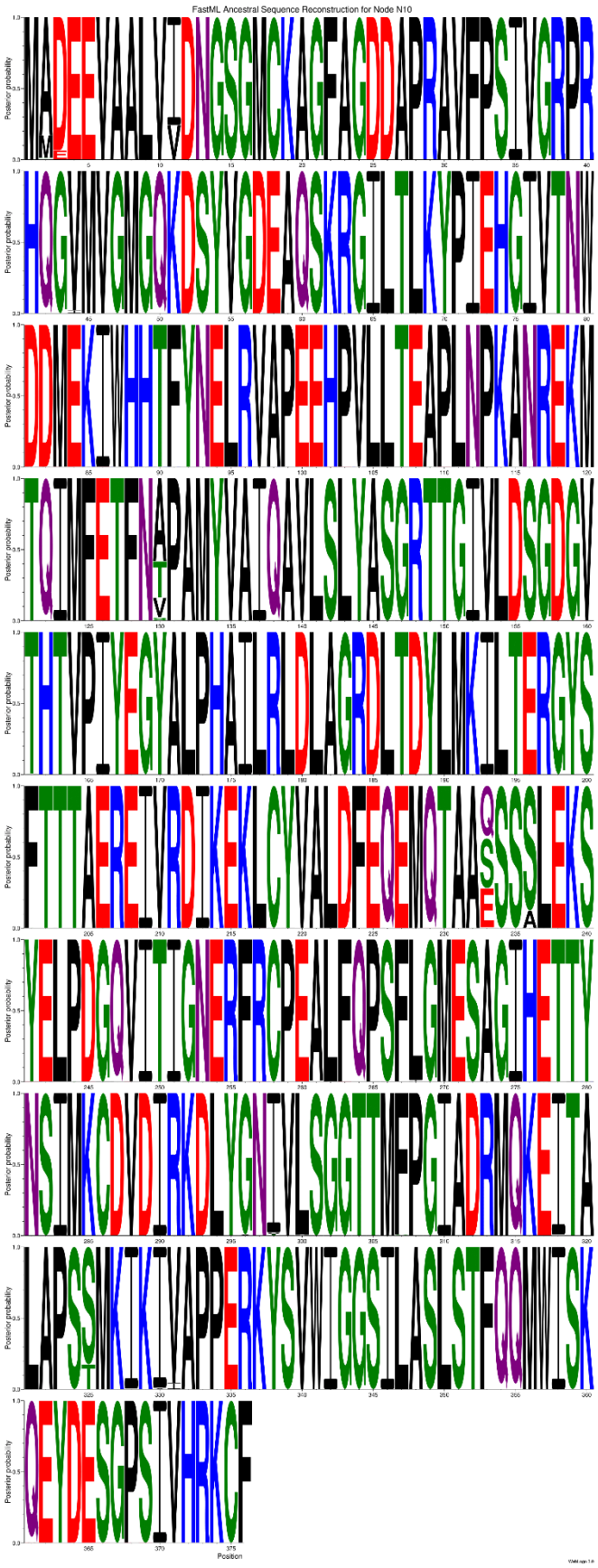
Node 2


FastML Ancestral Sequence Reconstruction for Node N18
Posterior probability
Position

Node 3


FastML Ancestral Sequence Reconstruction for Node N20
Posterior probability
Position

Node 4


FastML Ancestral Sequence Reconstruction for Node N10
Posterior probability
Position

Node 5

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COPY OF MANUSCRIPTS AND ARTICLES

Diversity from similarity: cellular strategies for assigning particular identities to actin filaments and networks

During the last year of my PhD, I wrote a review article alongside Dr. Adrien Antkowiak and Dr. Alphée Michelot. It discusses the molecular mechanisms that have arisen during evolution to segregate actin-binding proteins to the appropriate networks. This topic has been greatly discussed in the introduction of this manuscript.

Sizes of actin networks sharing a common environment are determined by the relative rates of assembly

During the course of my PhD, I also collaborated in another project of our group. This was the main project of a post-doc at the time, now maître de conference, Dr. Adrien Antkowiak.

Inside cells, the size of the different networks is controlled by many factors: the rates of nucleation and actin elongation at each network, the rates of network disassembly and the competition for actin monomers. Many proteins modulate the global organization of the actin networks, but the contribution of each protein is not fully understood.

The aim of this project was to understand how the different factors may regulate the global organization and size of the different actin networks. To answer this question, functionalized beads with a formin and the activator of the Arp2/3 complex were used simultaneously to reconstitute the branched- and the linear-networks in vitro. The set of essential proteins needed was determined, and then several experiments were done varying the concentration of one protein at a time to analyze the effect of this variation in branched- and linear-network assembly. The Arp2/3 complex, profilin, capping protein, ADF/cofilin and tropomyosin were analyzed. The results obtained show that all these ABPs impact the assembly of the actin networks in different ways. In other words, the assembly rates of each network strongly depended on the concentration of the ABPs in solution. These assembly rates should not be interpreted separately. The results obtained indicate that comparing the assembly rates of both networks side-by-side is sufficient to predict which actin network will be favored in cells, where there is homeostasis.

The *in vitro* scenarios were reproduced in *Saccharomyces cerevisiae* cells by either increasing or decreasing the intracellular concentration of the proteins by different techniques to verify the validity of those predictions. The actin cytoskeleton in these cells was observed by microscopy after fixation and phalloidin labeling, then the intensities of the branched- and linear-networks were quantified. When analyzing proteins that play a role in network assembly, the *in vivo* observations were predicted by the reconstitution assays. However,

in some cases, the *in vitro* and *in vivo* results did not fully correlate. A possible explanation is that in those cases, both network assembly and disassembly play an important role. To test this, the biomimetic system could be modified to include disassembly factors as well.

In most cases, the dependence of actin network assembly with protein concentration displayed a bell-shaped curve (Fig. 0.1, top). This result indicates that there are optimal concentrations of each accessory protein for the assembly of both networks. The assembly rate of both networks depends on a combination of the concentrations of each factor and the competition for actin monomers (Fig. 0.1, bot).

My contribution to this work was on the *Saccharomyces cerevisiae* experiments. I performed the molecular biology necessary to generate the tropomyosin overexpression strains. I also set up the phalloidin staining protocol in the lab, as well as the necessary parameters of the confocal microscope. Over the course of the project I was involved in many scientific discussions, and gave ideas on how to quantify the actin structures in yeast cells.

Besides sharing my knowledge about yeast and its protocols, I was interested in participating in this project because it complements my work very well. I believe that to properly understand the regulation of the sizes of the actin networks, it is necessary to tackle this question from different points of view. Whereas I put my focus in how differences in the actin orthologs contribute to the formation of the actin networks by modifying the interactions with ABPs, this work sought to understand this regulation by studying variations in the ABPs concentrations. These two studies aim to provide a better understanding on the complex regulation of the actin networks.

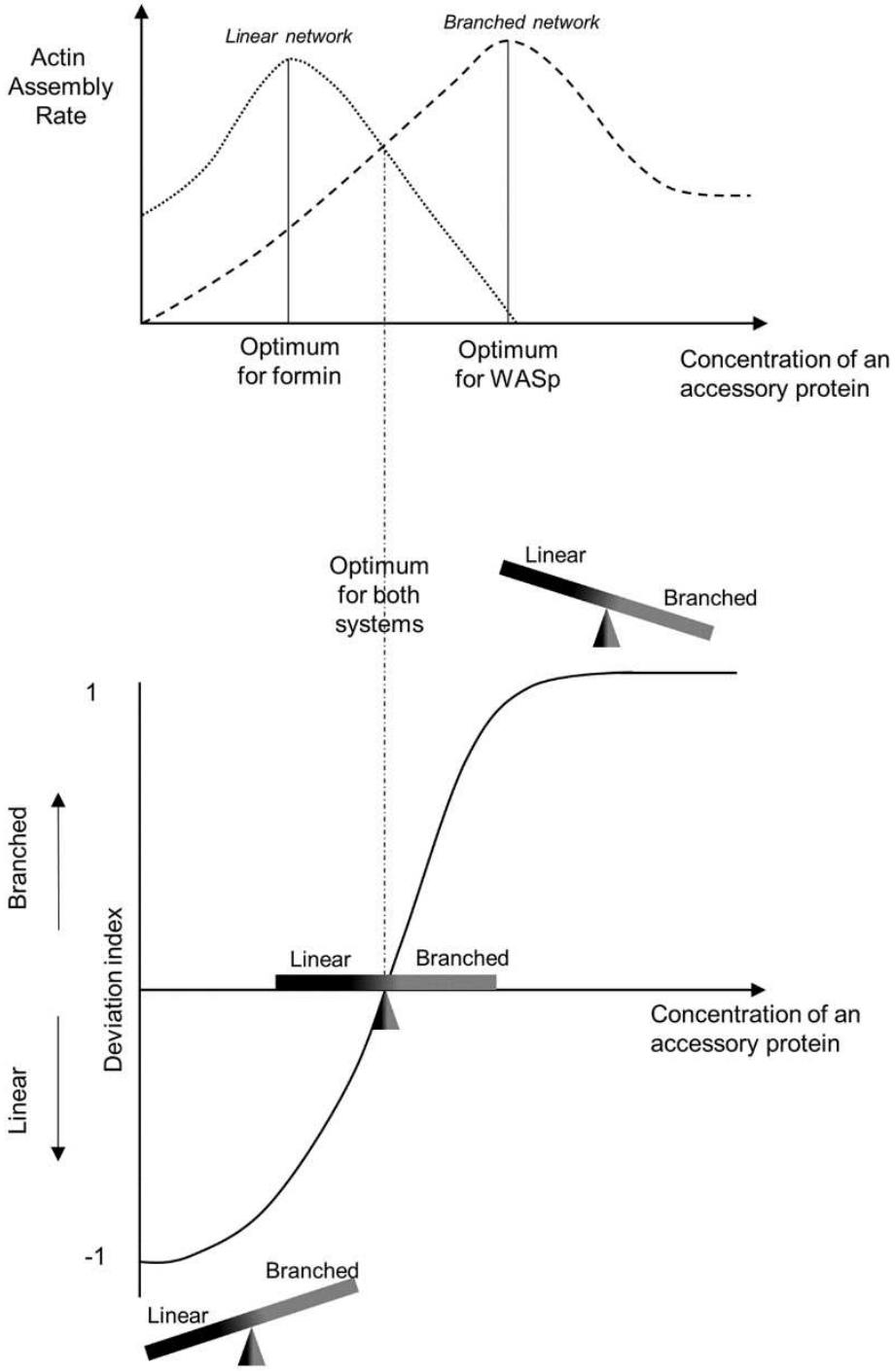

Fig. 0.1. Mechanism of network size control in a competing environment.

Mechanical stiffness of reconstituted actin patches correlates tightly with endocytosis efficiency

During my time in Alphée Michelot's lab, I also had the chance to participate in the project of Dr. Jessica Planade and Dr. Reda Belbahri.

An important property of the actin cytoskeleton is its ability to generate forces. This force generation is needed for many processes, among them clathrin-mediate endocytosis. Endocytosis is a process that regulates the internalization of extracellular components as well as the composition of the cellular membrane. This process is complex and has many steps. Actin in particular is recruited after a scaffold made of many proteins including clathrin is formed. Branched actin polymerization provides the force necessary for the invagination of the plasma membrane and the internalization of the vesicle. Among the many proteins that are recruited during vesicle formation, actin crosslinkers have been shown to act as a mechanical linkage between actin filaments in addition to the branched nucleated by the Arp2/3 complex.

The aim of this work was to gain insight in the link between the elasticity of actin networks and their ability to produce the necessary force for membrane deformation. For this, a combination of *in vitro* experiments to measure the mechanical properties of the actin meshwork and phenotypic observations in cells was used. The *in vitro* experiments were performed using cellular extracts lacking a protein of interest, which corresponds to a top-down approach. Three putative yeast actin crosslinkers were analyzed: Sac6, Scp1 and Abp140.

The results obtained in this study show actin crosslinking stiffens the branched actin network. From the three crosslinkers studied, Sac6 plays a dominant role in the rigidity of the actin networks. Absence of Sac6 in reconstitution experiments showed a decrease of the rigidity of the actin networks by 42%. This correlates as well with the observations in cells, where cells lacking Sac6 had patches in where actin assembled slower, have a longer lifetime and do not internalize properly. Together, these results show that a decrease in the stiffness of the actin branched-network is correlated with a decrease in endocytosis efficiency, which results in the accumulation of actin patches. This is illustrated in the proposed model (Fig. 0.2). A decrease in actin stiffness inside cells would result in patches that cannot internalize due to the lack of the necessary force, which would result in an accumulation of patches at the membrane and a reduced pool of available G-actin in the cell.

My contribution to this work was related to the *in vivo* observation of actin patches in *Saccharomyces cerevisiae* cells. For this, I used some strains that were available in the laboratory, but I also generated a new strain. I also verified the correct genomic modification in some of the strains. I set up the microscope parameters and I recorded the videos of the actin patch internalization, that were then analyzed by Dr. Reda Belbahri.

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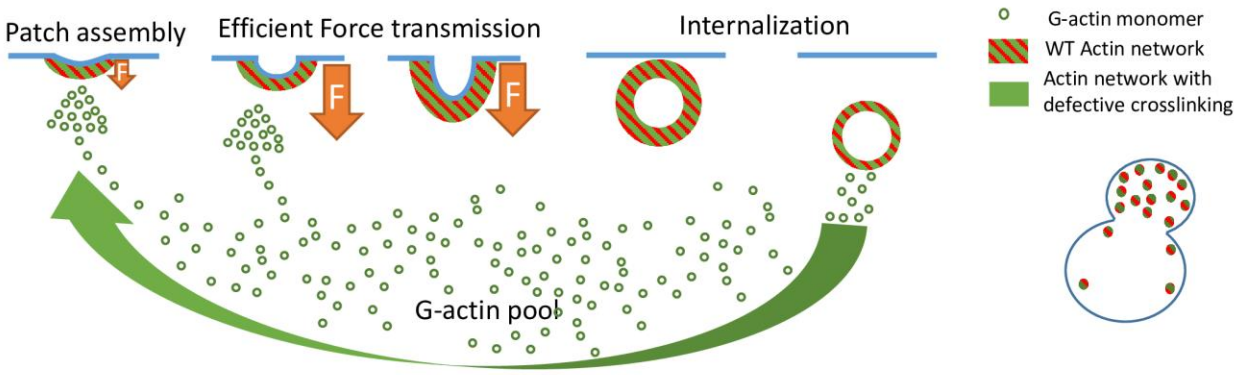

Defective actin crosslinking

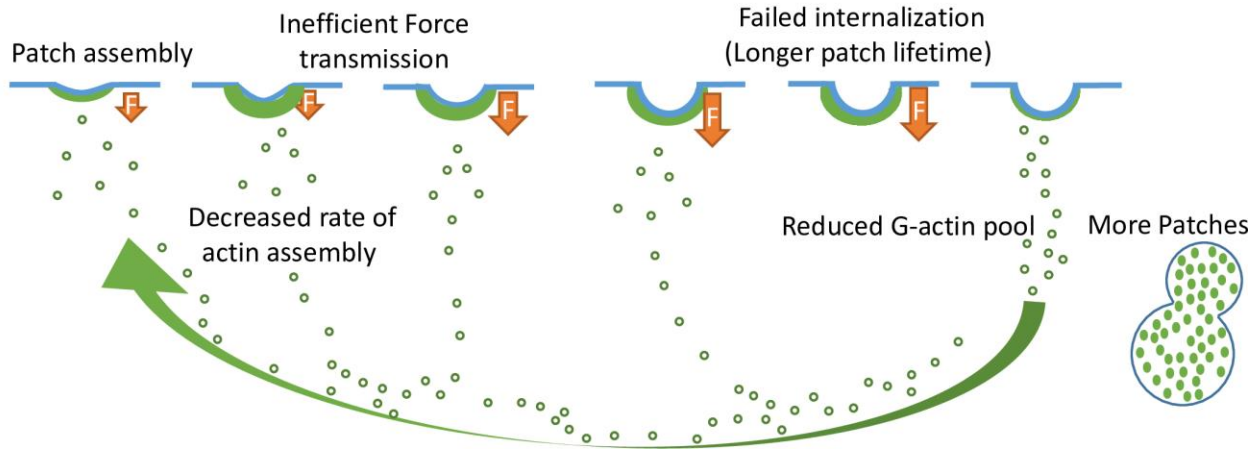

Fig. 0.2. Chain of consequence model for yeast endocytosis.

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Review

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Diversity from similarity: cellular strategies for assigning particular identities to actin filaments and networks

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The actin cytoskeleton has the particularity of being assembled into many functionally distinct filamentous networks from a common reservoir of monomeric actin. Each of these networks has its own geometrical, dynamical and mechanical properties, because they are capable of recruiting specific families of actin-binding proteins (ABPs), while excluding the others. This review discusses our current understanding of the underlying molecular mechanisms that cells have developed over the course of evolution to segregate ABPs to appropriate actin networks. Segregation of ABPs requires the ability to distinguish actin networks as different substrates for ABPs, which is regulated in three different ways: (1) by the geometrical organization of actin filaments within networks, which promotes or inhibits the accumulation of ABPs; (2) by the identity of the networks' filaments, which results from the decoration of actin filaments with additional proteins such as tropomyosin, from the use of different actin isoforms or from covalent modifications of actin; (3) by the existence of collaborative or competitive binding to actin filaments between two or multiple ABPs. This review highlights that all these effects need to be taken into account to understand the proper localization of ABPs in cells, and discusses what remains to be understood in this field of research.

1. Introduction

Actin plays a major role in many different biological processes such as cytokinesis, migration, vesicular trafficking and infection [1,2]. For each of these functions, actin filaments are organized into networks of optimized architectures, dynamics and mechanical properties. The main types of organizations include (but are not limited to) branched and linear networks of actin filaments [3,4]. Branched actin networks are generated by the association of a seven-subunit complex called the Arp2/3 complex, which nucleates short actin filament branches. Linear networks, where polar actin filaments are parallel or randomly organized, are generated from the de novo nucleation of actin filaments by factors such as formins, or from the debranching and reorganization of branched networks.

Actin networks are regulated by the association of different families of actin-binding proteins (ABPs). It is important to note that although all these proteins coexist in the cell cytoplasm, only a specific subset of ABPs interacts with each actin network while being excluded from the others [5–7]. Such an observation is surprising since it would be natural to assume that all actin filaments in the cell represent equivalent substrates for ABPs. On the contrary, these observations reveal the existence of complex mechanisms capable of precisely addressing the cell's ABPs, and research conducted in recent years has revealed a much more complex picture than anticipated. This work will review the multifarious strategies that cells use to guide ABPs to the appropriate actin

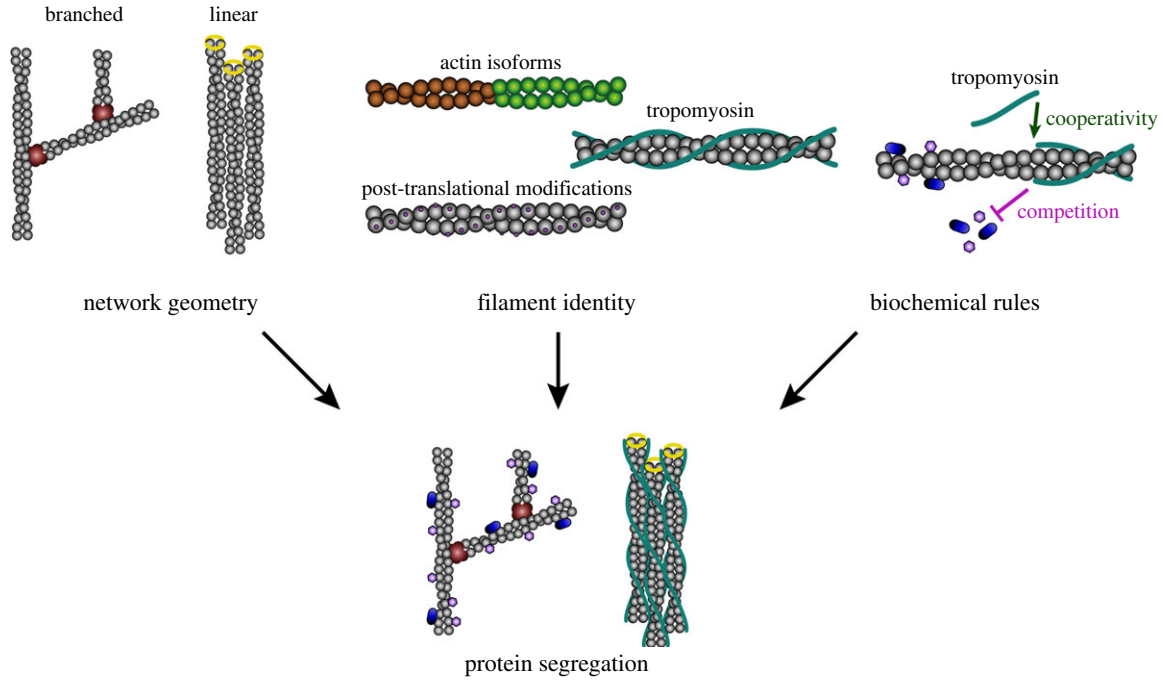

Figure 1. Three main mechanisms that account for the segregation of ABPs to different actin networks in cells. Schematic of the different molecular mechanisms described in this review. Actin networks are distinguished by different geometries. For example, the Arp2/3 complex generates branched networks and formins generate linear arrays. Actin filaments have different molecular identities based on the use of various actin isoforms, post-translational modifications and/or the presence of tropomyosin. Additionally, ABPs can compete or cooperate to restrict or promote their binding to actin filaments.

networks, the molecular mechanisms behind these processes, and discuss future directions for research in this area.

2. Cellular strategies for distinguishing actin networks as different substrates for actin-binding protein binding

There are multiple arguments to assert that the binding of most ABPs to specific actin subnetworks does not rely solely on their transport or on their local activation. First of all, actin networks in cells are often very close to each other. Sometimes an actin subnetwork can even form from a pre-existing one. This is the case, for example, of filopodia, which emerge through the elongation of actin filaments assembled in lamellipodia. In this context, the fast diffusion of proteins in the cytoplasm of cells (typical diffusion rates measured for globular proteins of 10 to 100 kDa range from around 10 to 100 $\mu m^2\, s^{-1}$) would prevent a precise and efficient targeting of ABPs [8,9]. Second, further evidence comes from the fact that local activation of specific actin assembly pathways in cells is often sufficient to induce the formation of functional actin networks. For example, the recruitment by optogenetic tools of specific RhoGTPases is sufficient to trigger actin assembly, and to initiate actin-dependent processes such as cell migration [10] or cytokinesis [11]. Similarly, triggering actin assembly from cellular extracts by specific factors such as WASp (which is an activator of the Arp2/3 complex) or formins, leads to the formation of actin filament networks with a composition of ABPs comparable to branched and linear actin networks, respectively [12–14].

All of these observations unambiguously indicate that, to a large extent, actin networks themselves represent different substrates for downstream protein interactions. This has led the community to ask what specific features could allow actin networks to distinguish themselves from each other, in order to be identified as different substrates for the cell's ABPs [15]. To date, two main hypotheses, not mutually exclusive, are guiding this field towards a better understanding of these principles. The first hypothesis is that the geometrical organization of filaments within actin networks is itself a sufficient characteristic to make these substrates distinct for ABPs (figure 1). The second hypothesis is that the actin filaments themselves within the actin networks could present different biochemical signatures, which could differentiate them for the different ABPs of the cell (figure 1).

3. The geometry of actin networks as an intrinsic feature of actin-binding proteins segregation

Actin filaments that are polymerized *in vitro* for standard actin-binding assays are generally between 1 and 100 µm in length, corresponding to approximately 360–36 000 actin subunits [16]. Since actin filaments are semi-flexible polymers with a persistence length of about 17 µm, this size range is of interest for studying certain biophysical aspects of actin filaments and their interactions with ABPs [17]. However, since most actin filaments *in vivo* are shorter than 1 µm, the results of these studies may bias our interpretation of how ABPs interact with actin filaments in cells.

