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Brigitte M. Jockusch *Editor*

The Actin Cytoskeleton

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This book is devoted to the memory of Klaus Weber (1936–2016).
His work on actin's expression and evolution stimulated cell biological research for decades.

Preface

This volume comprises 12 articles on the various functional tasks of actin. This protein, identified almost 160 years ago as specifically engaged in muscle contraction (see Mommaerts 1992 and Szent-Gyorgyi 2004), has made a dramatic career and is now considered a universal player in almost every motility phenomenon in all eukaryotic kingdoms. The history of actin research contains many interesting, even amusing details that are reported in Schleicher and Jockusch (2008) and Jockusch and Graumann (2011).

One of the turning points in considering the role of actin in life came in 1973 when Hugh Huxley, previously convinced that actin is a structural component specifically operating in the contractile machinery of striated muscle, was confronted with data on the cross-reactivity of muscle myosin with filamentous components in nonmuscle cells. In a well-received lecture, he then suggested that actomyosin components are generally responsible for cellular motility (Huxley 1973). One year later, it was shown that antibodies elicited against muscle actin decorate prominent filament bundles in nonmuscle cells (Lazarides and Weber 1974). The next decade revealed that most organisms contain not one, but many different actin genes leading to proteins with slightly different functions (see for example Vandekerckhove and Weber 1978) and actin genes then rapidly evolved in structure and number (Vandekerckhove and Weber 1984).

The chapters compiled in this volume shed light on the present state of the art in understanding the stunning functional versatility of actin—a small, rather compact protein of approximately 42 kDa. The introductory article by Ampe and Van Troys, revealing the present knowledge on actin isoforms, their differential expression and mutations, is followed by a detailed description of the precise organization of actomyosin filaments, the myofibrils, into sarcomeres, the functional units in striated muscle (Sanger JW et al.). Muscle myosin, the other prominent protein also originally identified in striated muscle (cf. Szent-Gyorgyi 2004)}, belongs to a large and very diverse family, many members of which show special adaptations to execute various motility processes in conjunction with actin (Masters, Kendrick-Jones and Buss). The conservation of the original architectural building plan of actomyosin filaments, modified by a large panel of actin binding partners, is highlighted in the article on the different cytoskeletal structures in migrating vertebrate cells (Lehtimäki, Hakala and Lappalainen).

Another spotlight is then switched on by the contribution by Steffen, Stradal and Rottner, who emphasize the many proteins involved in signaling cascades that mediate between membranes and the actin cytoskeleton. This is not restricted to the plasma membrane but is relevant also for internal membrane-enveloped vesicles. Thus, specific protein factors regulate the actin cytoskeleton in shape changes and vesicular trafficking.

The next three chapters reveal the interactions between pathogenic prokaryotes and the actin cytoskeleton of their hosts: to optimize bacterial multiplication, many different bacterial toxins can covalently modify the actin molecule itself, but also regulators of actin polymerization and organization (Aktories, Schwan and Lang). The articles on pathogenic bacteria (Tran Van Nhieu and Romero) and viruses (Marzook and Newsome) describe the crosstalk of these pathogens with their host cells. Adhesion, infection and intracellular multiplication depend on highly sophisticated ways invented by the pathogens to abuse the actin cytoskeleton—with disastrous consequences on cellular and tissue integrity.

The task of the actin cytoskeleton in organizing cells into functional tissues and organs is the topic of the next chapter which relates cell and tissue polarity to the intrinsic polarity of actin filaments and their associated adhesive structures (Luxenburg and Geiger). Notably, the vital role of actin polarity and dynamics in providing mechanical support for tissue development during morphogenesis is not confined to vertebrates, but also relevant for insects, as shown in the article on *Drosophila* development (Brüser and Bogdan).

Another complex of actin functions is presented in the chapter on nuclear actin (Viita and Vartiainen). This activity of actin has led a rather cryptic life for several decades, since the existence of nuclear actin had been doubted. Today, there is a wealth of solid and well-accepted evidence that actin operates also in the nucleus; however, the exact details of its function and molecular organization are still a matter of debate.

Finally, there remains a catalogue of unanswered questions. Many mechanistic details on actin filament generation, life span and regulation by the different binding partners are still left for future investigations (Pollard).

The progress in understanding the structure, function and regulation of the actin cytoskeleton made over the past seven decades reflects the enormous advance in techniques. Molecular genetics, refined biochemistry and structural biology, and live microscopy on cells expressing fluorescently tagged proteins have been employed in the studies reported here. Notably, pharmacology has contributed to many fundamental studies on actin dynamics, which is sensitive not only to bacterial toxins but also to a large and still growing number of toxins from sponges and fungi. The synthesis of specific inhibitors will be a platform for future pharmacological research in diagnosis and therapy of the numerous human diseases based on mutations in actin isoforms, actin regulators and myosin.

Braunschweig, Germany Brigitte M. Jockusch

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Contents

Mammalian Actins: Isoform-Specific Functions and Diseases

Christophe Ampe and Marleen Van Troys

Contents

Abstract

Actin is the central building block of the actin cytoskeleton, a highly regulated filamentous network enabling dynamic processes of cells and simultaneously providing structure. Mammals have six actin isoforms that are very conserved and thus share common functions. Tissue-specific expression in part underlies their differential roles, but actin isoforms also coexist in various cell types and tissues, suggesting specific functions and preferential interaction partners. Gene deletion models, antibody-based staining patterns, gene silencing effects, and the occurrence of isoform-specific mutations in certain diseases have provided clues for specificity on the subcellular level and its consequences on the organism level. Yet, the differential actin isoform functions are still far from understood in detail. Biochemical studies on the different isoforms in pure form are

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just emerging, and investigations in cells have to deal with a complex and regulated system, including compensatory actin isoform expression.

Keywords
Actin disease mutation • Actin genes • Baraitser-Winter syndrome • Cytoskeleton • Deafness • Isoform switching • Myopathy • Thoracic aortic aneurysms and dissection

1 Introduction

Eukaryotes have three types of cytoskeletal elements in their cytosol: tubulins, intermediate filaments, and actin filaments (Zampieri et al. 2014). The latter are the main component of the actin cytoskeleton that contain, next to actin molecules, actin-binding proteins and regulatory proteins. The actin cytoskeleton differs from cell type to cell type thereby gaining differential functions. For instance, in muscle cells, actin and myosin filaments form the contractile apparatus together with specific actin-binding proteins. Especially in striated muscle cells, this is organized in a highly regular manner (Sanger et al. 2016; Gautel and Djinovic-Carugo 2016). In (cultured) non-muscle cells, the actin cytoskeleton is more versatile and can be highly dynamic depending on the subcellular localization (Lehtimäki et al. 2016). Indeed, as we will describe below, actin filaments are formed via a complex polymerization cycle. The formation of higher-order cellular structures (i.e., actin-rich networks or bundles) is assisted by a broad range of actin-binding proteins with distinct and sometimes overlapping activities (Pollard 2016). These dynamic structures support the formation of membrane protrusions such as lamellipodia, filopodia, (micro)spikes, microvilli, podosomes, or invadopodia (Alblazi and Siar 2015; Blanchoin et al. 2014). With the exception of microvilli, these actin-rich structures are connected to migration or invasion of cells. In addition, in cells actin bundles may form various types of stress fibers in some cases emanating from focal adhesions that attach cells to the extracellular matrix (Vallenius 2013). In addition, in most cells cortical actin structures or networks exist (Gutierrez 2012; Eghiaian et al. 2015).

All eukaryotes express at least one actin, but it is not uncommon that species express more isoforms and this is the rule in vertebrates. What is known on how these isoforms differentially contribute to the functions of the mammalian actin cytoskeleton is reviewed here. We will mainly focus on actin isoforms in the cytoplasm, since in the nucleus their specific activities have hardly been investigated, although circumstantial evidence indicates there must also be nuclear actin isoform specificity (Tondeleir et al. 2012; Bunnell et al. 2011; Lechuga et al. 2014; Almuzzaini et al. 2016) (see below). For a general discussion of actin function in the nuclear compartment, we refer to another chapter (Viita and Vartiainen 2016).

2 Actin Isoforms: A Highly Conserved Family of Proteins

The number of actin-expressing genes varies between vertebrate species, but it appears that most mammals express six isoforms of actin and do so in a tissue-specific manner. In mouse and man, one distinguishes four muscle forms, namely, alpha-skeletal muscle actin, alpha-cardiac actin, alpha-smooth muscle actin, and gamma-smooth muscle actin, and two non-muscle actins referred to as beta- and gamma-cytoplasmic actin (Table 1 and Fig. 1). The designation of alpha, beta, or gamma comes from the electrophoretic mobility on 2D PAGE (Garrels and Gibson 1976) owing to a difference in isoelectric point. This difference is due to the number and type of acidic residues at the N terminus (Fig. 1) (Vandekerckhove and Weber 1978).

As can be appreciated from the aligned sequences (Fig. 1), actin is a very conserved protein. The six mammalian actins are extremely similar with highest

Table 1 Actin isoforms and associated mouse knockout models

Protein	Gene (mouse/ human)	KO phenotype (compensatory actin expression)	Rescue (*gene*) in KO context	Reference
Alpha-skeletal muscle actin	*Acta1* *ACTA1*	Neonatal lethal (Actc, Acta2)	Viable if high expression with lower myofiber force production (*Actc*)	Crawford et al. (2002) Nowak et al. (2009), and Ochala et al. (2013)
Alpha-cardiac actin	*Actc* *ACTC*	Perinatal lethal (Acta1, Acta2)	Hypodynamic and enlarged heart (*Actg2*)	Kumar et al. (1997)
Alpha-smooth muscle actin	*Acta2* *ACTA2*	Impaired vascular contractility (Acta1)	n.a.	Schildmeyer et al. (2000)
Gamma-smooth muscle actin	*Actg2* *ACTG2*	n.a.	n.a.	
			Conditional KO: (*organ*) *phenotype*	
Beta-cytoplasmic actin	*Actb* *ACTB*	E7 E10,5 (Actg1, Acta2)	(Motor neurons) no phenotype (CNS) hyperactivity (Skeletal muscle) quadriceps myopathy (AHC) impaired stereocilia maintenance[a]	Shmerling et al. (2005), Bunnell et al. (2011), Tondeleir et al. (2012), Cheever et al. (2011), Cheever et al. (2012), Prins et al. (2011), and Perrin et al. (2010)
Gamma-cytoplasmic actin	*Actg1* *ACTG1*	Progressive hearing loss (Actb)	Progressive myopathy Impaired stereocilia maintenance[a] (AHC)	Sonnemann et al. (2006) and Perrin et al. (2010)

AHC auditory hair cell, *CNS* central nervous system, *KO* knockout, *n.a.* not available
[a]Distinct patterns with distinct onset of hearing loss

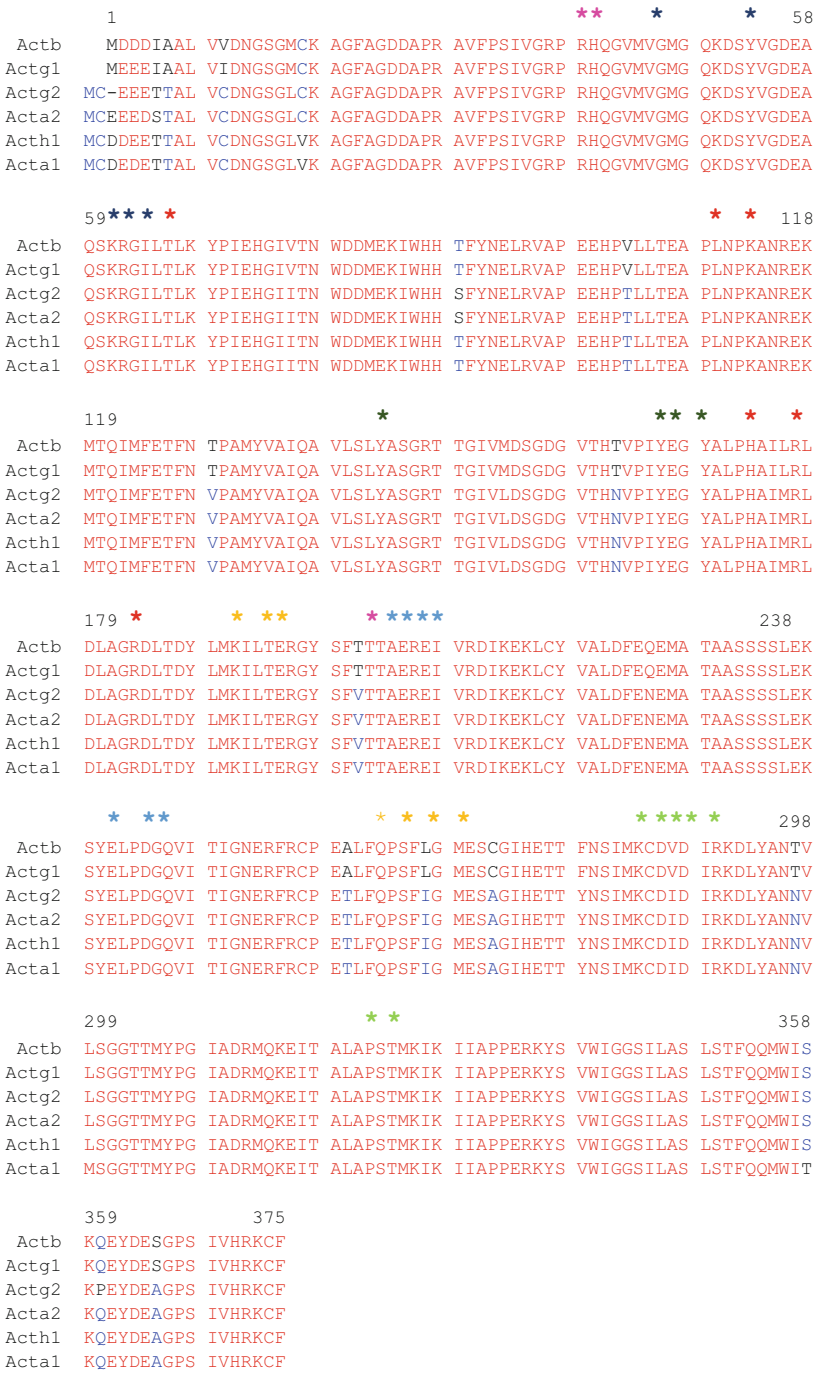

Fig. 1 Alignment of human actin sequences showing high sequence conservation: *red* indicates identical residues, and *blue* and *black* indicate that at that position at least one isoform is different,

divergence between cytoplasmic and muscle actins. It should be noted that the initiator methionine and the cysteine in muscle actins are posttranslationally removed and the first acidic residue is subsequently acetylated (the numbering used in the general section takes this into account and numbering starts with Asp1 of alpha-skeletal muscle actin; the numbering of mutated residues in disease variants is isoform specific). The non-muscle actins lack this cysteine and only the initiator methionine is removed prior to acetylation (references in Cook et al. 1991). Next to the well-established acetylation at the N terminus and the methylation of His73, mammalian actins may undergo a number of different posttranslational modifications. In general the role of these modifications is poorly understood, let alone isoform-specific effects. We refer the interested reader to Terman and Kashina (2013).

The conservation of the primary structures implies that the actin isoforms have the same fold and present similar surfaces. The actin molecule, also referred to as actin monomer or globular (G)-actin, adopts the hexokinase superfold (Bork et al. 1992; Kabsch and Holmes 1995). Traditionally it is divided in a large and small domain in between which the nucleotide (ATP or ADP) and the divalent metal ion (Mg^{2+} or Ca^{2+}) bind. Both domains are each subdivided into two structural subdomains (Fig. 2a) (Kabsch et al. 1990). Subdomain 1 contains the N and C terminus. In Fig. 2c the differential residues between the human actin isoforms have been mapped on the three-dimensional structure. This demonstrates no real hot spot domain of divergence exists with the exception of the N terminus.

Taking into account the ancient nature of the actin family, the combination of strong general conservation within all isoforms and the additional conservation within the cytoplasmic and muscle subgroups (e.g., at the N termini) is indicative of a high selective pressure both on the similarities and on the differences. This on the one hand reflects the shared properties of the actin isoforms, but on the other hand also underscores the importance of their uniqueness and functional specificity. As suggested by Gunning et al. (2015), the very limited sequence divergence in actin itself implies only subtle differences between the isoforms that may however be magnified by a more extensive divergence of the actin-associated proteins (Gunning et al. 2015). Together, this enables the diversity in dynamic actin-rich structures in specific cells or specific locations in cells, as mentioned above and further detailed below.

Fig. 1 (continued) but note that in these cases, it usually is a conserved substitution. Reference sequences were retrieved from the NCBI protein database: http://www.ncbi.nlm.nih.gov/protein/ and aligned with multalin: http://multalin.toulouse.inra.fr/multalin/multalin.html using the blosum62-12-2 and default settings. These sequences were also used for generating Fig. 2c. Residues with "*" are important in F-actin formation and are also displayed in space filling in Fig. 2b (same color code). Note that all residues marked with * are identical with the exception of the conserved substitution of valine or isoleucine at position 287. All actins are therefore assumed to form very similar polymer structures

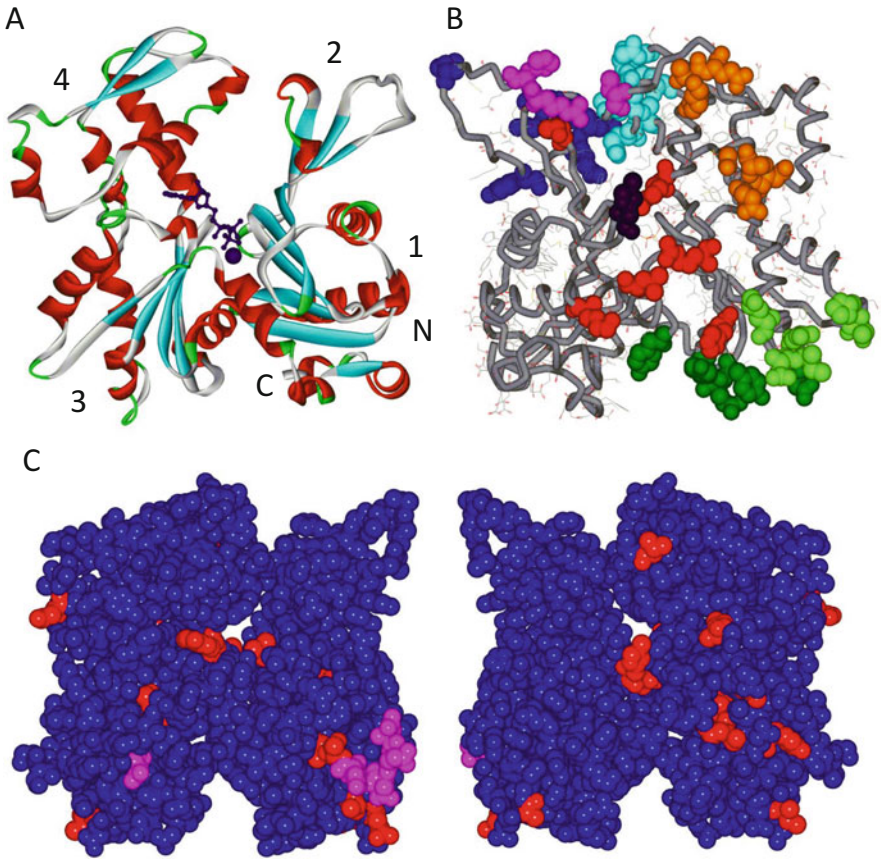

Fig. 2 Structural views of the actin molecule. (**a**) Ribbon presentation of monomeric actin with the alpha-helices in *red*, beta-strands in *blue*, beta-turns in *green* and loops in *gray*. This view is generally considered as depicting the front of the molecule. The molecule can be divided in subdomains 1–4. Subdomain 1 contains both the N and C termini. Subdomains 2 and 4 are at the pointed end; subdomains 3 and 4 are at the barbed end (based on their location at actin filament ends). ATP and the divalent ion are in *purple*. Taken from PDB entry 2BTF (Schutt et al. 1993) with omission of profilin. (**b**) The actin protomer in an orientation rotated 180° along a vertical axis in the plane relative to the molecule in 1A (thus viewing the back of the molecule). The main chain is in *gray*. The residues in space filing form contacts with neighboring protomers in the filament (as derived from Table 2). *Blue* and *green* indicate longitudinal contacts (light makes contact with light, and dark with dark); *red* and *orange* indicate contacts across the filament axis (residue making contacts via main chain atoms are not indicated; see Table 2). The residues in *magenta* (R39, H40, R183) make both types of contacts. The template structure was taken from PDB entry 3MFP (Fujii et al. 2010). (**c**) Scop3 visualization (Vermeire et al. 2015) of the variation of human actin isoactins plotted on the 3D structure: *blue*, identical residues; *red* and *magenta*, positions where mutations occur. Sites in magenta have a higher mutational tolerance. Two orientations are seen: on the left the actin molecule is in the same orientation as in Fig. 2a with the N-terminal residues (in *pink*) at the lower right. On the right is the same actin molecule in an orientation similar to 2b. The template structure of the actin molecule was taken from PDB entry 2BTF

Despite the identification of the actin isoforms nearly half a century ago, many questions still remain on how they actually differ. To what extent do we understand the isotype redundant and nonredundant functions? What is the role of isotype-specific expression and how is this regulated? Do isotype levels affect the cellular proteome? To what level do actin isoforms segregate in cells? What did the community learn from KO mouse models and isoform-specific mutations in disease? We will try to illustrate where this journey has led us thus far. To start with we briefly state common functions between the isoforms.

3 Actin Isoforms: General Properties

With general properties, we mean that all mammalian actin isoforms share them, although it does not exclude subtle differences.