Furthermore, actin filaments also differ significantly from one actin network to another. Primarily, they vary in length and relative orientation to each other. For example, the branched actin networks of the lamellipodium are composed of short actin filaments of 7–18 actin subunits [18], which are connected to each other by the Arp2/3 complex at an angle of 70°. At endocytic sites, filaments are also branched but appear to be longer, between 18 and 68 actin subunits [19–22]. At these scales, actin

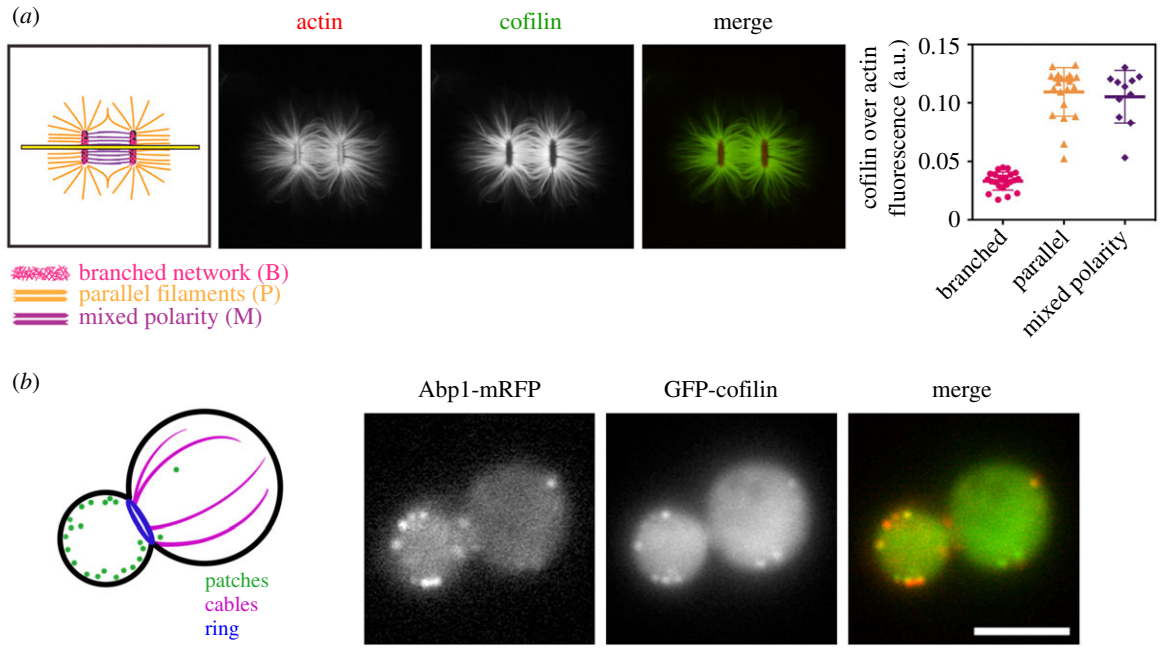

Figure 2. Actin network architecture as an important property to consider in explaining the accumulation of ABPs. (*a*) Profile of accumulation of ADF/cofilin on different actin network architectures reconstituted *in vitro* (adapted from [36]). Left: Schematic of the different actin network architectures assembled on a micropattern coated with an activator of the Arp2/3 complex (two vertical bars), in the presence of soluble actin and Arp2/3. Branched actin networks are assembled on the patterns, whereas linear actin networks (parallel or mixed polarity) are assembled from the elongation of the filaments away from the patterns. Middle: Localization of ADF/cofilin (in green) after its addition to polymerized actin network. Cofilin accumulates preferentially to linear actin networks. Right: Quantification of ADF/cofilin over actin intensities for each architecture. (*b*) Accumulation of ADF/cofilin on different actin network architectures *in vivo* (adapted from [38]). Left: Schematic of the different actin networks found in *S. cerevisiae* cells: branched networks in actin patches (dots), linear networks in actin cables (intra-cellular lines) and the cytokinetic ring (at the yeast bud neck). Right: ADF/cofilin (in green) co-localizes preferentially with the actin patch protein Abp1 (in red), indicating that other principles than network architecture are at play to account for the cellular localization of ADF/cofilin.

filaments are short enough to be considered totally rigid. Linear arrays, on the contrary, are often composed of longer filaments with up to 300 actin subunits [23,24]. The actin filaments are approximately parallel to each other, and may all have similar (e.g. in the case of filopodia) or random (e.g. in the case of cytokinetic rings or stress fibres) orientations.

Studying the relationship between the geometrical organization of actin networks and the apparent affinity of ABPs has required the development of more complex biomimetic systems than previously envisaged. The best description to date of the impact of actin network organization on the binding and activity of an ABP concerns myosins, particularly contractile myosins with multiple motor domains [25,26]. Several studies have shown that their recruitment and activity strongly depend on whether the actin networks are disorganized, branched by the Arp2/3 complex, parallel or antiparallel [27–29]. More particularly, these motors are capable of strong contractile activity, even to the point of disassembling actin networks, when actin filaments do not have the same orientation and polarity. These observations are very consistent with the effects of these molecular motors *in vivo*. This is the case, for example, at the sarcomeres of muscle fibres or at cell–cell junctions in tissues, where the contractile activity of the motors is exerted on actin filaments of opposite polarity [30]. This is also the case for many disorganized actin networks where myosin activity is capable of driving actin flows or pulsatile phenomena [31–33]. On the contrary, actin filament structures such as filopodia, where the actin filaments all have the same orientation, are not contractile structures [4,34]. On these structures, molecular motors are generally used for trafficking. It is important to note that the sensitivity of ABPs to different actin filament organizations does not seem to be limited to the case of molecular motors, but seems, on the contrary, to be quite general. For example, crosslinkers such as α-actinin bind preferentially when the spacing between two actin filaments is favourable [35]. Other proteins such as ADF/cofilin, which is involved in the disassembly of actin networks, accumulate on linear networks of actin filaments, but are not efficiently recruited on branched actin networks *in vitro* [36] (figure 2*a*).

The geometrical organization of actin networks is, therefore, a key parameter to consider when evaluating the affinity and activity of ABPs. However, other observations made at the cellular level show that the geometric organization of actin networks alone is not sufficient to fully address how ABP recruitment is carried out *in vivo*. Indeed, some proteins, such as ADF/cofilin mentioned above, are not found in cells on actin networks for which their affinity should be the strongest. While ADF/cofilin is clearly localized on branched actin networks such as endocytic actin patches, ADF/cofilin is hardly detectable on linear networks such as actin cables [37–39] (figure 2*b*). These observations indicate that additional principles, beyond the actin filament network architecture, need to be taken into account to obtain a global picture of how ABPs are addressed in cells.

4. Biochemical opportunities for generating different actin substrates

Although attractive, we saw that the segregation of ABPs observed in cells could not be explained solely by the geometric organization of actin filaments. The necessity to discriminate different populations of actin filaments in cells must

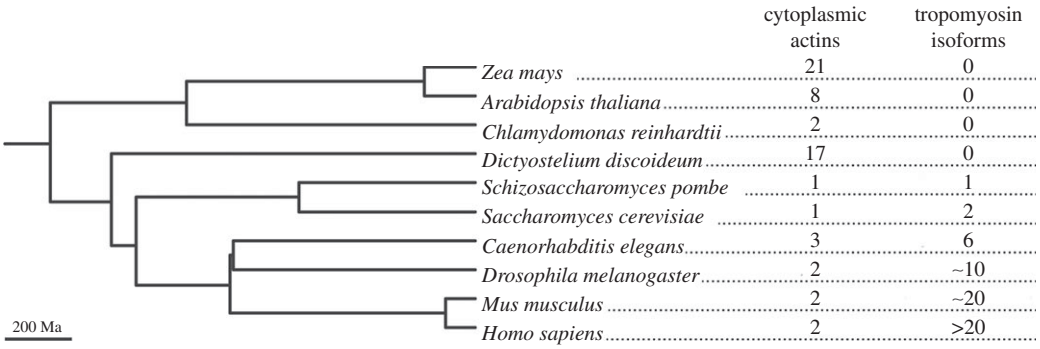

Figure 3. Phylogenetic tree showing how the number of cytoplasmic actin and tropomyosin isoforms changed over the course of evolution in eukaryotes. These numbers are strongly anti-correlated, suggesting that different lineages used different strategies to generate a complex actin cytoskeleton. For clarity, muscle actins which are specific to Metazoa and which are highly specialized were excluded.

lead us to consider other possibilities, including that small differences in structure and surface properties of the actin filaments themselves could modulate their affinity for certain ABPs. Our community has been working on a number of additional hypotheses to explain how actin filaments could be functionally different and acquire identities of their own. A first hypothesis is that cells could possibly use different actins, either through the expression of different actin isoforms or through post-translational modifications (PTMs) [40,41]. A second hypothesis is that specific ABPs could progressively decorate actin filaments to give them a specific identity, reinforcing or limiting the binding of other ABPs to the same filaments by steric effects or by stabilizing particular conformations of the filaments [5]. These two hypotheses are not mutually exclusive, meaning that cells could also use several strategies simultaneously to create the greatest possible diversity of actin substrates.

Proof that both of these strategies exist in cells is evident from a careful analysis of genomes of all eukaryotes (figure 3). Many species express a variable number of actin isoforms, which can be either cell-specific or expressed simultaneously in the same cell types. An extreme example is plants, which express multiple actin isoforms, that originated from genome duplications (figure 3). The number of actins varies for each plant species and can reach, for example, 21 isoforms in *Zea mays*. A potential limitation of this strategy is that the actin sequence must remain highly conserved in order to maintain its assembly properties and its ability to interact with the most essential ABPs. For example, even between two distant eukaryotes such as budding yeast and human, which diverged more than a billion years ago, actin sequences still retain around 90% identity. Actin mutations are generally rare, and usually lead in humans to serious diseases such as Baraitser–Winter syndrome [42]. Overall, while the existence of many actins in eukaryotes supports the possibility that cells can use a variety of actins to generate different actin-related functions, the difficulty of bringing mutations and generating variety also questions the effectiveness of such a mechanism to generate diversity of functions.

An alternative strategy to differentiate actin filaments without the need to mutate the actin itself is through the use of specialized ABPs. This hypothesis has gained considerable credibility with the comparison of eukaryotic genomes. Indeed, whereas some species, such as plants and amoebas, generally express dozens of different actin isoforms, other eukaryotes, for example, those from the kingdoms Animalia or Fungi, express only one or a very limited number of cytoplasmic actin isoforms (figure 3). Conversely, species expressing one or few actin isoforms express a multitude of tropomyosins, which are specific ABPs that wrap around actin filaments, whereas plants or amoebas do not express any (figure 3) [43]. This very strong anticorrelation is a signature that these two phenomena are probably related to each other. It suggests that while some species use multiple actin isoforms, representing different substrates for ABPs, in order to create different actin-related functions, other species that had gained tropomyosins in the course of evolution could use a limited number of actins, decorated by different tropomyosins, to generate functional diversity.

5. Generating a diversity of actin substrates by expressing a variety of actin isoforms

The question that will now be addressed is whether very similar eukaryotic actins are nevertheless able (1) to assemble separately within a common cytoplasm and (2) to form filaments of sufficiently specific molecular identity to interact differently with ABPs and carry specific cellular functions. The answer to this question is far from being clear today and is the subject of intense research.

5.1. Plant actins localization and functions

Plant actins were originally studied from the model organism *Arabidopsis thaliana*, which has 10 actin genes. Eight of these genes have been demonstrated to code for functional actin isoforms, grouped in two classes according to their sequence similarities and their tissue-specific expressions: vegetative (ACT2, 7 and 8) and reproductive (ACT1, 3, 4, 11 and 12) [40]. Vegetative and reproductive actins are involved in different cellular processes [44], and plant actin isoforms that are expressed in the same tissue can also assemble into isoform-specific structures. GFP-fusion proteins of ACT2 and ACT7, the main vegetative actin isoforms, co-localize only partially at the surface of chloroplasts, where ACT2 is mainly found in thinner and longer bundles, whereas ACT7 is organized into thick bundles [45]. Besides their differential expression and their spatial segregation, these isoforms are not functionally equivalent. Expression of the reproductive actin ACT1 in vegetative tissues causes dwarfing and altered morphology in most organs, showing that expression of ACT1 in these tissues is affecting the dynamics of actin and its associated proteins [46].

Another well-studied organism is the unicellular green algae *Chlamydomonas reinhardtii*, which carries two actin genes. The main isoform IDA5 is a conventional actin that is

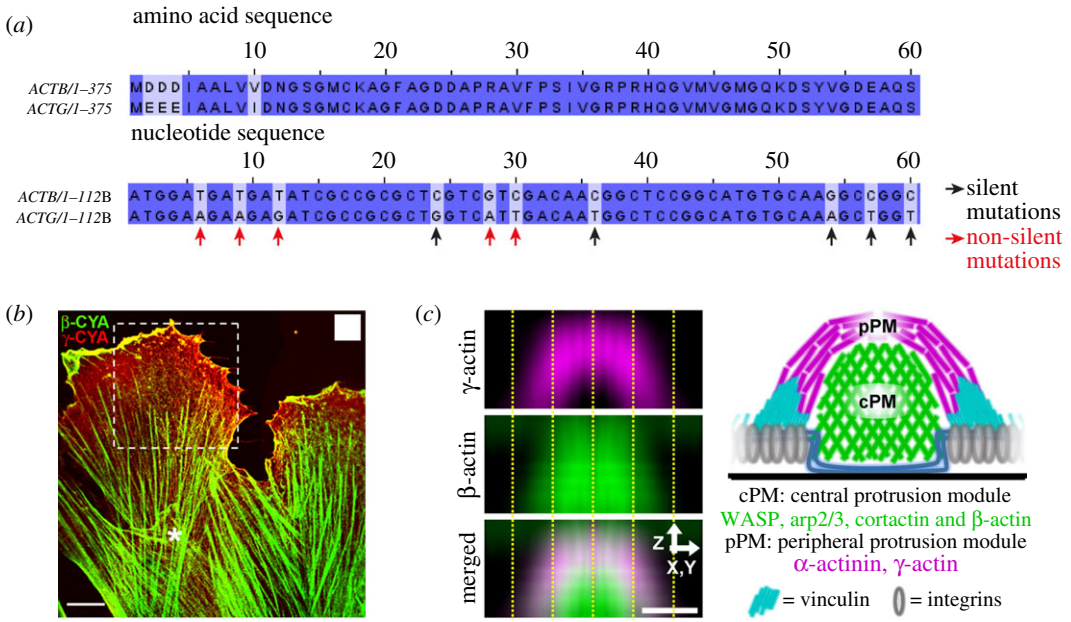

Figure 4. Evidence that highly similar cytoplasmic actins can nevertheless assemble into functionally different actin networks in cells. (*a*) Beginning of the nucleotide and amino acid sequence of β- and γ-actins. These proteins only differ in four amino acids located at the N-terminal end, although their nucleotide sequences have a much higher number of silent mutations (e.g. black arrows). (*b*) Example of the differential localization of β- (green) and γ-(red) actins at the cell scale, in migrating human subcutaneous fibroblasts (adapted from [54], scale bar, 10 µm) (*c*) Another example of the differential localization of β- (green) and γ-actins (magenta) within a same structure, podosomes (adapted from [57], scale bar: 0.5 µm). A linear network of γ-actin is surrounding a branched β-actin core.

expressed in normal conditions. The second actin, called NAP1 (for Novel Actin-like Protein 1), is highly divergent as it shares only 65% sequence identity with IDA5 [47]. The expression of NAP1 in wild-type cells is negligible, but it is highly upregulated in certain conditions, for example, when IDA5 is absent or after addition of the actin monomer sequestering drug latrunculin B [48,49]. This drug can prevent IDA5 polymerization, but surprisingly NAP1 generates latrunculin B-resistant structures. Despite being so different, essential actin functions can be performed by either of these actins, and cells lacking any of the actin genes can grow and divide normally. However, they also seem to maintain some specialized functions. The conventional actin IDA5 has a function in mating since it is involved in the elongation of the fertilization tube, a function that NAP1 cannot substitute [50–52]. Both IDA5 and NAP1 are found in the axoneme of the flagella but apparently in different structures. While IDA5 seems to be part of the inner dynein arms, NAP1 plays a role in flagellar formation independently of axonemal dyneins [52,53].

5.2. β- and γ-actin localization and functions

Generating actin structures from different actin isoforms is also possible in higher eukaryotes, including mammals. Mammalian organisms have six different actin isoforms: four muscle actins and two cytoplasmic actins. The latter, called β- and γ-actins, differ only in four amino acids at the N-terminal end (figure 4*a*) and are simultaneously expressed in cells. Due to their extreme similarities, determining the cellular localization of β- and γ-actin is challenging. Specific monoclonal antibodies, recognizing specifically the different N-terminal regions, are now available to visualize the localization of both isoforms in different cell types [54,55].

β-actin was originally found mainly in actin bundles of basal stress fibres, filopodia, at cell–cell contacts and in contractile rings, whereas γ-actin is present mainly in lamellar and dorsal cell regions (figure 4*b*) [54,55]. In epithelial cells, β-actin has been shown to play a role in adherens junction maintenance, and γ-actin in tight junction integrity [56]. In podosomes, which are actin-rich adhesive structures involved in migration and invasion, the use of better super-resolution microscopy techniques allowed a differential localization between actin isoforms to be distinguished. Actin filaments in podosomes are organized into two distinct networks, consisting of a β-actin core, composed of branched actin filaments nucleated with WASp, Arp2/3 and cortactin, and surrounded by a γ-actin envelope, composed of linear actin filaments bound to α-actinin and connected with myosins (figure 4*c*) [57].

These differences in localization suggest that these two isoforms, despite being so similar, could be associated with different cellular functions. During wound closure, cells assemble significantly more β-actin beneath the plasma membrane, which suggests a role for this actin in cell motility [58]. β-actin implication in cell motility was confirmed in fibroblasts where decreased β-actin protein levels lead to reduced motility [59,60]. The implication of γ-actin in cell migration is less clear. While γ-actin knocked-down cells are shown to migrate less in some studies [54,61], loss of γ-actin can also induce epithelial-to-mesenchymal transitions in another model [62]. In agreement with β-actin localization in the cytokinetic ring, β-actin knocked-down cells also show reduced proliferation and can be multinucleated [59,60,63,64]. In breast cancer cells, cycle entry and proliferation seem to be regulated by γ-actin, particularly in G1, while β-actin plays a role in later mitotic stages, especially in telophase for cytokinesis [64]. β-Actin implication in cell motility and proliferation can be explained by the direct activity of β-actin in the filaments of the structures controlling these processes, but also by the role that this specific actin plays in the regulation of transcription. β-Actin binds directly to chromatin remodelling proteins as well as RNA polymerases, a first indication of its role in nuclear processes [65]. This role is also confirmed as β-actin was shown to regulate the expression of cell cycle and actin dynamics related genes, as well as its own expression [59,66].

Cellular localization of these actins does not suggest any particular preference for a certain type of architecture. For instance, β-actin is localized at the linear structure of the stress fibres and contractile ring but it is also localized at the branched core of the podosomes. This lack of a general obvious rule complicates our understanding of the molecular mechanisms implicated in the assembly of these two actin isoforms into distinct networks. Moreover, this understanding is further complicated by functional tests at the whole organism level. Despite their amino acid sequences being so similar, the nucleotide sequences of beta and γ-actin genes possess silent mutations that affect 40% of the codons (figure 4*a*). By taking the β-actin gene, and changing only four codons to express γ-actin from this gene, it is possible to generate viable mice that are not expressing the β-actin protein [67]. This result is surprising, since β-actin knockout mice are reported to be embryonic lethal [68,69]. Therefore, essential functions of β-actin may not be related primarily to its amino acid sequence, but may also rely heavily on its nucleotide sequence. This difference in nucleotide sequence results in different translation speeds [70], which could lead to protein regulation at different levels: differential expression levels, alternative splicing and differential co- and PTMs.

5.3. Biochemical similarities and differences between actins

Differences in cell localization described above suggest that different actins, although very similar, must still have significantly different biochemical properties. However, while divergent actins expressed by prokaryotes have clearly distinguishable assembly properties, actins expressed in eukaryotes seem to have much more subtle biochemical differences [71,72]. The search for these subtleties has long suffered from the difficulty of purifying a variety of actin isoforms in order to study them independently. Most of our knowledge is based on studies using the same mammalian actin muscle isoform. A more limited number of studies have used the yeast actin *S. cerevisiae*, and only a few studies used mixtures of γ- and β-actin or actins from other species.

Actins from budding yeast and rabbit muscle are 87% identical, which indicates that these two actins are quite different comparatively to all actins expressed in eukaryotes. Budding yeast and rabbit muscle actins can nevertheless copolymerize [73]. Surprisingly, this is less clear for beta and γ-actin, despite being 99% identical. While these two isoforms were shown to copolymerize in some studies, other studies reported their ability to assemble into independent filaments [55,74,75]. Yeast and rabbit muscle actins show differences in flexibility, with a persistence length of rabbit muscle actin two-to-three-fold higher than yeast actin [76,77]. In the presence of magnesium, yeast actin polymerizes faster than muscle actin, which is due to a faster trimer nucleus formation rather than a faster elongation of the filaments [78–81]. This difference in rates of polymerization is also observed in plants, as the two vegetative actins ACT2 and ACT7 polymerize faster than the reproductive actins ACT1 and ACT11 [82]. Nucleotide hydrolysis, nucleotide exchange and Pi release are also faster for yeast actin compared to muscle actin filaments [80,81,83–85]. This correlates with the fact that the nucleotide-binding cleft of *S. cerevisiae's* actin appears more open than for muscle actin [86]. In summary, we can hypothesize from few well-characterized actins, that many biochemical and biophysical subtleties might overall account for important functional differences in cells.

Differences among actins are also sufficient to modulate some interactions with ABPs. Actin nucleators, which play an important role in architecture formation, are reported in few studies to favour specific actin isoforms. The formin DIAPH3, for example, has a preference for β-actin compared to γ-actin, suggesting that actin cables assembled from DIAPH3 could be enriched with β-actin [55]. The VCA domain of N-WASP, an activator of Arp2/3, does not show specificity for β- or γ-actin [87], but *S. pombe*'s Arp2/3 is reported to be a better nucleator of *S. pombe*'s actin than rabbit muscle actin [80]. In *Arabidopsis thaliana*, the binding affinity of profilins for actin monomers seems lower for a specific isoform, ACT2 [82]. Since profilin enhances formin-linear actin cable assembly, at the expense of Arp2/3-branched network assembly, it is tempting to speculate whether ACT2 would assemble more specifically within branched networks. Moreover, to achieve different actin functions, it appears that plant actins and ABPs have co-evolved to generate class-specific protein–protein interactions. The expression of the reproductive ACT1 isoform in vegetative tissues leads to aberrant cell and tissue morphology, a phenotype that is rescued by co-expression of the reproductive profilin (PRF4) and cofilin (ADF7) [88]. Evidence for coevolution of actin with ABPs can be also found by studying proteins from different species. For example, in both yeasts *S. cerevisiae* and *S. pombe*, profilin inhibits endogenous actin polymerization but has little effect on rabbit muscle actin polymerization [81,89,90]. Another example is vertebrate cofilin, which can bind to *S. cerevisiae's* actin but does not increase their flexibility nor promote severing [77,91].

These studies indicate to the scientific community that highly similar actin isoforms have subtle but significantly different properties to display preferential binding to a variety of ABPs. We are just beginning to identify the molecular mechanisms by which actin isoforms could assemble into distinct actin networks of specialized properties. However, we still do not have a satisfying overview of the variety of possible differences among all actin isoforms expressed in eukaryotes. Recently, new protocols have been developed [92–94], allowing for a wider variety of actin isoforms to be purified. It is likely that future comparisons of a greater diversity of actin isoforms, purified from similar protocols, will strengthen our knowledge of these mechanisms.

6. Actin's post-translational modifications

Co- and PTMs are covalent modifications to one or several amino acids of a protein, a process that is usually mediated by specific enzymes. These modifications can affect the interactions of the protein with its partners by changing its surface charge density, its structure, or by steric hindrance.

First, actin can be arginylated, which is the addition of an arginine residue at the N-terminal end. This modification is mediated by the arginyl-tRNA-protein transferase Ate1, a protein that has been identified in several organisms, including mammals, plants and budding yeast [95]. In *Dictostellium discoideum*, an organism expressing a large number of cytoplasmic actin isoforms, several actins (Act3, Act10, Act17, Act22, Act23 and the most abundant one Act8) are arginylated, and impairing Ate1 activity affects cell migration and substrate adhesion [96]. In mammals, arginylation is possible for β- and

γ-actins, but the latter is specifically degraded when it is arginylated [70,97]. As this PTM does not affect both actin isoforms equally, arginylation could be an important PTM to regulate specifically β-actin-dependent cellular processes. For example, arginylated β-actin, which corresponds to around 1% of total β-actin, is likely to be involved in lamella formation, as down-regulation of Ate1 reduces the formation of this structure [97–99].

Studies in Ate1 knocked-out cells indicate that actin arginylation is responsible for a decreased interaction with gelsolin, but for an increased recruitment of capping protein (CP) and twinfilin [100]. Arginylation adds positive charges to normally negative charged surfaces, a change that logically affects actin's interaction with binding partners such as gelsolin, whose binding relies on the first 10 amino acids of actin [101,102]. On the contrary, CP and twinfilin are not shown to bind to this area, but their increased binding could be explained by an absence of gelsolin which would leave excessive free actin filament barbed ends for these two proteins to bind to.

The most abundant PTM for β- and γ-actin is N-terminal acetylation, which is the addition of an acetyl group [103]. In animals, this PTM occurs after cleavage of the first one or two amino acids, and is modifying an important fraction of the actin [104–107]. It is mediated by the acetyltransferase NAA80, which is specific to actin and acetylates preferentially the monomeric actin-profilin complex [108,109]. Interestingly, plants and fungi do not express NAA80, but do express the general acetylase NatB, which acetylates many other proteins. In yeast, actin is co-translationally acetylated by NatB [110,111], but in plants, the role of NatB is less clear. Even though the lack of NatB affects plant growth, actin is not identified as a substrate for this protein [112]. As NatB targets the N-terminal part of proteins starting with Met-Glu-, Met-Asp-, or Met-Asn-, plant actins, which start with Met-Ala-, may not be modified or may be modified by a mechanism not yet identified [111]. Since plants like *Arabidopsis thaliana* already express several actin isoforms, we can also speculate that actin acetylation might be less important to generate different actin-based functions in this organism. In HeLa cells, actin acetylation affects cell motility and cytoskeletal organization [103]. Acetylated actin has a faster polymerization rate, including formin-induced polymerization, and a faster depolymerization rate, so filaments composed of acetylated actin are shorter lived [103]. The N-terminal residue of actin is not the only amino acid that can be acetylated. A complex of lysine-acetylated actin and cyclase-associated protein (CAP) was shown to promote the inhibition of the formin INF2 [113]. This proves that PTMs can not only regulate actin properties and its binding to other proteins, but also the activity of the other proteins themselves.

Arginylation and acetylation are two main PTMs of actin. Other PTMs, including phosphorylation and methylation, can also modify the chemistry of the actin molecule. For more details, we refer readers to a more detailed review [114].

7. Tropomyosins and the biogenesis of new actin substrates

7.1. Generating diversity from a limited number of actin isoforms: tropomyosin as the missing link

We will now study the more complex case where a cell is able to generate distinct actin networks from identical (or nearly identical) actin molecules. The distinction between actin networks can no longer be made on the basis of biochemical differences between the actin composing different networks, but on the basis of biochemical particularities of the ABPs composing each of the networks. For greater clarity, our discussion will distinguish two different cases: the first case corresponds to the de novo generation of new actin networks from determined actin nucleation factors; the second case corresponds to the reorganization of pre-existing networks into networks with different properties.

In the first case, the assembly of new actin filament networks suggests that filaments acquire particular identities at the moment when they are generated by nucleation factors. The idea that this function is carried by factors such as the Arp2/3 complex or formins is *a priori* tempting. However, the coincubation of the Arp2/3 complex, its VCA activator and a formin (FMNL2) leads to the formation of mixed actin networks (i.e. having both Arp2/3 branches and formin-bound filaments) and not of distinct actin networks [115]. It should be noted that in the experiment described, the actin filament branches are much longer than the branches present in the cells, and that we could not exclude the possibility that formin cannot bind to very short branches. It is also possible that formin FMNL2 is a peculiar isoform that can bind to branched networks [115,116]. Nevertheless, this experiment rather suggests that another regulator is needed to effectively segregate formins and the Arp2/3 complex on separate networks. We have already seen in paragraph 4 that careful genomic analysis strongly suggests that proteins of the tropomyosin family are responsible for functional diversity of the actin cytoskeleton in higher eukaryotes [43]. We shall see that genetics, cell biology and biochemistry have also provided additional evidence for the importance of tropomyosins.

The second case corresponds to situations where actin networks undergo major dynamic reorganizations, independently of any nucleation of new actin filaments. For instance, linear actin structures found beneath the lamellipodia are not exclusively generated by formins, but also emerge to a large extent from pre-existing lamellipodial actin networks [117–120]. Interestingly, in this case, where actin filaments are not generated de novo, tropomyosin recruitment correlates also very well with filament debranching and the re-organization of actin filaments into new linear actin structures [121,122]. These observations suggest that regardless of the mechanism by which actin networks are formed, tropomyosins are consistently of key importance in giving actin filaments a new identity.

7.2. Tropomyosins localization and functions

Most cells express multiple tropomyosin isoforms and splicing variants, and those proteins have been proposed to provide actin filaments specific identities [5,123–125]. This concept has been reinforced by the observation of cellular localization of tropomyosins, which is highly dependent on the type of tropomyosin isoform [126,127] (figure 5*a*). Among the tens of isoforms that exist in metazoans, individual actin structures usually interact with a subset of tropomyosins. Most actin networks in cells are decorated by specific families of tropomyosins, including filopodia, lamella and stress fibres, with the exception of branched networks such as lamellipodia or endocytic actin patches which do not recruit tropomyosins (figure 5*b*) [124,125,127,128]. Some tropomyosins do not form copolymers

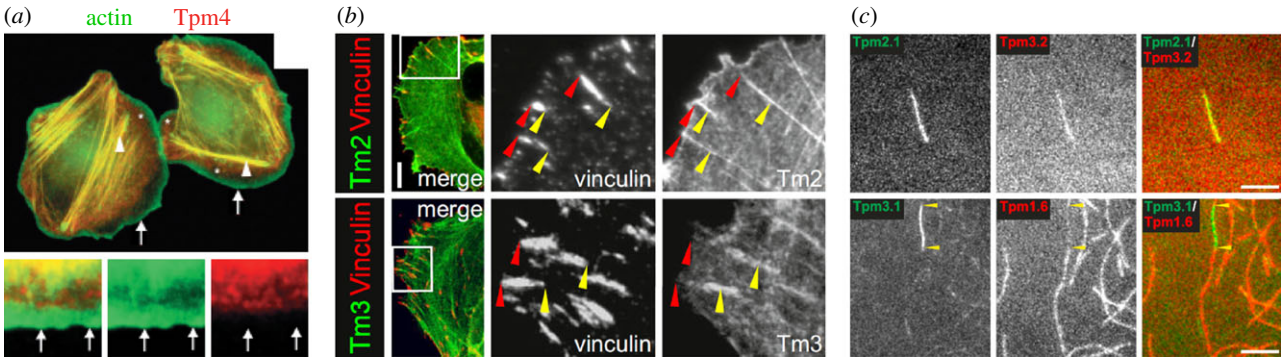

Figure 5. Functional differentiation of actin networks by tropomyosins. (*a*) Example of the specific localization of tropomyosin 4 (in red) in MTLn3 cells. Tropomyosin localizes with actin (in green) in stress fibres and in lamellar structures (arrowheads), while it is absent from the branched actin structures at the leading edge (arrows) (adapted from [128]). (*b*) Example of the differential localization of tropomyosin isoforms (in green) in U2OS cells. While Tpm2 shows a strong colocalization with the focal adhesion-specific protein vinculin (in red), Tpm3 localizes proximally to focal adhesions (adapted from [131]). (*c*) *In vitro* single-filament scale imaging reveals that while some tropomyosin isoforms (top images: Tpm2.1 in green and Tpm3.2 in red) can copolymerize with actin filaments, others cannot (bottom images: Tpm3.1 in green and Tpm1.6 in red) (adapted from [134], scale bar, 5 µm).

(figure 5*c*), indicating that they are therefore involved in many different cellular functions [126,129], and modulation of the expression of tropomyosins triggers specific cellular responses. For example, some cancer cell lines can remarkably recover rigidity sensing and rigidity-dependent growth, when a single tropomyosin isoform (Tpm2.1) is over-expressed [127,130]. However, structures such as stress fibres are highly sensitive to the expression level of any isoform of tropomyosin [127,131]. Recent data also suggest that modulation of any tropomyosin isoform impacts the whole myosin organization in cells, thus acting on both tension and traction forces driven by focal adhesions [132]. Therefore, some actin networks might bind to multiple families of tropomyosins simultaneously, which is coherent with the high concentration of tropomyosin present in cells, and with the fact that some tropomyosin isoforms have the ability to copolymerize as demonstrated *in vitro* (figure 5*c*) [133,134].

The presence of tropomyosins on linear actin networks suggests a preference for formin-generated filaments. Indeed, incubation of the pan-formin inhibitor SMIFH2 in oocytes decreases the cortical tropomyosin level [135]. Tropomyosin depletion promotes the expansion of lamellipodia while its overexpression inhibits this branched structure while promoting linear networks [136–138]. Moreover, it seems that some formins assemble actin filaments bound to specific tropomyosins. This is beautifully illustrated in fission yeast, where an exchange of the localization of the fission yeast formins For3 and Cdc12 results in an exchange in localizations of the tropomyosin forms on the corresponding actin networks [139]. Also, the absence of CP in fission yeast cells induces simultaneously ectopic recruitment of the tropomyosin Cdc8 and of both formins Fus1 and Cdc12 [140]. However, specific downregulation of some formins (mDia1 and mDia3) does not affect the localization of tropomyosins, indicating that some formins may not share this specificity for tropomyosins [141].

7.3. Impact of tropomyosins on actin filament nucleation, debranching and the binding of other actin-binding proteins

Tropomyosins are dimers of α-helices forming parallel coiled-coils that span several actin subunits [123]. A biochemical link between formins and tropomyosins has been described *in vitro*, and cooperativity between these proteins is established [136,142,143]. In budding yeast, the presence of tropomyosin can specifically increase the nucleation rate of a formin. Conversely, tropomyosins are generally strong inhibitors of Arp2/3-induced actin nucleation and branch formation [144,145]. Debranching and re-organization of actin networks into linear arrays is also favourable to tropomyosins, as this process generates more actin pointed ends from where tropomyosins can bind [121,122]. These observations agree well with the localization of tropomyosin in cells.

The binding of tropomyosin around actin filaments contributes directly to the recruitment of particular families of ABPs, and the dissociation of others. Tropomyosins regulate the activity of the different families of myosins by modifying their binding to actin filaments and their enzymatic kinetics [146,147]. This mechanism is important because it allows cargoes to be directed to appropriate locations and regulates contractility. Elegant *in vitro* studies confirm at the level of single actin filaments that tropomyosin excludes other ABPs, such as fimbrin or ADF/cofilin, therefore preventing filaments from disassembly [129,134,148,149]. Interestingly, tropomyosin is not required *per se* to assemble cables *in vitro* in the absence of disassembling factors but it becomes necessary to maintain cable assembly in biomimetic assays where treadmilling has been reconstituted [136,150]. Tropomyosins are hence major biochemical regulators that define the identity of actin filaments and regulate the binding of many families of ABPs, thereby leading to the segregation of these proteins to different actin networks.

8. Regulation of actin networks protein composition by competition between ABPs

Numerous lines of evidence indicate that not only tropomyosins, but most ABPs, display cooperative or competitive binding effects to actin filaments, and that these effects need to be taken into account to understand globally how an appropriate ABP composition of actin networks is reached [5]. A number of cellular biology studies demonstrate unambiguously that the removal of a given ABP from one actin network may trigger a global relocation of ABPs from other actin networks [6,140]. As a consequence, phenotypes observed in cells are not only due to the absence of

the ABP of interest, but also to the mislocalization of other ABPs. Several hypotheses could explain this phenomenon. First, it is possible that the absence of a protein in a network may open a binding site for other proteins, or allow the binding of competing proteins. For instance, in yeast, removal of fimbrin from actin patches, which are Arp2/3-branched networks, leads to an ectopic localization of tropomyosin to those networks [6]. Second, it is possible that ectopic protein localization triggers the cooperative binding of additional proteins. For instance, loss of CP from actin patches creates free actin filament barbed ends, where formins can bind, which in turn favours the ectopic binding of tropomyosin [140]. Finally, the absence of an ABP could also have consequences on the geometry of the network, which would consequently impact its ABP composition. Overall, these results indicate that although tropomyosins are key regulators for addressing ABPs to appropriate networks, proper segregation of ABPs on specific actin networks in cells also relies on a global and complex biochemical equilibrium, involving many different families of ABPs. Addressing these questions in the future will require to integrate all these parameters into a comprehensive model.

9. Conclusion

The aim of this review was to describe our current knowledge of the different molecular mechanisms involved in the definition of the identity of actin filaments and networks for a proper segregation of ABPs in cells. We conclude this work by emphasizing that these mechanisms are often not purely distinct from each other, but interrelated. A clear example is the fact that a protein like tropomyosin gives an identity to actin filaments, but is also involved in competitive binding with other ABPs. As many different protein–protein interactions and molecular mechanisms are simultaneously involved, a comprehensive understanding of these complex systems requires non-superficial analysis.

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RESEARCH ARTICLE

Sizes of actin networks sharing a common environment are determined by the relative rates of assembly

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Abstract

Within the cytoplasm of a single cell, several actin networks can coexist with distinct sizes, geometries, and protein compositions. These actin networks assemble in competition for a limited pool of proteins present in a common cellular environment. To predict how two distinct networks of actin filaments control this balance, the simultaneous assembly of actin-related protein 2/3 (Arp2/3)-branched networks and formin-linear networks of actin filaments around polystyrene microbeads was investigated with a range of actin accessory proteins (profilin, capping protein, actin-depolymerizing factor [ADF]/cofilin, and tropomyosin). Accessory proteins generally affected actin assembly rates for the distinct networks differently. These effects at the scale of individual actin networks were surprisingly not always correlated with corresponding loss-of-function phenotypes in cells. However, our observations agreed with a global interpretation, which compared relative actin assembly rates of individual actin networks. This work supports a general model in which the size of distinct actin networks is determined by their relative capacity to assemble in a common and competing environment.

Introduction

Eukaryotic cells assemble a range of filamentous actin (F-actin) structures from actin monomers (globular actin [G-actin]) to accomplish diverse processes. Actin assemblies may be formed generally through two different mechanisms: (1) branched networks of actin filaments, for which actin filaments are nucleated and elongate from the side of pre-existing ones by a 7-subunit complex called the actin-related protein 2/3 (Arp2/3) complex and (2) linear networks of actin filaments, for which actin filaments are capped at their dynamic barbed ends by a homo-dimer of formin and elongate processively by the insertion of actin monomers [1]. In addition, different actin networks have distinct geometrical organizations, sizes, dynamics, and mechanical properties, which are adapted to their precise function. These parameters are tightly controlled by specific accessory proteins. The accessory proteins may have one or

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Abbreviations: ABP, actin-binding protein; ADF, actin-depolymerizing factor; ADP, adenosine diphosphate; Arp2/3, actin-related protein 2/3; ATP, adenosine triphosphate; Bni1p, bud neck involved protein 1; Cap1/2, capping protein 1 and 2; CK-666, Arp2/3 complex inhibitor I; F-actin, filamentous actin; FH1-FH2, formin homology domain 1 and 2; G-actin, globular actin; Las17p, budding yeast WASp ortholog; Pfy1, profilin; WASp, Wiskott–Aldrich syndrome protein.

multiple effects on actin filaments, which may include nucleating new filaments from free actin monomers, cross-linking filaments into specific geometries, controlling elongation rates, and severing and disassembling actin filaments [2]. The presence of all of these variables within a dynamic cell raises a number of challenges to our understanding of actin biology.

The size of actin networks is controlled by the balance between their specific rates of actin filament nucleation, rates of assembly at actin filament barbed ends, and rates of network disassembly. Originally, it was thought that each cellular actin network was assembling independently of the others due to the presence of a large and unlimited pool of actin monomers. As a consequence, this original model proposed that rates of actin polymerization and disassembly were constant and that the size of each actin network was mainly determined by the rate of actin nucleation. However, recent studies challenged this idea and demonstrated that different actin networks in cells were in tight competition for a limited pool of actin monomers [3–5]. This discovery of homeostatic actin networks suggests that beyond the regulation of actin nucleation, the growth rate and size of actin networks was also controlled by the concentration of polymerizable actin left in a competitive environment. For instance, cells in which the Arp2/3 complex activity is diminished, the disappearance of branched networks is tightly correlated with the assembly of an unusually high number of formin-dependent actin cables [3,6]. Conversely, formin inhibition does not change the level of F-actin but promotes the assembly of Arp2/3-branched networks in cells. With this new model, any growth of an actin network beyond its usual extent partially depletes the amount of G-actin that is available in the cytoplasm and therefore reduces the size of other actin networks. This concept was recently formalized theoretically under the name of global treadmilling [7].

The actin accessory protein profilin, which tightly binds to G-actin, was demonstrated to regulate F-actin network homeostasis by favoring the formin assembly pathway over the Arp2/3 complex assembly pathway [6,8]. Control of profilin expression is a powerful mechanism to regulate the formation of dynamic actin-rich protrusions and collective cell migration [9]. Such an observation suggests that fine-tuning the activity or expression of actin accessory proteins is an efficient and physiological mechanism for cells needing to reorganize rapidly their actin cytoskeleton. Besides profilin, most other actin accessory proteins have been shown to impact globally the organization of the actin cytoskeleton. This is the case for cultured cells [10,11] as well as in more complex processes such as epithelial-to-mesenchymal transitions [12]. However, how specific factors modulate globally the organization of the actin cytoskeleton in a homeostatic environment is not yet clear.

Understanding the global impact of an accessory protein is a challenge, as most proteins have multiple effects on actin assembly. For example, actin-depolymerizing factor (ADF)/cofilin binds to G-actin as well as to F-actin, with multiple effects on actin assembly, disassembly, and monomer recycling [2,13,14]. Furthermore, understanding how the activity of these accessory proteins modulates branched versus linear network assembly is confounded by the fact that they have different consequences on the formin and Arp2/3 assembly pathways. To circumvent this problem, the assembly of both actin assembly pathways were reconstituted in this study from a minimal number of essential components in a shared experimental environment in which the steady state is rapidly reached. Although both types of actin networks assembled independently, this system enabled us to test and compare side-by-side the effect of various families of proteins on both actin assembly pathways. Our results indicate that all actin accessory proteins potentially impact actin assembly pathways differently. This work demonstrates that the interpretation of these differences in the context of a competitive environment provides a general explanation of how the size of linear and branched networks is balanced in cells.

Results

Reconstitution of actin-based motility from WASp and formin-coated beads in a common environment

Previous studies reconstituted separately Arp2/3-based and formin-based actin motilities in vitro [15–17]. Typical assays use polystyrene microbeads coated by a nucleation-promoting factor of the Arp2/3 complex or coated by formins in an environment in which actin filament elongation is funneled at the bead surface to generate propulsive forces. Arp2/3-based actin motility is characterized by the assembly of dense tails of branched actin networks, in which beads are pushed by the continuous elongation of uncapped actin filament barbed ends at their surface [15]. Formin-based actin motility is characterized by the assembly of dense cables of linear actin filaments, in which beads are pushed by the processive elongation of actin filaments capped at their barbed ends by the formins [16,17].

Simultaneous Arp2/3-based and formin-based actin nucleation was reconstituted in vitro [8,18], but the set of essential accessory proteins that is required to obtain a simultaneous steady-state actin network assembly from Wiskott–Aldrich syndrome protein (WASp) and formin-coated beads has not yet been determined. Polystyrene microbeads of 2-μm diameter were coated with the budding yeast WASp ortholog (Las17p), and 4-μm diameter microbeads with the formin homology domains 1 and 2 (FH1-FH2) of a budding yeast formin, bud neck involved protein 1 (Bni1p) (Fig 1A). A sustained and organized actin assembly occurred immediately at the surface of the beads when they were mixed in a buffer solution containing solely 8 μM prepolymerized F-actin, 250 nM Arp2/3 complex, 1 μM capping protein, and 15 μM profilin (Fig 1B). Such experimental conditions were sufficient to generate actin networks of visibly distinct architectures. WASp-coated beads were propelled by assembling actin networks at rates that are comparable with other studies (typically up to 2 μm/min) [15,19], showing that the system is competent for actin-based motility and, more generally, for force generation. In the case of formin-coated beads, the beads often stalled rapidly, but force generation of actin networks is inferred from the buckling of some actin cables polymerizing at their surface [17] (Fig 1C). The use of a fluorescent actin reporter shows that the amount of polymer around both types of beads increases linearly from the beginning of the experiment and for several tens of minutes, suggesting a constant and measurable rate of actin assembly for all conditions tested (S1A Fig). Actin assembly rates on WASp-coated beads were systematically measured on comet tails after symmetry breaking, and experiments performed with submicron size beads, which undergo symmetry breaking more rapidly, gave similar results (S1B Fig). The design of this biomimetic system presents the advantage to compare the efficiency of actin assembly for two distinct assembly pathways in equivalent conditions.

WASp/formin biomimetic assay recapitulates the effect of an inhibition of the Arp2/3 complex

We wanted to confirm that beyond visibly distinct actin architectures, both WASp and formin-coated beads were mimicking appropriately the assembly of branched and linear actin networks in vivo. We investigated first the sensitivity of actin network assembly in the biomimetic assay to variable concentrations of Arp2/3 complex. The concentrations of profilin and capping protein were fixed at values that are optimal for both branched and linear network assembly, and the concentration of Arp2/3 complex was varied between 0 and 500 nM (Fig 2A and 2B). As expected, increasing concentrations of Arp2/3 complex enhanced actin nucleation on WASp-coated beads and increased the rate of actin assembly on these beads (Fig 2C).

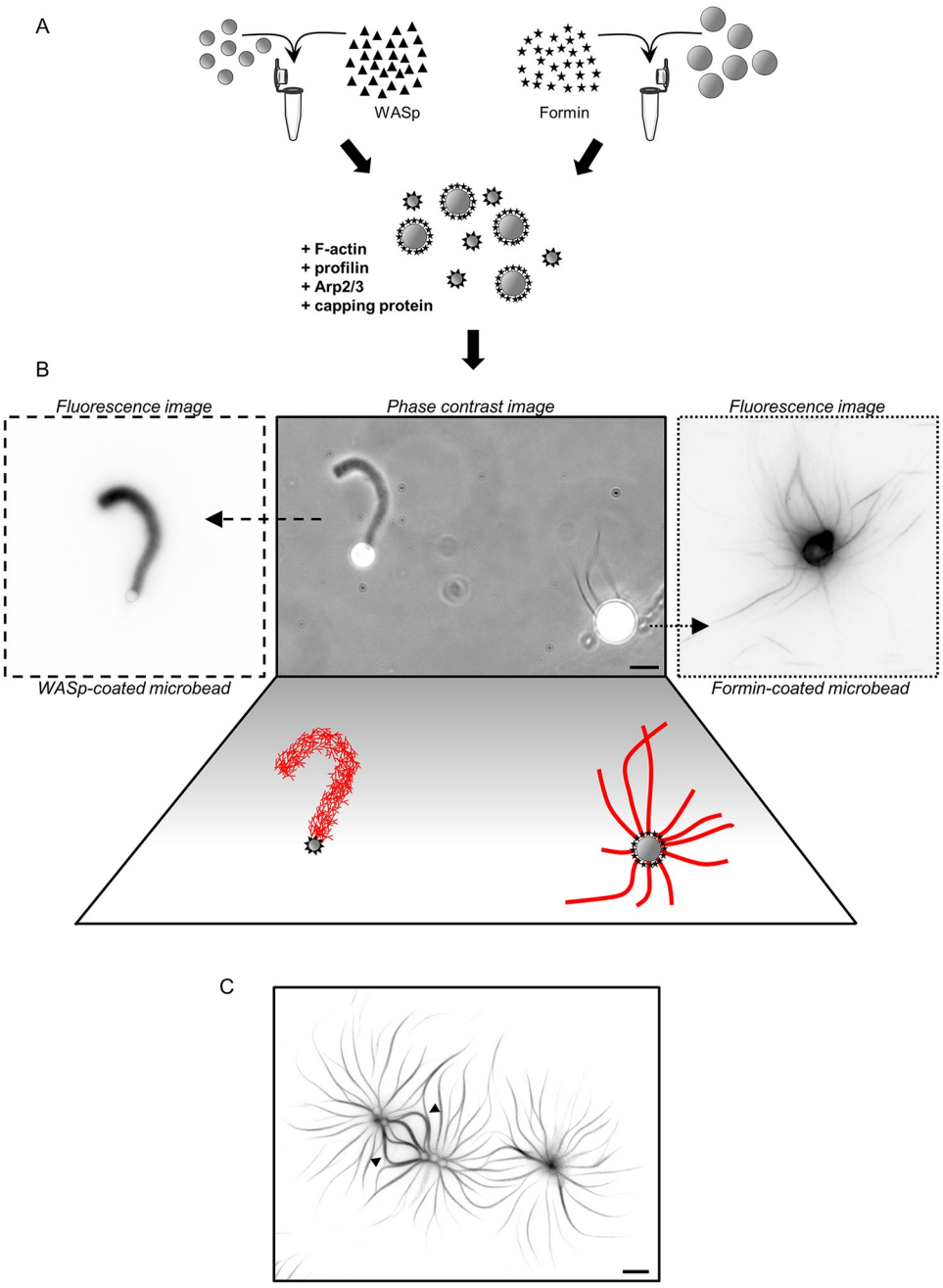

Fig 1. Simultaneous assembly of branched and linear networks of actin filaments in vitro. A. Schematic of experimental bead assay setup. B. Phase contrast and fluorescence snapshots of branched actin networks assembled around 2-μm diameter WASp-coated microbeads and linear actin networks assembled around 4-μm diameter formin-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, and capping protein. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. C. Fluorescence snapshot of actin networks assembled around multiple formin-coated microbeads 45 min after the initiation of the experiment. Buckling events indicated by the black arrowheads. Scale bar: 10 μm. Arp2/3, actin-related protein 2/3; WASp, Wiskott–Aldrich syndrome protein.