All isoforms are assumed to require the chaperonin CCT to reach the native state. This has been experimentally shown for beta-cytoplasmic actin, alpha-skeletal actin, and alpha-cardiac actin (Vang et al. 2005; Rommelaere et al. 1993; Neirynck et al. 2006; Costa et al. 2004; Grantham et al. 2000). Because there is an absolute requirement for CCT to fold actin and because multiple sites of actin interact with CCT (Neirynck et al. 2006), the high sequence conservation between the isoforms allows assuming that also gamma-cytoplasmic actin and alpha- and gamma-smooth muscle actins require chaperonin-assisted folding.

The next shared property obviously is the core business of the actin molecule: the capacity to form polymers. Actin monomers self-associate in a head to tail fashion and thus form polarized filamentous structures, termed F-actin. Polymerization dynamics have initially been deciphered in vitro, mostly using alpha-skeletal muscle actin, and corroborated in vivo. We refer to Carlier et al. (2015) for a detailed description. When inducing polymerization of purified Ca^{2+}-ATP-actin monomers (by adding KCl and $MgCl_2$ to relatively low concentrations), one observes a lag phase, termed nucleation phase. Interestingly, when starting from Mg^{2+}-actin monomers, this phase is shortened (Carlier et al. 1986). The lag phase is followed by a phase in which both ends elongate, albeit at different rates. Polymerization is accompanied by hydrolysis of ATP to ADP-P_i, but subsequent P_i release lags behind. Together, this finally leads to a steady state in which treadmilling occurs; this is the cycling of actin protomers (subunits within the polymer) through the filament. ATP-actin monomers preferentially add to the ATP side of the actin filament (also termed the barbed or fast polymerizing end), whereas ADP-actin protomers preferentially dissociate from the ADP filament end (also termed the pointed or slow depolymerizing end). Obviously, ADP needs to be exchanged by ATP if an actin molecule reenters the polymerization cycle (Tondeleir et al. 2011).

The first model of F-actin was based on fiber diffraction of oriented gels of actin filaments and on the original G-actin structure (Kabsch et al. 1990; Holmes et al. 1990; Lorenz et al. 1993); see details in Dominguez and Holmes (2011).

Two high-resolution cryo-EM structures of alpha-skeletal F-actin have been published in 2010 (Fujii et al. 2010; Murakami et al. 2010) and a high-resolution structure with tropomyosin in 2015 (von der Ecken et al. 2015). The paper by Fujii et al. focused on the contact sites between the protomers within the filament and the paper by Murakami et al. on nucleotide binding, inorganic phosphate, and Mg^{2+} interaction. We will use these structures in relation to the differences in the amino acid sequence of isoactins.

In all recent models, the structure of the actin protomer is more flattened than the G-actin structure as derived from various crystallographic models (reviewed in Dominguez and Holmes 2011) indicating that a conformational change is associated with polymer formation. The structure of the filament can be described either as a two-stranded helix with two strands intertwined in a right-handed fashion or as a single left-handed helix (rotation of −166.6° and a translation of 27.6 Å in Fujii et al. 2010; compared to values reviewed in Dominguez and Holmes 2011). Viewing it as a double strand, one should consider contacts along the filament axis and across. We list these contacts in Table 2, show them on the monomer structure (Fig. 2b), and indicate them with color-coded asterisks in the alignment (Fig. 1).

Table 2 Contacts in F-actin based on Fujii et al. (2010), Murakami et al. (2010), and Dominguez and Holmes (2011)

Longitudinal		Across	
Dark blue	*Dark green*	*Orange*	*Red*
V45	Y143	E270	R39[a]
H40[a]	Y169[b]	S265	H40[a]
Y53	E167[b]	G268	H40
K61	E167	267/268 MC[c] ox	H173
R62	D288	K191	L110
I64	Y166	T194	R177
Light blue	*Light green*	E195	K113
Ala 204, Ile208	Ile 287	194 MC ox	110 MC ox (H_2O)
E205	D286, R290	195 MC ox	110 MC ox (H_2O)
R39	D286[b]	Q263/S265 via Mg^{2+}	T66
D244	R290	T202[a]	R183[b] (via P_i at site 1)
E241	T324		
G245	P322		
E207/Q59 via Mg^{2+}	D288[b] D286		
T202[a]	K284		

Color codes refer to Fig. 2b

[a]Magenta in Fig. 2b

[b]The longitudinal contacts at E167, Y169, D286, and D288 are different in the F-actin-tropomyosin structure (ADP) (von der Ecken et al. 2015) and the cryo-EM structures (Fujii et al. 2010) (Murakami et al. 2010) either due to the presence of tropomyosin or because of the presence of the phosphates (probably reflecting ADP + P_i). Most notably in the former there is an intramolecular salt bridge between R183 and Y169, whereas both residues participate in longitudinal contacts in (Murakami et al. 2010)

[c]MC ox: interaction via main chain oxygen (and H_2O)

What has been underappreciated prior to the elucidation of the cryo-EM structure presented by Murakami et al. (2010) is that inorganic phosphate and Mg^{2+} also have a structural role in the filament, on top of their role in regulating ATP hydrolysis and filament turnover (Table 2). Indeed, Glu207 and Gln59 in one protomer coordinate a Mg^{2+}-ion that also interacts with Asp88 along the filament axis. Similarly, it was pointed out that also two inorganic phosphates contribute to F-actin contacts as this ion could stabilize ternary interactions (Table 2). A first such site is near Thr202 and near Arg183 of the actin protomer across the filament and also near Lys284 of the actin molecule along the longitudinal axis. Arg183 also binds a second inorganic phosphate together with Arg206 of a neighboring protomer across the axis. One of these inorganic phosphates could even result from the hydrolysis of ATP to first ADP-P_i (P_i still at the position where it was originally bound, near Ser14) to ADP and P_i which is then transferred to the residues at the first site (Arg183, Thr202, Lys 284). This is consistent with data showing that the methylated His73 (purple in Fig. 2b and near Arg183 in magenta) is a kind of gating residue for the release of the hydrolyzed phosphate (Nyman et al. 2002). The structural model of Murakami may also explain why the hydrolysis rates are different between Mg^{2+} and Ca^{2+}-ATP actin (Carlier et al. 1986) because Ca^{2+} would prevent nucleophilic attack from a water molecule.

Having mapped the filament contacts of skeletal muscle actin on the 3D structure (Table 2 and Fig. 2b), we can now extrapolate this to the information in the primary structures of the other actin isoforms (Fig. 1, asterisks). As can be appreciated from the alignment, all residues suggested as intrafilament contacts are, with exception of residue 287 (Val or Ile), absolutely conserved in the six mammalian isoforms. This is not surprising, as all isoforms are capable of polymerization which has been shown experimentally by in vitro polymerization experiments (Kuroda and Maruyama 1976; Bergeron et al. 2010; Muller et al. 2013). At the same time, it explains that observed isoform-specific differences in polymerization kinetics (see below) are subtle and suggests these are caused indirectly by the differential amino acids. It is also consistent with the notion that actins can form mixed filaments in vitro in these cases that have been studied.

Since the largest divergence between actin isoforms lies in the N terminus, its biophysical properties are potentially important. In the G-actin crystal structure, there is no electron density for the N terminus (Kabsch et al. 1990). In the F-actin models, the N terminus is however well resolved and exposed (Fujii et al. 2010; Murakami et al. 2010) in agreement with the notion that this region is involved in binding myosins as originally proposed for the myosin II-actin structure derived by modeling of the individual structures of actin and the motor domain of myosin II and low-resolution cryo-EM data (Rayment et al. 1993).

A third function shared between actins is that they interact with an extraordinary number of partner proteins (reviewed in Pollard 2016). These partners may bind either the monomer or the polymer (or both) and influence various aspects of the polymerization process or use the filaments for localization or transport purposes. An extensive overview of these proteins is beyond the scope of this review, but some actin-binding proteins will be mentioned below if differences in relation to

actin isoforms have been documented. In addition, mammalian actins bind a number of small molecules from diverse natural sources that have been substantial in studying actin in cells or in vitro. To our knowledge, no actin isoform-specific differences have been observed on the effects of these compounds on actin properties. Actin polymer-interacting agents include the widely used fungus-derived phalloidin and cytochalasin D (Cooper 1987) and jasplakinolide (Bubb et al. 1994). Phalloidin is, in its fluorescent version, used as probe for F-actin in cells and cytochalasin D as an agent for binding the barbed end. Latrunculins (Yarmola et al. 2000) are used to inhibit actin polymerization by monomer sequestration. Apart from being tools for (cellular) functional studies, they have assisted structural studies (Dominguez and Holmes 2011). Given their cytotoxicity, some of these molecules are even considered in antitumor therapies (Kita and Kigoshi 2015). More recently alternative F-actin probes, such as LifeAct, Utrophin, or F-tractin (respectively, containing calponin homology domains of human ubiquitous dystrophin, the actin-binding domain from yeast ABP140 and from rat inositol trisphosphate-3-kinase) fused to a fluorescent protein, are increasingly used in live cell imaging (Burkel et al. 2007; Riedl et al. 2008; Johnson and Schell 2009). Also nanobody-based tools for following cytoplasmic actin dynamics (in plants) have recently been developed (Rocchetti et al. 2014). The capacities of these new tools to aid in elucidating actin dynamics are promising, but this will need to be accompanied by detailed insight in their specificities, since several studies already point out preferential binding to different F-actin-rich structures on the subcellular level or effects on actin dynamics (Courtemanche et al. 2016). Currently, it is not clear whether these tools display actin isoform specificity.

4 Actin Isoforms: The Things that Make Me Different Are the Things that Make Me[1]

4.1 Differential Expression of Actin Isoforms

The expression patterns of actin isoforms are temporally and spatially regulated during development (reviewed in Tondeleir et al. 2009). The names of the actin isoforms are in part derived from the tissue types in which they were first detected (Vandekerckhove and Weber 1978, 1979). From microarray data, it is now evident that most if not all tissues express more than one type (Fig. 3).

Striated muscles in skeletal muscle and the heart each have a different type of alpha-actin. The major form in adult skeletal muscle is alpha-skeletal muscle actin (Vandekerckhove et al. 1986) encoded by the *ACTA1* gene in humans (*Acta1* in mouse, Table 1). Alpha-cardiac actin, encoded by *ACTC*, is the major form in adult heart, but some regions contain considerable amounts of alpha-skeletal muscle actin as well. Figure 3 demonstrates this on the mRNA level. This has also been

[1] A. A Millne (Piglet in Winnie The Pooh).

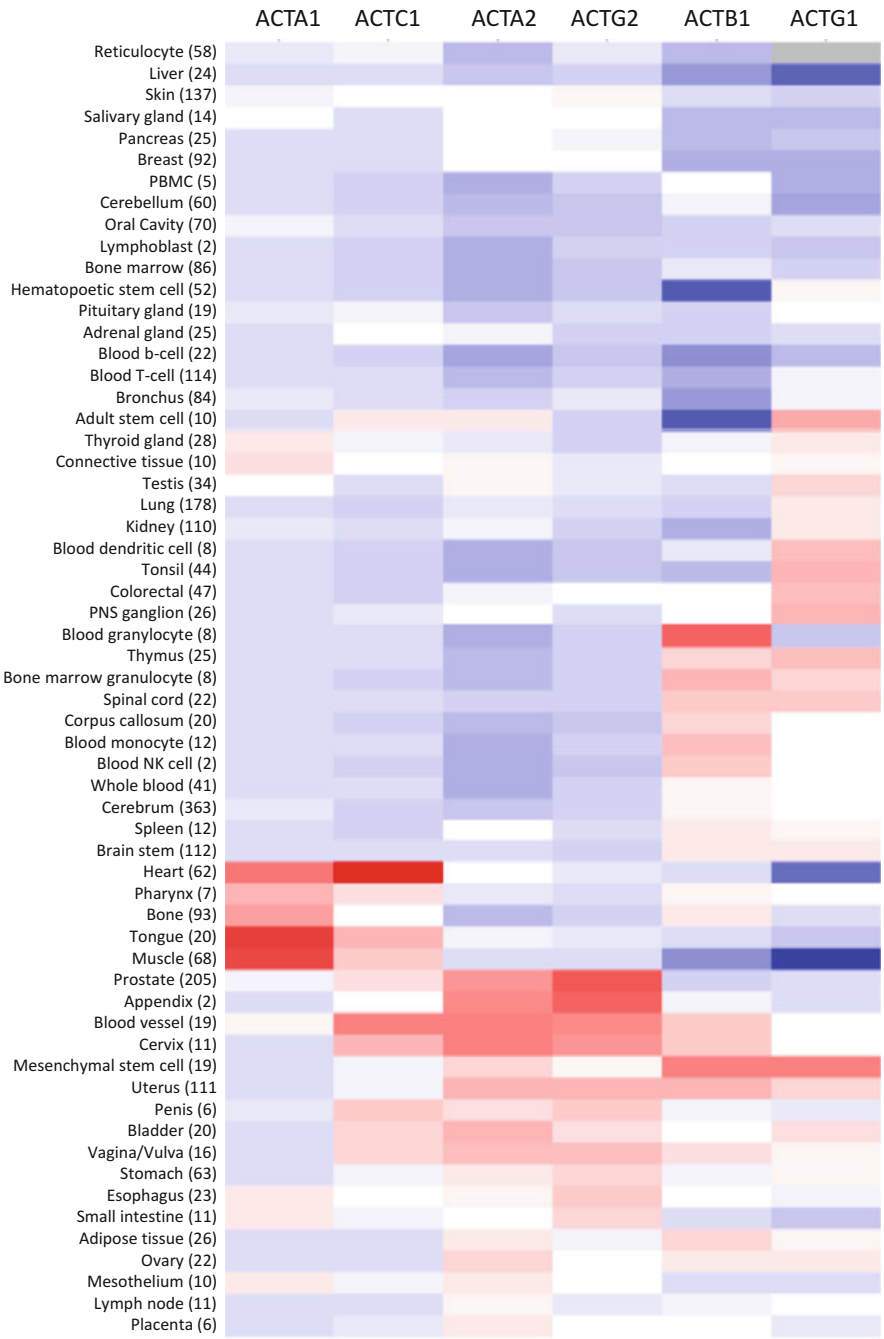

Fig. 3 Tissue-specific differences in actin isoform transcript level. The heat map of actin isoform mRNA levels expressed in the human body was generated in medisapiens in silico transcriptomic

observed at the protein level in healthy hearts (Vandekerckhove et al. 1986; Suurmeijer et al. 2003; Orlandi et al. 2009).

Nonstriated muscle also has typical actin isoforms: *ACTA2* and *ACTG2*, that are often co-expressed as evident from the cluster analysis (Fig. 3), consistent with Fatigati and Murphy (1984). On the protein level, alpha-smooth muscle actin has been shown as the major form in vascular smooth muscle tissues and gamma-smooth muscle actin in enteric tissues (for references, see the introduction of Arnoldi et al. 2013).

For the cytoplasmic actins, the view is variable (Fig. 3). In some tissues or cells, the mRNA for beta-cytoplasmic actin (*ACTB*) is the dominant form (e.g., blood granulocyte, spinal cord corpus callosum), while in others gamma-cytoplasmic actin (*ACTG1*) (e.g., adult stem cell, PNS ganglion, tonsil). Some tissues or cells have nearly similar mRNA levels of the cytoplasmic actins.

These data on tissue- or cell-specific mRNA expression levels evidence tissue-specific regulation of the actin gene promoters. This promoter regulation has mainly been studied in the context of the skeletal and smooth muscle phenotypes and for switches in isoform expression during myogenesis. The important role of the CArG box – serum response factor (SRF) – myocardin (cotranscription factor) controlling axis was established in smooth muscle cells but falls outside the scope of this review (Small 2012; Pipes et al. 2006; Owens et al. 2004; McDonald et al. 2006). However, overall the knowledge of transcriptional control for all six actin isoforms is far from complete.

Obviously the expression data on the mRNA level (Fig. 3) and regulation of the actin promoters are valuable when trying to make correlations of actin expression with diseases such as cancer, but one has to keep in mind that, in general, the correlation between actin mRNA and protein levels has not been made. This is of relevance because in the case of non-muscle actins, various control mechanisms for actin mRNA stability and actin mRNA translation have been described. In mouse two beta-actin transcripts with different lengths of the 3′ untranslated region are expressed in a tissue-specific manner, and translation of the longer transcript is under regulation of a miRNA (Ghosh et al. 2008). Both transcripts contain the so-called zipcode: a 54-nucleotide sequence immediately 3′ of the stop codon (Kislauskis et al. 1997) that targets the transcripts to the periphery of cells where they are locally transcribed upon stimulation (Bassell et al. 1998; Leung et al. 2006; Yao et al. 2006; Huttelmaier et al. 2005). The zipcode is recognized by the zipcode-binding protein 1 (ZBP1) and ZBP2 (Ross et al. 1997; Gu et al. 2002). ZBP2 mainly acts in the nucleus and ZBP1 in the cytoplasm. The translocation through and local activation of translation of beta-actin mRNA in the cytoplasm are dependent on ZBP1 (Huttelmaier et al. 2005; Leung et al. 2006; Yao et al. 2006) and the ZBP1-

Fig. 3 (continued) tool: bodymap (http://ist.medisapiens.com/). Only expression data from healthy tissues were used. Mean expression values were used for clustering tissues and genes. *Blue* indicates low expression levels and *red* high expression levels (*gray* no expression level known)

binding microtubule motor KIF11. Disruption of the interaction of KIF11 with ZBP1 delocalizes β-actin mRNA and affects cell migration (Song et al. 2015). The zipcode is specific to the beta-actin isoform. The RNA-binding protein Sam68 (Src associated in mitosis) may cooperate with ZBP1, regulating localized translation of beta-actin mRNA in dendrites (Klein et al. 2013). The RNA-binding protein HuR stabilizes beta-actin mRNA via a site immediately 3′ of the zipcode sequence and silencing HuR reduces beta-actin mRNA levels (Dormoy-Raclet et al. 2007). Downregulation of β-actin and HuR also affects cell migration of human corneal fibroblasts (Joseph et al. 2014). In addition, downregulation of beta-actin mRNA during myogenesis was attributed to a conserved nucleotide sequence more downstream in the 3′ UTR of the beta-actin mRNA (a sequence located approximately 600 nucleotides 3′ of the stop codon) (DePonti-Zilli et al. 1988). Further details on regulation of beta-actin mRNA transport, stability, and regulation are reviewed in Artman et al. (2014).

In differentiating C2C12 myoblasts, a different type of regulation was proposed to control gamma-cytoplasmic actin expression. During differentiation and fusion, an alternative, noncoding mRNA splice variant is increasingly expressed and its expression correlates with reduction of translation of gamma-cytoplasmic actin protein. This alternative transcript, containing an extra exon that is conserved in Mammalia and is situated between the regular exons 2 and 4, contains an in-frame stop codon, but no corresponding shorter protein was observed using an anti-gamma-cytoplasmic actin antibody. This transcript is muscle specific as it was only present in the skeletal and heart muscle and diaphragm and not in other investigated non-muscle tissues (Drummond and Friderici 2013). In the discussion of this paper, it was suggested that downregulation in the muscle of beta-cytoplasmic actin would be controlled by a similar type of regulated unproductive splicing and translation (RUST) regulation.

Collectively, this indicates that, on top of their differences in primary structure (Fig. 1), the specific functions of actin are also governed by expression regulation and by their ratios in cells when present together.

4.2 Isoform Specificity from Biochemical Experiments

Evidently, obtaining pure actin isoform in sufficient quantities is a prerequisite to study specific biochemical properties of the isoforms. Although this sounds trivial, it has been very cumbersome to recombinantly express actin in the classical expression systems. Because of its requirement for the folding chaperone CCT (Rommelaere et al. 1993), which is not present in prokaryotes, actin cannot be produced in a functional form in bacteria. Beta-cytoplasmic actin can be produced in yeast, but because this organism does not have the appropriate methyltransferase, actin is not methylated at His73 and this affects the polymerization kinetics (Nyman et al. 2002). In addition, in this organism, actin may be incompletely processed N terminally (Cook et al. 1991; Kalhor et al. 1999). Although purification of yeast-produced mammalian actin was valuable for studying mutant actins relative to similarly produced wild type (Nyman et al. 2002; Schuler et al. 1999, 2000a, b), it

cannot be used to fully compare properties of mammalian isoactins because of the potential functional importance of the (differentially) processed N terminus. Along similar lines, tags for easier purification may interfere with proper folding or functioning of actins (Rommelaere et al. 2004; Rutkevich et al. 2006).

People have therefore mostly relied on using alpha-skeletal muscle actin which can be relatively easily purified from skeletal muscle (Spudich and Watt 1971). Beta-cytoplasmic actin has been purified from the profilin-actin complex isolated from calf thymus (Lindberg et al. 1988), but this is difficult to achieve in a truly preparative manner. Given that actins are usually co-expressed, mixed populations are obtained when purifying them from tissue (Gordon et al. 1977; Strzelecka-Golaszewska et al. 1985; Coue et al. 1982), and also commercially available actin protein preparations are consequently isoform mixtures. Thus from a technical point, producing pure actin isoforms in bulk for in-depth biochemical characterization remained challenging for a long time.

The recent progress by producing alpha-cardiac and beta- and gamma-cytoplasmic actin in baculovirus is certainly a breakthrough (Bergeron et al. 2010; Muller et al. 2013; Bookwalter and Trybus 2006; Rutkevich et al. 2006) although in these actin preparations, a measurable amount of insect actin is present for which it is necessary to assume it does not significantly interfere within the experiments. Baculovirus-produced alpha-cardiac actin and classically purified bovine cardiac actin have been compared in relation to their interaction with tropomyosin and troponin, and in this case no difference was observed in contractile and regulatory functions (Bai et al. 2014).