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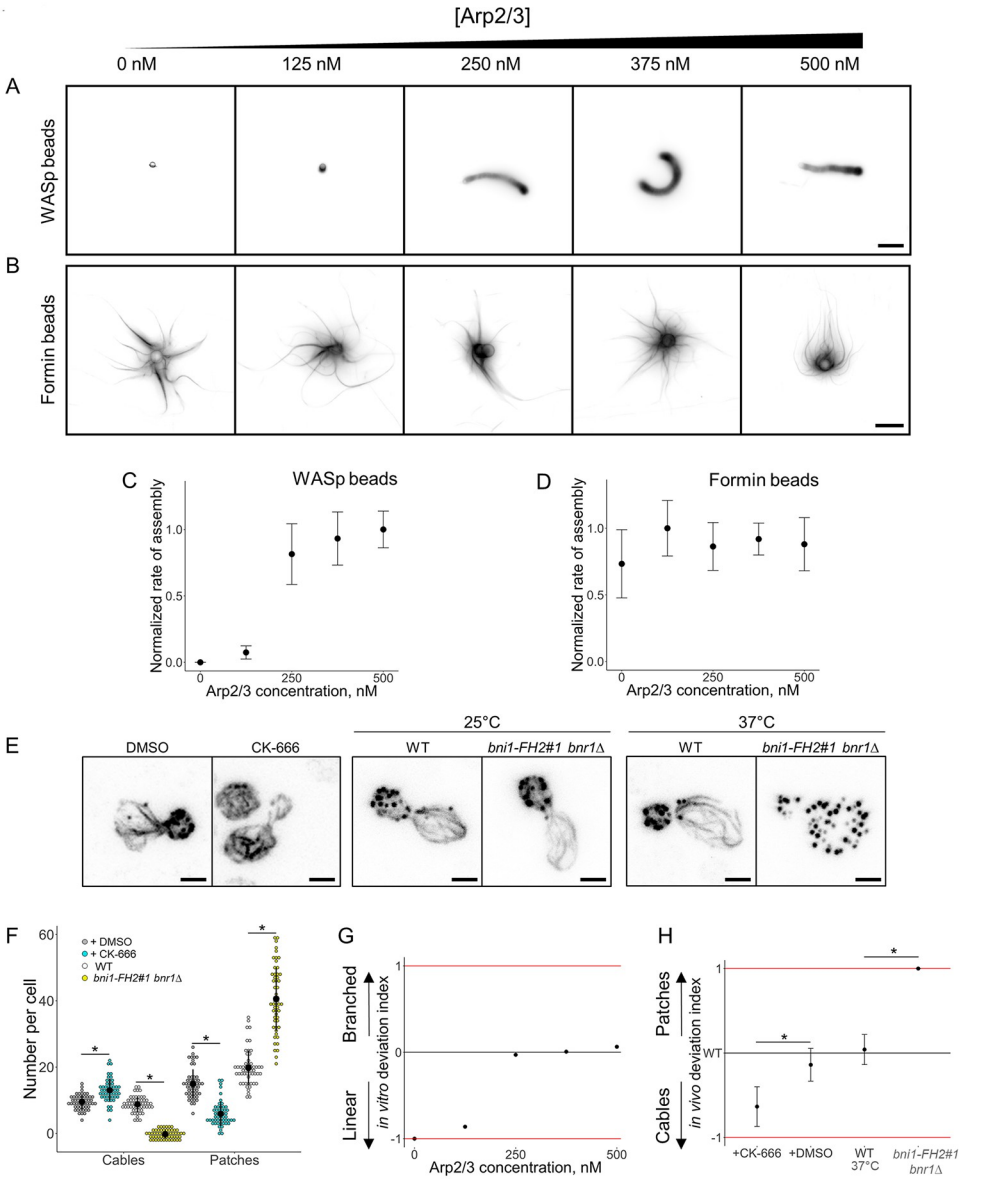

Fig 2. Modulation of the branched-to-linear actin network balance by the Arp2/3 complex. The underlying data can be found within S1 Data. A. Fluorescence snapshots of actin networks assembled around WASp-coated microbeads in the presence of fluorescent actin, profilin, capping protein, and variable concentrations of Arp2/3 complex. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. B. Fluorescence snapshots of actin networks assembled around formin-coated microbeads in the presence of fluorescent actin, profilin, capping protein, and variable concentrations of Arp2/3 complex. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. C. Quantification of (A). Rate of actin assembly around WASp-coated microbeads as a function of the Arp2/3 complex concentration, normalized to the maximum value. D. Quantification of (B). Rate of actin assembly around formin-coated microbeads as a function of the Arp2/3 complex concentration, normalized to the maximum value. E. Snapshots of the actin cytoskeleton organization in budding yeast cells fixed and labeled with fluorescent phalloidin, in the presence or in the absence of 200 μM CK-666 (left images), and for the formin defective mutant *bni1-FH2#1 bnr1*Δ at nonrestrictive temperature (25 ˚C; center images) or restrictive temperature (37 ˚C; right images). Scale bars: 2 μm. F. Quantification of (E). Average number of actin patches and cables per cell. G. In vitro deviation index, calculated as a function of the Arp2/3 complex concentration. This index compares how actin assembly rates around WASp and formin-coated beads deviate from a balanced situation in which both types of networks assemble optimally. H. In vivo deviation index, based on structures number, calculated in the presence of DMSO, 200 μM CK-666, at 37 ˚C for wild-type cells or at 37 ˚C for *bni1-FH2#1 bnr1*Δ cells. This index compares how the number of actin patches and cables deviate from the wild-type condition. Arp2/3, actin-related protein 2/3; CK-666, Arp2/3 complex inhibitor I; WASp, Wiskott–Aldrich syndrome protein.

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However, the Arp2/3 complex is not expected to affect actin nucleation on formin-coated beads, and actin assembly on formin-coated beads was constant over a large range of Arp2/3 concentration (Fig 2D).

We next determined if the reconstitution mimics the effect of an Arp2/3 inhibition in vivo. Budding yeast is a convenient system for such comparison as only two spatially distinct actin networks are present during most of its cell cycle. The Arp2/3 assembly pathway is represented by the presence of endocytic actin patches, while the formin assembly pathway is represented mainly by the presence of spatially distinct actin cables and in a lesser proportion by the presence of a cytokinetic actin ring [10,20]. Addition of the Arp2/3 complex inhibitor I (CK-666) in fission yeast reduces dramatically the number of patches, while the number and size of cables increases [3]. We stabilized, labeled with fluorescent phalloidin, and imaged the actin cytoskeleton of budding yeast cells in the presence or absence of CK-666 (Fig 2E and S2A Fig). On average, 15 actin patches per cell were visible in control conditions, but their number decreased to 6 actin patches on average in the presence of CK-666 (Fig 2F). The quantification of the total patch intensity per cell indicates a similar tendency, with an actin patch intensity per cell reduced on average by 75% in the presence of CK-666 (S2B Fig). The situation is inversed for actin cables. The number of visible cables per cell increases from 9 to 13 in the presence of CK-666, and cables assembled per cell are on average 240% brighter with CK-666 than in control conditions (Fig 2F and S2B Fig). These results demonstrate a similar effect of CK-666 in budding yeast as in fission yeast. Conversely, inhibition of formins in the temperature sensitive yeast mutant *bni1-FH2#1 bnr1*Δ [21] increases the number of actin patches to 41, while no actin cables are visible anymore (Fig 2E and 2F).

A close comparison of the results from the biomimetic assay and in cells shows a correlated increase in branched network assembly. However, the Arp2/3-independant linear actin network assembly in the biomimetic assay does not match with the variation in cable assembly observed in cells. The reason is that if the biomimetic assay allows an understanding of how two different networks of actin filaments assemble when encountering similar conditions, it does not take into account how they may compete for a common pool of proteins as they do in cells [3,5]. This discrepancy imposed a second type of analysis of our results in order to evaluate the capacity of each actin network to assemble relatively to the other actin network. This comparison was made by defining a deviation index, which compares the efficiency of actin assembly for both branched and linear networks of actin filaments for a given biochemical condition in vitro. This index measures how actin assembly between branched and linear networks deviates from an equilibrium situation in which actin assembles equally well for both networks and is defined as follows:

$$\text{In vitro deviation index } ([ABP]) = \frac{r_{branched([ABP])} - r_{linear([ABP])}}{r_{branched([ABP])} + r_{linear([ABP])}},$$

in which r is the rate of actin assembly for branched or linear networks for a given concentration of a given accessory protein (actin-binding protein [ABP]).

This index varies between the values −1 and +1, in which −1 represents an extreme case in which only formin-coated beads assemble actin networks in vitro; 0 represents a balanced situation in which formin-coated beads assemble as much actin as WASp-coated beads; and +1 represents the other extreme case in which only WASp-coated beads assemble actin networks in vitro (Fig 2G).

Similarly, an in vivo index comparing the deviation in the number of actin patches and cables in yeast cells is defined as follows:

$$\text{In vivo deviation index}([CK666]) = \frac{\frac{N_{patches,CK666}}{N_{patches,DMSO}} - \frac{N_{cables,CK666}}{N_{cables,DMSO}}}{\frac{N_{patches,CK666}}{N_{patches,DMSO}} + \frac{N_{cables,CK666}}{N_{cables,DMSO}}},$$

in which N is the number of actin patches and cables in the presence or in the absence of CK-666 (Fig 2H). Defining an in vivo deviation index based on the intensity actin patches and cables gave us similar results throughout this study (S2C Fig). The in vivo deviation index also varies between the values −1 and +1, in which −1 represents an extreme case in which only actin cables are visible in cells; 0 represents the wild-type equilibrium; and +1 represents the other extreme case in which only actin patches are detected.

A comparison of the in vitro and in vivo indexes (Fig 2G and 2H) indicates that an appropriate interpretation of the biomimetic assay is able to predict how the balance between Arp2/3 and formin networks is modified by a change of the Arp2/3 complex activity. Both indexes indicate trends, thus should not be compared quantitatively, as a value 0 for the in vivo index is equivalent to the wild-type condition, while a value 0 for the in vitro index is equivalent to a condition in which both linear and branched network assemble equally well relative to their respective optimal condition of assembly. A more thorough analysis would require the precise knowledge of the cytoplasmic concentrations of the proteins used in these assays.

WASp/formin biomimetic assay recapitulates the biochemical effect of the actin assembly regulator profilin

Profilin is an established regulator for the homeostasis of actin networks [6,8,22]. Profilin binds tightly to ATP-G-actin ($K_d \approx 100$ nM) and prevents the spontaneous nucleation and the elongation of actin filaments at their pointed ends [1]. Profilin also binds directly to the poly-L-proline domain of formin and enhances processive barbed end elongation rates. In contrast, profilin reduces nucleation and branching by the Arp2/3 complex [8]. Profilin also binds to actin filament barbed ends, competing with other barbed end interactors, therefore triggering a multitude of effects on actin filament barbed-end dynamics [22]. Profilin also contributes to actin recycling by promoting the exchange of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) on actin monomers.

Profilin was added in the biomimetic assay at various concentrations ranging from 0 to 60 μM (Fig 3A and 3B). Actin assembly curves for both types of networks displayed bell-shaped curves but with different characteristics (Fig 3C and 3D). Actin assembly and bead motility were modestly efficient for WASp-coated beads in the absence of profilin but were inhibited by the presence of large amounts of profilin (Fig 3C). On the contrary, actin assembly into cables was inefficient on formin-coated beads in the absence of profilin but efficient in the presence of up to 60 μM of profilin (Fig 3D). Another difference between these curves is that optimal conditions for actin assembly were reached at different concentrations of profilin, i.e., about 15 μM for WASp-coated beads and 30 μM for formin-coated beads (Fig 3C and 3D). These assays are performed at low bead densities, and we verified that the assembly of one type of actin network does not influence the assembly of the other (S3 Fig). Therefore, as for the Arp2/3 complex, the side-by-side comparison of these curves does not show intuitively whether branched network or linear network assembly is favored for a given concentration of profilin. Consequently, we plotted for each concentration of profilin the in vitro deviation index (Fig 3E). This deviation index varies between +0.94 in the absence of profilin to −0.75 for a high concentration of profilin, indicating nonambiguously the ability of this protein to

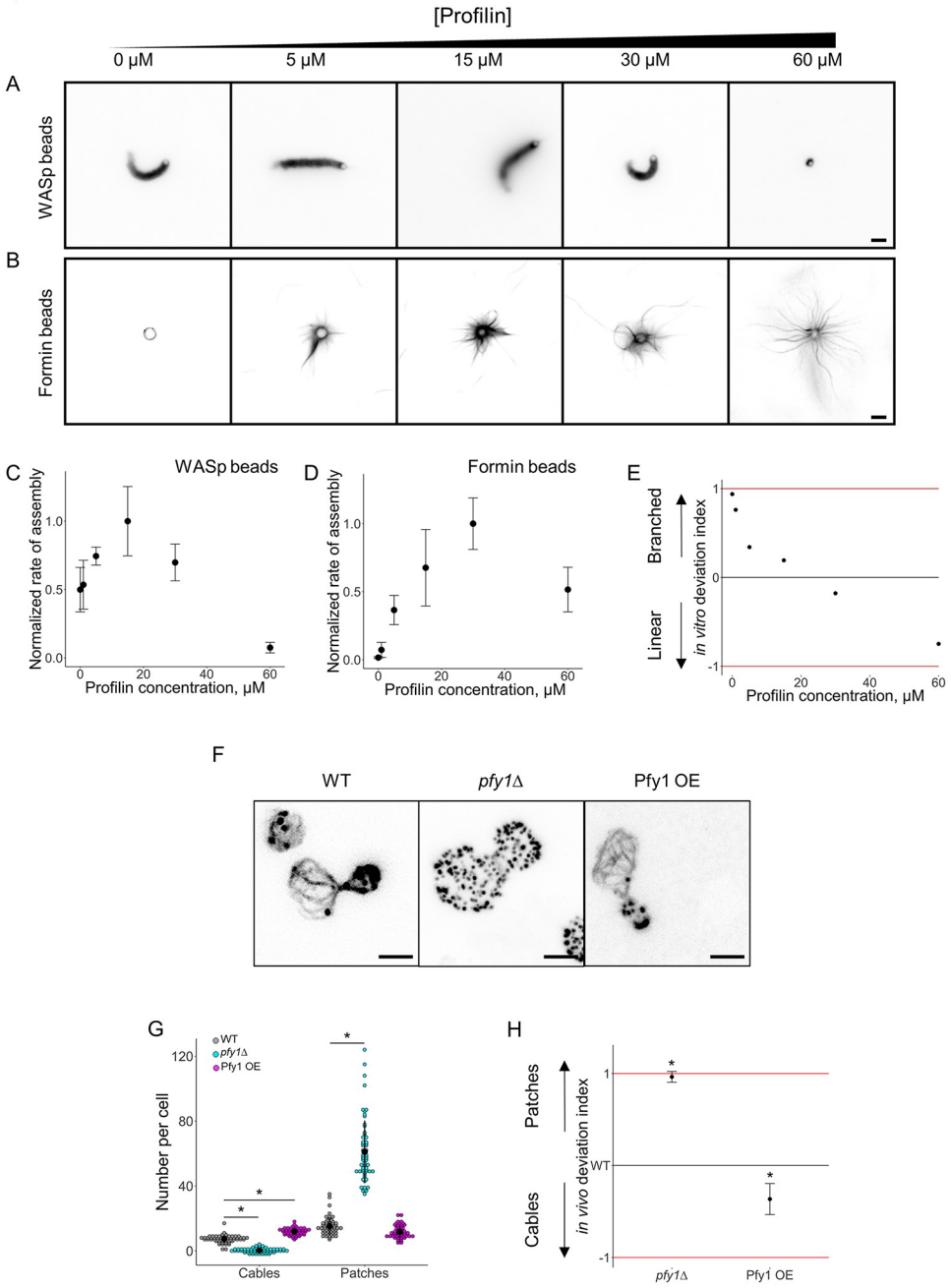

Fig 3. Modulation of the branched-to-linear actin network balance by profilin. The underlying data can be found within S1 Data. A. Fluorescence snapshots of actin networks assembled around WASp-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, capping protein, and variable concentrations of profilin. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. B. Fluorescence snapshots of actin networks assembled around formin-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, capping protein, and variable concentrations of profilin. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. C. Quantification of (A). Rate of actin assembly around WASp-coated microbeads as a function of the profilin concentration, normalized to the maximum value. D. Quantification of (B). Rate of actin assembly around formin-coated microbeads as a function of the profilin concentration, normalized to the maximum value. E. In vitro deviation index, calculated as a function of the profilin concentration. F. Snapshots of the actin cytoskeleton organization in wild-type, *pfy1*Δ, and Pfy1 overexpressing budding yeast cells fixed and labeled with fluorescent phalloidin. Scale bars: 2 μm. G. Quantification of (F). Average number of actin patches and cables per cell. H. In vivo deviation index for *pfy1*Δ and Pfy1 overexpressing cells. Arp2/3, actin-related protein 2/3; Pfy1, profilin; WASp, Wiskott–Aldrich syndrome protein.

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switch actin assembly from branched networks on WASp-coated beads to cable assembly on formin-coated beads.

We compared our results with the effect of profilin in budding yeast cells [23]. Labeling of actin structures with fluorescent phalloidin indicated that profilin null (*pfy1*Δ) cells do not assemble any visible actin cables but assemble >5 times more actin patches than wild-type cells (Fig 3F and 3G and S2A Fig). On the contrary, overexpression of profilin reduced the number of actin patches from 15 in wild-type cells to 12 on average, while the number of actin cables increased from 7 to 12. As above, an in vivo deviation index is defined as follows:

$$\text{In vivo deviation index} = \frac{\frac{N_{patches,mutant}}{N_{patches,wild\ type}} - \frac{N_{cables,\ mutant}}{N_{cables,wild\ type}}}{\frac{N_{patches,mutant}}{N_{patches,wild\ type}} + \frac{N_{cables,mutant}}{N_{cables,\ wild\ type}}},$$

in which N is the number of actin patches and cables for wild-type or mutant cells. Calculation of the in vivo deviation index for wild-type, *pfy1*Δ, and profilin overexpressing cells shows a similar trend than the in vitro deviation index (Fig 3E and 3H), demonstrating again that the biomimetic assay and its analysis recapitulate the physiological effect of profilin in vitro.

Effect of capping protein on Arp2/3-based and formin-based actin assembly pathways

Our previous results demonstrated that proteins such as Arp2/3 and profilin modulate branched and linear actin network assembly differently and, therefore, impact the balance between branched and linear networks of actin filaments in vivo. We followed for the rest of this study the hypothesis that any other protein implicated in actin assembly was also likely to affect actin assembly pathways differently and, therefore, impact the branched/linear actin network balance [7].

We first tested this hypothesis by focusing our attention on capping protein. Capping protein, which is a heterodimer of Cap1 and Cap2, binds tightly to the barbed end of actin filaments and inhibits their elongation [1]. On branched networks of actin filaments, capping protein limits the size of individual actin filaments, contributes to the densification of actin networks [24], and promotes filament nucleation by the Arp2/3 complex [25]. On linear networks of actin filaments, capping protein is able to bind simultaneously with formin to actin filament barbed ends [26,27]. However, this mutual binding is weak and enables rapid displacement of one by the other. As a consequence, capping protein competes with formin to bind actin filament barbed ends [28,29]. Capping protein also increases the steady-state concentration of monomeric actin in these assays, which can influence both pathways [30,31].

The concentration of capping protein was varied in the biomimetic assay between 0 and 15 μM (Fig 4A and 4B). As for profilin, both actin assembly pathways display bell-shaped curves with optimal concentrations of capping protein around 1 μM for both types of actin networks (Fig 4C and 4D). Actin network assembly occurred noticeably faster on WASp-coated beads than on formin-coated beads. At high concentration of capping protein, both networks were found to assemble equally well. Preincubation of proteins for 2 h at room temperature before introduction of the beads did not change our observations, indicating that steady-state actin assembly in these assays is reached rapidly from the disassembly of the pool of filamentous actin (S4A and S4B Fig). Overall, the determination of the in vitro deviation index for capping protein predicts that the assembly of branched networks is favored over linear networks for low concentrations of capping protein (<1 μM) and reaches a stable equilibrium between both structures for concentrations of capping protein above 1 μM (Fig 4E, S4A and S4B Fig). We compared these results with the effect of an absence of capping protein in

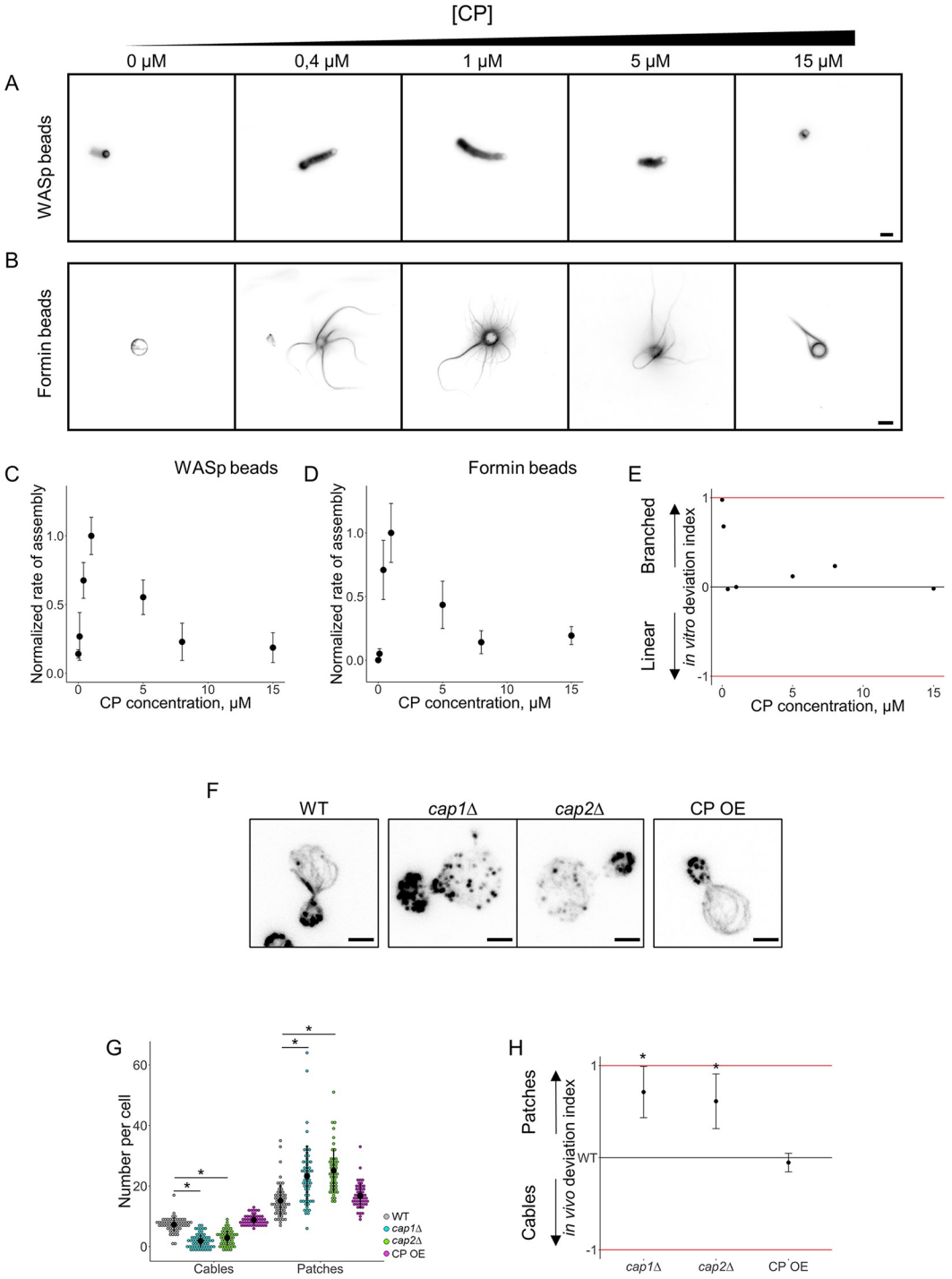

Fig 4. Modulation of the branched-to-linear actin network balance by capping protein. The underlying data can be found within S1 Data. A. Fluorescence snapshots of actin networks assembled around WASp-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, and variable concentrations of capping protein. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. B. Fluorescence snapshots of actin networks assembled around formin-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, and variable concentrations of capping protein. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. C. Quantification of (A). Rate of actin assembly around WASp-coated microbeads as a function of the capping protein concentration, normalized to the maximum value. D. Quantification of (B). Rate of actin assembly around formin-coated microbeads as a function of the capping protein concentration, normalized to the maximum value. E. In vitro deviation index, calculated as a function of the

capping protein concentration. F. Snapshots of the actin cytoskeleton organization in wild-type, *cap1*Δ, *cap2*Δ, and capping protein overexpressing budding yeast cells fixed and labeled with fluorescent phalloidin. Scale bars: 2 μm. G. Quantification of (F). Average number of actin patches and cables per cell. H. In vivo deviation index for *cap1*Δ, *cap2*Δ, and capping protein overexpressing cells. Arp2/3, actin-related protein 2/3; WASp, Wiskott–Aldrich syndrome protein.

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yeast. As described previously, *cap1*Δ and *cap2*Δ cells have a similar phenotype because capping protein requires the expression of both subunits for its function [32]. Absence of capping protein in yeast was also correlated with higher amounts of actin polymer in cells, suggesting that capping protein is also important in cells to increase the concentration of polymerizable actin [33]. In both *cap1*Δ and *cap2*Δ cells, an elevated number of patches and very few actin cables were detectable (Fig 4F and 4G and S2A Fig). Remaining actin cables may be present due to the presence of other inhibitors of actin filament barbed-end elongation in yeast [34,35]. As a consequence, the in vivo deviation index was higher for both strains, as predicted by the biomimetic assay (Fig 4E and 4H). We also investigated the effect of an overexpression of capping protein in yeast cells. Overexpression was induced from a multicopy plasmid derived from the plasmid used for the endogenous expression and purification of a functional capping protein [36] (S5 Fig). Surprisingly, despite the strong overexpression of capping protein in these cells, their actin cytoskeleton appears to be normal (Fig 4F and 4G).

As low rates of actin assembly for weak barbed-end capping are partly due to a depletion of the monomeric actin pool in the biomimetic assay [31], we also investigated a situation in which actin networks were initiated from a fixed concentration of G-actin. However, low concentrations of capping protein in the presence of high amounts of G-actin leads to an uncontrolled barbed-end assembly from WASp beads and the formation of nonpolarized actin networks whose fluorescence signal was difficult to quantify (S4C Fig [25,37]). The geometry of such actin networks also does not reflect the well-defined structure of the actin patches observed in the *cap1*Δ or *cap2*Δ cells (Fig 4F). Overall, the different conditions tested for these experiments (Fig 4C, 4D and 4E, S4A, S4B and S4C Fig) suggest that the pool of actin monomers reaches a deterministic steady state rapidly when experiments are initiated from F-actin and mimics best what is observed in cells.

Effect of ADF/cofilin on Arp2/3-based and formin-based actin assembly pathways

Another protein that is expected to impact actin assembly is ADF/cofilin. While ADF/cofilin is mostly known for its importance on actin disassembly [2,13,14,31], it is also identified as a strong competitor of the Arp2/3 complex [38]. ADF/cofilin inhibits actin nucleation and branch formation by the Arp2/3 complex, but ADF/cofilin does not have, to our knowledge, any reported direct effect on actin nucleation or elongation by formins.

ADF/cofilin at various concentrations ranging from 0 to 10 μM was added in the biomimetic assay, and the net rates of actin assembly were measured (Fig 5A and 5B). Similar to profilin, optimal concentrations of ADF/cofilin are different for the assembly of actin on WASp-coated beads (around 0.3 μM of ADF/cofilin) and for the assembly of actin on formin-coated breads (around 1 μM of ADF/cofilin) (Fig 5C and 5D). Low concentrations of ADF/cofilin favored the assembly of actin on WASp-coated beads. On the contrary, concentrations of ADF/cofilin above 1 μM reduced sharply actin assembly on WASp-coated beads, while actin assembly remained possible on formin-coated beads. Overall, the in vitro deviation index for ADF/cofilin indicates that while low concentrations of ADF/cofilin (<1 μM) favor branched network assembly over linear network assembly, higher concentrations of ADF/cofilin (>1 μM) progressively favor linear network assembly over branched network assembly

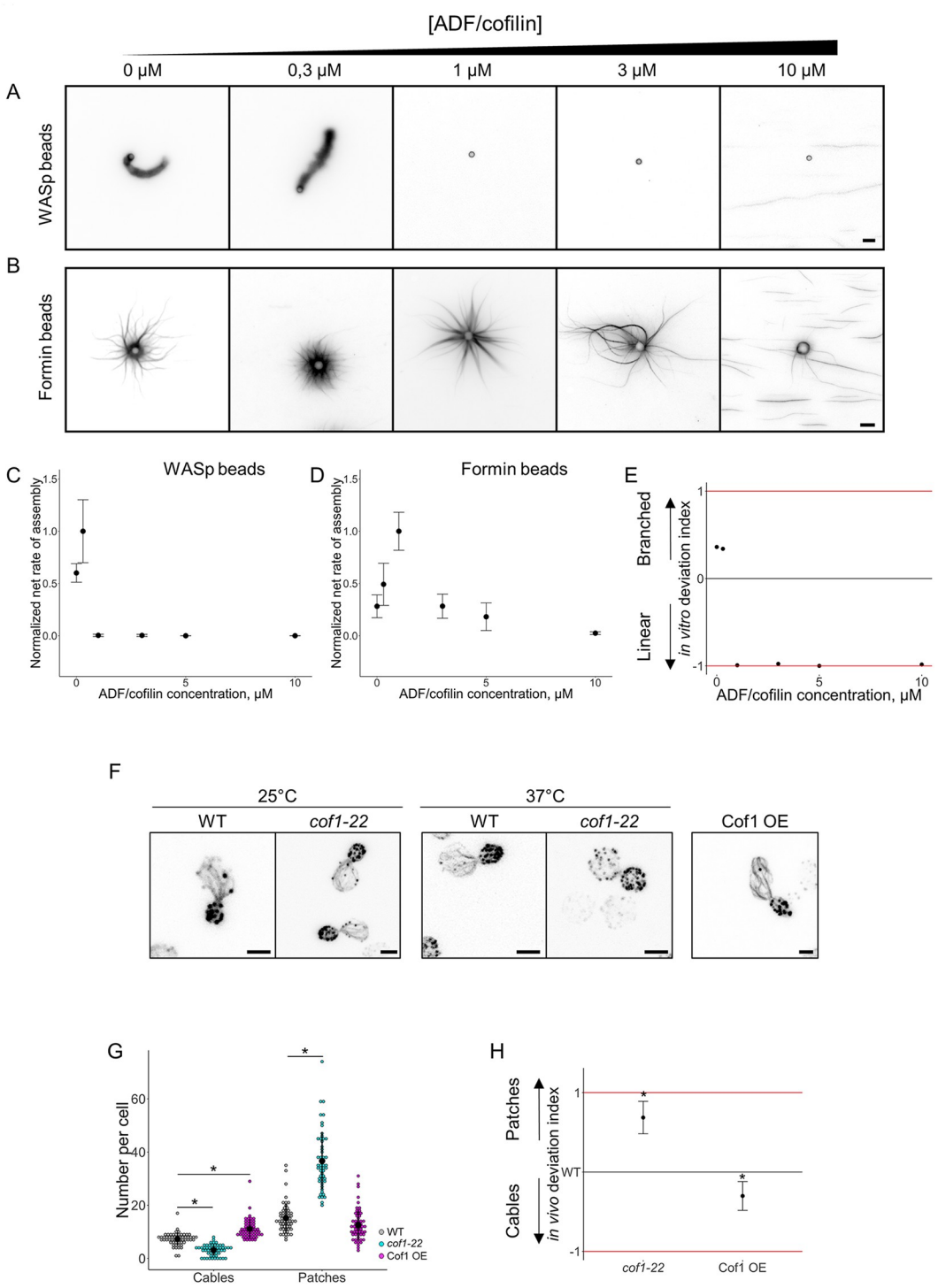

Fig 5. Modulation of the branched-to-linear actin network balance by ADF/cofilin. The underlying data can be found within S1 Data. A. Fluorescence snapshots of actin networks assembled around WASp-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, capping protein, and variable concentrations of ADF/cofilin. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. B. Fluorescence snapshots of actin networks assembled around formin-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, capping protein, and variable concentrations of ADF/cofilin. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. C. Quantification of (A). Net rate of actin assembly around WASp-coated microbeads as a function of the ADF/cofilin concentration, normalized to the maximum value. D. Quantification of (B). Net rate of actin assembly around formin-coated microbeads as a function of the ADF/cofilin concentration, normalized to the maximum value. E. In vitro deviation index,

calculated as a function of the ADF/cofilin concentration. F. Snapshots of the actin cytoskeleton organization of budding yeast cells fixed and labeled with fluorescent phalloidin for ADF/cofilin overexpressing cells (right image) and for *cof1-22* cells at nonrestrictive (25 ˚C; left images) and restrictive (37 ˚C; center images) temperatures. Scale bars: 2 μm. G. Quantification of (F). Average number of actin patches and cables per cell in the wild-type, *cof1-22* mutant, and ADF/cofilin overexpressing conditions. H. In vivo deviation index of ADF/cofilin overexpressing cells and *cof1-22* cells at 37 ˚C. ADF, actin-depolymerizing factor; Arp2/3, actin-related protein 2/3; WASp, Wiskott–Aldrich syndrome protein.

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(Fig 5E). We compared these results with the effect of an inhibition of a temperature-sensitive mutant of ADF/cofilin (*cof1-22*) [39] and of an overexpression of ADF/cofilin in yeast. As expected, while *cof1-22* cells did not show any visible defect in actin patch or cable assembly at nonrestrictive temperature (25 ˚C), *cof1-22* cells had an abnormally high number of patches and less cables at 37 ˚C. On the contrary, overexpressing cells had a small decrease of patch numbers and significantly more actin cables (Fig 5F and 5G and S2A Fig). Comparison of the in vivo and in vitro deviation indexes show a similar trend for ADF/cofilin and predicts that a higher concentration of ADF/cofilin in cells would progressively imbalance cells toward even more cable assembly (Fig 5E and 5H).

Effect of tropomyosin on Arp2/3-based and formin-based actin assembly pathways

Finally, we tested the impact of tropomyosin in the biomimetic assay. Tropomyosin also strongly modulates actin assembly. First, as a competitor of the Arp2/3 complex, it inhibits actin nucleation and branch formation by the Arp2/3 complex [40–42]. Second, tropomyosin cooperates with profilin to enhance formin-dependent nucleation of actin cables [43].

Various concentrations of tropomyosin ranging from 0 to 40 μM were added in the biomimetic assay and we measured the net rates of actin assembly (Fig 6A and 6B). Tropomyosin progressively reduced the rate of actin assembly on WASp-coated beads, while it progressively increased actin assembly on formin-coated beads (Fig 6C and 6D). As a consequence, the in vitro deviation index predicts a shift from a favorable branched actin assembly in the absence of tropomyosin to a favorable actin cable assembly in the presence of increasing concentrations of tropomyosin (Fig 6E). We compared these results with the effect of an inhibition of a temperature-sensitive mutant of tropomyosin (*tpm1-2 tpm2*Δ) [44] and with an overexpression of tropomyosin in yeast. As previously reported, while *tpm1-2 tpm2*Δ cells did not show any visible defect in actin patch or cable assembly at nonrestrictive temperature (25 ˚C), *tpm1-2 tpm2*Δ cells presented a higher number of patches and a severe decrease of cables number at 37 ˚C. On the contrary, overexpression of tropomyosin induced a dramatic phenotype where cells are often misshaped, with a much-reduced number of actin patches and the formation of numerous actin cables (Fig 6F and 6G and S2A Fig). Side-by-side analysis of the in vitro and in vivo deviation indexes reveal a similar trend for both indexes (Fig 6E and 6H). A small shift between these indexes in the absence of tropomyosin also suggests a possible slight overestimation of cable stability in the bead assay at low concentration of tropomyosin (see Discussion).

Discussion

In this work, we measured quantitatively for a range of communal biochemical conditions the efficiencies of actin assembly on dually present branched (WASp/Arp2/3) networks and on linear (formin) networks. For most actin accessory proteins tested in this work, actin network assembly rates were strongly dependent on the concentration of the actin accessory proteins present in solution. Assembly rates displayed bell-shaped curves almost systematically, indicating the existence of optimal concentrations of these accessory proteins for the assembly of

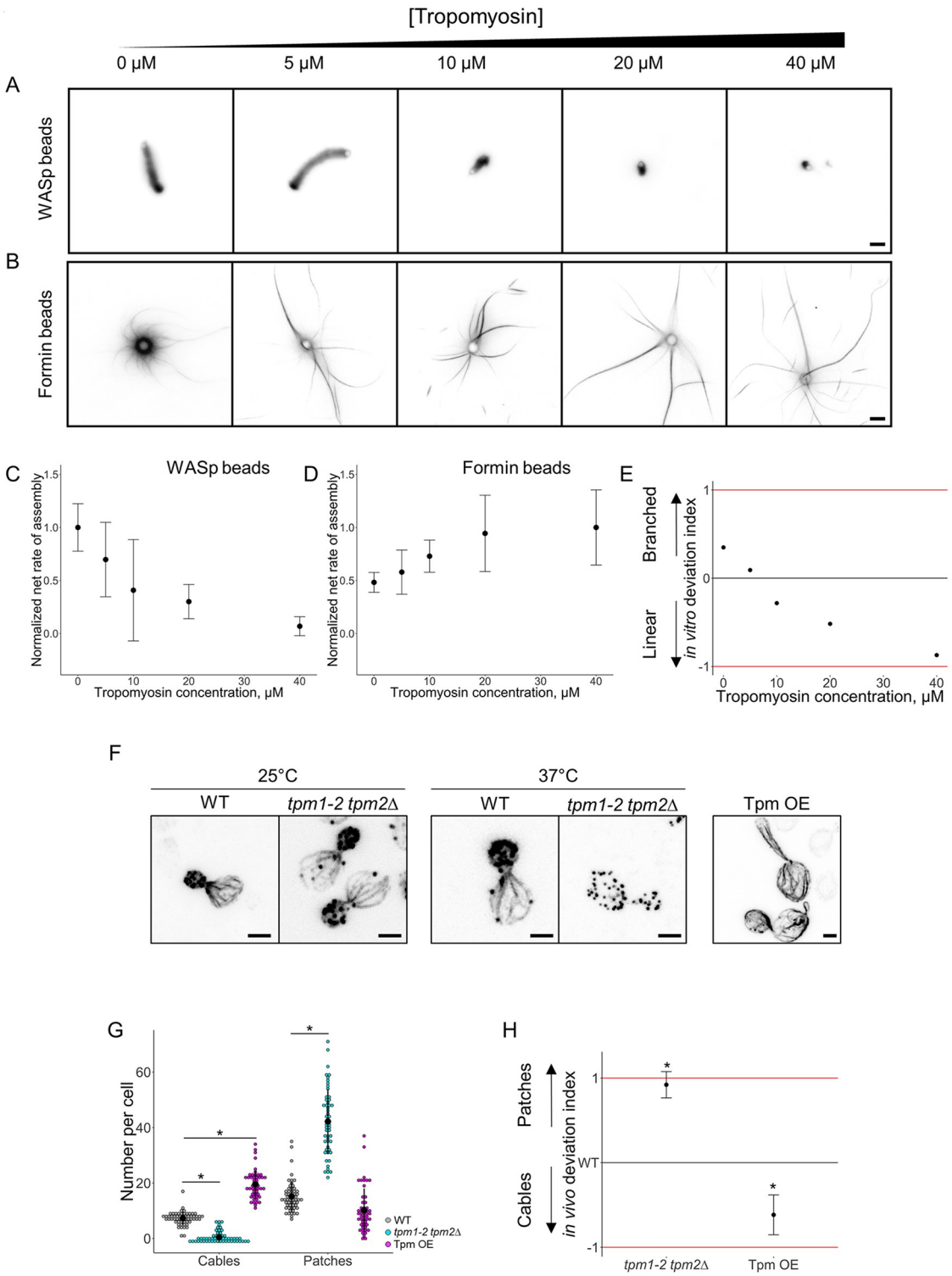

Fig 6. Modulation of the branched-to-linear actin network balance by tropomyosin. The underlying data can be found within S1 Data. A. Fluorescence snapshots of actin networks assembled around WASp-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, capping protein, and variable concentrations of tropomyosin. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. B. Fluorescence snapshots of actin networks assembled around formin-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, capping protein, and variable concentrations of tropomyosin. Images were taken 30 min after the initiation of the experiment. Scale bar: 5 μm. C. Quantification of (A). Net rate of actin assembly around WASp-coated microbeads as a function of the tropomyosin concentration, normalized to the maximum

value. D. Quantification of (B). Net rate of actin assembly around formin-coated microbeads as a function of the tropomyosin concentration, normalized to the maximum value. E. In vitro deviation index, calculated as a function of the tropomyosin concentration. F. Snapshots of the actin cytoskeleton organization of budding yeast cells fixed and labeled with fluorescent phalloidin for tropomyosin overexpressing cells (right image) and for *tpm1-2 tpm2*Δ at nonrestrictive (25 ˚C; left images) and restrictive (37 ˚C; center images) temperatures. Scale bars: 2 μm. G. Quantification of (F). Average number of actin patches and cables per cell. H. In vivo deviation index of tropomyosin overexpressing cells and *tpm1-2 tpm2*Δ cells at 37˚C. Arp2/3, actin-related protein 2/3; WASp, Wiskott–Aldrich syndrome protein.

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actin networks. The effect of these accessory proteins is different for the assembly properties of each actin networks (Fig 7, upper panel), which suggests that all accessory proteins control the amount of actin polymer assembled on each pathway. Actin regulators generally exhibited their characteristic differential effects on branched and linear networks even in the absence of competition for soluble factors. The main differences observed were that (1) an accessory protein may be essential for the assembly of an actin network but not another network and (2) that the range of optimal concentrations for actin assembly is not necessarily the same for both networks (Fig 7, upper panel).

Our results indicate that rates of actin assembly for each individual actin assembly pathway should not be interpreted separately. The existence of a global competition between actin networks requires an interpretation of these results in the light of how a common pool of components is shared in a given biochemical environment (Fig 7, lower panel).

Predicting the shape of these curves is difficult because accessory proteins often have multiple effects on actin assembly. However, our results indicate that in most cases, a side-by-side comparison of actin assembly rates between these structures is sufficient to predict which actin network will be favored in cells, i.e., in an environment in which the competition for a limiting pool of actin monomers exists (Fig 7, lower panel). If we compare now the effect of all the actin accessory proteins tested in this study, we notice that profilin is the most efficient regulator to switch actin assembly from branched to linear networks, i.e., in which the in vitro deviation index varies to its extreme values. Other accessory proteins also regulate actin network homeostasis but only partially.

We also verified the validity of our predictions by evaluating the numbers and intensities of actin patches and actin cables in yeast for a variety of mutant conditions. We generally found an exact correlation between the predictions made with the in vitro reconstitution assay and the observations in cells, indicating that actin assembly rules the balance between branched and linear networks for most accessory proteins. Such biomimetic system can also help us to make predictions that would be difficult to test experimentally. For example, our model predicts that an overexpression of Arp2/3 in cells would not prevent a remaining population of cables to assemble. In the absence of tropomyosin, we found that observations in the biomimetic overestimated slightly the number of actin cables. A possible explanation is that in the case of tropomyosin specifically, the size of actin structures may not be only determined by the effect of tropomyosin on actin assembly but may also be partially controlled by its effect on actin disassembly. This hypothesis is strongly supported by previous models, suggesting that tropomyosin protects actin filaments from the cellular actin disassembling machinery [45,46]. It would be interesting in the future to integrate principles of actin disassembly in the biomimetic assay developed in this study. For instance, the addition of factors such as Aip1 and coronin would determine to which extent actin disassembly may also regulate the size of actin networks in cells [47–49].

General principles highlighted in this work are likely to apply to other cellular models, although the existence of multiple overlapping actin structures in higher eukaryotes makes these principles harder to decipher. Notably, the inhibition or the depletion of the Arp2/3

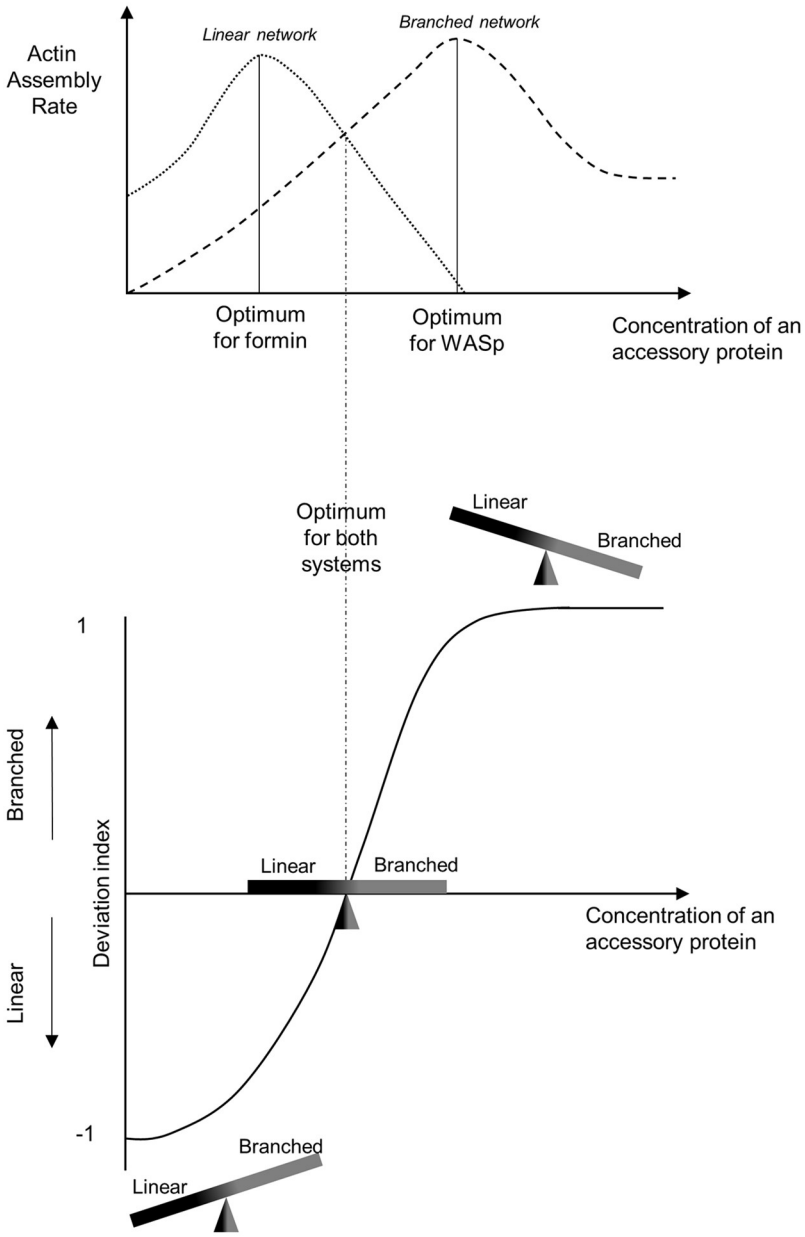

Fig 7. Cartoon representing how the size of actin networks is controlled in a common and competing environment. Results from this study indicate that actin assembly pathways (e.g., branched Arp2/3 and linear formin) do not have the same sensitivities to variable concentrations accessory proteins (upper panel). Principally, actin assembly rates vary differently and can span from cases in which only one type of network is able to assemble to cases in which they both assemble with similar efficiencies. Optimum concentrations of accessory proteins are also generally different for both pathways. Arp2/3, actin-related protein 2/3.