The scientific challenge of biochemical characterization should not be underestimated. Actins have a beautiful but inherently complex biochemistry because of a multistep polymerization process linked to the nucleotide status and because of the multitude of actin-binding proteins. Given the very slight variations in sequence and 3D structure between isoforms, this results in subtle differences in the polymerization process or differential affinities for given actin-binding proteins that require very careful comparative studies, often requiring more than one dedicated assay. This is already evident from the pioneering studies presented below.

Using recombinantly produced beta- and gamma-cytoplasmic actin from insect cells, a number of similarities and differences have been observed. Both cytoplasmic actins are equally thermostable and form similar filaments as judged by electron microscopy. The nucleotide exchange rates of Mg^{2+}-beta- and gamma-cytoplasmic actins were very similar (Bergeron et al. 2010). However polymerization studies showed that gamma-actin (starting from the Mg^{2+}-ATP form) polymerized slower compared to beta-actin. Interestingly, gamma-cytoplasmic actin had a somewhat slower P_i release which was interpreted that treadmilling for beta-actin is slightly faster. It was intriguing that the differences between beta- and gamma-cytoplasmic actin polymerization properties were larger when starting from the Ca^{2+}-ATP actin form. Although in living cells Mg^{2+} is considered the most relevant divalent ion, these authors suggest that, upon activation, local Ca^{2+} increases may be sufficient to affect isoactins differentially. This study using light

scattering, and another one using FRET, also showed that both cytoplasmic forms may copolymerize (Muller et al. 2013; Bergeron et al. 2010).

The Manstein group also showed differential interactions between polymers from alpha-skeletal muscle actin and beta- or gamma-cytoplasmic actin with non-muscle myosins. The cytoplasmic actins displayed more extensive stimulation of myosin ATPase activity, compared to alpha-skeletal muscle actin. Beta-cytoplasmic actin most potently activated non-muscle myosin 2C1, and gamma-cytoplasmic actin preferentially activated the unconventional myosin 7A (Muller et al. 2013).

For some other actin-binding proteins, preferential partnering with an actin isoform has been suggested. BetaCap73, for example, was shown to preferentially bind beta-cytoplasmic actin over alpha-skeletal muscle actin (Shuster et al. 1996). Cofilin 1, which can bind to F-actin in a cooperative and stoichiometric manner, has comparative affinities for binding to an isolated site on filaments composed of alpha-skeletal muscle actin or human cytoplasmic actin (85% beta, 15% gamma), but the cooperative free energy is larger for binding beta-/gamma-cytoplasmic actin filaments (Prochniewicz et al. 2005). Using co-IP, it was shown that the Arp2/3 complex and cofilin 1 may preferentially associate with gamma- over beta-cytoplasmic actin in lung adenocarcinoma A459 cells (Dugina et al. 2015). Similarly, gamma- but not beta-cytoplasmic actin was found in a complex with annexin V in platelets (Tzima et al. 2000). Although this suggests that beta- and gamma-cytoplasmic actin may reside in different supermolecular complexes, it was not shown whether these actin-binding proteins have a different binding affinity using purified components. The ratio of beta- versus gamma-cytoplasmic actin may in any case be a determining factor in such experiments. Indeed, in A549 cells, a higher amount of gamma-cytoplasmic actin was present or the gamma-/beta-actin ratios were altered by selective silencing (Dugina et al. 2015). It was similarly shown that the transcription factor MRTFA preferentially interacts with gamma-cytoplasmic actin in A549 cells. However, upon depletion of the latter isoform, beta-cytoplasmic actin was also found in complex with this protein (Lechuga et al. 2014).

By contrast tropomyosin-bound tropomodulins 1 and 3 showed no difference in pointed end capping capability of alpha-skeletal muscle actin versus platelet beta-/gamma-actin although tropomodulin 3 preferentially sequestered beta- and gamma-cytoplasmic actin over alpha-skeletal muscle actin in the absence of tropomyosin (Yamashiro et al. 2014). Such differential regulatory activity may be of importance in striated muscle cells where the three actin isoforms are present (Sonnemann et al. 2006). Indeed it was shown that in the sarcoplasmic reticulum of skeletal muscle, tropomodulin 3 and gamma-cytoplasmic actin define a distinct microdomain (Gokhin and Fowler 2011).

From this limited number of studies, it is clear that differential interaction between actin-binding proteins and specific actin isoforms may occur. This is a story of affinities linked to cellular concentrations and, likely, regulation. A major biochemical challenge is therefore to further understand differential interaction between isoactins and the myriad of actin-binding proteins.

4.3 What Did We Learn from Mouse Models and from Genetics?

Table 1 lists the mouse knockout (KO) models and conditional KO models that have been generated for actin isoforms. A gamma-smooth muscle KO has not yet been reported. The nonconditional striated muscle and beta-cytoplasmic actin KOs are lethal (Table 1). Below we discuss evidence for the lack of functional redundancy of actin isoform based on these genetic models and research on cells derived from these mouse models. A striking but recurring observation is the compensatory regulation of the loss of one actin isoform by expression of one or more other actin isoforms.

4.3.1 Cytoplasmic Actin KO Mice Demonstrate That Beta- and Gamma-Cytoplasmic Actins Have Different Functions

The *Actb*$^{-/-}$ mice are embryonically lethal despite the fact that compensatory actin expression is strongly apparent (Table 1). The knockout of beta-cytoplasmic actin indeed results in an increase in expression of the other actin isoforms. In mouse embryonic fibroblasts (MEFs) derived from these KO animals, this compensation even ensures that the total level of actin is kept constant relative to wild-type MEFs. This is achieved by a slight upregulation of gamma-cytoplasmic actin and a moderate to strong upregulation of alpha-smooth muscle actin (Tondeleir et al. 2012; Bunnell et al. 2011) (note this occurs in MEFs from independently generated KO mice). The *Actb*$^{-/-}$ MEFs, presented in Tondeleir et al., also had increased expression of gamma-smooth muscle and contain detectable amounts of a striated muscle actin isoform. Alpha-smooth muscle actin expression was also observed in select populations of cells in the hippocampus of embryos with conditional beta-actin KO in the brain (Cheever et al. 2012), in all tissues of heterozygous *Actb*$^{+/-}$ mice and in *Actb*$^{-/-}$ embryos (Bunnell et al. 2011; Tondeleir et al. 2012). This was not observed in CD4-positive T cells of a conditional beta-cytoplasmic actin knockout (Bunnell et al. 2011). As also discussed below, these knockout T cells and the knockout MEFs display impaired migration (Bunnell et al. 2011; Tondeleir et al. 2012) suggesting a specific function of beta-actin in this important process. Yet, Tondeleir et al. (2012) showed that the situation is more complex. Indeed, the beta-cytoplasmic actin knockout MEFs not only have altered expression levels of isoactins, but they in addition have extensive changes in their entire proteome: many actin cytoskeletal proteins associated with stress fiber formation are differentially expressed, and the population is enriched for proteins containing LIM domains, EF hand structures, and calponin homology domains (Ampe et al. 2013). Pathway analysis of two independent beta-cytoplasmic actin KO MEF cell lines showed 154 common proteins with up- or downregulation, merging down on Rho signaling. Based on this result, the prediction was made that the Rho pathway was overactivated in the absence of beta-cytoplasmic actin. This was indeed validated by various experiments which also implicated increased TGF-beta signaling resulting in enhanced contractility (Tondeleir et al. 2012). *Actb*$^{-/-}$ cells also show more stress fibers and focal adhesions (Bunnell et al. 2011; Tondeleir et al. 2012).

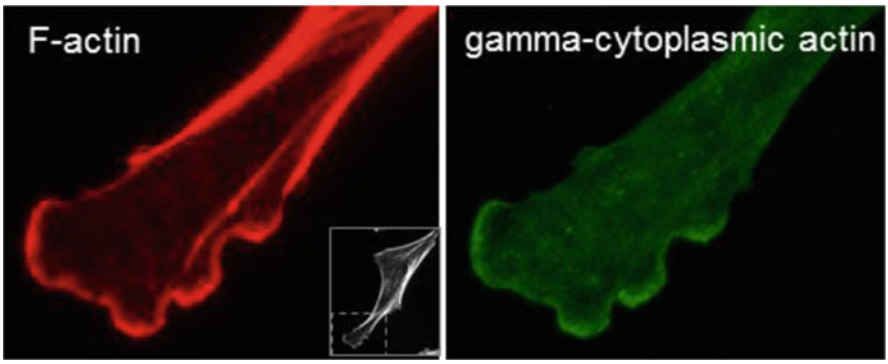

Fig. 4 Gamma-cytoplasmic actin in lamellipodia upon beta-cytoplasmic knockout. Mouse embryonic fibroblast derived from a *Actb*$^{-/-}$ mouse show prominent staining in lamellipodia using a gamma-actin cytoplasmic antibody (Chemicon) (*green*). The phalloidin staining (*red*) on the side of the cell likely represents stress fibers formed by alpha-smooth muscle actin which is strongly upregulated in these cells (Tondeleir et al. 2011). Images courtesy of Dr. Anja Lambrechts

The most striking result in relation to actin isoform function, however, was that treating beta-cytoplasmic actin knockout cell lines with the ROCK inhibitor Y27632 restored cell migration. This suggests that the initially observed impaired cell migration was not due to the absence of polymerization capacity produced specifically by beta-cytoplasmic actin but that the other actins present can provide this. In line with this, gamma-cytoplasmic actin is enriched in lamellipodia in the *Actb*$^{-/-}$ MEFs (Fig. 4). The impaired migration is then rather an indirect effect from stronger adhesion and contractility (Tondeleir et al. 2012). In ex vivo *Actb*$^{-/-}$ neural crest cells, prominent stress fibers were also observed and cell migration is similarly impaired. Cells moved in sheets and not as individual cells. Unlike MEFs, ROCK treatment could not fully restore cell migration in these cells. In addition, impaired cell migration of neural crest cells was not only observed in vitro in derived cells but also in the *ActB*$^{-/-}$ embryos. In vivo, these cells moved in clumps in the embryos, likely by a reduced expression of cadherin 11 and lack of membrane-bound N-cadherin (Tondeleir et al. 2014).

From the embryonic lethality of the *Actb* KO mice (Shawlot et al. 1998; Bunnell et al. 2011; Shmerling et al. 2005), it is clear that in the developing embryo, beta-cytoplasmic actin is essential. It is however unclear why. This may be direct or indirect (i.e., by altering the cellular proteome), caused by a general defect in cell migration (Bunnell et al. 2011) or in particular cell populations like neural crest cells (Tondeleir et al. 2014). However, other studies point to a different cause of lethality. In line with altered gene expression (Tondeleir et al. 2012), to some extent confirmed by data in (Bunnell et al. 2011), it may well be that the nuclear function of beta-actin is the more important one. Indeed it was shown that expression of Gata2, a transcription factor in early erythropoiesis, was dramatically reduced in *Actb*$^{-/-}$ embryos leading to defective primitive and definitive erythropoiesis

(Tondeleir et al. 2013). Specifically expressing Gata2 in the erythroid lineage from the Rosa promoter rescued lethality of the $Actb^{-/-}$ embryos to some extent.

The observation that, in contrast to $Actb^{-/-}$, $Actg^{-/-}$ animals are viable clearly indicates that gamma-cytoplasmic actin has a different function than beta-cytoplasmic actin. The $Actg^{-/-}$ animals however also displayed reduced postnatal survival and had reduced litter numbers, suggesting some uncharacterized embryonic lethality. From the gamma-cytoplasmic and the conditional beta-cytoplasmic actin knockout models, it is evident that both isoforms function in the ear. However, the pathology associated with each actin isoform is distinct (Perrin et al. 2010; Belyantseva et al. 2009) and suggests specific roles in auditory hair cells (AHC). Indeed, it was recently demonstrated in wild-type mouse embryos (E16,5), using antibodies and electron microscopy, that both beta-actin and gamma-actin are present in actin-based structures in cochlear and vestibular hair cells. In stereocilia and junctions, there is an excess of gamma-cytoplasmic actin over beta-actin, whereas their amounts are approximately equal in cuticular plates (Andrade 2015). Interestingly, per compartment, the ratios evolve over time with gamma-cytoplasmic actin becoming the minor form in vestibular hair cells of very old mice. This suggests that the proportions of actin isoforms may vary during the lifetime, at least in auditory hair cells. To our knowledge, such an evolution over lifetime has not been studied for the cytoplasmic actins in other tissues where they are co-expressed.

Along the same lines, old $Actg1^{-/-}$ animals presented hearing loss despite upregulation of beta-actin (Perrin et al. 2010) and despite the fact that gaps in the F-actin structures of auditory stereocilia from AHC where filled up with beta-cytoplasmic actin. Clearly there is a different function, but at present it is unknown whether this is due to a specific partner or specific intrinsic properties of gamma-cytoplasmic actin related to polymer formation or stability in function of mechanical repair. Expressing a mutant fascin 2 in the conditional $Actb^{-/-}$ in AHC aggravated the phenotype on stereocilia maintenance; however, it was shown that the affinity of fascin 2 for beta- and gamma-actin was similar (Perrin et al. 2013).

Another study showing the importance of gamma-actin in maintaining health is that of Sonnemann et al. (2006). A conditional $Actg^{-/-}$ knockout in the muscle resulted in progressive myopathy. Gamma-cytoplasmic actin is a very minor form in the muscle, yet its ablation results in a specific phenotype. By contrast, a similar $Actb^{-/-}$ knockout resulted in quadricep myopathy (Prins et al. 2011) emphasizing that the cytoplasmic actins have different functions in muscle cells and lead to different disease-related phenotypes.

4.3.2 Muscle Actin KO Mice Have Distinct Phenotypes, but Partial Rescue May Occur

Mouse models with a full deletion of the alpha-skeletal or alpha-cardiac muscle actin isoform result in perinatal or neonatal mortality, respectively (Table 1), indicating that these are essential genes (Crawford et al. 2002; Kumar et al. 1997). Also here, compensatory expression of other isoforms is observed. The *Acta1* hemizygous and homozygous null mice have increased levels of alpha-

cardiac and alpha-smooth muscle actin mRNA and protein in limb muscles. The total actin levels of WT, hemizygous, or knockout animals were even similar, suggesting that the other actins that are present cannot take over the function of alpha-skeletal muscle actin in the KO. Thin and thick filament organization appeared normal in *Acta1*$^{-/-}$ mice, yet force production was reduced. This was interpreted that newborn *Acta1*$^{-/-}$ null mice have a competitive disadvantage during feeding resulting in malnutrition and ultimately death (Crawford et al. 2002). Transgenic expression of alpha-cardiac actin in skeletal muscle of *Acta1*$^{-/-}$ mice rescued the lethal phenotype (Table 1). This may come as a surprise because upregulation of alpha-cardiac actin was not sufficient to rescue the *Acta1*$^{-/-}$ mice, but it was noted that only transgenes with high expression levels of alpha-cardiac actin survived for a long time (Nowak et al. 2009). In addition, myofibers of these transgenically rescued animals with alpha-cardiac actin fully replacing skeletal muscle actin generated less force. Mechanical analyses of the myofibers showed lower stiffness and differences in actin-associated reflections in the X-ray fiber diffraction pattern, together suggesting different structures of thin filaments in this transgene mouse relative to wild type. This was interpreted in a scenario that limiting the actin conformational changes during contraction affect the myosin cross-bridge cycle (Ochala et al. 2013).

Similar to *Acta1* deletion, *Actc* deletion results in increased levels of alpha-skeletal actin and alpha-smooth muscle actin mRNA. At the protein level, alpha-vascular smooth muscle actin was increased in newborn mice and even incorporated in myofibrils. Despite this compensation, the total amount of actin was reduced in the heart of *Actc*$^{-/-}$ newborn mice and heart sarcomere ultrastructure was disorganized (Kumar et al. 1997). Transgenic expression of gamma-smooth muscle actin in the heart rescued the lethal phenotype. The total amount of actin was restored, but the hearts were hypodynamic with reduced rates of contraction and relaxation (Kumar et al. 1997) again suggesting subtle differences in isoform-specific functions.

The compensatory expression observed in the striated muscle KO mice may be reminiscent of the switch in muscle actin isoform expression that is known to occur during muscle development (reviewed in Tondeleir et al. 2009). Myoblasts contain beta- and gamma-cytoplasmic actin (Schwartz and Rothblum 1981). Upon differentiation, initially alpha- smooth muscle actin expression is upregulated (Springer et al. 2002; Lancioni et al. 2007) and subsequently alpha-cardiac actin and then alpha-skeletal actin in a coordinated process (Cox et al. 1990; Biben et al. 1996; Lancioni et al. 2007; Kislinger et al. 2005).

Mice without expression of alpha-smooth muscle actin (*Acta2*$^{-/-}$ mice) are viable and relatively healthy, but they have impaired vascular contractility and blood pressure homeostasis (Schildmeyer et al. 2000). Vascular smooth muscle of these mice expressed alpha-skeletal muscle actin which is usually not present in these muscles. This mild phenotype of the alpha-smooth muscle actin whole-body knockout mouse strongly contrasts with the severe and often lethal phenotypes of diseases associated with alpha-smooth muscle actin (see below).

4.4 Actin Isoform Differences and Similarities from Cellular Studies

For studying isoform-specific function in cells, one has relied on antibody staining, overexpression studies, or using cells in which one isoform was silenced or knocked out. In relation to actin isoforms, all of these approaches come with their specific flaws, either of a technical or biological nature:

1. Given the high conservation of the actin isotypes, reliable (commercially available) isoform-specific antibodies have only gradually become available. In addition, variability in fixation procedures for monitoring distinct subcellular structures using these antibodies may influence specific parts of the dynamic actin cytoskeleton differently (e.g., in fragile thin-membrane structures such as lamellipodia) and thus hamper comparisons between studies.
2. Overexpression studies mostly use tagged actins and this tagging is only possible at the N terminus which is where isoforms differ to the largest extent.
3. Similarly as described above for KO models and cells derived thereof, silencing the expression of one actin isoform in a cell using RNAi often induces compensatory upregulation of other actin isoforms thus impacting the cellular properties. This obviously complicates analysis of isoform-specific function. The necessity to monitor the other actin isoforms systematically is only slowly gaining awareness. However in recent papers, this is mostly done for the isoform of the "same expression pair" (e.g., the cytoplasmic pair, the smooth muscle pair) but not for other actins. For example, upon beta-cytoplasmic actin silencing, gamma-cytoplasmic actin level is measured (or vice versa), but whether expression levels of, e.g., alpha-smooth muscle actin are altered is not consistently investigated.
4. Complete RNAi of actin is not trivial due to the high stability of actin proteins.
5. Cell-type-dependent differences are expected. Indeed, actin isoforms act differently in a cellular content by virtue of their interplay with actin-binding proteins, but the repertoire of the latter may substantially differ from cell type to cell type.

Notwithstanding these issues, we here report on (recent) studies that have added pieces to the complex actin isoform puzzle at the cellular level, either for the cytoplasmic or for the muscle actins.

4.4.1 Cytoplasmic Actins Can Segregate in Different Subcompartments in Cells

Whereas a beta-cytoplasmic actin antibody exists for an appreciable time (Gimona et al. 1994), obtaining specific antibodies for gamma-cytoplasmic actin took a longer time (Dugina et al. 2009; Sonnemann et al. 2006). Several of these are now on the market. The conclusion from antibody-based studies is however double and thus far from clear: overall staining for beta- and gamma-actin often overlaps to a large extent, but specific areas of enrichment of either of the two isoforms have sometimes been observed, often in a cell-type-specific manner. Simiczyjew et al.,

using two different sets of commercially available beta- and gamma-cytoplasmic actin antibodies, showed that both isoforms are present in lamellipodia and other peripheral or protrusive actin-rich structures related to migration and invasion in an invasive cancer cell line. In addition, they specifically showed the simultaneous presence of both actin cytoplasmic isoforms in invadopodia (Simiczyjew et al. 2014, 2015). This occurrence of beta- and gamma-cytoplasmic actin in the same substructures in a cell does not necessarily mean they are doing the same at these locations, and sometimes actual segregation has been observed. Indeed, in epithelial, endothelial, and neuroblastoma cells, beta-cytoplasmic actin was shown to be enriched in ruffles and in radial stress fibers and gamma-cytoplasmic actin was observed in the cortical meshwork (Dugina et al. 2009; Shum et al. 2011; Pasquier et al. 2015). In confluent epithelial cells, both beta- and gamma-cytoplasmic actins are associated with apical junctional complexes, but, interestingly, they differ in time of recruitment to these structures and in their dynamics (Baranwal et al. 2012). In the endothelial cells, hCMEC/D3 gamma-actin localized in the submembranous fibrous network, around the nucleus and in the periphery, whereas beta-actin was in the periphery. Both isoforms were also present in dense perinuclear structures. Interestingly upon stimulation with TNF, both isoforms relocalized to stress fibers, but do this in an asymmetric distribution: beta-cytoplasmic actin on the basal side of the cell and gamma-cytoplasmic actin apically in a submembranous network (Latham et al. 2013).

Another striking differential staining of the cytoplasmic actins has been observed in oocytes during meiosis and early embryogenesis. In 2- to 8-cell embryos, beta-actin was in the cell-cell contact zones and at the cleavage plane of mitotic blastomeres, whereas gamma-cytoplasmic actin was in the cortex (Brockmann et al. 2011). Using gamma- or beta-cytoplasmic actin-specific antibodies, it was shown that in striated muscle, beta- and gamma-cytoplasmic actins segregate away from the myofibrils that contain alpha-skeletal muscle actin. Beta actin associates with the sarcolemma where it colocalizes with dystrophin (Prins et al. 2011), whereas gamma-actin localized, together with tropomodulins, in the intermyofibrillar compartments (Gokhin and Fowler 2011).