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complex leads to an excess of F-actin linear networks such as bundles and transverse arcs at the expense of lamellipodial networks and ruffle formation in insect and animal cells [50–53]. Conversely, profilin depletion increases the branching density and enhances Arp2/3 complex localization to the lamellipodium, whereas F-actin linear structures are strongly impaired [6]. Strikingly, microinjection of profilin in cells rescues the phenotype and induces the formation of F-actin bundles to the expense of the peripheral lamellipodia length [6]. Generally, ADF/

cofilin and tropomyosin depletions cause the expansion of branched networks [11,54]. On the contrary, elimination of any of the tropomyosin isoforms fatally compromises stress fiber formation [45]. Unexpectedly, our results indicate that a low barbed-end capping activity favors actin assembly on branched networks in conditions in which the pool of polymerizable actin drops. This is likely due to the fact that nucleation by the Arp2/3 complex generates a large number of barbed ends, which remain free to elongate in the absence of capping. In an apparent contradiction, depletion of capping protein in many cell types abolishes the lamellipodium and promotes the formation of filopodial structures [28,55,56]. However, these data do not indicate to our knowledge whether actin filaments elongating in these structures are nucleated and elongated by formins or if they simply elongate due to the absence of sufficient capping in the lamellipodium.

As the size, shape, and activity of many other cellular structures are tightly regulated by a large number of factors in a competitive environment, we expect that a better understanding of their regulation will emerge from the principles presented in this work.

Material and methods

Plasmid constructions

Plasmids for protein expression and purification. Standard methods were employed for DNA manipulations. DNA fragments corresponding to gene coding sequences were obtained by PCR amplification (Phusion High-Fidelity DNA Polymerase, Finnzymes) of *Saccharomyces cerevisiae* genomic DNA. For WASp overexpression (Gst-Las17(375-Cter)-6xHis), the coding sequence was cloned in pGEX-4T-1 plasmid between BamHI and NotI. This construction of Las17 keeps the poly-L-proline domains necessary for the interaction with profilin. For capping protein overexpression (6xHis-Cap1/Cap2), the coding sequence of Cap1 was cloned in pRSFDuet-1 plasmid between BamHI and NotI and the coding sequence of Cap2 was cloned between BglII and XhoI. For tropomyosin overexpression, the coding sequence of Tpm1 was cloned in pRSFDuet-1 plasmid between NdeI and PacI with an Ala-Ser extension at the N-terminal end in order to increase actin affinity [57]. For formin overexpression (Gst-Bni1(1215-Cter)-TEV-9xHis, corresponding to the proline rich FH1 and the FH2 domains) the coding sequence of Gst and Bni1 were cloned simultaneously in a yeast multicopy plasmid (2 μ URA3) under the control of a GAL1 promoter between BamHI and PacI [48].

Plasmids for overexpression and phalloidin labeling. The coding sequences of profilin, ADF/cofilin, tropomyosin (Tpm1 and Tpm2 with an Ala-Ser extension), and capping protein (Cap1 and Cap2) were cloned in yeast 2 μ multicopy plasmids under the control of a GAL1 promoter. The plasmids are derived from the pRS425 and pRS426-based constructs that we use for protein purification in yeast, but a stop codon was kept at the end of the coding sequences. Control of capping protein overexpression was also verified with a 9 myc-tagged Cap2.

Protein expression, purification and labeling

Actin. *S. cerevisiae* actin was purified from commercially purchased baker's yeast (Kastalia, Lesaffre, Marcq-en-Baroeul, France), as described in [48,58]. As labeling on cysteines impacts profilin binding, rabbit muscle actin was purified and labeled on lysines with Alexa Succinimidyl Ester dyes, as described in [59,60].

Arp2/3 complex. *S. cerevisiae* Arp2/3 complex was purified from commercially purchased baker's yeast (Kastalia, Lesaffre, Marcq-en-Baroeul, France) based on a protocol modified from [61,62]. Droplets of liquid yeast culture were frozen in liquid nitrogen and ground in a

steel blender (Waring, Winsted, CT, USA). 50 g of ground yeast powder was mixed with a lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT supplemented with protease inhibitors (Set IV, Calbiochem, Merck4Biosciences, Darmstadt, Germany). The yeast extract was cleared by centrifugation at 160,000 g for 30 min and fractioned by a 50% ammonium sulfate cut. The insoluble fraction was dissolved and dialyzed in HKME buffer (25 mM Hepes pH 7.5, 50 mM KCl, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT, 0,1 mM ATP) overnight at 4 ˚C. This fraction was then loaded onto a 2-ml Glutathione-Sepharose 4B (GE Healthcare Life Sciences, Piscataway, NJ, USA) column pre-charged with GST-N-WASp-VCA [61,62]. Bound Arp2/3 complex was purified with HKME buffer and eluted with 20 mM Tris-HCl pH 7.5, 25 mM KCl, 200 mM $MgCl_2$, 1 mM EGTA and 1 mM DTT. Fractions of interest were detected by Bradford assay, pooled, concentrated with an Amicon Ultra 4-ml device (Merck4-Biosciences, Darmstadt, Germany), and dialyzed against HKG buffer (20 mM Hepes, pH 7.5; 200 mM KCl; 6% glycerol).

Formin. *S. cerevisiae* formin was overexpressed in yeast (MATa, leu2, ura3-52, trp1, prb1-1122, pep4-3, pre1-451) under the control of a GAL1 promoter for 12 h at 30 ˚C with 2% galactose. Cells were harvested by centrifugation, frozen in liquid nitrogen, and ground in a steel blender (Waring, Winsted, CT, USA). For protein purification, 5 g of ground yeast powder was mixed with 45 ml of HKI10 buffer (20 mM Hepes, pH 7.5; 200 mM KCl; 10 mM Imidazole, pH 7.5), supplemented with 50 μl of protease inhibitors (Set IV, Calbiochem, Merck4Biosciences, Darmstadt, Germany), and thawed on ice. The mixture was centrifugated at 160,000 g for 30 min, and the supernatant was incubated with 500-μl bed volume of Nickel-Sepharose 6 Fast Flow (GE Healthcare Life Sciences, Piscataway, NJ, USA) for 2 h at 4 ˚C. Bound protein was batch purified with HKI20 buffer (20 mM Hepes, pH 7.5; 200 mM KCl; 20 mM Imidazole, pH 7.5) and was TEV-cleaved from Nickel-Sepharose for 1 h at room temperature. The protein was concentrated with an Amicon Ultra 4 ml device (Merck4Biosciences) and dialyzed against HKG buffer.

WASp and capping protein. *S. cerevisiae* WASp were overexpressed in Rosetta 2(DE3) pLysS cells. Bacteria were lysed in 20 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 200 mM NaCl, 0,1% Triton X-100, 5% glycerol and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). After clearing the bacterial lysate by centrifugation at 160,000 g for 20 min, the protein was subjected to a first-step purification on Glutathione-Sepharose beads following the manufacturer's recommendations. It was then eluted with 100 mM L-glutathione reduced and further purified by the addition of Nickel-Sepharose beads 6 Fast Flow (GE Healthcare Life Sciences, Piscataway, NJ, USA). An elution was performed using HKI500 buffer (20 mM Hepes, pH 7.5; 200 mM KCl; 500 mM Imidazole, pH 7.5). The protein was concentrated with an Amicon Ultra 4-ml device (Merck4Biosciences) and dialyzed against HKG buffer.

S. cerevisiae capping protein was purified with a similar protocol including only a Nickel-Sepharose purification step in HKI20 buffer (20mM Hepes pH 7.5, 200 mM KCl, 20 mM imidazole pH 7.5, 0,1% Triton X-100, 10% glycerol).

Tropomyosin. *S. cerevisiae* tropomyosin was overexpressed in Rosetta 2(DE3)pLysS cells and purified based on a protocol modified from [63]. Briefly, cells were lysed in extraction buffer (50 mM imidazole-HCl, pH 6.9, 300 mM KCl, 5 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche)) by sonication and boiled for 10 min. Cell debris and insoluble proteins were pelleted at 300,000 g for 20 min. The clear supernatant containing pure tropomyosin was finally dialyzed into 50 mM KCl, 10 mM Tris-HCl pH 7.5 and 0.5 mM DTT overnight at 4 ˚C.

Profilin and ADF/cofilin. *S. cerevisiae* profilin and cofilin were overexpressed in Rosetta 2(DE3)pLysS cells and purified as described in [48,64,65].

Actin assembly assays

Functionalization of beads. Polystyrene microspheres (2 μm diameter, 2.5% solids [w/v] aqueous suspension, Polysciences, Inc) were diluted 10 times in HK buffer (20 mM Hepes pH 7.5, 150 mM KCl) and incubated with 100 nM Las17 for 30 min on ice. Beads were saturated with 1% bovine serum albumin (BSA) for 15 min, washed and eventually stored on ice in HK buffer supplemented with 0,1% BSA. Bni1 (1 μM) was coated on glutathione-coated particles (4.37 μm diameter, 0.5% solids [w/v] aqueous suspension, Spherotech, Inc) with a similar protocol.

Bead motility assays. Unlabeled and labeled actins were mixed to reach a final labeling percentage of 2%. Actin was prepolymerized in KMEI buffer (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM Imidazole pH 7.8) for 1 h at room temperature. To initiate actin network assembly, Las17 and Bni1-coated beads (10^4 beads of each type per μl) were incubated with F-actin and other proteins in a motility buffer (20 mM Hepes pH 7.5; 100 mM KCl; 2 mM EGTA; 2 mM $MgCl_2$; 50 mM DTT; 5 mM ATP; 0.3 mg/ml glucose; 0.03 mg/ml catalase; 0.15 mg/ml glucose oxidase; 0.8% methylcellulose 1,500 cP and 0.5% BSA). Standard optimal protein concentrations are 8 μM F-actin, 15 μM profilin, 1 μM capping protein, and 250 nM Arp2/3 complex unless otherwise stated. These values were kept as reference values for all experiments related to the titration of accessory proteins. For every titration experiment, the range of concentration of the tested protein was chosen so that its effect could be analyzed from its absence up to saturating amounts for both actin networks. Highest concentrations therefore represent situations that are out of the physiological regime.

Image acquisition, processing and analysis. For in vitro actin assembly assays, images were acquired on a Zeiss Axio Observer Z1 microscope equipped with a 100x/1.4NA Oil Ph3 Plan-Apochromat objective and a Hamamatsu ORCA-Flash 4.0LT camera. Images were acquired with Zen 2.3 blue edition.

Data quantification. All set of images were taken using the same light intensity and exposure time. Intensity values were measured over time before reaching the steady state using Fiji (Version 1.52e). Fluorescence of the background was subtracted for each value. Actin assembly rates r were calculated as follows:

$$r = \frac{I_{t_2} - I_{t_1}}{t_2 - t_1},$$

in which I is the intensity value at a given time point t. Actin assembly rates were normalized to their maximal values. Data were quantified and statistically analyzed with GraphPad Prism 6.05, and plotted with R using the package ggplot2 (https://ggplot2.tidyverse.org). For all conditions, experiments were repeated independently at least 3 times. Data presented in the manuscript correspond to one set of experiments, which includes a minimum of $n = 30$ data points per condition from a minimum of 6 independent beads. In all plots of actin assembly rates, error bars indicate standard deviations.

Actin organization in yeast

Yeast cell fixation and phalloidin staining. Yeast strains used in this study were obtained from previous published studies, including [21,36,39,44,66]. To assess actin organization, yeast cells were fixed and stained with fluorescently labeled phalloidin as described in [43,67]. Briefly, strains were grown in YPD medium at 25 ˚C to early/mid log phase and fixed at 25 ˚C with 4% formaldehyde for 1 h. Thermosensitive mutants were cultivated for 1 h at 37 ˚C before fixation. For protein overexpression, wild-type and cells carrying the plasmid of interest were grown in synthetic yeast medium supplemented with 2% galactose before fixation. Cells were

then stained overnight at 25 ˚C with AlexaFluor-568-phalloidin and washed three times with PBS before imaging.

Cell imaging. Cells were imaged in PBS– 70% glycerol on a Leica TCS SP8 STED inverted confocal microscope using a 63x, 1.4 NA oil Plan Apochromatic objective lens in combination with a hybrid detector. Full z-stacks were acquired with LAS X software.

Data quantification. Number of actin cables and patches was scored from a maximal intensity projection done with Fiji. For all conditions, a minimum of 50 cells were imaged and analyzed. In all plots, error bars indicate standard deviations and symbols * indicate significant statistical differences compared to the wild-type conditions using a Student *t* test ($p < 0.0001$). All data used to draw conclusions are available in S1 Data.

Supporting information

S1 Data. Data for Figs 2, 3, 4, 5 and 6 and S1, S2, S3 and S4 Figs.
(XLSX)

S1 Fig. Fluorescence intensity analysis of branched and linear actin networks. The underlying data can be found within S1 Data. A. Fluorescence intensity of F-actin networks were quantified over time for standard conditions (in black) and for the most extreme perturbations performed (lowest protein concentration in green; highest protein concentration in red). Lines indicate linear regressions. B. Rate of actin assembly around 0.5-μm diameter WASp-coated microbeads in the presence of fluorescent actin, profilin, and capping protein as a function of the Arp2/3 complex concentration, normalized to the maximum value. Arp2/3, actin-related protein 2/3; F-actin, filamentous actin; WASp, Wiskott–Aldrich syndrome protein.
(TIF)

S2 Fig. Wide field snapshots of the actin cytoskeleton organization in yeast strains presented in this study. Scale bars: 5 μm. The underlying data can be found within S1 Data. A. Budding yeast cells fixed and labeled with fluorescent phalloidin at the indicated temperatures. B. Quantification of Fig 2E based on total intensities and not numbers of actin structures. C. In vivo deviation index, based on structures intensities, calculated in the presence of DMSO and 200 μM CK-666. CK-666, Arp2/3 complex inhibitor I.
(TIF)

S3 Fig. Branched and linear actin networks emerging from bead surfaces do not influence each other in these reconstituted assays. The underlying data can be found within S1 Data. A. Rate of actin assembly around WASp-coated microbeads as a function of the profilin concentration when formin-coated beads are not present. B. Rate of actin assembly around formin-coated microbeads as a function of the profilin concentration when WASp-coated beads are not present. C. In vitro deviation index, calculated as a function of the profilin concentration, measured from data obtained in (A) and (B). WASp, Wiskott–Aldrich syndrome protein.
(TIF)

S4 Fig. Modulation of the branched-to-linear actin network balance by capping protein at steady state and in conditions in which actin assembly is initiated from G-actin. Quantification of actin networks assembly at steady-state around WASp-coated and formin-coated microbeads in the presence of fluorescent actin, Arp2/3 complex, profilin, and variable concentrations of capping protein. Left plots indicate rates of actin assembly around WASp-coated and formin-coated microbeads as a function of the capping protein concentration, normalized to the maximum value. Right plot indicates the in vitro deviation index calculated as a function of the capping protein concentration. The underlying data can be found within S1 Data. A.

Condition in which the accessory proteins were incubated for 2 h at room temperature with prepolymerized actin (F-actin) before addition of the microbeads. B. Condition in which 8 μM G-actin, 15 μM profilin, and 250 nM Arp2/3 were incubated for 2 h at room temperature before addition of the microbeads. C. Condition in which 4 μM of G-actin, 12 μM of profilin, 250 nM Arp2/3, and the microbeads were incubated at room temperature simultaneously. Red dots indicate that the intensity of actin networks was difficult to quantify due to the uncontrolled barbed-end assembly around WASp-coated beads at low concentration of capping protein. The image is a fluorescence snapshot of an actin network assembled around WASp-coated microbeads in the presence of 4 μM fluorescent G-actin, 250 nM Arp2/3 complex, 12 μM profilin, and 100 nM capping protein, taken 30 min after the initiation of the experiment. Scale bar: 5 μm. Arp2/3, actin-related protein 2/3; F-actin, filamentous actin; G-actin, globular actin; WASp, Wiskott–Aldrich syndrome protein.
(TIF)

S5 Fig. Overexpression of capping protein in yeast. Western blot control of capping protein overexpression with a 9 myc-tagged Cap2. Pgk1 is a loading control. Cap2, capping protein 2.
(TIF)

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Supervision: Alphée Michelot.

Validation: Adrien Antkowiak.

Visualization: Adrien Antkowiak.

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PLOS BIOLOGY

SHORT REPORTS

Mechanical stiffness of reconstituted actin patches correlates tightly with endocytosis efficiency

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Abstract

Clathrin-mediated endocytosis involves the sequential assembly of more than 60 proteins at the plasma membrane. An important fraction of these proteins regulates the assembly of an actin-related protein 2/3 (Arp2/3)-branched actin network, which is essential to generate the force during membrane invagination. We performed, on wild-type (WT) yeast and mutant strains lacking putative actin crosslinkers, a side-by-side comparison of in vivo endocytic phenotypes and in vitro rigidity measurements of reconstituted actin patches. We found a clear correlation between softer actin networks and a decreased efficiency of endocytosis. Our observations support a chain-of-consequences model in which loss of actin crosslinking softens Arp2/3-branched actin networks, directly limiting the transmission of the force. Additionally, the lifetime of failed endocytic patches increases, leading to a larger number of patches and a reduced pool of polymerizable actin, which slows down actin assembly and further impairs endocytosis.

Introduction

Endocytosis is a key process that regulates the internalization of extracellular material and the homeostasis of the plasma membrane. Clathrin-mediated endocytosis, which is the main pathway to endocytosis in yeast, has been characterized as a multistage process implicating more than 60 different proteins [1–3]. The process begins with the assembly at the membrane of an inner layer coat of adaptor proteins, among which clathrins form a scaffold for the forming vesicle. Nucleation promoting factors of actin assembly are later recruited at endocytic sites before the assembly of a network of actin filaments branched by the actin-related protein 2/3 (Arp2/3) complex [1,4,5]. These actin networks, which surround the forming vesicle, have a diameter of about 200 nm and therefore appear as small patches at the resolution of a fluorescence microscope [6]. Actin polymerization at the plasma membrane is essential in yeast to counteract the high turgor pressure present in these cells and to provide the force necessary for the deformation of the membrane and its internalization [7,8]. Any perturbation brought

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Abbreviations: Abp140, actin-binding protein 140; Arp2/3, actin-related protein 2/3; CH, calponin homology; CLR, calponin-like repeat; DoG, difference of Gaussian; GFP, green fluorescent protein; Las17, yeast homolog of WASp; NPF, nuclear promoting factor; PRD, proline-rich domain; Sac6, yeast homolog of fimbrin; Scp1, yeast homolog of calponin; WASp, Wiskott-Aldrich syndrome protein; WT, wild type.

to actin assembly, for example, by the addition of latrunculin A, has dramatic effects on the rate and on the efficiency of endocytosis [9].

Actin patch assembly is correlated with the recruitment of numerous accessory proteins which decorate actin filaments [2]. These proteins affect actin filament organization and dynamics and are crucial in providing actin networks with optimized properties for efficient endocytosis. Among these accessory proteins, actin crosslinkers have been identified as a potential mechanical linkage between actin filaments in addition to the Arp2/3 complex branches. In budding yeast, three putative actin crosslinkers have been identified and localized to actin patches [2]: 1) the yeast homolog of fimbrin (Sac6), which is composed of four tandem calponin homology (CH) domains organized in two distinct actin-binding regions; 2) the yeast homolog of calponin (Scp1), which is composed of an N-terminal CH domain, a proline-rich domain (PRD), and a C-terminal calponin-like repeat (CLR). Calponin actin bundling occurs through two separate actin-binding regions in the PRD and CLR regions; 3) actin-binding protein 140 (Abp140), which is a much less studied yeast-specific actin-bundling protein. Sac6 is reported to be an important protein for endocytosis in yeast, particularly for the initiation of membrane bending and for reaching scission stage [10]. On the contrary, absence of Scp1 or Abp140 causes no obvious change in cells. However, a strong genetic interaction between Sac6 and Scp1 highlights a partially redundant function of these two proteins [11,12].

The impact of crosslinkers on the mechanics of entangled actin filaments has been extensively studied by rheometry in the past decades [13–15]. Creating permanent bonds between entangled filaments drastically increases the elastic moduli from a value on the order of 1 Pa to a value in the 100 Pa vicinity. More recently, several teams have been able to measure the mechanics of reconstituted branched actin networks that are polymerized from a surface and crosslinked by the Arp2/3 complex [16–19]. These networks, which are closer to the ones present in yeast endocytosis, differ from entangled filaments in their much higher density because of the growth process that occurs from a surface. They also already possess mechanical bonds between their filaments (the Arp2/3 complex), although each of these bonds is connected to three strands of filament instead of four in the case of crosslinkers. In the case of endocytic actin patches, it is not clear where crosslinking of filaments occurs within a highly branched network of actin filaments, in which the average branch-to-branch distance is 50 nm (which corresponds to only 20 actin subunits) [20]. Bieling and colleagues showed a moderate effect of crosslinkers on branched networks grown from a mix of purified proteins [17]. The strongest effect was obtained on the linear elasticity in presence of alpha-actinin or filamin (3.1 kPa for both to be compared to 1.6 kPa without crosslinkers). To our knowledge, no measurement has been conducted before this study on the effect of crosslinkers on networks reconstituted from cell extracts.

The link between actin networks' elasticity and their ability to invaginate the membrane is still unclear. The general idea is that a soft actin network will inefficiently transmit the polymerization force necessary to bend the membrane and surpass turgor pressure [8]. While many theoretical models have been proposed to describe force production in endocytosis [8,21], very few take explicitly into account the rigidity of the actin meshwork. The exception is the work of Tweten and colleagues [22], which uses a continuous mechanics approach to model actin growth leading to endocytosis. In this model, the actin network cannot efficiently produce endocytosis if the elastic modulus is below 80 kPa. Beyond the rigidification that allows for the transmission of force, crosslinkers could possibly store in their deformation elastic energy that could be released to aid endocytosis. Computational modeling demonstrates that because of the twisting of actin filaments, fimbrin crosslinkers could store as much as one-sixth of the energy needed for endocytosis [23]. Additional elastic energy could be also stored in the bending of filaments to be released upon endocytosis through crosslinkers unbinding.

While actin crosslinkers rigidify actin networks in vitro, recent genetics and cell biological studies suggest additional effects of these proteins in cells. Binding of fimbrin to actin filaments is competitive with other actin-binding proteins such as tropomyosin [24,25]. Consequently, loss of function of fimbrin in fission yeast correlates with a mislocalization of tropomyosin to actin patches, therefore complicating the interpretation of endocytic phenotypes [24]. Indeed, multiple direct and indirect effects of actin crosslinkers make their precise contribution to clathrin-mediated endocytosis difficult to isolate. Our goal here is to achieve a better understanding of endocytosis through the combination of phenotypic observations in cells and direct measurements of the mechanical properties of actin patches in the presence or in the absence of these crosslinkers. In this study, we took advantage of the possibility to reconstitute endocytic actin patches from yeast protein extracts [26]. We combined this approach with a high-throughput mechanical measurement technique using chains of magnetic microbeads [27]. We used a top-down approach to compare the mechanical properties of actin patches assembled from various mutant yeast strains with the corresponding endocytic defects in cells.

Results

Three putative actin crosslinkers impact differently membrane invagination during clathrin-mediated endocytosis

Previous studies demonstrated that a careful study of actin patch dynamics provides meaningful information about the formation and internalization efficiency of vesicles during clathrin-mediated endocytosis [1,28]. Defects in actin network assembly impact force generation at the membrane and result, in some cases, in ineffective or abortive endocytic events [29–31].

Because effects of mutations can vary in different yeast backgrounds, we aimed at quantifying precisely actin patch dynamics in wild-type (WT) cells and for a variety of mutant cells in the *Saccharomyces cerevisiae* S228C strain used in this study. Selected mutants include single knockouts for genes encoding all the proteins described as actin crosslinkers, namely *sac6Δ*, *scp1Δ*, *abp140Δ*, and the double-mutants *sac6Δ abp140Δ* and *sac6Δ scp1Δ*. We first analyzed precisely the timing and trajectories of actin patches for all strains by recording the fluorescence intensity of the actin-binding protein 1 (Abp1)–green fluorescent protein (GFP) actin reporter over time (Fig 1). We performed this analysis manually over a small number (15–30) of well-defined actin patches (S1A Fig), as well as with an automatic detection method that was less precise but enabled us to analyze phenotypic differences over a much larger number of actin patches (>300). The two methods obtained similar results (S1B Fig). Our results show that actin patches in *abp140Δ* cells have a similar median lifetime to WT cells ($p = 0.6$) (Fig 1B). In agreement with a previous study [12], we found that actin patches in *sac6Δ*, *sac6Δ scp1Δ*, and *sac6Δ abp140Δ* cells have a longer lifetime than WT cells (33%, 33%, and 30% more than WT respectively, $p < 10^{-3}$), but contrary to this study, we found no effect of the deletion of Scp1 on the lifetime of actin patches ($p = 0.81$). Such differences with this previous publication could be explained by the use of different yeast backgrounds between the two studies. Increased patch lifetimes are signatures of a delayed actin network assembly and are correlated with defective internalization of actin patches (Fig 1C and 1D). Analysis of maximum displacements indicates that most patches in WT, *scp1Δ*, and *abp140Δ* cells migrate efficiently up to a median value of 0.3 μm, 0.26 μm ($p = 0.16$), and 0.27 μm ($p = 0.2$), respectively. On the contrary, *sac6Δ* and the double-mutant *sac6Δ scp1Δ* and *sac6Δ abp140Δ* cells have limited movements up to a median value of 0.11 μm, 0.14 μm, and 0.11 μm ($p < 10^{-3}$ as compared to WT) (Fig 1C). The fraction of patches undergoing displacements larger than 200 nm, which is considered as a typical distance above which an endocytic event has successfully occurred, drops from 66% for WT to 33% in *sac6Δ* cells (Fig 1D). Mutants lacking only Scp1 or Abp140 behave

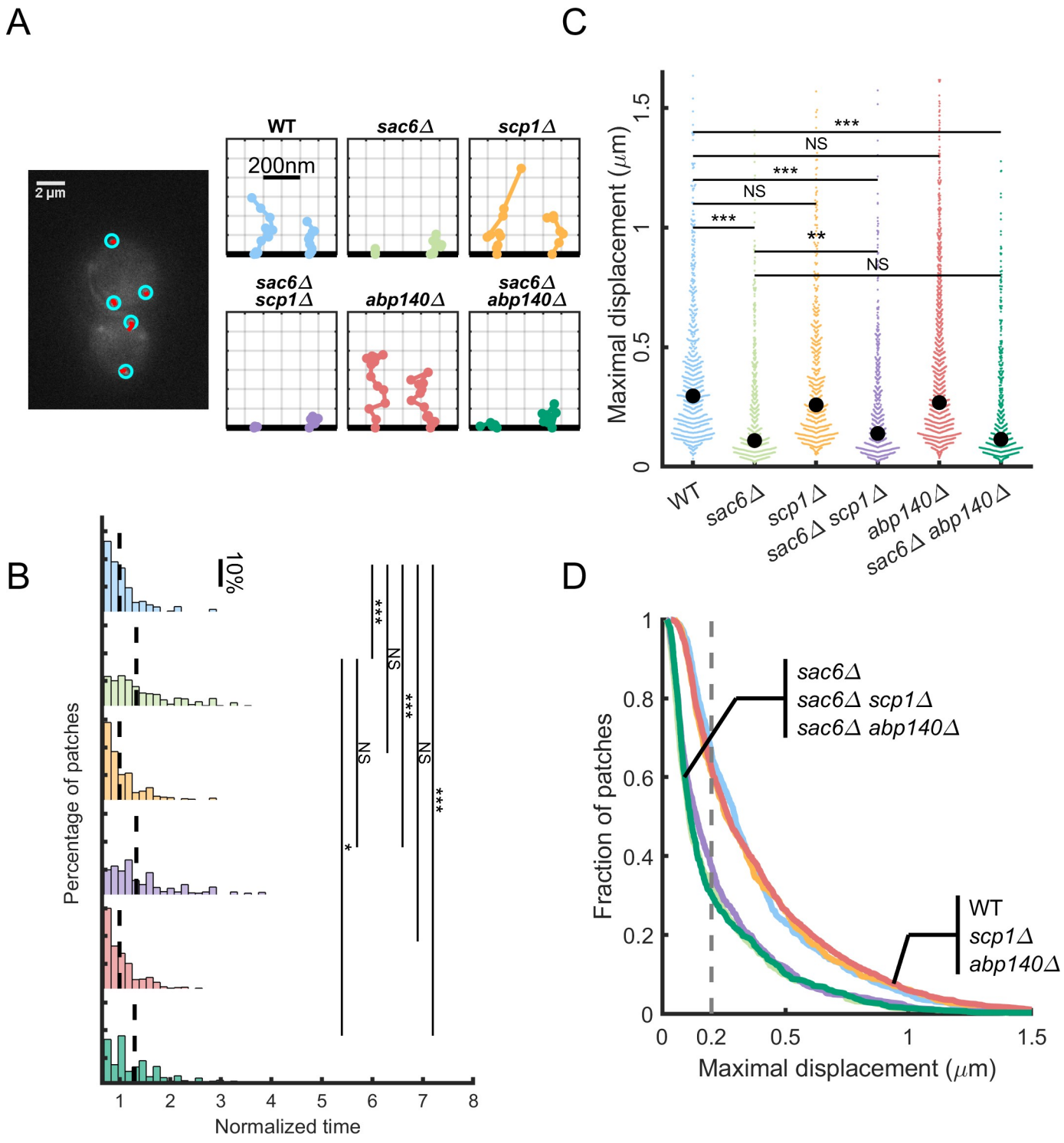

Fig 1. Endocytic phenotypes in *S. cerevisiae* cells lacking putative crosslinkers. The underlying data can be found within S1 Data. (A) Observation and tracking of endocytic patches in the different strains used in this study. Left: Epifluorescence image of a representative yeast cell expressing Abp1 fused to GFP (Abp1–GFP) and analyzed to obtain the trajectories of its endocytic actin patches. Actin patches are detected (blue circles) and are progressively tracked (red line) using the plugin TrackMate. Right: Plots showing, for the six different strains analyzed in this study, two typical examples of patch trajectories towards the interior of the cell. The plasma membrane is represented by the horizontal bold line tangent to the *x* axis, while the *y* axis represents the normal to the membrane and is oriented towards the interior of the cell. Identical scales have been used for the two axes. (B) Histogram of actin patch normalized lifetimes extracted from the analysis for the different strains. Lifetimes are normalized by the median of the WT strain for each replicate. Dashed line is the median. (C) Maximal displacement of the actin patches. The maximal displacement represents the distance between the first point of the trajectory and the furthest point inside the cell. Each colored dot

represents a patch. Black dots indicate the median. (D) Endocytosis efficiency. Proportion of patches that have a maximal displacement larger than a given value (*x* axis) for each strain (same color code as in B). The dashed line at 0.2 μm indicates the typical displacement above which endocytic events have successfully occurred. Abp1, actin-binding protein 1; Abp140, actin-binding protein 140; GFP, green fluorescent protein; NS, not significant; Sac6, yeast homolog of fimbrin; Scp1, yeast homolog of calponin; WT, wild type.

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similarly to the WT (61% and 62%, respectively), whereas the double mutants *sac6Δ scp1Δ* and *sac6Δ abp140Δ* show an impaired rate of success (37% and 32%, respectively), which is comparable to *sac6Δ* (33%).

Overall, trajectories of actin patches suggest that while Sac6 has a major impact on actin patch internalization and efficient endocytosis, the absence of Scp1 or Abp140 has little to no effect on endocytosis.

Actin patches of all mutants lacking Sac6 assemble slower and are more numerous

Membrane deformation during endocytosis is powered by the local growth of an actin gel attached to the plasma membrane by adaptor proteins [2]. Defective endocytosis in mutant strains can derive from multiple effects on the actin cytoskeleton. As the level of recruitment of Abp1–GFP varies significantly among strains [10,12], we first aimed at evaluating whether rates of actin assembly in individual endocytic patches were affected in cells.

The patch assembly was monitored in cells by following the fluorescence increase of Abp1–GFP. The rate of actin assembly, which is the slope of the intensity versus time curve during the assembly phase (see Materials and methods), decreases significantly from WT cells to *sac6Δ abp140Δ* and *sac6Δ scp1Δ* cells (68% and 65% of WT rates, respectively; $p < 10^{-4}$), and to *sac6Δ* cells (59% of WT rates, $p < 10^{-4}$) (Fig 2A). The rates of *abp140Δ* cells and *scp1Δ* cells were also lower but to a lesser extent (91% and 87% of WT rates, respectively; $p < 10^{-4}$).

Such effects are unexpected for mutants of proteins usually described as putative crosslinkers and require further investigation to interpret the endocytic phenotypes. We took advantage of a previous protocol in which the formation of actin patches is reconstituted in vitro from yeast cellular extracts around artificial microbeads. Most of the proteins involved in endocytosis, including actin, are soluble and remain present in large amount in these extracts. The networks assembled from the extracts have a similar protein composition to cellular actin patches and include in particular all the crosslinkers mentioned in this study [26]. We investigated whether the slowed-down actin assembly is directly due to the absence of Sac6 by following actin assembly from the extracts in vitro on beads. We grafted superparamagnetic beads of 4.5 μm diameter with the yeast homolog of Wiskott–Aldrich syndrome protein (WASp), Las17, which is a nucleating promoting factor (NPF) of the Arp2/3 complex. These beads are mixed with passivated beads and yeast protein extracts, which triggers the growth of an actin shell from the functionalized beads. During the growth, a low homogenous magnetic field (2 mT) drives the self-organization of the beads into linear chains (Fig 2B). Because of the nanometer resolution on the bead displacements [18], the evolution of the actin shell thickness can be precisely monitored. As previously observed with HeLa cell and *Xenopus* egg protein extracts [32,33], the thickness of the shell reaches a plateau after a few minutes (Fig 2B). We compared the growth of actin networks reconstituted from protein extracts generated from WT, *sac6Δ*, and *scp1Δ sac6Δ* cells (Fig 2B). When reconstituted from a WT protein extract, the growth starts after a delay, and the thickness reaches a plateau within 8 to 10 minutes. In *sac6Δ* protein extracts, actin assembly occurs much faster, and the thickness plateaus within 2 to 3 minutes. This time window is short, and the plateau is usually reached before data acquisition is possible with our experimental setup. The double-mutant protein extract assembles actin

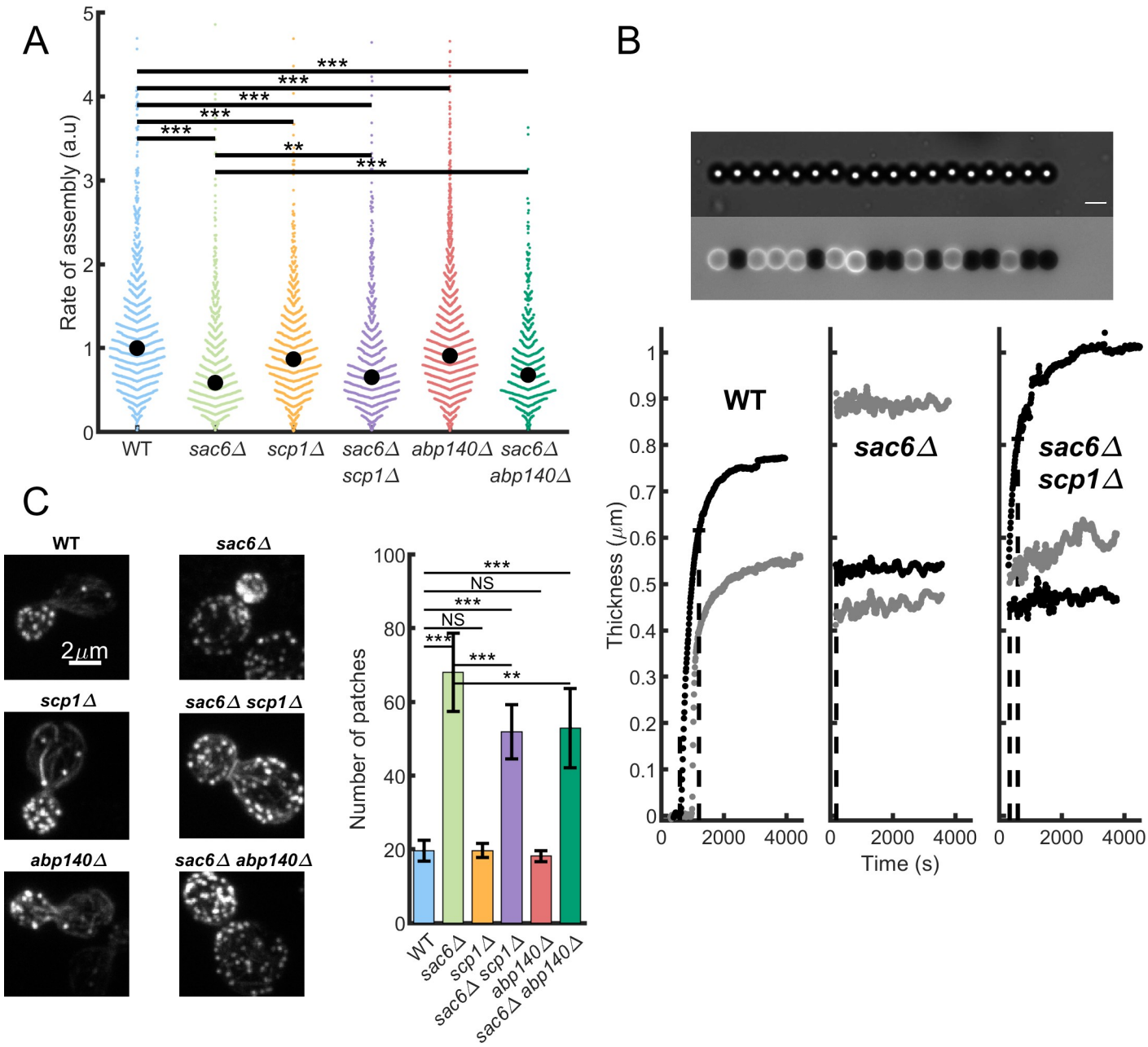

Fig 2. Dynamics of actin assembly. The underlying data can be found within S2 Data. (A) Net rate of actin patch assembly in cells. The rates are obtained from the curves of the fluorescence intensity as a function of time during the phase of patch assembly for all strains used in this study. Each colored dot is a measured patch. Black dot is the median. (B) Dynamics of reconstituted actin patch assembly. Growth of actin networks from protein extracts at the surface of magnetic beads. Top: Chain of magnetic beads in the bright field (top) and fluorescence (bottom) channels. On the fluorescence image, actin shells grown around magnetic beads (4.5 μm diameter) are visible in light gray, whereas black beads are nonactivated beads grafted with BSA. The image in bright field is focused below the equatorial plane of the beads to obtain nanometer resolution on the position of the center of the bead. Scale bar is 5 μm. Bottom: Evolution of the thickness of the shell with time for WT, *sac6Δ*, and *sac6Δ scp1Δ* protein extracts. For each strain, a few curves have been represented (gray and black). (C) Number of actin patches for the different strains. Left, typical images used to count the number of patches per cell. Such images are the result of the z-stack projection of the maximum intensities. Actin has been labeled by phalloidin–Alexa568. Right, average number of actin patches in the different strains. Error bars are standard errors. Abp140, actin-binding protein 140; a.u., arbitrary unit; BSA, bovine serum albumin; NS, not significant; Sac6, yeast homolog of fimbrin; Scp1, yeast homolog of calponin; WT, wild type.

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shells faster than the WT extract but slower than the *sac6Δ* extract. This qualitative description is confirmed by quantitative measurements of the typical timescale of growth: WT, $\tau = 830$

seconds ± 70 seconds ($N = 27$); *sac6Δ scp1Δ*, τ = 360 seconds ± 40 seconds ($N = 17$); *sac6Δ*, τ = 195 ± 10 seconds ($N = 30$). These results show that actin assembly on the beads is slower in WT extracts than in extracts generated from *sac6Δ* cells. This effect could be explained by a competition between Sac6 binding and Arp2/3 branching observed in vitro on single filaments [34]. The fast growth of actin gels in *sac6Δ* extracts is in opposition to the measured rates of actin assembly in *sac6Δ* cells (Fig 2A) and suggests strongly that the differences observed in the patch assembly dynamics between WT cells and *sac6Δ* cells cannot be attributed to a direct effect of Sac6 on actin nucleation or polymerization.

Another possibility is that the filamentous/globular actin ratio could be altered in some strains, reducing rates of actin assembly through the reduction of the available pool of polymerizable actin. To test this hypothesis, we fixed cells and stained the actin cytoskeleton with fluorescent phalloidin, and the cells were imaged by confocal microscopy (Fig 2C). In agreement with this hypothesis, we found that while the number of actin patches per cell is similar for WT, *abp140Δ*, and *scp1Δ* cells, with, respectively, 19.6 ± 2.8, 19.7 ± 1.9, and 18.2 ± 1.5 patches per cells on average, the number of patches is increased to 68.1 ± 10.6, 51.8 ± 7.4, and 52.9 ± 10.7 patches per cells for *sac6Δ*, *sac6Δ scp1Δ*, and *sac6Δ abp140Δ* cells, respectively (mean ± standard error). Our results show that the slower patch assembly observed in mutants lacking Sac6 is possibly due to an excessive assembly of actin in cells rather than to a direct effect of Sac6 on the actin polymerization dynamics. An increasing number of patches with a constant pool of actin should lead to a reduced amount of monomeric actin available and a slowed-down actin patch assembly.

Networks reconstituted from yeast protein extracts are mechanically stiffer than those reconstituted from a minimal mix of purified yeast proteins

We investigated next the mechanical properties of actin patches reconstituted from the different extracts, expecting that the absence of crosslinkers may have a quantifiable impact on the elastic properties of the gels. These measurements were performed with the same superparamagnetic beads as the ones used in the previous section. After growth at low magnetic field (2 mT) that drives the self-organization of the beads into linear chains (Fig 2A), the magnetic field is ramped up to 80 mT for 7.5 seconds. This timescale has been chosen to match the typical timescale of endocytosis measured in vivo (see previous section). Increasing the magnetic field increases the dipolar magnetic force between each pair of microbeads from a few piconewtons to about one nanonewton. This force deforms the shell of actin present on one of the two beads, and the shell thickness is measured by video microscopy during deformation (Fig 3A). We analyzed the deformation of the shell as a function of the imposed force to get the elastic properties of the shell. The procedure includes fitting the force-deformation curve with a Hertz model of the contact between elastic spheres, modified to take into account the limited thickness of the elastic shell [35].

Actin networks reconstituted from WT yeast extracts have an elastic modulus of median value of 5.7 kPa ($n = 107$). The distribution of measured values is rather broad and is best represented by a logarithmic distribution (Fig 3A). The broad distribution validates our experimental approach, the throughput of which, as compared to other techniques, is relatively high because of the self-organization of the beads [18].

We compared networks reconstituted from yeast protein extracts to networks reconstituted from a minimal mix of purified yeast proteins. This minimal mix is composed of actin, profilin, Arp2/3 complex, and capping protein [36–38]. We used concentrations of actin, Arp2/3, and capping protein that were measured in the extracts by western blot [39]. Profilin was added at a 3-fold excess over actin. Magnetic bead experiments showed that these networks

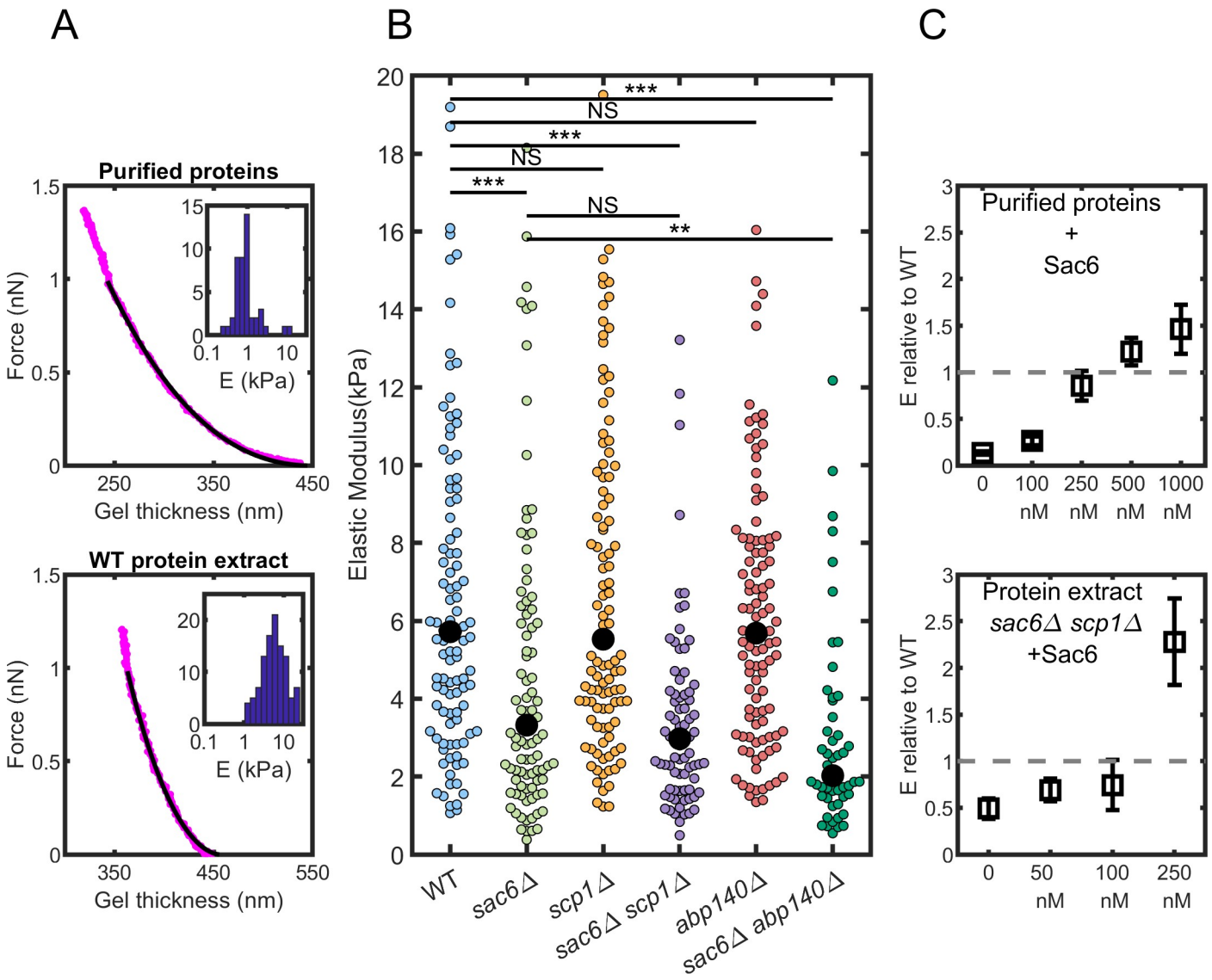

Fig 3. Elastic properties of reconstituted actin networks. The underlying data can be found within S3 Data. (A) Principle of the measure. Force-distance curves obtained on an actin network and distribution of the elastic moduli extracted from many force-distance curves (inset). Gels are assembled from a mix of purified proteins (top, 46 gels) or from WT protein extracts (bottom, 107 gels). (B) Elastic moduli of the actin gels assembled from protein extracts. Colored symbols are individual measurements; black dots are the median values. (C) Effect of the addition of purified Sac6 on the elastic modulus. Elastic moduli normalized by the elastic modulus of WT protein extract when Sac6 is added to mix of purified proteins (top) or *sac6Δ scp1Δ* protein extract (bottom). Error bar calculation is described in the Materials and methods section. Abp140, actin-binding protein 140; NS, not significant; Sac6, yeast homolog of fimbrin; Scp1, yeast homolog of calponin; WT, wild type.

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were much softer (Fig 3A). The median elastic modulus was measured at 0.86 kPa ($n = 46$), which is widely different from the elastic modulus of gels assembled in yeast extracts ($p < 10^{-4}$). This difference can be due to the absence of structural proteins such as crosslinkers or to a change in the network architecture due to other accessory proteins present in the extract. This value is also significantly smaller than previously published measurements on actin networks grown in a similar system of purified mammalian proteins [17,27]. This is, however, not very surprising because actin filaments purified from budding yeast are known to be more flexible than actin filaments purified from skeletal muscles [40].

Sac6, Scp1, and Abp140 impact actin patch stiffness differently

To investigate the effect of Sac6, Scp1, and Abp140 on the elasticity of the actin networks, we reconstituted networks with protein extracts from mutant knockouts (Fig 3B). The elastic modulus for actin networks reconstituted in *sac6Δ* extracts is significantly softer than for networks assembled in WT extracts, with a median at 3.3 kPa ($p < 10^{-4}$, $n = 98$). On the contrary, our measurements show no significant softening for actin networks reconstituted in *scp1Δ* and *abp140Δ* extracts, with respective elastic modulus medians of E = 5.5 kPa ($n = 109$) and E = 5.7 kPa ($n = 106$). To test whether the presence of Sac6 could mask an effect on the elasticity from Scp1 and Abp140, we also tested extracts from double knockout mutants lacking Sac6 and either Scp1 or Abp140. Actin networks reconstituted from the double knockout mutant *sac6Δ scp1Δ* were measured at a median modulus E = 3.0 kPa ($n = 79$), which is softer than for WT extracts ($p < 10^{-4}$) but not significantly different from *sac6Δ* extracts ($p < 0.2$). On the contrary, actin networks assembled from the double knockout *sac6Δ abp140Δ* extracts were measured at a median modulus E = 2.0 kPa ($n = 51$), which is softer than both WT ($p < 10^{-4}$) and *sac6Δ* extracts ($p < 0.005$).