The effect of beta- or gamma-cytoplasmic actin overexpression has been tested in myoblast cell lines, in keratinocytes, and in various cancer cells. In C2 myoblasts, expression of the beta form resulted in approximately twofold increase in cell surface area, and gamma-cytoplasmic actin expression resulted in a decrease relative to control cells. This correlated with the amount of stress fibers, with the gamma-cytoplasmic actin-expressing cells having fewer stress fibers and more diffusive actin staining (Schevzov et al. 1992). In another myoblast cell line, overexpression of beta-cytoplasmic actin resulted in increased migration (Peckham et al. 2001). The increased speed was mainly observed for poorly spread cells and was attributed to a myosin effect rather than to having more beta-actin for polymerization. Overexpression of gamma cytoplasmic actin was not assayed in this cell line. More recently, overexpression of gamma-cytoplasmic actin in HaCaT keratinocytes, in the lung cancer A549, and in colon cancer cell line HCT116 cancer was associated with increased (in vitro and in vivo) proliferation and

in vitro invasiveness. Overexpression of beta-cytoplasmic actin in contrast had opposing effects (Dugina et al. 2015). In a breast adenocarcinoma cell line, transiently transfected with GFP-tagged beta- or gamma-actin, both isoforms increased invasion velocity; however, the invasive capacity is consistent with the observation that they are both present in invadopodia (Simiczyjew et al. 2015).

Silencing an actin isoform in a cell is expected to be informative on its role in actin-dependent cellular properties such as morphology or migration. Given the possible extensive functional redundancy between the isoforms, it may however rather inform on whether the other isoform(s) present can completely or partially functionally complement the silenced isoform. When the effect of silencing is (as described for the KOs) an increase in expression of the other actin isoforms, it is even harder to deduce whether the observed effect is caused by loss or gain of an isoform. We describe examples of beta- or gamma-cytoplasmic actin silencing in different cell types, the observed compensatory actin expression (if monitored), and the effects on actin-dependent cell properties.

In HaCaT keratinocytes and in A549 (lung epithelium) and HCT116 (colon) cancer cell lines, silencing of beta-cytoplasmic actin leads to an increase in gamma-cytoplasmic actin expression and vice versa (stable sh-RNA-based silencing; level of (smooth) muscle actin isoforms was not checked). Silencing the cytoplasmic actin isoforms separately resulted for both in a strong reduction in cell proliferation (Dugina et al. 2015). Whereas for silencing of gamma-cytoplasmic actin, this could be expected via the upregulation of beta-cytoplasmic actin (since beta-actin overexpression negatively affects proliferation in these cells, see above), it is unclear why silencing of beta-actin, resulting in an increase in gamma-cytoplasmic actin, also results in reduction of proliferation (Dugina et al. 2015). It suggests that both absolute and relative expression levels of the two cytoplasmic actins determine the outcome. The silencing of either isoform in HaCaT, A5549, or HCT116 induced distinct morphological differences. Beta-cytoplasmic actin downregulation (with compensatory gamma upregulation) induced a spread or fanlike phenotype; by contrast downregulation of gamma-cytoplasmic actin resulted in a contractile phenotype (Dugina et al. 2015). In beta-actin knockout MEFs, gamma-actin was also observed in protrusions (Tondeleir et al. 2012) (Fig. 4): this was interpreted that also gamma-smooth muscle actin (or alpha-smooth muscle actin which was also present in this cell) can provide the driving force for cell migration since, upon ROCK inhibition, these *Actb*$^{-/-}$ MEFs migrated faster than untreated WT-MEFs. The effect on cell invasion in vitro was also investigated upon beta- or gamma-cytoplasmic silencing in A549 and HCT116 cancer cell lines with as main outcome (and in line with overexpression studies in these cells) an increased invasive capacity upon beta-cytoplasmic actin downregulation (compensated by gamma-cytoplasmic actin upregulation). Based on this result and on the relative levels of the two actin isoforms in lung and colon tumors and the effects on proliferation, the authors connect more gamma-cytoplasmic actin and less beta-cytoplasmic actin with a tumorigenic and invasive state and provide an onset to possible underlying mechanisms involving actin-binding and signaling proteins (Dugina et al. 2015). The possible impact of the loss of beta-cytoplasmic actin on the cellular program, as

shown in Tondeleir et al. 2012, may however also play a determining role here. Both gamma- and beta-cytoplasmic actins are thus likely to function in cell migration, but the observation that in a cancer cell context there may be isoform differences in invasiveness (linked to actin concentration) merits further study. The fact that both gamma- and beta-cytoplasmic actins simultaneously populate invadopodia in a mesenchymal (triple negative) cancer cell line (Simiczyjew et al. 2015) may also point at isoform-specific roles in invasion that are either context dependent or different for specific "subfunctions."

In endothelial cells, silencing gamma-cytoplasmic actin was shown to be required for neovessel maintenance (Pasquier et al. 2015). Also chemotactic cell migration was inhibited in these endothelial cells and, interestingly, the degree of inhibition was dependent on the type of chemoattractant. Furthermore random cell migration was also reduced. This clearly establishes a role for gamma-cytoplasmic actin in these cells, since in this system beta- cytoplasmic actin was not upregulated. The phenotypes observed may still be due to a general reduction of the concentration of actin, but arguing against this is that stress fibers in the endothelial cells with reduced gamma-cytoplasmic actin were thickened as a result from changing the beta- to gamma-cytoplasmic actin ratio (Pasquier et al. 2015). Unfortunately the role of beta-cytoplasmic actin could not be investigated in this endothelial model as its silencing resulted in cytotoxicity.

The same group reported on silencing of gamma-cytoplasmic actin in the neuroblastoma cell line SH-EP. It was found that silencing of only approximately 40% was achieved (Shum et al. 2011). There was no effect on beta-cytoplasmic actin levels, but it was observed that total actin levels were similar as in nontreated cells. The identity of the compensatory actin isoform was however not determined. Despite the relatively high levels of residual gamma-cytoplasmic actin, the cells displayed reduced migration capacity in wound closure assays and transwell assays. This was attributed to loss of polarity, although the microtubule system was visibly not affected, and in line with the observation that partial depletion of gamma-actin reduces tubulin dynamics (Shum et al. 2011; Po'uha et al. 2013). Silencing of gamma-cytoplasmic actin also resulted in an increase in the average size and number of focal adhesions. In addition, an increase in stress fibers was noted, but this can be due to increased expression of another actin isoform, similar to what was observed in beta-cytoplasmic actin MEFs that show prominent stress fiber staining as a consequence of compensatory expression of alpha-smooth muscle actin (Tondeleir et al. 2012). Another parallel between the Shum and Tondeleir paper is that both systems displayed increased Rho-ROCK signaling and that inhibition of this pathway rescued the aberrant phenotypes. This again indicates careful studies must be done to make a distinction between direct effects of the reduction of a particular isoform or an indirect effect by newly expressed isoforms.

In another study in A549 lung epithelial cells, an upregulation of alpha-smooth muscle actin was also seen upon silencing of gamma-cytoplasmic (but not upon silencing beta-actin). As a result, the cells underwent a special form of epithelial mesenchymal transition (EMT): epithelial to myoblast transition (EMyT) (Sandbo and Dulin 2011). This is characterized by upregulation of, in addition to alpha-

smooth muscle actin, SM22, L-caldesmon, calponin-1, and tropomyosin, i.e., other smooth muscle cell markers (Lechuga et al. 2014). Similar to the studies presented above, this was accompanied by decreased wound closure and increased stress fiber content. Interestingly, these authors pinpointed a specific interaction between MRTF-A [a transcription factor involved in driving muscle-specific gene expression (Wang et al. 2003)] and gamma-cytoplasmic actin by coIP, whereas the presence of beta-actin in the coIP-ed complex could only be observed after gamma-cytoplasmic actin silencing.

Baranwal et al. (2012) studied effects of silencing beta- or gamma-cytoplasmic actin on the dynamics of adherens junctions in human colonic epithelial cells. Again upregulation of the other isoform was observed but not to the level of total actin of control cells. In both cases, this resulted in a reduction of barrier function of epithelia. Interestingly, beta-cytoplasmic actin knockdown resulted in disrupted adherens junctions, but left the tight junctions intact and the inverse was seen for gamma-actin-silenced cells. Remarkably assembly of tight junctions and adherens junctions was affected to similar extends in either of the silenced cells (Baranwal et al. 2012). The authors conclude that the two cytoplasmic actins have unique roles in regulating the steady-state integrity of AJs and TJs, but are both essential for normal barrier function of epithelial monolayers, rapid AJ/TJ reassembly, and formation of three-dimensional cysts.

4.4.2 Smooth Muscle Actin Isoforms in Cells: Differential Localization and Function

As already mentioned above, striated and smooth muscle actins are often pairwise co-expressed. This has been well documented on the tissue level using immunohistochemistry. The compensatory expression of gamma-smooth muscle actin that was present in smooth muscle cells of the *ACTA2*$^{-/-}$ mice (in bladder smooth muscle cells or myofibroblast) (see above) (Tomasek et al. 2013; Zimmerman et al. 2004) was however not observed in myofibroblasts of the stromal reaction or those derived from other fibrotic conditions (Arnoldi et al. 2013). The same authors showed that alpha- and gamma-smooth muscle actins differentially localize by forming two distinct filamentous systems in cultured vascular smooth muscle cells. Gamma-smooth muscle actin localized more centrally and did not connect to focal adhesions, whereas alpha-smooth muscle actin fibers extended throughout the cell and anchored to vinculin containing adhesions.

Silencing or overexpression of smooth muscle actins has been little explored. In cultured vascular smooth muscle cells, Arnoldi et al. (2013) showed that silencing of the alpha-smooth muscle form also increased the amount of gamma-smooth muscle actin, whereas the silencing of the latter form was not compensated by the former. It goes without saying that these observations complicate isoform-specific analysis, especially since a gamma-smooth muscle actin antibody only recently be came available. Interestingly, the silencing of alpha-smooth muscle actin, and upregulation of gamma-smooth muscle actin, was associated with a reduction of cell size. It was put forward that alpha-smooth muscle actin is involved in cell contraction, whereas gamma-smooth muscle actin appeared not directly involved

herein (Arnoldi et al. 2013). In a separate study, using a different antibody, Benzoubir and co-workers proposed gamma-smooth muscle actin as a marker for EMT in hepatocellular carcinoma (Benzoubir et al. 2015). We note however that also alpha-smooth muscle actin has been proposed as a marker for EMT and in particular of a subtype: EMyoT (Sandbo and Dulin 2011). Recently, Chen et al. investigated effects of alpha-smooth muscle actin overexpression and silencing in vascular smooth muscle cells on proliferation and migration via a negative effect on Rac activation (Chen et al. 2016). They suggest this regulation may keep vascular smooth muscle cells quiescent in healthy arteries where alpha-smooth muscle actin expression is high. When this expression declines after arterial injury, hyper-activation of Rac may occur and promote smooth muscle cell migration and proliferation, thereby contributing to a thickening of the inner layer of blood vessels (intimal hyperplasia).

4.5 Mutant Actin Isoforms Associated with Disease

For each of the six human actin isoforms, mutations in the coding sequence have been identified in diseases. Research has mainly focused on how the mutations affect actin function and on how this is related to the pathology. We refer to (Rubenstein and Wen 2014) for a review of the various model systems used to study this. We do not provide a complete list of mutants but only discuss these mutants that were biochemically studied or showed pronounced cellular defects.

Both beta- and gamma-cytoplasmic actin mutations have been found in patients with Baraitser-Winter syndrome (Riviere et al. 2012). This is a rare disease but with complex clinical features including facial dysmorphism, deafness, and in some cases dystonia. Although mutations in both actins lead to this disease arguing that the cytoplasmic actins function in similar processes, it was noticed that mutations in beta-cytoplasmic actin were associated with the more severe forms (Di Donato et al. 2014). Lymphoblastoid cell lines of these patients showed an increased F-actin content and an increased perimeter. In Baraitser-Winter syndrome, Arg195 in beta-cytoplasmic actin and Ser154 in gamma-cytoplasmic actin were predominantly mutated resulting in dominant negative effects (Riviere et al. 2012). Another beta-cytoplasmic actin mutation R182W results in developmental malformations, juvenile onset dystonia, and deafness (Procaccio et al. 2006). This mutant was biochemically studied. It assembled more slowly into filaments, had an increased ATP hydrolysis rate in the filament, and depolymerized faster. Moreover its capacity to activate the non-muscle myosin NM2A ATPase activity was impaired (Hundt et al. 2014). Beta-cytoplasmic actin mutant E116K (mimicked in yeast actin) polymerized faster than wild-type (WT) actin, and at high concentration, it formed filament structures under conditions that usually maintain G-actin. Filaments were also more resistant to latrunculin-A-induced polymerization (Johnston et al. 2013). By contrast a beta-actin mutant from a patient with neutrophil dysfunction (Nunoi et al. 1999) showed reduced F-actin assembly compared to WT beta-actin.

In line with the phenotype of the KO in mice (see above), mutations in gamma-cytoplasmic actin lead to progressive hearing loss (Baek et al. 2012; van Wijk et al. 2003; Rendtorff et al. 2006; Morin et al. 2009; Park et al. 2013; Miyagawa et al. 2013, 2015; Mutai et al. 2013; Kemerley et al. 2016), but also beta-actin mutations may result in deafness (Procaccio et al. 2006).

Mutations in alpha-skeletal muscle actin (*ACTA1*) lead to a group of diseases called actinopathies (Nowak et al. 2013). These come with variable severities and have heterogeneous clinical manifestations, but death of patients within the first year is unfortunately no exception. Over 400 mutations in *ACTA1* are listed at the Leiden Open Validation database: http://dmd.nl/ (select ACTA1). Over 200 of these are missense mutations resulting in presumably defective proteins. Indeed the majority of the mutations (approximately 90%) are dominant and expected to arise de novo (Laing et al. 2009). These patients express mutant actin molecules that have adverse effects on the remaining WT molecules and on their function, likely within the thin filament, but in view of the complex biochemistry of actin (see above), it is unlikely that only one function is affected. In addition, it is thought that the ratio between the mutant actin and WT actin (including alpha-cardiac muscle actin (*ACTC*) which is sometimes upregulated in skeletal muscles from patients) codetermines severity of the disease (Nowak et al. 2013; Ravenscroft et al. 2011). Patients having recessive mutations do not produce functional protein. This can be either due to frame shift mutations or to missense mutations resulting in protein that is not capable of folding (Costa et al. 2004).

Muscle biopsies from patients with mutations in alpha-skeletal muscle actin (*ACTA1*) most commonly display nemaline bodies, but also other aberrant structures have been observed such as aggregates, caps, core-like areas, dystrophic changes, fiber-type disproportion, intranuclear rods, minimal change, and zebra bodies (reviewed in Nowak et al. 2013). However no clear correlation can be made between mutation and histochemical phenotype or pathophysiology. Patients having the same alpha-skeletal actin mutation frequently display a different degree of severity and may have different ultrastructural aberrations in their muscle (Hutchinson et al. 2006; Laing et al. 2009).

Mutations in alpha-cardiac actin lead either to hypertrophic (HCM) or dilated cardiomyopathy (DCM) (Mogensen et al. 1999; Olson et al. 1998). Compared to alpha-skeletal actin, fewer mutants have been identified, but some of these have been characterized biochemically using baculovirus-produced proteins (Mundia et al. 2012; Bai et al. 2014; Bookwalter and Trybus 2006). A range of biochemical defects was discovered. Mutants A230V (HCM) and R312H (DCM) were less thermally stable than WT actin, whereas E99K (HCM) was more stable (probably by forming contact with other actin molecules under nonpolymerizing conditions). M305L (HCM) and R312H had increased rates of phosphate release suggesting altered polymerization kinetics and less stable filaments. Mutants Y166C (HCM) and R312H had a lower amount of filamentous actin in polymerization experiments suggesting polymerization defects (Mundia et al. 2012). Mutant A331P was investigated in reconstitution experiments with tropomyosin/troponin and myosin. Purified mutant actin displayed faster polymerization kinetics than WT; thus,

polymerization was not negatively affected. Tension generated by acto-myosin interaction was however affected both in the absence and presence of tropomyosin/troponin (Bai et al. 2014), and it was calculated that cross-bridge force was weakened. Another mutant, E99K, has a reduced affinity for myosin and consequently a decreased ability to generate contractile force (Bookwalter and Trybus 2006).

Alpha-smooth muscle actin is highly expressed in vascular SMCs and the major form in arteries (Arnoldi et al. 2013). Mutations in alpha-smooth muscle actin (ACTA2 in humans) cause cerebrovascular or thoracic aortic disorders and in particular thoracic aortic aneurysms and dissection (TAAD) (Georgescu et al. 2015; Bradley et al. 2016). The TAAD diseases have heterogeneous clinical manifestations with many subtypes. Over 30 mutations in alpha-smooth muscle actin have been associated with these diseases (Guo et al. 2007, 2009; Campens et al. 2015). Patients have aortic pathologies such as medial degeneration and smooth muscle cell hyperplasia in the vasa vasorum. Smooth muscle cells from TAAD patients with mutation R116Q and T351N still have phalloidin-binding stress fibers, but filamentous costaining with an alpha-smooth muscle actin antibody was greatly reduced (Guo et al. 2007). The R256H mutant was reproduced in yeast actin and shown to result in a polymerization defect (Malloy et al. 2012). Also mutant R256C was investigated biochemically and displayed defective properties of relevance for the disease (Lu et al. 2015): mutant filaments were less stable and more easily severed by cofilin, also in the presence of tropomyosin. There was reduced contractile force by slower and less productive interactions of myosin with actin-tropomyosin filaments.

A mutation at R148S in gamma-smooth muscle actin results in familial visceral myopathy, a life-threatening disease (Lehtonen et al. 2012) [interestingly a mutation at equivalent position has been found in Acta1 and Acta2 (Laing et al. 2009; Guo et al. 2007)]. Patients showed aggregates in enteric muscle cells. Expressing a V5 tagged version of this mutant in U2OS sarcoma cells resulted in diffuse cytoplasmic staining, and the mutant could be more easily extracted from the insoluble pellet fraction compared to wild-type gamma-smooth muscle actin suggesting this mutant lacks efficient polymerization capacity. However, also a dominant negative effect on myosin-based contractility was observed in these cells (Lehtonen et al. 2012). Also another disease, megacystis-microcolon-intestinal hypoperistalsis syndrome has been associated with ACTG2 mutations (Wangler et al. 2014; Thorson et al. 2014; Tuzovic et al. 2015).

Collectively these studies suggest that disease symptoms may arise from a variety of defective actin properties and show again that the intrinsic complex biochemistry necessitates careful analysis to understand the dysfunction of the mutants in relation to the disease. Such studies, often comparing WT to mutant actin, parallel the difficulty in finding differences between WT-isoactins (which can to some extent be considered as natural mutants). Underscoring actin isoform specificity, mutations at a same position in different isoforms result in different diseases. Next to the fact that mutations may result in a disease phenotype, also expression levels of actin isoforms have been connected to malignancy. Indeed,

comparing normal and neoplastic non-small cell lung cancer or colon cancer, it was found that beta-cytoplasmic actin was decreased in the malignant tissue, whereas gamma-cytoplasmic actin expression was doubled (Dugina et al. 2015). Alpha-smooth muscle actin expression is also used in the context of cancer due to its expression in activated myofibroblasts in the tumor environment (also termed cancer associated fibroblasts: CAFs) (Otranto et al. 2012).

5 Conclusion

We presented the complex story of mammalian actin isoforms. The mouse genetics and the human diseases provide evidence that these isoforms do not have entirely redundant functions and that a proper functioning actin molecule is necessary for maintaining a healthy state. However linking the phenotypes upon (conditional) ablation or arising from a disease with the molecular or cellular level remains difficult. This is in part due the recurring observation that the expression level of one actin isoform communicates with that of other actin isoforms. This is clear from both genetic ablation and silencing experiments. How this communication occurs is, however, unresolved and is in our opinion a major future challenge. This involves both studying isoform-specific signaling mechanisms to the nucleus, preferably in a tissue-specific manner, and understanding control elements in the actin gene promoters.

The biochemistry on recombinant and thus pure isoactins (or derived mutants) other than alpha-skeletal muscle actin is only emerging. Given the vast array of actin-binding proteins (which as a rule also come in different isoforms), a significant amount of research lays ahead of us to map differential properties of the interactions. Although this work on purified components is essential, we need to go even further as the complex biochemistry obviously occurs in a cellular environment. Measuring co-occurrence or segregation of particular actin isoforms in subcellular structure continues to be a prerequisite. The new superresolution microscopy technologies combined with (improved) specific antibodies or other visualization tools will contribute to this. We should however not forget that the actin cytoskeleton is a dynamic structure with changing compositions of multicomponent systems. Understanding the spatiotemporal regulation of the actin cytoskeleton and the contribution of particular isoforms in this will require intensive meticulous research in various model systems.

This will ultimately allow us to understand “why the things that make the actin isoforms different are part of the things that make us.”

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Assembly and Maintenance of Myofibrils in Striated Muscle

Joseph W. Sanger, Jushuo Wang, Yingli Fan, Jennifer White, Lei Mi-Mi, Dipak K. Dube, Jean M. Sanger, and David Pruyne

Contents

Abstract

In this chapter, we present the current knowledge on de novo assembly, growth, and dynamics of striated myofibrils, the functional architectural elements developed in skeletal and cardiac muscle. The data were obtained in studies of myofibrils formed in cultures of mouse skeletal and quail myotubes, in the somites of living zebrafish embryos, and in mouse neonatal and quail embryonic cardiac cells. The comparative view obtained revealed that the assembly of striated myofibrils is a three-step process progressing from premyofibrils to nascent myofibrils to mature myofibrils. This process is specified by the addition of new structural proteins, the arrangement of myofibrillar components like actin and myosin filaments with their companions into so-called sarcomeres, and in

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their precise alignment. Accompanying the formation of mature myofibrils is a decrease in the dynamic behavior of the assembling proteins. Proteins are most dynamic in the premyofibrils during the early phase and least dynamic in mature myofibrils in the final stage of myofibrillogenesis. This is probably due to increased interactions between proteins during the maturation process. The dynamic properties of myofibrillar proteins provide a mechanism for the exchange of older proteins or a change in isoforms to take place without disassembling the structural integrity needed for myofibril function. An important aspect of myofibril assembly is the role of actin-nucleating proteins in the formation, maintenance, and sarcomeric arrangement of the myofibrillar actin filaments. This is a very active field of research. We also report on several actin mutations that result in human muscle diseases.