In conclusion, the absence of Sac6 has an effect on the elasticity in every combination tested here (*sac6Δ*, *sac6Δ scp1Δ*, and *sac6Δ abp140Δ*). The absence of Scp1 does not change the elasticity of reconstituted networks even in the absence of Sac6, whereas the absence of Abp140 softens the networks, but only in the absence of Sac6.

Addition of purified Sac6 restores the rigidity of reconstituted actin patches

We wanted to control whether the softening of actin gels was really due to an absence of the proteins from the meshwork and not to any other indirect effect during the assembly. To verify the central role of Sac6 in the rigidity of actin networks, we purified Sac6 and added it to both biomimetic assays, i.e., when actin gels are assembled from a minimal mix of protein or from the *sac6Δ scp1Δ* cell extract (Fig 3C). In both cases, the elastic modulus increases as a function of the Sac6 concentration in a dose-dependent manner.

On actin gels assembled from cell extracts, the elastic modulus is not significantly different from actin gels assembled in WT extracts when *sac6Δ scp1Δ* extracts are supplemented with 100 nM Sac6 prior to the experiments. They become significantly more rigid at 250 nM ($p < 10^{-6}$). On actin gels assembled with purified proteins, the elastic modulus reaches the value of WT extracts actin gels when [Sac6] = 250 nM, which is a value larger than the value measured in the extracts (25 nM). Above 250 nM of Sac6, the elastic modulus overpasses the value of the WT (Fig 3C). The fact that the amount of Sac6 that needs to be added to the mix of purified proteins is larger than the value measured in the extracts highlights the role of the other accessory proteins present in the cell extract. These proteins may impact the architecture of the networks; among them, one can think of unidentified crosslinkers or other side-binding proteins that will modify the flexibility of the filaments.

Discussion

Sac6 plays a dominant role in actin networks rigidity

In the past, most studies have investigated the impact of actin accessory proteins on the mechanical properties of actin networks separately from their phenotypic effect in cells. The experimental setup presented in this study allows for the closest comparison possible between the effect of actin crosslinkers for clathrin-mediated endocytosis and their quantitative contribution to actin network stiffening.

Our results demonstrate that actin crosslinking stiffens actin networks at endocytic sites. Our results also reveal that the three putative actin crosslinkers stiffen actin networks with different efficiencies. Sac6 plays a major role in the stiffening of actin patches, and its absence is sufficient to decrease dramatically the rigidity of actin networks by 42%. Abp140 also seems to contribute to the stiffening of actin networks, although to a lesser extent: its absence in the presence of Sac6 does not soften actin patches, but its absence combined with an absence of Sac6 has a clear softening effect on actin networks (65%). A possible explanation could be that Sac6 dominates the stiffening of actin networks so that any contribution of Scp1 or Abp140 is not detectable in its presence. Another explanation could be that Abp140 and Sac6 compete to a certain extent in their binding to actin, for example, if Sac6 binds to actin filaments with a higher affinity than Abp140. Abp140 may then act as a mechanical rescue that would compensate for an absence of Sac6. The situation is very different for Scp1, for which we could not detect any significant reduction of actin network stiffness in its absence even in the absence of Sac6. These observations do not necessarily prove that Scp1 has no crosslinking activity but that, in a physiological context, its effect is negligible when compared to Sac6. It is possible that the affinity of Scp1 for actin filaments is lower than that of Abp140 and Sac6. Alternatively, Scp1 could have a typical timescale of binding and unbinding much smaller than the timescale probed here (7.5 seconds), which was chosen to be as close as possible to the duration of endocytosis. Scp1 may attach and detach many times during the lifetime of the patch, resulting in the absence of rigidification of the actin network both in vivo and in vitro.

Relation between patch rigidity and endocytosis efficiency

In yeast endocytosis, actin networks contribute to the invagination of the plasma membrane, and the force exerted by the polymerization of the actin filaments is needed to counteract the turgor pressure. In current mechanistic models, for this force to be effective, it is postulated that the network is attached to the deformed membrane via the coat proteins and polymerizes from the plasma membrane, pushing the network and the invaginated membrane inside the cell [41,42]. The turgor pressure acts as a force by unit surface that opposes the growth of the actin network. Because the network is elastic, the opposing force can compress the growing network, limiting its lengthening. If the network developing the force is too soft, most of the growth will be counteracted by this compression. In the following, we outline a simple model to support this idea. We consider an elastic material that gains a slab of thickness l in a time t because of the growth process and that is submitted to a compressive force by unit area $-\sigma_{op}$. Because the new slab is deformed, the actual increase in thickness (x) is going to be smaller than l. In a linear elastic material, the compressive force and the strain are related by Hooke's law: $\sigma = E\,\varepsilon$, where σ is the force by unit area (called stress in mechanics), E the Young modulus of the material, and $\varepsilon = \log(1 + \delta l/l)$ the true strain experienced by the material with $\delta l = -(l - x)$. From the previous expression, we get $-\sigma_{op} = E \log(x/l)$. Hence the increase in thickness of the growing gel is $x = l \exp(-\sigma_{op}/E)$. In term of gel growth speed, this increase leads to $v = l/t \exp(-\sigma_{op}/E)$ by dividing the previous equation by t.

If the opposing force by unit area is small compared to the elastic modulus ($\sigma_{op} << E$), the deformation is limited, and the effect of the gel elasticity is minimal on the growth speed. One can simplify the equation to $v = l/t(1 - \sigma_{op}/E)$. However, if the opposing stress is of the same magnitude as the elastic modulus (σ_{op} approximately E), the compression in the gel is important, and the speed of growth will depend exponentially on the elasticity of the network.

In this paper, we have measured the elastic modulus of an actin network resembling the actin structure assembled during yeast endocytosis. We obtained values of 5.7 kPa for gels assembled from WT extracts and 3.3 kPa for gels assembled from *sac6Δ* extracts. These moduli

can be compared to the force by unit area opposing the growth. In the case of endocytosis in yeast, the opposing force is mainly due to the turgor pressure that hampers invagination of the membrane inside the yeast. Pressure as high as 0.6 MPa has been reported [43]. If one would directly take the turgor pressure as the opposing stress, an unrealistic large deformation would be obtained: the actin network is too soft to grow under such pressure. A few factors can, however, limit the magnitude of the opposing stress. If the surface on which the turgor pressure is acting, i.e., the patch of clathrin that will form the vesicle, is smaller than the surface of the actin network, the stress opposing the growth will be reduced by the ratio of these areas. This seems to be the case, as recent super-resolution imaging of endocytic patches measured the coat proteins extending to 20–50 nm outer radius, while the actin-associated proteins extended to 80–100 nm [5]. Another factor that has been proposed to mitigate the opposing stress is the lever effect [44]: if the actin filaments are pushing at an angle on the coat proteins, the force opposing the growth will be lowered. The tight organization of the endocytic patch with actin activators at the periphery of the patch [5] would also facilitate this effect. Although a precise estimation of the opposing force in endocytosis is not within our grasp yet, it is reasonable to assume this stress is of the same order of magnitude as the typical elastic moduli of actin networks. The elasticity of the network is thus going to heavily impact its growth speed.

Consequences of defective actin crosslinking on clathrin-mediated endocytosis

The precise characterization of the contribution of actin crosslinkers to actin network rigidity enabled us to investigate possible correlations with the corresponding phenotypes in yeast cells. We analyzed the effects of an absence of crosslinkers at the patch scale and at the whole cell scale.

We observed that loss of actin patch stiffness is well-correlated with a decreased efficiency of endocytosis. This is quantified by the increased patch lifetime and the reduced internalization efficiency, which are both clear signatures of defective endocytosis. In addition, the rate of actin assembly is also lower in mutant cells. Therefore, defective endocytosis could be the result of softer actin networks, slower rates of actin assembly, or both. Increased patch lifetime is linked to the presence of a larger number of actin patches in mutant cells, and it is therefore likely that a larger fraction of actin is polymerized in these cells. It is now well-accepted in the field that the pool of polymerizable actin is not in large excess but is, on the contrary, limited to the point that the different actin networks assembled in the cell compete for this limited reservoir [45,46]. In this context, it is likely that a larger number of patches in the mutant cells correspond to a larger amount of polymerized actin and therefore to a lower amount left of polymerizable actin.

Conclusion

Overall, we suggest that our data are consistent with a chain-of-consequences model (Fig 4). In this model, we propose that the first effect of a decrease in actin crosslinking is to impact the stiffness of actin networks. As a consequence, actin networks are less efficient in transducing forces to the membrane, and failed endocytosis increases actin patch lifetime. A longer lifetime of actin patches for an equivalent rate of endocytic events initiation explains why more actin patches are present in cells on average. As actin networks compete for a limited pool of actin monomers, an increase in the number of actin patches increases the amount of polymerized actin and decreases the pool of monomeric actin. Lower amounts of monomeric actin therefore reduce the rate of actin assembly, which causes further failures of endocytosis.

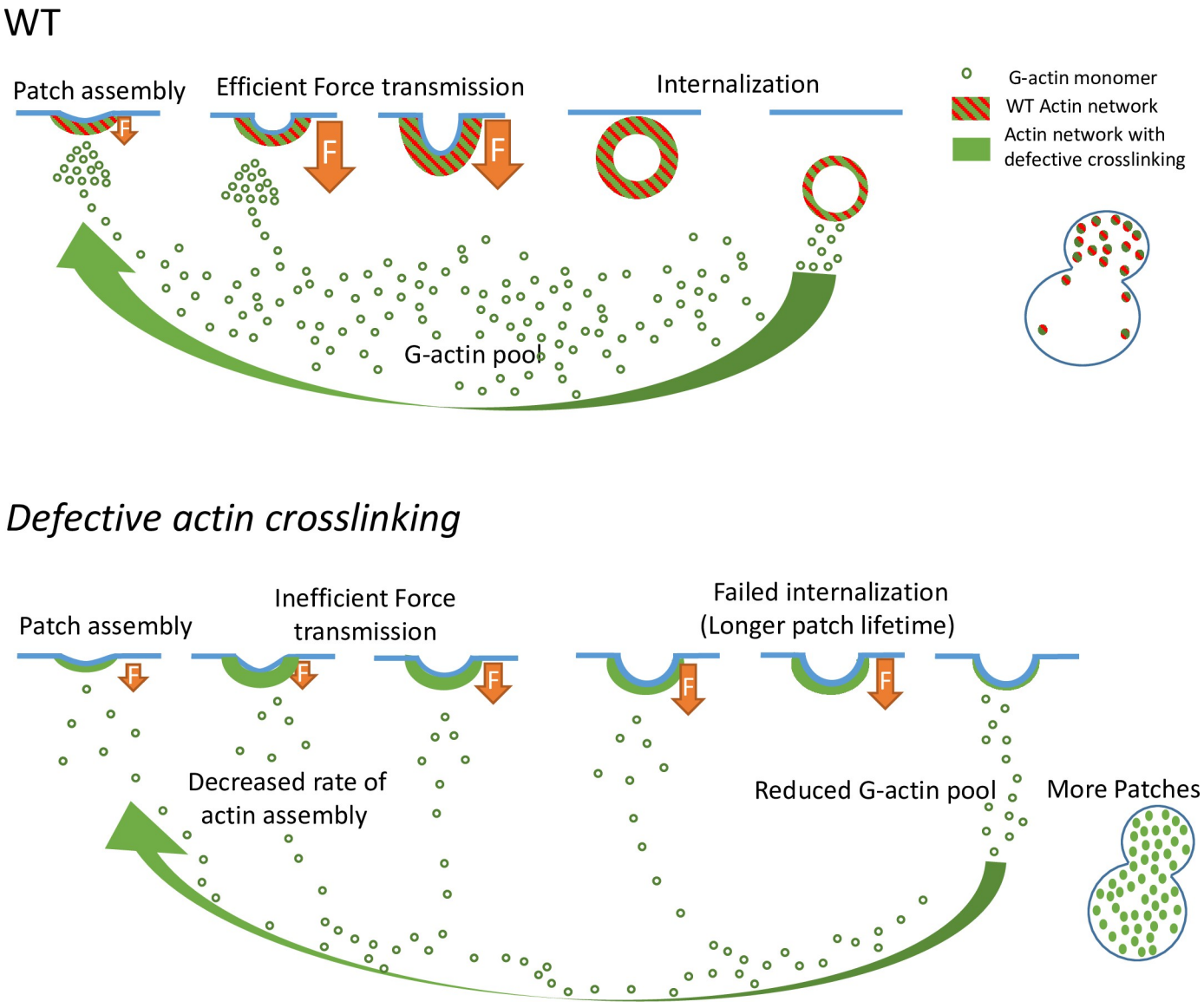

Fig 4. Schematic of a chain-of-consequence model for yeast endocytosis. This model proposes that in WT cells (upper panel), the mechanical properties of actin patches (green filled dots) are optimized for efficient force transmission at the plasma membrane (in blue) and a rapid internalization of the endocytic vesicles. Subsequently to the internalization of an endocytic vesicle, the actin network disassembles and contributes to replenishing the limiting pool of actin monomers (green open dots) up to a normal level. In mutant strains, defective actin crosslinking (crosslinkers are in red) impacts the stiffness of actin networks. Force transduction to the membrane is less efficient, increasing actin patch lifetime and the proportion of failed endocytic events. Longer lifetime of actin patches for an equivalent frequency of endocytic events initiation accounts for a higher number of actin patches in these mutant cells. Because the pool of actin monomers is limiting, an increased number of actin patches means that more actin is polymerized in cells and that less monomeric actin is present. Depletion of the pool of monomeric actin leads to a reduced rate of actin assembly, which causes further failures of endocytosis. WT, wild type.

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Materials and methods

Yeast strains

Yeast mutants were generated with standard procedures as described in [47] and are derived from a S228C strain.

Yeast cell imaging and analysis

Live cell imaging. Cells were cultured at OD 0.5 to 0.7 and immobilized on coverslips coated with 0.1 mg · ml^{-1} of concanavalin A. Cells were imaged using a Zeiss Axio Observer Z1 microscope equipped with a 100×/1.4 NA oil Ph3 Plan-Apochromat objective (Zeiss, Oberkochen, Germany) and a Hamamatsu ORCA-Flash 4.0 LT camera (Hamamatsu, Hamamatsu City, Japan). Images were acquired with Zen 2.3 blue edition software. Endocytic actin patch tracking analysis was performed by following the centroid intensity of Abp1-GFP patches using the Fiji plugin TrackMate [48] (https://imagej.net/TrackMate). Individual patches were detected with a median filter and a difference of Gaussian (DoG) particle detection algorithm for spots with a diameter of approximately 0.5 μm. A selection of particles was subsequently done for particles with a contrast above 0.1. With the Linear Assignment Problem tracker, the patch tracks were reconstituted. From the tracks files, we plotted the lifetime and the maximum displacement, which is the distance between the initial and the furthest position on the trajectory. The data were processed through custom scripts in MATLAB R2017b (The MathWorks, Natick, MA, USA). For each patch, position, lifetime, maximum displacement, and intensity were recorded for the analysis, and only patches with a lifetime longer than 5 seconds and displaying an increase followed by a decrease in intensity were kept for the analysis [49]. From the intensity over time curve, we measured the slope of the intensity during the actin assembly (from 4 seconds before the maximum of the intensity). Because the patch lifetime and the rate of actin assembly significantly differed between replicates, we normalized them by the median of the WT observed on the same day, pooled the normalized data of the different strains, and tested the statistical significance of the pooled data with Wilcoxon rank test (Fig 1B).

Phalloidin staining. Cells were cultured at 25˚C in standard rich media (YPD) and collected at OD 0.5–1, fixed in 4% formaldehyde/YPD for 2 hours, washed twice in PBS, and stained overnight with 250 nM phalloidin-Alexa568 (Invitrogen Ref. A12380; Carlsbad, CA, USA). Cells were imaged using a Leica TCS SP8 X White Light Laser confocal microscope (Leica, Wetzlar, Germany) equipped with an HC PL APO CS2 100×/1.4 NA oil objective and a hybrid detector. Z-stack images were collected every 0.3 μm with Las X 3.5.5.19976 software. Patches were counted manually from the maximum intensity z-stack projections.

Yeast extract preparation

Yeast extracts were prepared based on a published protocol [50]. Briefly, yeast strains were cultured in standard rich media (YPD) at 30˚C to an OD_{600} of 1.5–2. Cells were harvested by centrifugation, resuspended in cold water, and centrifuged again. Pellets were flash-frozen in liquid N_2 and ground by mechanical shearing in a Waring blender. To each gram of yeast powder were added 100 μl of Hepes buffer (100 mM [pH 7.5]) and 10 μl of protease inhibitors (Protease Inhibitor Cocktail Set IV, Calbiochem, Merck4Biosciences). Yeast powder was gently mixed on ice with the buffer, progressively thawed, and centrifuged for 20 minutes at 50,000 × *g*. The cleared supernatant was collected, kept on ice, and used within 3 hours.

Protein expression, purification, and labeling

Yeast actin purification and labeling. *S. cerevisiae* actin was purified from commercially purchased baker's yeast (Kastalia, Lesaffre, Marcq-en-Baroeul, France) as described in [51] and labeled with Alexa dyes as described in [52].

Yeast WASp (Las17), profilin (Pfy1) and capping protein (Cap1 and Cap2). *S. cerevisiae* WASp, profilin, and capping protein were expressed and purified from *Escherichia coli* Rosetta 2(DE3)pLysS cells as described in [53].

Yeast Arp2/3 complex. Endogenous *S. cerevisiae Arp2/3* complex was purified from a myc-tagged yeast strain as described in [54].

Yeast fimbrin (Sac6). *S. cerevisiae*'s fimbrin was overexpressed from a multicopy plasmid (2 μ URA3 *Pgal1-SAC6-9×HIS*) in yeast (*MATa*, *leu2*, *ura3-52*, *trp1*, *prb1-1122*, *pep4-3*, *pre1-451*) under the control of a GAL1 promoter. Protein expression was induced for 12 hours at 30˚C with 2% galactose. Cells were harvested by centrifugation, frozen in liquid nitrogen and grinded in a steel blender (Waring, Winsted, CT, USA). For protein purification, 5 g of ground yeast powder was mixed with 45 ml of HKI10 buffer (20 mM Hepes [pH 7.5], 200 mM KCl, 10 mM imidazole [pH 7.5]) supplemented with 50 μl of protease inhibitors (Set IV, Calbiochem, Merck4Biosciences, Darmstadt, Germany), and thawed on ice. The mixture was centrifugated at 370,000 × *g* for 40 minutes, and the supernatant was incubated with 500 μl bed volume of Ni-Sepharose 6 Fast Flow (GE Healthcare Life Sciences, Piscataway, NJ, USA) for 2 hours at 4˚C. Bound protein was batch purified with HKI500 buffer (20 mM Hepes [pH 7.5], 200 mM KCl, 500 mM imidazole [pH 7.5]), concentrated with an Amicon Ultra 4 ml device (Merck4Biosciences), dialyzed for 2 hours in HKG buffer (20 mM Hepes [pH 7.5], 200 mM KCl, 6% glycerol), aliquoted, and flash-frozen in liquid nitrogen.

Actin assembly on superparamagnetic beads

Functionalization and passivation of beads. Superparamagnetic microspheres (Dynabeads M-450 Epoxy, 4.5 μm diameter; Thermo Fisher Scientific, Waltham, MA, USA) were diluted 10 times in HK buffer (20 mM Hepes [pH 7.5], 150 mM KCl) and incubated with 100 nM Las17 for 30 minutes on ice. Tubes were rotated during incubation to avoid the sedimentation of the beads. Beads were then saturated with 1% BSA for 15 minutes, washed, and eventually stored on ice in HK buffer supplemented with 0.1% BSA.

Actin assembly from yeast extracts. 0.75 μl of Las17-functionalized microbeads and 0.75 μl of BSA-passivated microbeads were added to 28.5 μl of yeast extract supplemented with 0.1 to 0.3 μM of Alexa568-labeled actin (10%–5% labeled) to induce formation of branched actin networks. Actin networks were assembled for 20–30 minutes before introduction into homemade flow chambers. Chambers were sealed with a mix of 1/3 Vaseline, 1/3 lanolin, and 1/3 paraffin.

Actin assembly from purified proteins. 0.75 μl of Las17-functionalized microbeads and 0.75 μl of BSA-passivated microbeads were added to a protein mix containing 1.2 μM Alexa568-labeled actin, 3.6 μM profilin, 25 nM Arp2/3 complex, and 400 nM capping protein in a motility buffer containing 20 mM Hepes (pH 7.5), 100 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 3 mM ATP, 5 mM DTT, and 1.5% BSA.

Timescale of actin network growth around beads

For each pair of beads, we measured the gel thickness at the plateau by averaging the thickness over the last 500 seconds. For gels assembled from WT protein extracts, the time t_0 at which the growth starts and the time t_{80} at which the thickness is 80% of the final thickness are measured from the curves. The timescale of the growth τ is the difference between t_{80} and t_0. When gels were assembled from *sac6Δ* extracts, the plateau was reached before the recording of the thickness was possible: τ was then considered as the first time point, even if this is an overestimation as compared to WT. This was also the case for some gels assembled from the double-mutant protein extracts. For these gels, when possible, t_{80} was measured, and τ is directly chosen to be t_{80} because t_0 is not measurable.

Mechanical measurements

The mechanical experimental setup consists of a Zeiss Axio A1 inverted microscope with a modified stage hosting two coils of diameter 88 mm and width 44 μm, with a soft iron core

and 750 turns of copper wire. A Bipolar Operational Power supply (Kepco, New York City, NY, USA) feeds the coils with up to 5 A electrical current corresponding to an 80 mT homogenous magnetic field just above the objective. The objective is a 100× oil immersion Apochromat with 1.4 NA. Timelapse images were recorded with an ORCA-Flash 4.0 CMOS camera (Hamamatsu). A Labview (National Instruments, Austin, TX, USA) custom program allows controlling of the field with simultaneous image acquisition.

All chains of beads present in the experimental chamber undergo deformation during the ramp up of the field. To avoid any plastic effect that would deform permanently the zone of contact between the beads, we used a new experimental chamber with fresh actin shells for each repetition.

Data analysis was performed with Image J (NIH, Bethesda, MD, USA) and MATLAB. The position of the bead center was determined with the center of mass of the white pixels on images taken at a focus below the equatorial plane of the beads. The force was calculated from the value of the field, the magnetic susceptibility of the beads, and their position. The value of the elastic modulus was obtained with a fit of the deformation of a shell by a bead as a function of the force. A criterion of goodness of fit ($R^2 > 0.9$) was used to remove aberrant curves. The comparison between the networks reconstituted from different mutant was made with a Wilcoxon–Mann–Whitney nonparametric test. For the experiments with an increasing concentration of purified crosslinkers, a random effect model from meta-analysis [55] was used to take into account a higher variability of measure between different extract preparations than within the same extract preparation. This model uses the inverse variance inside a sample as a weight for the calculation of the mean. Standard errors were computed from both these weights and the variance between samples.

Supporting information

S1 Fig. Comparing the TrackMate method with a manual tracking of the patches. (A) Example of the intensity of an actin patch over time. The manual and automatic tracking lifetimes are indicated with a black arrow and gray arrow, respectively. The higher threshold on detection explains the shorter lifetime measured with TrackMate. The slope of the red dashed line represents the rate of actin assembly. (B) Manual tracking of patch lifetime in the different strains. Each colored dot represents a patch. Black dots indicate the median. (C) Patch lifetime distribution. Patches with a lifetime lower than 5 seconds (dashed line) were not taken into account to avoid detection artifacts.
(TIF)

S2 Fig. (A) Histogram of patch lifetime extracted from the analysis for the different strains. The median (represented by a dashed line) is 8.0 seconds for WT, 7.2 seconds for *scp1Δ* and *abp140Δ*, 10.4 seconds for *sac6Δ* and *sac6Δ abp140Δ*, and 9.6 seconds for *sac6Δ scp1Δ*. The data are pooled from several replicates and non-normalized. (B) Rate of actin patch assembly in the cells. The rates are obtained from the curves of the fluorescence intensity as a function of time during the phase of patch assembly for all strains used in this study. Each colored dot is a measured patch. The median (represented by a black dot) is 1,800 a.u. for WT, 2,100 a.u. for *scp1Δ*, 1,900 a.u. for *abp140Δ*, 1,200 a.u. for *sac6Δ*, 1,000 a.u. for *sac6Δ abp140Δ*, and 1,600 a.u. for *sac6Δ scp1Δ*. The data are pooled from several replicates and non-normalized. Abp140, actin-binding protein 140; a.u., arbitrary unit; Sac6, yeast homolog of fimbrin; Scp1, yeast homolog of calponin; WT, wild type.
(TIF)

S1 Data. Raw data for Fig 1.
(XLSX)

S2 Data. Raw data for Fig 2.
(XLSX)

S3 Data. Raw data for Fig 3.
(XLSX)

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