Keywords

α-Actinin • Actin • DNase1 • Formin • Gelsolin • I-bands • Jasplakinolide • Latrunculin A • Leiomodin • Mature myofibril • Muscle myosin II • Muscle myosin-binding protein C • Myofibrillogenesis • Myomesin • Myosin • Nascent myofibril • Nonmuscle myosin II • Premyofibril • Tropomodulin • Tropomyosin • Vitamin D-binding protein • Z-bands • Z-bodies

1 Introduction

The basic organization of proteins that make up the repeating pattern of the structural units, the sarcomeres, in vertebrate cardiac and skeletal muscles persists for a lifetime of contractions despite changes in the size and contractile properties of the muscle during exercise, growth, aging, and illness. The initial assembly of sarcomeres and myofibrils has long puzzled scientists (Haeggqvist 1920; Crick 1977; Sanger and Sanger 2014). Since the classic biochemical work of Zak and his collaborators, it has been known that the half-lives of sarcomeric proteins range from 5 days to 2 weeks (Clark and Zak 1981). It was surprising, therefore, that fluorescently tagged proteins, when microinjected into cultured neonatal and embryonic skeletal muscle cells, were incorporated within hours, as opposed to days, into all sarcomeres and myofibrils (reviewed in Sanger et al. 1986a, b, 2004). Similar observations were obtained with the microinjection of fluorescently dye-coupled muscle proteins into freshly isolated adult rat cardiomyocytes: The rapid exchange of sarcomeric proteins is seen in both embryonic and adult cardiomyocytes (LoRusso et al. 1992). Measuring the rates of *f*luorescence *r*ecovery *a*fter *p*hotobleaching (FRAP) of such cells showed that of five sarcomeric Z-band proteins tested each had a characteristic exchange rate in cultured quail embryonic skeletal muscle cells (Wang et al. 2005a, b), and in vivo measurements in zebrafish embryos expressing fluorescently labeled fusions of the five Z-band proteins in skeletal muscle confirmed these exchange rates (Sanger et al. 2009; Wang et al. 2014). We have speculated that these observations reflect a constitutive

process of dynamic protein exchange in muscle cells that allows efficient replacement of old or damaged sarcomeric proteins by new sarcomeric proteins without disassembly of the myofibrils (Sanger et al. 2010). Understanding the details of assembly and maintenance of myofibril architecture is still a major challenge in the muscle field (Piccirillo et al. 2014).

2 The Three-Step Model of Myofibrillogenesis

The first alignment of sarcomeric proteins seen during myofibrillogenesis in skeletal myotubes of avian and mouse cultures is detected at the elongating ends and sides of thin premyofibrils composed of actin filaments with small bands of nonmuscle myosin II that alternate with puncta containing muscle-specific α-actinin (Z-bodies) (Figs. 1 and 2). However, even before myotubes form, muscle-specific α-actin, together with nonmuscle actin isoforms β and γ, is present

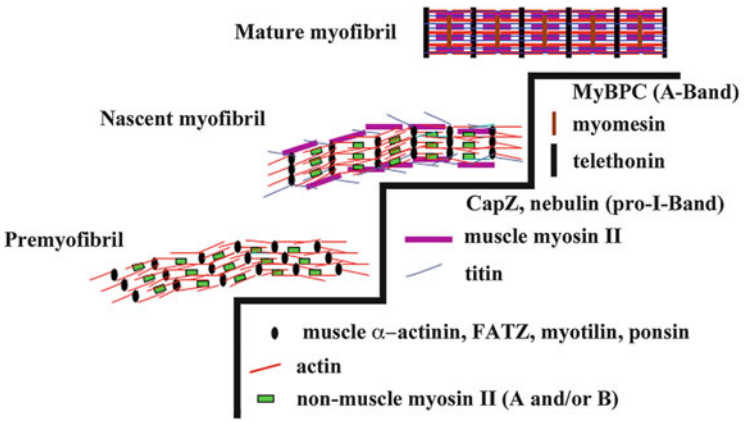

Fig. 1 Diagram of the premyofibril model for de novo skeletal myofibrillogenesis: premyofibrils to nascent myofibrils to mature myofibrils. The Assembly of premyofibrils is initiated at the spreading ends or sides of muscle cells. **Premyofibrils** are composed of minisarcomeres that contain sarcomeric proteins in the α-actinin enriched Z-Bodies, and in the attached thin filaments (F-actin and their associated proteins tropomyosin and troponins). Myosin filaments, containing non-muscle myosin IIA and/or IIB, are present in the mini A-Bands of the premyofibrils. In **nascent myofibrils**, Z-Bodies in adjacent fibrils align and farm beaded Z-Bands. Titin molecules and muscle myosin II thick filaments are first detected in nascent myofibrils. Nascent myofibrils possess two different types of myosin II, i.e., non-muscle myosin II and muscle myosin II. The thick muscle filaments in the nascent myofibrils overlap each other, exhibiting continuous anti-muscle myosin II staining in fixed cells. Titin molecules appear, but are not well aligned with the thin filaments, which already contain CapZ and nebulin (Pro-I-bands). During the maturation in to mature myofibrils, the beaded Z-Bands transform into continuous Z-Bands. The muscle myosin II binding proteins, Myosin Binding ProteinC (MyBPC) and M-Band proteins (e.g., myomesin), appear as late components, presumably aiding the stability (MyBPC), and alignment of thick filaments side by side by cross-linking them (myomesin) into A-Bands. Telethonin is another late assembling protein, present exclusively in the Z-Bands of the mature myofibrils. Non-muscle myosin II proteins are absent from the mature myofibrils (Diagram modified from White et al. 2014)

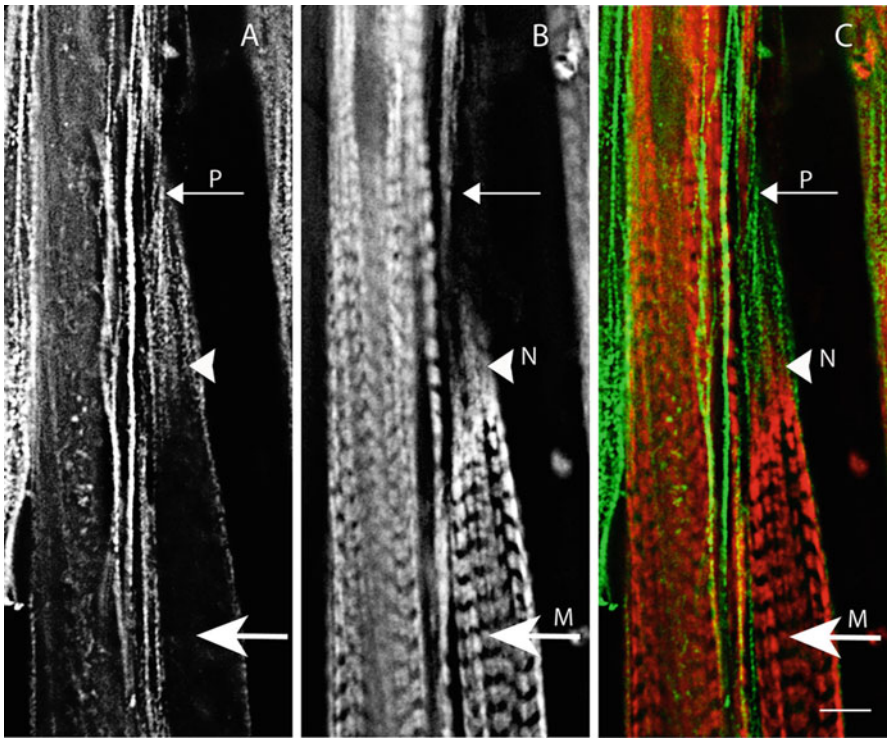

Fig. 2 Distribution of two isoforms of myosin II during myofibril assembly in cultivated mouse skeletal myotubes. Myotubes were co-stained with antibodies to nonmuscle myosin IIA, with *green* fluorescence, and muscle myosin II, with *red* fluorescence. (**a** and *green* in **c**) Detection of the nonmuscle myosin; (**b** and *red* in **c**) detection of muscle myosin II. Thin fibrils (premyofibrils, P) with small bands of the nonmuscle IIA isoform of myosin are present in the tips of myotubes (*small arrows* **a**, **c**) and along the sides of myotubes. Distal to the tip, muscle myosin II is organized in A-bands in mature myofibrils (M) in the shaft of the myotube (*large arrows* **b** and **c**). The *large arrow* in (**a**) points to the absence of nonmuscle myosin IIA in the A-bands. The *small arrow* in **b** points to the absence of muscle myosin II in premyofibrils. Both isoforms of myosin II are present in a nascent myofibril (N) zone with unbanded muscle myosin II (*arrowhead* in **b**) and nonmuscle myosin IIA (*arrowhead* in **a**) localized between premyofibrils (P) in the myotube tip and mature myofibrils (M). The image is a single plane from a Z-stack deconvolved with Leica AF6000 software (Modified from White et al. 2014. Bar = 5 μm)

in dividing myoblasts in avian cultures (Devin and Emerson 1978, 1979). As myofibrils assemble in embryonic skeletal muscle, two isoforms of muscle-specific actin, differing only in two amino acids, cardiac α-actin (ACTC1) and skeletal α-actin (ACTA1), are expressed in unequal amounts in the muscle cell (Vandekerckhove et al. 1986). In 11-day embryonic skeletal muscle, the level of cardiac α-actin expressed is substantially higher than skeletal α-actin. But the relationship switches as development proceeds, leading to an excess of skeletal α-actin in 18-day embryonic skeletal muscle (Vandekerckhove et al. 1986).

Figure 1 presents in a model the major changes in protein composition and arrangement during the maturation process from premyofibrils to nascent and mature myofibrils of skeletal muscle. These sequences are largely based on staining with specific antibodies, for example, for the different myosins (Sanger et al. 2004, 2006, 2010) and Z-band components (Rhee et al. 1994; Wang et al. 2005a, 2007, 2008, 2014; White et al. 2014). When premyofibrils are formed, nascent myofibrils assemble behind the zone of premyofibril formation. During myofibrillogenesis, nonmuscle myosin II and muscle myosin II both are seen in premyofibrils and nascent myofibrils, respectively (Fig. 2). Finally, mature myofibrils form distal to the nascent myofibrils. Here, muscle-specific myosin filaments are aligned in register in A-bands with myosin-binding protein C and myomesin. Z-bodies fuse to form Z-bands, and nonmuscle myosin II is lost from the mature myofibrils by an unknown process.

Similar evidence for a three-step model of myofibrillogenesis was obtained in cat and rat cardiomyocytes isolated from adult hearts and grown in culture (LoRusso et al. 1997). Premyofibrils and nascent myofibrils were not detected initially in the freshly isolated adult cardiomyocytes. However as the cardiomyocytes spread and increase in size, premyofibrils appear at the spreading cell margins, and new mature myofibrils form. Such re-expression of fetal proteins in adult cardiomyocytes might also occur as reinitiation of the embryonic program for de novo myofibrillogenesis in diseased hypertrophic hearts.

3 Myofibrillogenesis in the Skeletal Muscle Cells of Zebrafish Embryos

Zebrafish embryos are translucent and therefore ideal subjects for the study of the myofibril formation in the somites of live animals along their trunk (Sanger et al. 2009). One-day embryos already have 25–30 somites that stretch from the tail to the thorax area. Skeletal muscle assembly proceeds along a time course from the oldest somites near the head to the youngest somites at the tail, providing a clear indicator of the assembly sequence. The youngest somites are initially shorter and increase in length over several days. Figure 3 is derived from a one-day embryo with 30 somites and shows the actin filament arrangement as seen by staining with the natural toxin phalloidin in somites ranging from 25 μm caudal to 60 μm at the head region. Figure 4 depicts the development of the Z-bands as displayed by staining for the main Z-band component α-actinin. In the shortest somites, α-actinin-positive Z-bodies are seen in premyofibrils that are continuous from end to end in the youngest muscle cells. The minisarcomeres bounded by Z-bodies in these premyofibrils range from 0.5 to 1.2 μm. In older somites, α-actinin antibody staining reveals primarily Z-bands that mark the mature sarcomere lengths of 2.4 μm. These mature sarcomeres do not stretch completely from one end of the muscle cells to the other end, but rather between the ends of each mature myofibril and the boundary of the somite where punctate Z-bodies, characteristic of minisarcomeres of premyofibrils, are still visible. Thus, as the somites increase

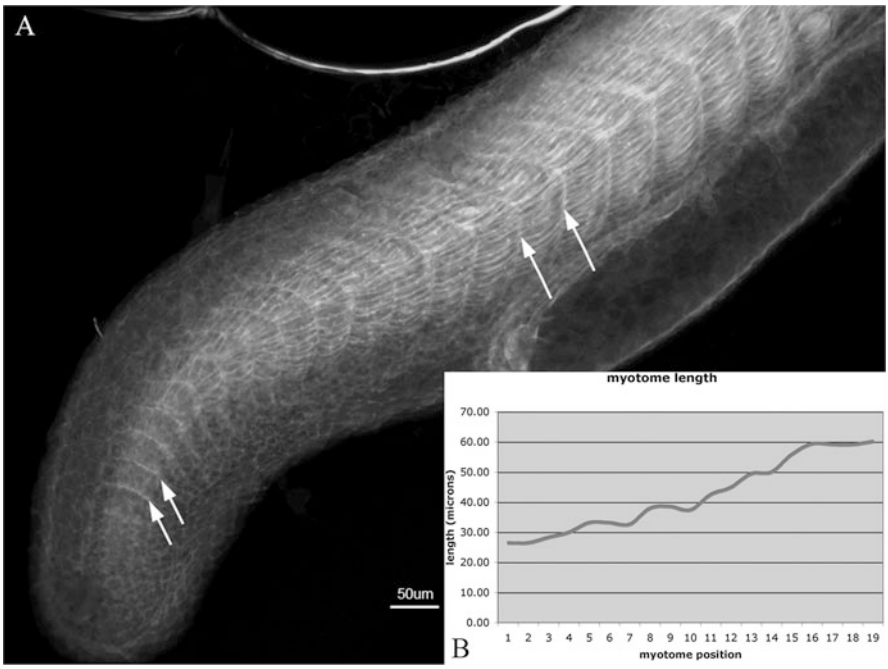

Fig. 3 Actin organization and somite length distribution in a 1-day-old zebrafish embryo. (**a**) The youngest somites of the total of 30 somites present at this time point are shown in this image. The actin filaments are decorated with a fluorescent variant of the natural actin-binding toxin phalloidin. The chevron boundaries (*arrows*) of the somites are visualized, and the myofibrillar actin fibers extend from the one-somite boundary to the boundary of the next somite in series. Note the doubling of both the somite and continuous myofibrillar lengths between one of the youngest and shortest caudal somites (*pair of short arrows*) versus an older and longer cephalic somite (*pair of longer arrows*). Scale = 50 μm. Image first published in Sanger et al. (2009). (**b**) Graph of the lengths of the youngest somites in (**a**). Note the stepwise increase in the somite length distribution which range from a caudal 25 μm to a cephalic 60 μm

their lengths during early development, the formation of premyofibrils and their maturation into new mature sarcomeres occur at the ends of the muscle cells. This process results in elongation of existing mature myofibrils and resembles the sequence of myofibril assembly observed in cultures of avian, cat, mouse, and rat muscle cells.

Figure 5 shows the changing distribution of muscle-specific myosin during skeletal myofibril assembly in three groups of somites, in a 1-day-old embryo, immune stained with a muscle myosin-specific antibody. In the two youngest somites, a few aggregates of muscle myosin are present. In the two adjacent older somites, muscle myosin-positive fibers are running longitudinally, as characteristic of nascent myofibrils (Fig. 5a). The fifth somite (Fig. 5b) shows overlapping muscle myosin fibers, continuously decorated by the antibody, as typical for nascent myofibrils, as well as some banded fibers. In the oldest somites near the zebrafish

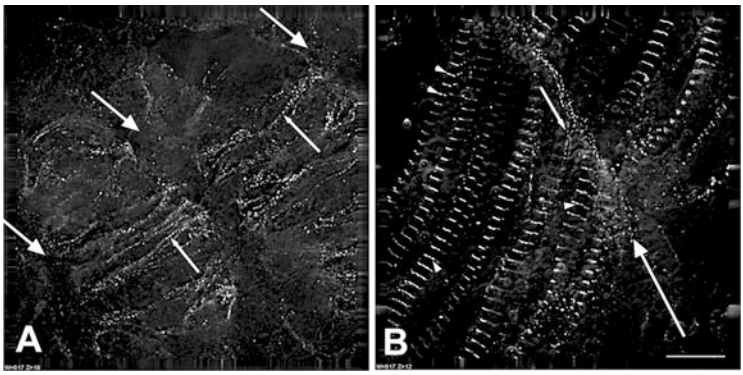

Fig. 4 Distribution of Z-bodies and Z-bands in somites in the tail of a 25-somite-stage zebrafish embryo. Immune staining for α-actinin was used to visualize Z-bodies and Z-bands. (**a**) Two somites near the caudal end of the zebrafish. The *three large arrows* point to the boundaries or myosepta of two somites. The Z-bodies of premyofibrils (*thin arrows*) are aligned linearly from one myoseptum to the other. These Z-bodies mark the boundaries and lengths (0.5–1.2 μm) of the minisarcomeres present in the premyofibrils. (**b**) Two somites near the cephalic end of the tail of the same zebrafish embryo. The Z-bands of the mature myofibrils have sarcomeric separations of about 2.0–2.4 μm. Premyofibrils (*short arrow*) are detected at the ends of some mature myofibrils and at the boundary between the two somites (*large arrow*). The *arrowheads* indicate some still beaded Z-bands. Scale, valid for (**a**) and (**b**), = 10 μm (Modified from Sanger et al. 2009)

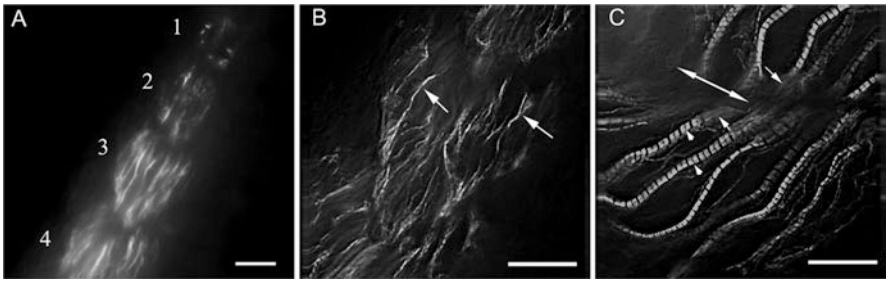

Fig. 5 Assembly patterns of muscle-specific myosin in a 1-day-old zebrafish embryo. The somites were immune stained with a muscle-specific myosin II antibody. (**a**) Somites at end of tail. Note that muscle myosin has already been deposited in the newest somite labeled 1. In somites 3 and 4, the myosin molecules are already more aligned into solid fibers (evidence of nascent myofibrils). (**b**) Somite number 5 of the same embryo, at higher magnification. The *arrows* indicate fibers with overlapping arrays of myosin filaments, i.e., in nascent myofibrils. (**c**) One of the oldest somites near the head of the fish. The *double-arrowheaded line* indicates the myoseptum between two adjacent somites. Note the aligned A-bands (*arrowheads*) in the mature myofibrils in the middle of the somite. The *small arrows* at the ends of the mature myofibrils indicate overlapping myosin filament staining. These images suggest that mature myofibrils can still increase in length by the addition of sarcomeres near the somite boundaries. Scales = 10 μm (Modified from Sanger et al. 2009)

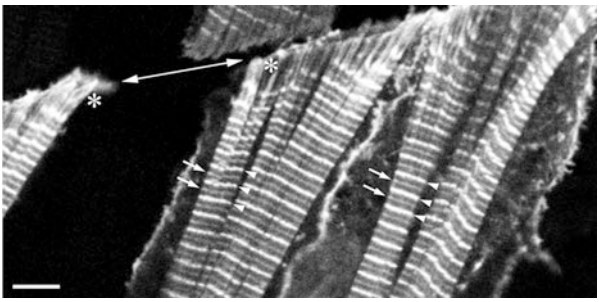

Fig. 6 Expression of YFP-actin in sarcomeres of mature myofibrils in skeletal muscles of a living zebrafish, 5 days postfertilization. The plasmids encoding YFP-actin were previously injected into the yolk of the zebrafish embryos 10–20 min postfertilization. The *double arrow line* marks the somite boundary. In these living muscle cells, mature myofibrils show actin deposits in sarcomeres about 1.9 μm in length. In addition to the sarcomeric I-bands are narrow bands of actin (*short arrows*). These patterns support the interpretation that the thin filaments from adjacent Z-bands (*arrowheads*) of a common sarcomere overlap in the middle of the sarcomere, implying that this myotube is partially contracted. In addition, at the ends of the mature myofibrils and bordering the boundaries of the somites are YFP-actin filaments in a continuous arrangement (*asterisks*), indicating the existence of premyofibrils and nascent myofibrils in this location. These are the sites where mature myofibrils elongate as the somites grow, passing a maturation process from premyofibrils to nascent myofibrils to mature myofibrils. Scale = 5 μm (Modified from Sanger et al. 2009)

head, the A-band alignment of mature myofibrils predominates (Fig. 5c). However, at the boundary between somites, near the ends of these mature myofibrils, unbanded fibers of myosin seen in nascent sarcomeres are present. This is the site where elongating myofibrils will form (Fig. 5c).

The arrangement of actin filaments during maturation of skeletal muscle in the zebrafish was analyzed in embryos expressing YFP-actin, 5 days after fertilization. Figure 6 shows that most of the actin filaments are already organized in sarcomeric units, as typical for mature myofibrils. However, adjacent to somite boundaries are also actin filaments still deposited in an unbanded pattern, as characteristic for premyofibrils and nascent myofibrils. Thus, the results obtained in living zebrafish embryos confirm that the three-step model of myofibrillogenesis (Fig. 1), originally based on studies of cultured avian and mammalian striated muscle cells (Rhee et al. 1994; Dabiri et al. 1997; Du et al. 2003; Sanger et al. 1986a, b; Wang et al. 2005a), also operates in fish development.

4 Interfering with Actin Polymerization: The Effect on Myofibrillogenesis

Many nonmuscle cells, including fibroblasts cocultivated in cultures of cardiac and skeletal muscle cells, and endothelial cells in the lining of blood vessels develop so-called stress fibers, which comprise filamentous actin, myosin, and associated

proteins arranged in minisarcomeres (Sanger et al. 1983) that are reminiscent of premyofibrils. Similar fibers can also be seen in the cleavage furrows of dividing cells (Sanger and Sanger 1980). The resemblance of stress fibers and premyofibrils led to the suggestion that stress fibers or stress fiber-like structures in muscle cells might serve as a reusable template on which myofibrils could be assembled (Dlugosz et al. 1984). However, Z-bodies of premyofibrils containing fluorescently labeled α-actinin were not static during myofibrillogenesis and could be followed, aligning as they grew apart and fused to form Z-bands of mature myofibrils, without a stress fiber-like template (Sanger et al. 1984, 1986a, b, 1989; Dabiri et al. 1997).

The response of stress fibers and premyofibrils to actin-binding proteins like vitamin D-binding protein (VDBP) and DNase1 also differs. Injection of these proteins caused disassembly of stress fibers in nonmuscle cells, but had no effect on premyofibrils and sarcomeres of myofibrils in muscles (Sanger et al. 1990). This indicates that premyofibrils are neither identical with stress fibers nor reusable templates for the formation of mature myofibrils (reviewed in Sanger et al. 2004, 2006, 2010).

Exposure to latrunculin A (Lat-A), an inhibitor of actin polymerization (Morton et al. 2000), also elicited disparate responses from stress fibers in nonmuscle cells and premyofibrils and mature myofibrils in muscle cells (Wang et al. 2005b, 2014). Concentrations of 0.2–0.5 μmol Lat-A reversibly induced the disassembly of stress fibers in fibroblasts and myoblasts in cultures of quail primary muscle cells, but this concentration had no effect on premyofibrils or myofibrils in later stages of myofibrillogenesis. Figure 7 illustrates the effects of increasing concentrations and exposure times of Lat-A on myotubes expressing YFP-α-actinin. The same transfected myotube was followed over time in three different regions: the growing end of the myotube where premyofibrils first formed, a transition zone where the first mature myofibrils were detected, and the rounded middle of the myotube in which mature myofibrils dominated. As the concentrations of Lat-A were raised gradually over one-hour time periods, premyofibrils started to disassemble when the inhibitor concentration reached 1 μM after 3 h and were completely disassembled by 6 h when Lat-A reached 10 μM. In the absence of premyofibrils, myofibrillogenesis was halted. In the transition zone, the first forming mature myofibrils proved more resistant to the drug but also disassembled eventually after longer exposure times with higher concentrations. In contrast, older mature myofibrils were stable even at the highest Lat-A concentration (10 μM) and exposure time (10 h) tested. Removal of Lat-A led to the reformation of premyofibrils in the leading edge of the myotube, and the restart of the formation of new mature myofibrils (Wang et al. 2005b), probably by the increase of the pool of actin monomers. Figure 8 suggests in a diagram how insertion of additional sarcomere components such as actin capping proteins might increase the stability of the actin thin filaments during the progression from premyofibrils to nascent and mature myofibrils.

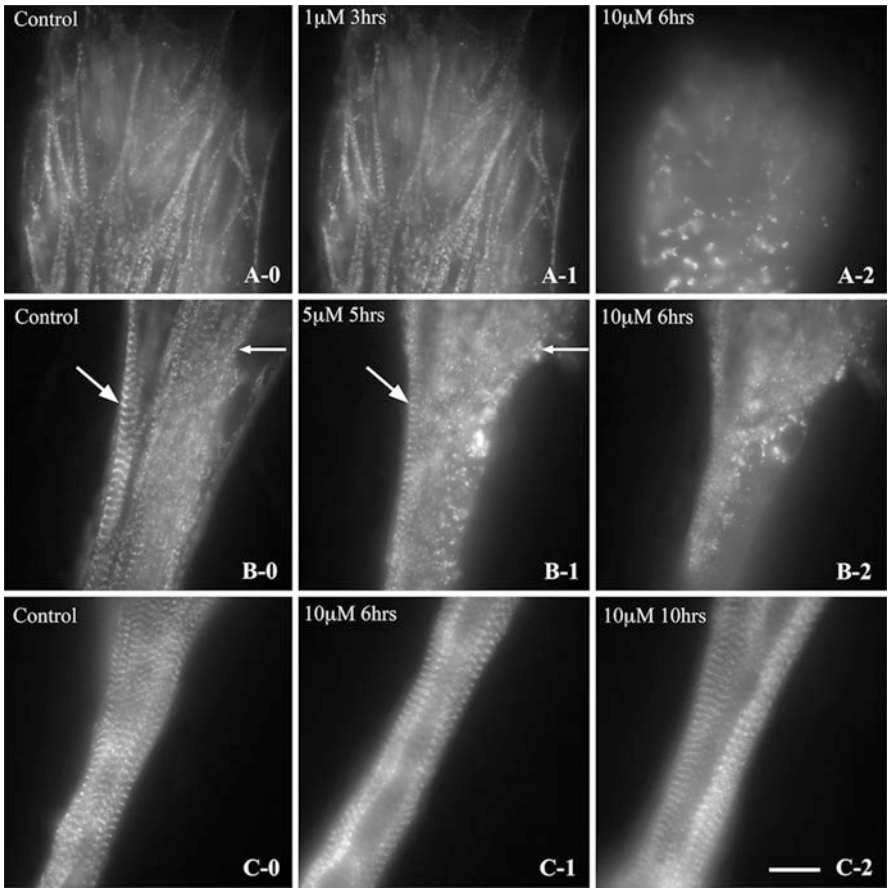

Fig. 7 Differential effects of Lat-A on myofibril assembly in cultured embryonic quail myotubes expressing YFP-α-actinin. The natural toxin latrunculin A (Lat-A) was used to interfere with actin polymerization into filaments, monitored in three different regions of the same myotube. The exact Lat-A concentrations and exposure times in all three different regions of the same transfected myotube are listed in each picture. (**A-0**, **A-1**, **A-2**) Images from the growing tip. (**A-0**) Premyofibrils in the control. (**A-1**) Partially disrupted premyofibrils. (**A-2**) Completely disassembled premyofibrils. (**B-0**, **B-1**, **B-2**) Images from the transition region between the tip and the central part. (**B-0**) Mature myofibrils (*large arrow*) and assembling myofibrils (*small arrow*) in a transition region near the tip of the myotube. (**B-1**) Disassembled premyofibrils (*small arrow*) and remaining mature myofibrils (*large arrow*). (**B-2**) At higher concentration of Lat-A and longer exposure, mature myofibrils are also disassembled in the transition zone. (**C-0**, **C-1**, **C-2**) Images from the central zone. (**C-0**) Mature myofibrils. (**C-1**) The older mature myofibrils in the central zone of the myotube are more stable (cf. identical treatment for **B-2** and **C-1**). (**C-2**) At the highest drug concentration and longest exposure time tested, mature myofibrils in the central zone still remain intact (Modified from Wang et al. 2005b, Bar = 10 μm)

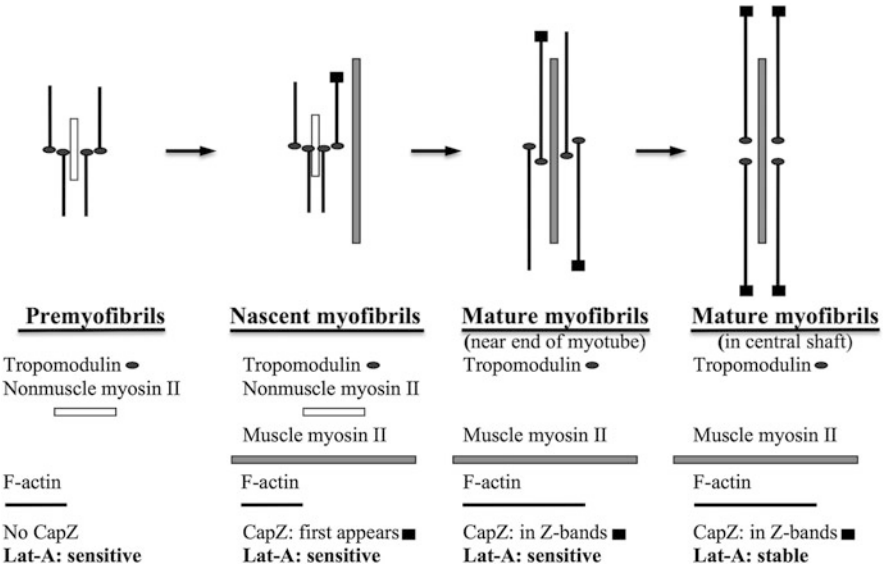

Fig. 8 Diagram illustrating the Lat-A effect on the assembly of premyofibrils, nascent and mature myofibrils in skeletal muscle cells. Two candidate proteins that may stabilize the actin filaments by capping opposite ends of the filaments are shown: tropomodulin on the slow-growing end (*minus* or *pointed end*) and CapZ on the fast-growing end (*plus* or *barbed end*). Tropomodulin is present at actin filament ends at all stages of assembly (Almenar-Queralt et al. 1999). In contrast, CapZ appears first associated with actin filaments in nascent myofibrils. This may explain the increased sensitivity of premyofibrils to Lat-A when compared to nascent or mature myofibrils (see Fig. 7). Intermediate in sensitivity were the nascent myofibrils located in the transition region of the myotube between the ends and in the central shaft. Furthermore, the first mature myofibrils in these transition regions were also more sensitive than mature myofibrils in the central shaft (see Fig. 7). This suggests that the thin filaments of the freshly formed mature myofibrils may not have their full complement of CapZ or other actin-associated proteins. Mature myofibrils in the central shaft of the myotube with well-defined banded concentrations of CapZ were stable in even 50 μM Lat-A (Diagram and text modified from Wang et al. 2005b)

5 Myofibrillar Dynamics Followed by Fluorescence Recovery After Photobleaching

Changes in the dynamic properties of the sarcomeric proteins during the formation of the three myofibril stages were detected by fluorescence recovery after photobleaching (FRAP) (Wang et al. 2005a, 2014; Sanger and Sanger 2008). Comparison of the photobleaching recoveries of YFP expressed in Z-bodies of premyofibrils, nascent myofibrils, and mature myofibrils showed that exchange of sarcomeric proteins between the cytoplasmic pool and sarcomere subunits takes place in each myofibril stage, but is more dynamic in the early stages of Z-band assembly (premyofibrils and nascent myofibrils) than in the Z-bands of mature

myofibrils. This supports the idea that as additional proteins localize in Z-bands in mature myofibrils, a more stable protein complex forms (Wang et al. 2005a). FRAP measurements of YFP-actin and YFP-tropomyosin in premyofibrils and mature myofibrils of both cardiac and skeletal muscle cells showed similar changes (Wang et al. 2008, 2014). Two proteins, cypher and myotilin, that have similar total recovery rates in premyofibrils and in mature myofibrils both show a decrease in their fast mobile fractions and an increase in slow mobile fractions (Fig. 9). This implies a tighter binding of these molecules to cross-linking partners in the Z-bands, as the Z-bodies of premyofibrils align and fuse to form the Z-bands of mature myofibrils (Fig. 10). The increasing number of cross-links between Z-band proteins may cause the decrease in the dynamic behavior of sarcomeric proteins during myofibril maturation (Stout et al. 2008).

The two groups of actin filaments in each sarcomere are oppositely oriented in polarity. This reflects different rates of polymerization at the two ends of the individual filaments, originally measured in isolated actin filaments (Pollard 1984). The faster-growing ends (+) are embedded in the Z-bands, and the slower-growing ends (−) are in the middle of sarcomeres, where they can overlap with the filaments from the next Z-band when sarcomeres shorten. In quail myofibrils, transfected with YFP-actin, the insertion of actin monomers from a soluble pool into the thin filaments was measured at the Z-band (+ end of filaments) and in the mid-sarcomere (− end of filaments), by monitoring the recovery of fluorescence after photobleaching (Fig. 11). It takes many hours for the full recovery of fluorescence of YFP-actin to match the prebleached images. Recovery is faster at the Z-bands of mature myofibrils than in their center part. Remarkably, we did not detect any fluorescence movement or flow of YFP-actin from the Z-bands to the middle of the sarcomeres that would suggest a treadmilling process of actin (Wang et al. 2014). Therefore, we suspect that actin monomers may be exchanging laterally between the fast- and slow-growing ends of the thin filaments in the mature myofibrils (Wang et al. 2014). Comparing the relative recoveries of fluorescence after photobleaching at the Z-band for actin with four actin-binding proteins of the Z-band (myotilin, FATZ, α-actinin, and telethonin) revealed that they differ for each protein, but the order of overall exchange follows the same sequence in quail myotubes, in zebrafish skeletal muscles, and in embryonic mouse cardiomyocytes (Fig. 12) (Wang et al. 2014).

6 Interfering with Actin Filament Stability: The Effect on Myofibrillogenesis

Jasplakinolide, in contrast to Lat-A, binds to and stabilizes F-actin (Bubb et al. 1994, 2000; Hagiwara et al. 2011) and has a dramatic effect on the dynamics of actin exchange in both the early and late stages of myofibrillogenesis (Wang et al. 2014). Figure 13 shows quail myotubes formed in tissue culture stained with anti-α-actinin, to reveal the myofibrillar development in immunofluorescence. When exposed to 0.1 μM jasplakinolide, the myofibrils present in 4-day cultures

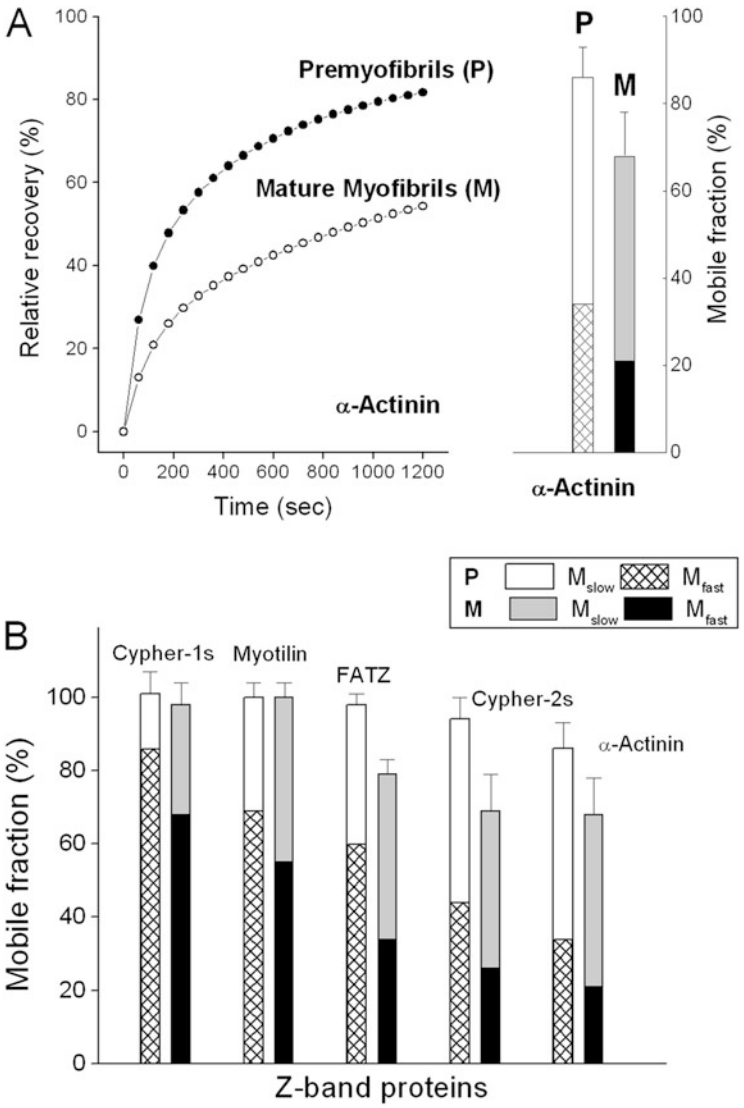

Fig. 9 Fluorescence recovery rates of different Z-band proteins in Z-bodies and in Z-bands after photobleaching (FRAP) of cultured quail myotubes. Embryonic quail myoblasts were transfected with fluorescent probes for the proteins indicated, and the myotubes formed in culture were exposed to photobleaching. The recovery times of the photobleached spots are shown. (**a**) The FRAP curves of sarcomeric α-actinin in the Z-bodies of premyofibrils (P, *black circles*) and in the Z-bands (M, *white circles*) of mature myofibrils. Note that the recovery rate for α-actinin in the Z-bodies of premyofibrils is faster than in the Z-bands of the mature myofibrils. (**b**) Comparison of the fast (M_{fast}) and slow mobile fractions (M_{slow}) of different proteins, all labeled with YFP and analyzed by FRAP in Z-bodies and Z-bands. Values are the means ± SDs. In all cases, the fast mobile fractions are higher in the Z-bodies as compared to the Z-bands

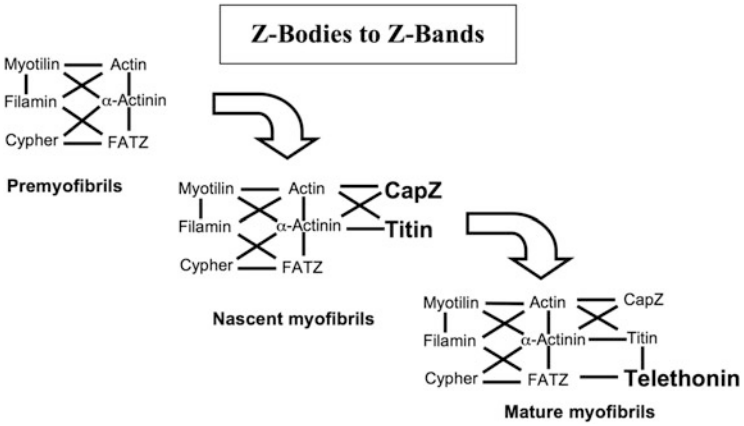

Fig. 10 A model of the progression in complexity from Z-bodies in premyofibrils and nascent myofibrils to Z-bands of mature myofibrils. Premyofibrils already contain a complex of interacting proteins responsible for the architecture and stability of their Z-bodies. Early during the transition to nascent myofibrils, two more proteins, CapZ and titin, were detected as additional components. During the early phase of maturation to mature myofibrils, telethonin is added, as seen in transfection experiments with YFP-telethonin (Wang et al. 2005a, b) and later with immunofluorescence (White et al. 2014). The exchange of early components should permit the remodeling of Z-bodies in their maturation process, and the addition of new proteins will ensure the stability of Z-bands of the contracting mature myofibrils needed to form Z-bands during myofibrillogenesis

are heavily affected: premyofibrils disassemble and nascent myofibrils are reduced in number. In 5-day-old myotubes, the same dose of jasplakinolide causes also disassembly of premyofibrils, but the mature myofibrils already formed in the center of a myotube remain intact (Fig. 13). Removal of jasplakinolide results in reformation of the premyofibrils and new formation of mature myofibrils (Wang et al. 2014). Similar results were obtained on myotubes expressing either YFP-actin or YFP-FATZ (Fig. 14).

The effect of jasplakinolide was also demonstrated in FRAP time-course experiments with cultured quail skeletal myotubes expressing YFP-actin or YFP-tropomyosin 1α (TPM1α, a muscle-specific tropomyosin isoform). The recovery of fluorescence after photobleaching of YFP-actin is strongly suppressed in both premyofibrils and mature myofibrils (Fig. 15). Analysis of quail skeletal myotubes transfected with YFP-TPM1α with the same technique revealed that exposure of premyofibrils to jasplakinolide also reduced the dynamics and mobile fractions of tropomyosin, but had no effect on the dynamics of this protein in mature myofibrils (Fig. 16). This result may reflect the fact that mature skeletal myofibrils contain two additional actin-binding proteins, CapZ and nebulin, which both can stabilize the thin filaments (see model in Fig. 17). Jasplakinolide's effect on tropomyosin dynamics in premyofibrils is much smaller than its effect on actin dynamics in premyofibrils (cf. Fig. 15 vs. Fig. 16). Removal of jasplakinolide restores the dynamics of YFP-actin and YFP-tropomyosin to control values in premyofibrils

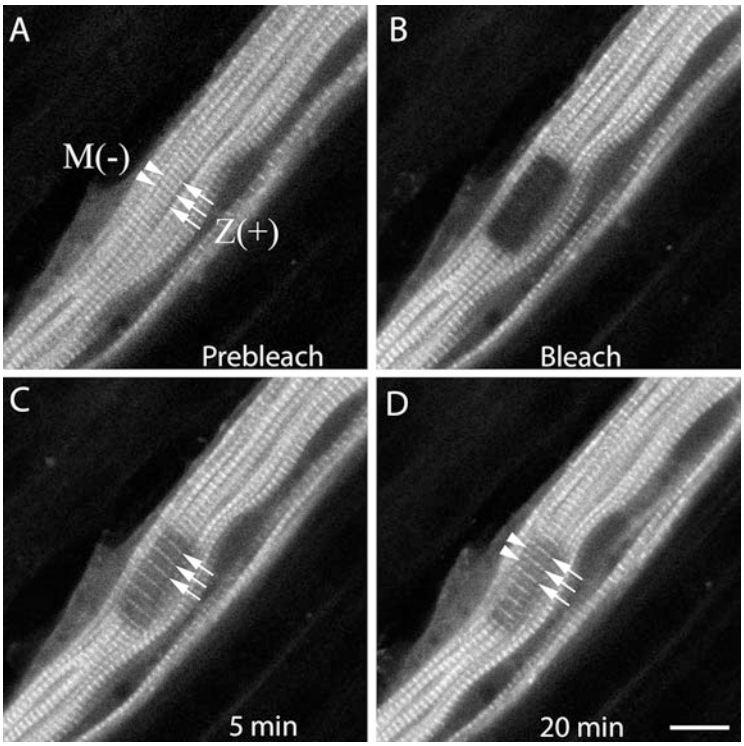

Fig. 11 FRAP in a cultured quail myotube expressing YFP-actin. The myotube was formed by fusion of transfected embryonic quail myoblasts in tissue culture. Actin fluorescence was photobleached selectively in a group of mature myofibrils in control medium, and then the recovery of the fluorescence was measured over the next 20 min (**a**–**d**). (**a**) Prebleached image. The *short arrows* indicate Z-bands of the mature myofibrils prior to photobleaching. The fast-growing ends (i.e., the *plus* or *barbed ends*) of the actin filaments are embedded in the Z-bands [Z (+)], where they overlap with the plus ends of the actin filaments extending into the adjacent sarcomere. The *minus* or *pointed ends* of the actin filaments terminate in the middle of the sarcomeres [M (−)]. The sarcomere lengths in this living quail myotube are 1.9 μm; thus, the ends of the one-micron-long thin filaments overlap in the middle of the sarcomeres (positions indicated by *arrowheads*). (**b**) First image recorded after photobleaching. Note the loss of the fluorescence in the bleached spot. (**c**) Image recorded 5 min after photobleaching. The initial recovery of the YFP-actin fluorescence is detected in the Z-bands, indicated by *three arrows*. (**d**) Twenty minutes after photobleaching, fluorescence is also detected in the middle of the sarcomere (indicated by *arrowheads*). Thus, incorporation of YFP-actin first occurs at the Z-band insertions of actin filaments and later at the overlap regions of the actin filaments in the middle of the sarcomeres (*arrowheads*). Scale = 10 μm

and mature myofibrils (Wang et al. 2014). In contrast to its effect on actin and tropomyosin, jasplakinolide had no effect on the dynamics of the troponin complex (YFP-troponin-T, YFP-troponin-C), or two Z-band proteins (YFP-α-actinin; YFP-FATZ), in either premyofibrils or mature myofibrils in muscle cells (Wang et al. 2014).

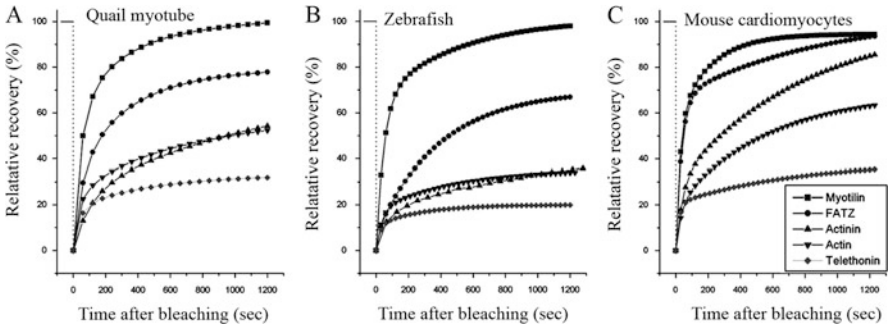

Fig. 12 Comparison of the dynamics of five protein components in the Z-bands of mature myofibrils. Three different cell types were transfected with five different plasmids and analyzed by FRAP. (**a**) Embryonic quail myotubes formed in tissue culture. (**b**) Zebrafish myotomes developed from yolk injected shortly after fertilization. (**c**) Mouse neonatal cardiomyocytes. In all three muscle types, the fastest exchanging protein is YFP-myotilin and the slowest exchanging protein is YFP-telethonin. Note that the relative recoveries of fluorescence of YFP-FATZ, YFP-α-actinin, YFP-actin, and YFP-telethonin are higher in cardiomyocytes than the exchange rates of their counterpart Z-band proteins in the two different types of skeletal muscle cells (Modified from White et al. 2014)

Jasplakinolide treatment of cardiomyocytes provided additional insights into the dynamics of YFP-actin and YFP-TPM1α in premyofibrils versus mature myofibrils (Wang et al. 2014). Jasplakinolide treatment and FRAP time-course experiments, performed on mouse neonatal cardiomyocytes transfected with either YFP-actin (Fig. 18) or YFP-TPM1α (Fig. 19), revealed some differences as compared to skeletal muscle. As expected and similar to the results obtained with quail skeletal muscle (Fig. 15), the dynamics of actin decreases as premyofibrils become mature myofibrils in control cardiomyocytes (Fig. 18), while jasplakinolide reduces the dynamics of YFP-actin in both premyofibrils and mature myofibrils in both types of muscle (cf. Figs. 18 with 15). (Furthermore, jasplakinolide decreased also the dynamics of YFP-TPM1α in cardiac premyofibrils Fig. 19.) However, in cardiac cells jasplakinolide did decrease the dynamics of TPM1α even in mature myofibrils (Fig. 19). This is in sharp contrast to the lack of a jasplakinolide inhibition on TPM1α dynamics in skeletal mature myofibrils (cf. Figs. 19 with 16). This difference may at least in part reside in the different complements of actin-binding and actin-stabilizing proteins. While skeletal myofibrils contain two long nebulin molecules per thin filament (Fig. 17), cardiac muscle contains at most one nebulin molecule per 50 thin filaments (Witt et al. 2006). Instead, cardiac muscle cells express nebulette, which is a much smaller molecule (MW = 107 kD) than nebulin (MW – 60,800 kD) (Millevoi et al. 1998). Thus, while nebulin accompanies virtually the entire length of thin filaments, nebulette covers only 8.6% of their surface in cardiac muscle (Millevoi et al. 1998). Therefore, it seems plausible to assume that nebulin suppresses the dynamics of tropomyosin in mature myofibrils in skeletal muscle (Pappas et al. 2010; Wang et al. 2007, 2008), while in cardiac cells the amount of nebulin and also nebulette is insufficient for this protective effect.

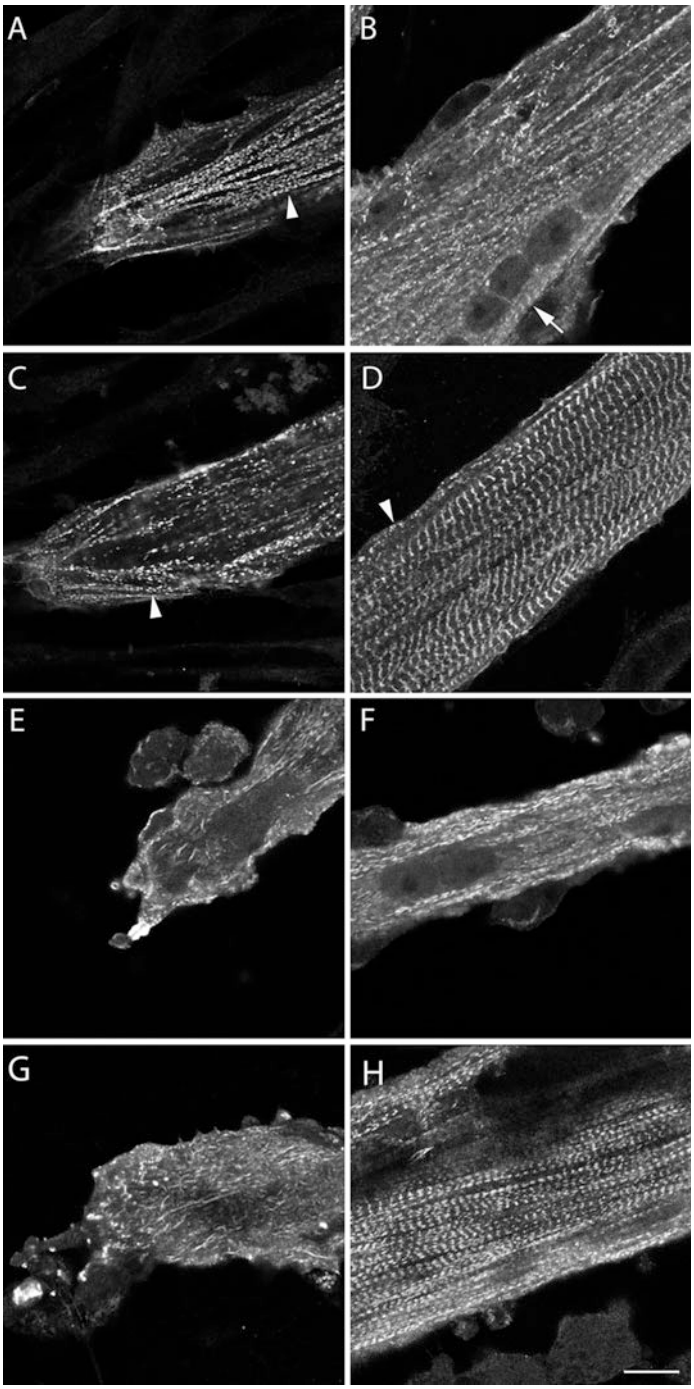

Fig. 13 Effect of jasplakinolide on myofibril formation in cultivated quail myotubes. The cultures were stained with α-actinin antibodies. (**a**–**d**) Myotube maturation in control medium. (**a**, **b**) Four-day-old myotube displaying Z-bodies in premyofibrils in the myotube end (*arrowhead*, **a**) and,

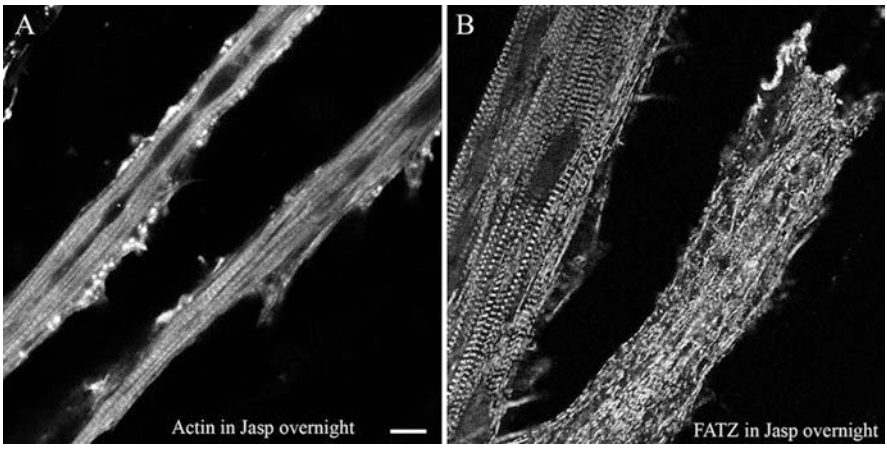

Fig. 14 Effects of jasplakinolide on sarcomeric architecture in living embryonic quail myotubes formed in tissue culture. (**a**) The drug was added to myotubes expressing YFP-actin on the fifth day in culture and observed 1 day later. Mature myofibrils are present, but there are no signs of premyofibrils or nascent myofibrils along the shaft of the two myotubes, as seen in control myotubes. (**b**) Two myotubes expressing YFP-FATZ after 1 day in the presence of jasplakinolide (6-day-old myotubes). FATZ is a component of both Z-bodies and Z-bands. The mature myofibrils in the left myotube appear unaffected by exposure to jasplakinolide. In contrast, the spreading end of the left myotube has lost its linear arrays of premyofibrils typically seen in control myotubes. Scale = 10 μm

Based on these data, Wang et al. (2014) suggested that tropomyosin may exchange from the thin filaments in two different ways: either laterally, from the intact sides of the thin filament, or from areas of the thin filament where actin monomers had been removed laterally by another actin-binding protein, cofilin (Fig. 20). Opposing roles of cofilin (destabilizer) and tropomyosin/nebulin (stabilizer) in myofibrillar integrity had already been discussed previously (Ono 2010), and cofilin-2, the striated muscle isoform of cofilin, is required for the maintenance of actin exchange in myofibrils (Agrawal et al. 2012). In lamellipodia of nonmuscle cells and in vitro, it was shown that jasplakinolide blocks cofilin-actin complex formation (Tsuji et al. 2009; Minamide et al. 2010). Assuming that this is similar for the muscle-specific isoforms in myofibrils, the loss of actin would be inhibited by

Fig. 13 (continued) additionally, mature myofibrils in the central region (*arrow*, **b**). (**c**, **d**) Images obtained on the fifth day: myofibrils have aligned in the end of the myotube (*arrowhead*, **c**). In the central region, a few premyofibrils line the margin of the myotube (*arrowhead*, **d**) which is filled with mature myofibrils. (**e**–**h**) drug-treated myotubes. (**e**, **f**) Jasplakinolide (0.1 μM) was added to myotubes on the fourth day of culture for 24 h. There are very few premyofibrils (**e**) and mature myofibrils (**f**) visible. (**g**, **h**) Jasplakinolide was added to 5-day-old cultures for 24 h. Aligned premyofibrils were absent from the end of this myotube (**g**), but mature myofibrils, which had probably been formed before the addition of the drug, were seen resistant in the central region (**h**) (Text modified from Wang et al. 2014. Bar = 10 μm)

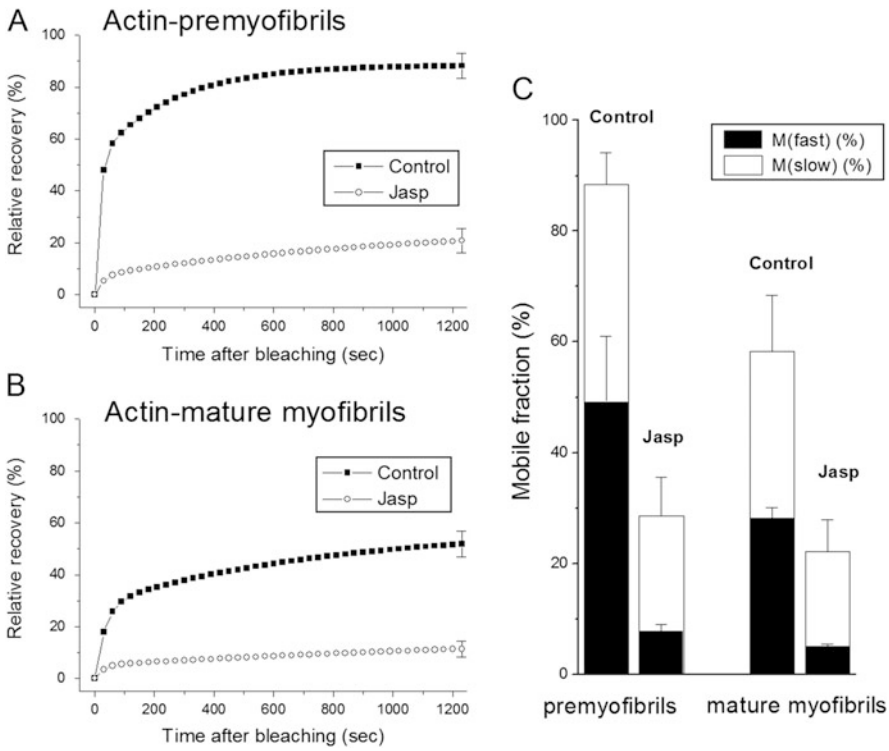

Fig. 15 Fluorescence recovery in premyofibrils (**a**) and mature myofibrils (**b**) of cultured quail skeletal myotubes expressing YFP-actin, in controls and in the presence of jasplakinolide. (**a**, **b**) Note the dramatic decrease in the rates of recovery after photobleaching in the presence of jasplakinolide (*lower curves*) as compared to controls (*upper curves*). (**c**) Calculations of mobile fractions of YFP-actin reveal a prominent decrease of both fast and slow mobile fractions in the presence of jasplakinolide, in premyofibrils as well as in mature myofibrils (Reproduced with permission from Wang et al. 2014)

jasplakinolide, but exchange of tropomyosin from the stabilized areas of F-actin could still take place (Fig. 20). Thus, Wang et al. (2014) interpreted the smaller loss of YFP-tropomyosin in premyofibrils in the presence of jasplakinolide to be due to the continuation of lateral exchange of tropomyosin.

In summary, the interference with actin filament polymerization and the interference with actin filament stability, as seen by treatment with Lat-A and jasplakinolide, respectively, have dramatic consequences on myofibril architecture. The premyofibril is the most sensitive structure in both types of striated muscles.

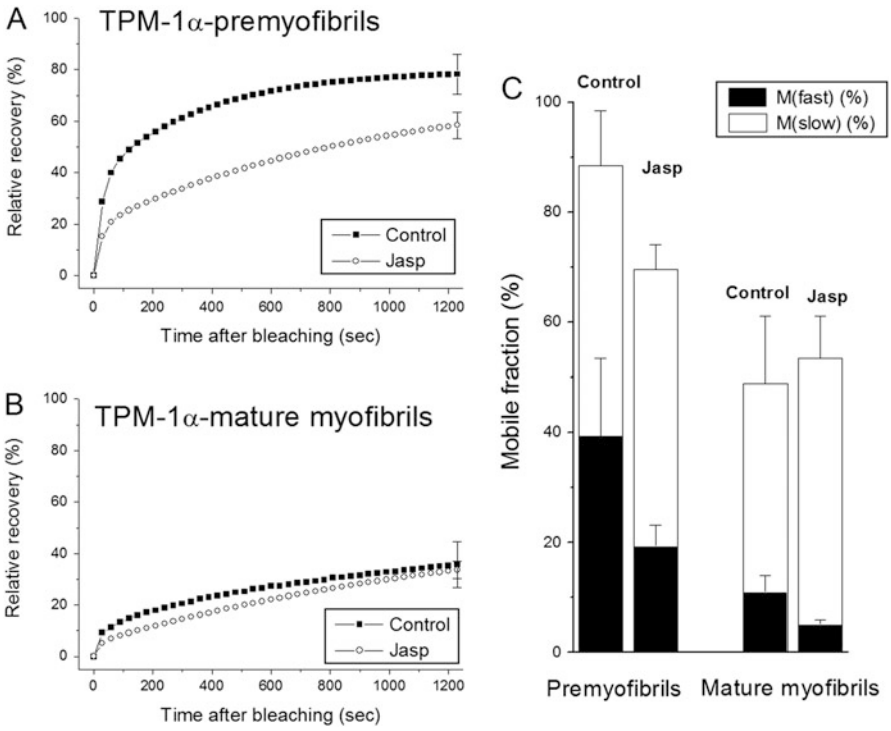

Fig. 16 Fluorescence recovery in premyofibrils (**a**) and mature myofibrils (**b**) of cultured quail skeletal myotubes expressing YFP-tropomyosin, in controls and in the presence of jasplakinolide. (**a**) In premyofibrils, the effect of the drug on YFP-tropomyosin (TPM1α) fluorescence recovery is similar to that of YFP-actin (cf. Fig. 15a). (**b**) In contrast, in mature myofibrils, the recovery of YFP-TPM1α did not significantly differ between control and drug-supplemented medium. (**a**, **b**) In control medium, the recovery rates in premyofibrils of YFP-TPM1α (**a**, *upper curve*) were faster than in mature myotubes (*upper curve*, **b**). (**c**) Calculations of mobile fractions of YFP-TPM1α show similar effects as depicted for YFP-actin (cf. Fig. 15c) (Reproduced with permission from Wang et al. 2014)

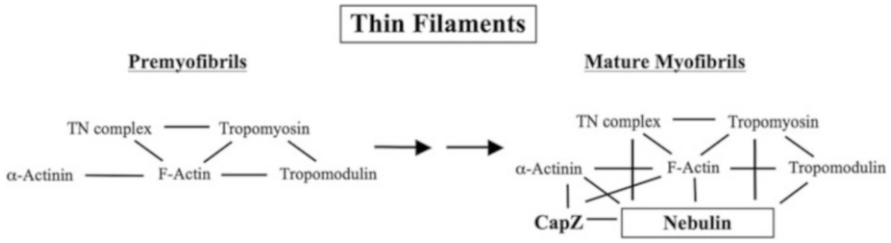

Fig. 17 Model to account for the decrease in the dynamics of actin and TPM1α during transformation of thin filaments in premyofibrils into the thin filaments of mature myofibrils. The addition of two new proteins (CapZ and nebulin) to the actin filaments during this differentiation process provides additional cross-links of actin and tropomyosin in mature myofibrils of skeletal muscle (Modified from Wang et al. 2007)

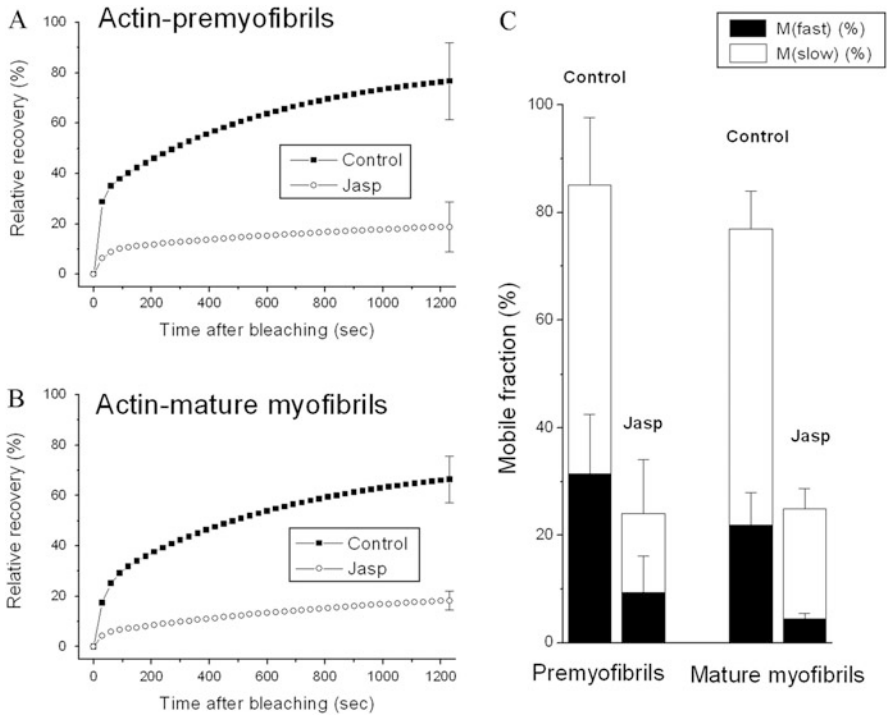

Fig. 18 Fluorescence recovery in premyofibrils (**a**) and mature myofibrils (**b**) of cultured mouse neonatal cardiomyocytes expressing YFP-actin, in controls and in the presence of jasplakinolide. (**a**, **b**) Like seen in Fig. 15 for quail skeletal myotubes, the fluorescence recovery of YFP-actin is more sensitive to jasplakinolide in premyofibrils than in mature myofibrils. (**c**) Again similar to the results obtained for mouse skeletal myotubes, mobile fractions of YFP-actin react dramatically to the drug. Jasplakinolide suppresses the dynamics of actin in both premyofibrils and in mature myofibrils by about 75% (Modified from Wang et al. 2014)

7 Growth and Maturation of Myosin Filaments

From studies of vertebrate striated muscles, it has been generally assumed that the sarcomeric A-bands assemble from thick myosin filaments that are about 1.5 μm long, which is the precise length of A-bands of mature myofibrils (Huxley 1963). However, while analyzing the formation of new myofibrils in the first cardiomyocytes in early embryonic avian hearts in situ, Du et al. (2008) discovered groups of isolated muscle myosin filaments in the embryonic myocytes that were much shorter than 1.5 μm. Similar short filaments of muscle myosin were also detected in cultures of quail skeletal muscle cells (Du et al. 2008). Muscle myosin II forms bipolar filaments with crossbridges to actin filaments at either end of the filament and no cross bridges in the center of the filaments. Both antibodies to

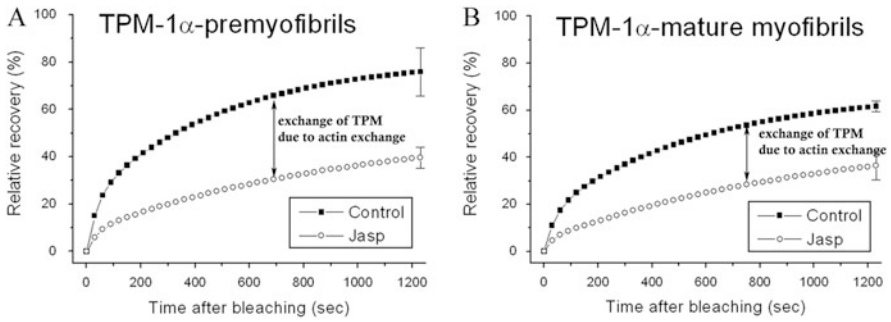

Fig. 19 Fluorescence recovery in premyofibrils (**a**) and mature myofibrils (**b**) of cultured mouse neonatal cardiomyocytes expressing YFP-TPM1α, in controls and in the presence of jasplakinolide. The dynamics of YFP-TPM1α decreases about 10% from premyofibrils to mature myofibrils in control cells. (**a**) Like seen in Fig. 16 for quail skeletal fibers, the drug decreases the dynamics of tropomyosin by about 50% in premyofibrils. (**b**) In contrast to the situation in skeletal myotubes, jasplakinolide induced also a prominent decrease of TPM1α fluorescence recovery in mature cardiac myofibrils (cf. Fig. 16b with panel b of this figure). For possible explanation, see text and Fig. 20. The differences in the dynamics of YFP-TPM1α between the control *upper curves* versus the jasplakinolide *lower curves* (marked by *double arrows*) are suggested due to the action of actin exchange releasing tropomyosin in the control cells (Modified from Wang et al. 2014)

muscle myosin II used in the studies reported here react exclusively with epitopes in the crossbridge regions of the myosin II molecules, i.e., near the N-terminal region of the myosin heavy chain (Wachsberger et al. 1983; Miller and Stockdale 1986), leaving the center of the muscle myosin filaments when aligned in A-bands unstained. This pattern of staining allowed the detection of short myosin filaments in avian cardiac cells (Du et al. 2008), as well as in early somites of zebrafish embryos (Sanger et al. 2009). Measurements of these shorter myosin II filaments and the longer myosin II filaments present in the A-bands of mature myofibrils in zebrafish skeletal embryonic muscles revealed filament length distributions almost identical with those reported by Du et al. (2008) for avian cardiomyocytes: the smaller myosin filaments ranged from 0.4 to 1.1 μm in the youngest somites, while the aligned myosin II filaments in the A-bands of the mature myofibrils in older somites showed a narrower distribution, with an average length of 1.5 μm (Fig. 21).

In the avian cardiomyocytes, the shorter myosin II filaments were not associated with titin, as revealed by antibody staining (Du et al. 2008). This gave some support to the suggestion that titin might determine the final lengths of the thick filaments in the mature A-bands. However, it seems more likely that titin tethers the muscle myosin filaments to the nascent myofibrils (see Fig. 1), permits the subsequent alignment of these thick filaments into A-bands, and regulates the dynamics of myosin molecules in mature myofibrils (Turnacioglu et al. 1997). It is also tempting to speculate that in the absence of competing short nonmuscle myosin II filaments

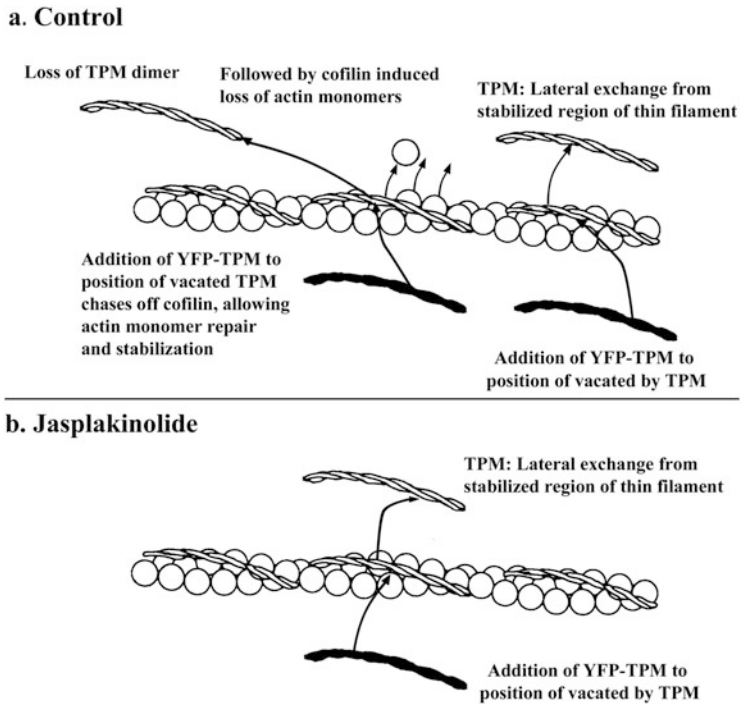

Fig. 20 Diagram illustrating two possible types of dynamic TPM1α exchange from the thin filament. (**a**) In control muscle cells, tropomyosin dimers, each covering seven actin molecules along the thin filament, could exchange for new molecules either (1) by lateral release or (2) by the activity of cofilin which promotes the loss of actin monomers from the filament. Subsequent addition of TPM1α to the cofilin-affected site would chase off cofilin, allowing actin polymerization and stabilization of the site. (**b**) Stabilization of thin filaments by jasplakinolide should not inhibit TPM1α lateral exchange (**a**, first possibility), but any activity of cofilin (**a**, second possibility) seems unlikely, as jasplakinolide-stabilized actin filaments should be protected against cofilin activity (Reproduced with permission from Wang et al. 2014)

in premyofibrils, short muscle myosin II filaments could substitute these and proceed to grow in length in mature myofibrils. In invertebrate muscles, short muscle myosin filaments are initially aligned into short A-bands. For example, the lengths of the A-bands (1.5 μm) and sarcomeres (2.5 μm) in embryonic mites grow gradually over 30 h to reach their final A-band lengths of five microns in mature myofibrils, with 10-μm-long sarcomeres (Aronson 1961). In other invertebrate and vertebrate organisms, the initial muscle myosin filaments were also first detected in overlapping linear patterns, preceding the alignment of the muscle myosin filaments into birefringent A-bands (reviewed in Aronson 1961 and in Sanger et al. 2004).

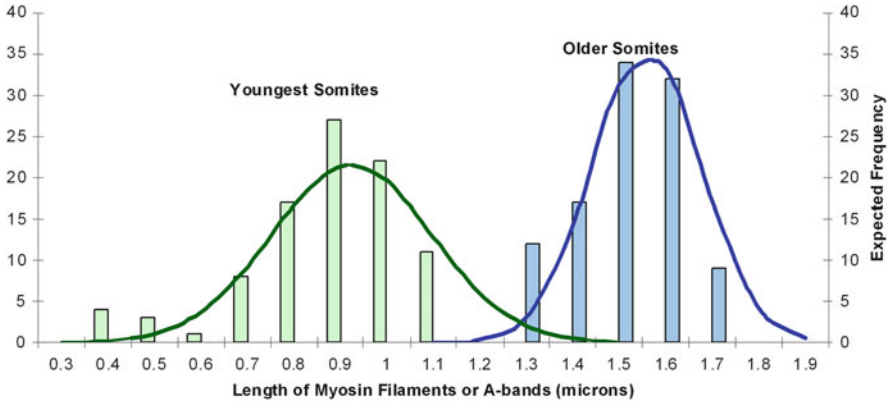

Fig. 21 Length of myosin filaments in recently formed and older somites in skeletal muscle cells in zebrafish embryos. The embryos were stained for indirect immunofluorescence with antibodies against muscle myosin II. *Bars* show the frequency distributions versus lengths of short muscle myosin II filaments in the last two or youngest caudal somites and in older rostral somites. The detected short filaments in the youngest formed somites have an average length of 0.9 (±0.17) μm, while the average length of the A-bands in older somites is 1.5 (±0.11) μm. The *smooth curves* illustrate the expected frequencies versus length distributions (Modified from Sanger et al. 2009)

8 Comparison of the Formation of Synthetic Polymers Versus Myofibrillogenesis

In synthetic polymers composed of identical long molecules, quick stretching and spinning of the initially unaligned molecules dissolved in a solvent leads to the tight packing and lack of entanglements of the long polymers, which results in strength of the polymer as the solvent evaporates (Schaller et al. 2015). In contrast to such long synthetic polymers, myofibrils are composed of multiple interacting groups of myofilaments arranged longitudinally in linked contractile units, the sarcomeres, which run the length of the myotube. Stability is provided along the length of myofibrils through lateral connections between sarcomere filaments. The initial myofibril axis is formed in the premyofibril stage by groups of actin filaments tethered at their fast-growing ends to Z-bodies and interacting with filaments of nonmuscle myosin II in minisarcomeres. The addition of titin and muscle myosin II filaments to premyofibrils results in nascent myofibrils containing two myosin isoforms, nonmuscle and muscle specific (see Fig. 1) (Du et al. 2003, 2008; White et al. 2014). In mature myofibrils, Z-bands, I-bands, and A-bands become aligned across groups of sarcomeres, creating the paracrystalline arrays of myofibrils that produce the great strength of the striated muscle. How the overlapping myosin II filaments align to form mature myofibrils with uniform A-bands is still unclear. As stretching promotes the formation of long synthetic polymers, pulling during muscle contractions may provide and maintain the alignment of sarcomeric actin and myosin II filaments in mature I- and A-bands.

Inhibitors of contraction have been shown to block reversibly myofibrillogenesis at the premyofibril-nascent myofibril boundary (Golson et al. 2004). The elongation of the early myotubes parallels the time course of myofibrillogenesis. The growing ends of the myotube can be considered as two independent units coordinating the initial actin filament assembly and alignment toward the future mature myofibrils. The three-step process, leading from premyofibril and nascent myofibril toward mature myofibrils, impedes any entanglements of the many macromolecules involved in the construction of the sarcomere.

9 Actin Nucleation Factors and Myofibrillogenesis

As already mentioned above, myofibrillogenesis requires massive assembly of actin filaments and their placement into sarcomeres to serve as thin filaments. This begins with the initial incorporation of polymerized actin into premyofibrils, continues with addition of filaments during maturation of myofibrils, and can be further stimulated after development by hypertrophic cues, such as insulin-like growth factor 1 (IGF-1). Actin filaments likely play additional roles during myofibrillogenesis, such as contributing to costameres, the protein complexes involved in the transfer of the power of contraction to the extracellular matrix, or assisting in the organization of triads which serve to rapidly transmit electric signals within the striated muscle fibers (Ervasti 2003; Falcone et al. 2014). Factors that influence the lengths or stability of thin filaments have been reviewed elsewhere (Littlefield and Fowler 2008; Ono 2010). Here, we discuss actin nucleation factors (ANFs) that initiate new filament assembly from actin monomers and the evidence that these proteins contribute to myofibrillogenesis.

Formins are highly conserved dimeric proteins present in nearly all eukaryotes (Higgs and Peterson 2005; Chalkia et al. 2008). Formins were identified as ANFs that also have the unique ability to shepherd growth of the filament barbed end by remaining attached as elongation occurs (Pruyne et al. 2002; Sagot et al. 2002a; Kovar and Pollard 2004). This "processive capping" allows formins to shield the growing barbed end from the inhibitory effects of capping proteins, such as CapZ (Kovar et al. 2003; Zigmond et al. 2003; Moseley et al. 2004), and in some circumstances allows formins to accelerate filament elongation (Romero et al. 2004; Paul and Pollard 2008). Formins with such activities are expected to favor the formation of relatively long, unbranched actin filaments, making them ideal candidate ANFs for thin filaments. However, it has become clear that not all of the 15 mammalian formins share these activities. Some formins have only a poor nucleating activity but strong capping, severing, or bundling activities on actin filaments (reviewed in Goode and Eck 2007).

The first formin identified as a regulator of myofibrillogenesis was Fhod3. Fhod3 is expressed most abundantly in the heart (Kanaya et al. 2005), and its knockdown via RNAi blocks establishment of myofibrils and disrupts preassembled myofibrils in cultured neonatal rat cardiomyocytes (Taniguchi et al. 2009; Iskratsch et al. 2010). Consistent with this, mice homozygous for a null *Fhod3*

mutation die during embryogenesis due to failed myocardial development (Kan-o et al. 2012a). While cardiomyocytes in these embryos initially develop normally to form premyofibrils, these never mature into myofibrils (Fig. 22) (Kan-o et al. 2012a). Similarly, loss of a Fhod3-related formin from the nematode *Caenorhabditis elegans* prematurely arrests sarcomere formation in striated

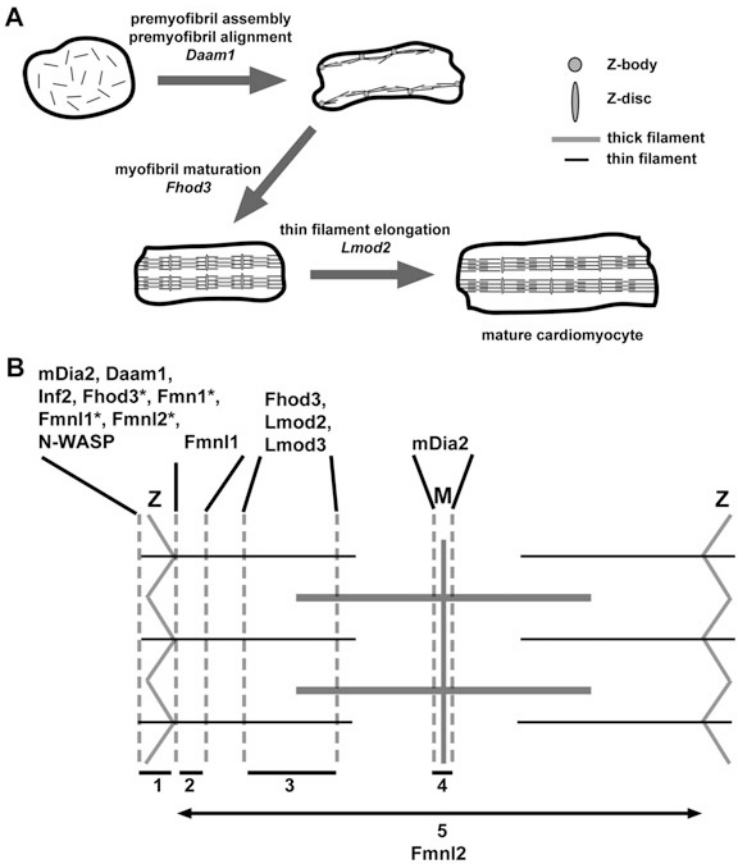

Fig. 22 Actin nucleation factors (ANFs) and myofibrillogenesis. (**a**) ANF-coding genes implicated in cardiomyocyte myofibril development. The formin gene *Daam1* is not required for assembly of premyofibrils, but it is required for the proper alignment of premyofibrils along the developing cardiomyocyte's long axis. The formin gene *Fhod3* is required for premyofibrils with thin filament-anchoring Z-bodies, to mature into myofibrils containing thin filaments anchored to Z-disks. The leiomodin gene *Lmod2* is required for elongation of thin filaments to their mature length. (**b**) The distribution of ANFs in the muscle sarcomere. In a model sarcomere, Z-bands (Z) and the M-line (M) are marked, *thin black lines* represent thin filaments, and *thick gray lines* represent thick filaments. Myofibril-associated ANFs are found in five distributions: (*1*) at the Z-band, (*2*) flanking the Z-band, (*3*) along the distal portions of thin filaments but not at the pointed ends, (*4*) at the M-line, and (*5*) at the entire region between two Z-bands. ANFs marked by *asterisks* (*) are those present at the indicated location only in fully mature myofibrils

muscles, suggesting that this is a widely conserved formin function (Mi-Mi et al. 2012). Null *Fhod3* mutations have not been observed in humans, but a coding variant, V1151I, is associated with increased incidence of hypertrophic cardiomyopathy (Wooten et al. 2013).

However, it is unclear how Fhod3 contributes to myofibril maturation. The localization of Fhod3 in myofibrils has been controversial, alternatively being observed at Z-disks in mouse and human adult heart sections and extracted myofibrils (Iskratsch et al. 2010; Rosado et al. 2014) or along thin filaments near to, but not precisely at, the pointed ends in cultured neonatal cardiomyocytes and in mouse and human adult heart sections (Fig. 22) (Taniguchi et al. 2009; Kan-o et al. 2012b). Also, in vitro assays have failed to demonstrate ANF activity for Fhod3, but instead revealed that Fhod3 caps barbed ends to inhibit filament elongation (Taniguchi et al. 2009; Kan-o et al. 2012b). A closely related formin, Fhod1, is also present in cardiomyocytes, primarily at intercalated disks and costameres (Dwyer et al. 2014; Al Haj et al. 2015), but this formin also caps filaments while lacking nucleating activity in vitro (Schönichen et al. 2013). Thus, while Fhod-class formins are present in cardiac muscle, they might not direct actin filament assembly, but instead have some more subtle role in myofibril maturation.

Mice with a hypomorphic mutation of another formin gene, *Daam1*, also have severe heart developmental defects, including cardiomyocytes with narrow myofibrils that are disorganized with respect to their orientation (Fig. 22) (Li et al. 2011). These mutant cardiomyocytes also have defective cell-cell contacts, patchy loss of cell surface adhesion molecules, and abnormal plaques at the intercalated disks. Again, these phenotypes do not directly reveal a role for this formin in directing thin filament assembly, but leave open the possibility that the primary defect in *Daam1* mutant cardiomyocytes may be in cell adhesion, which can indirectly have an impact on myofibrillogenesis. A more comprehensive survey identified Fmnl1, Fmnl2, mDia2, Fmn1, and Inf2 as five additional sarcomere-associated formins in cultured neonatal mouse cardiomyocytes or extracted adult mouse myofibrils (Rosado et al. 2014), with variable localizations with respect to sarcomeric positions (Fig. 22). Unlike Fhod3 or Fhod1, all these formins are capable of nucleating actin filaments in vitro (Goode and Eck 2007). RNAi-mediated knockdown of Daam1, Fmnl1, and mDia2 in cultured cardiomyocytes each individually results in strongly, though not completely, disrupted myofibril organization, while knockdown of Fmnl2 eliminates recognizable striations, suggesting each of these formins plays some unique role in myofibrillogenesis (Rosado et al. 2014). However, loss of not a single one of these isoforms eliminated thin filaments. Even the Fmnl2 knockdown cells that lack striations still have a large abundance of actin filaments.

Roles for formins in skeletal muscle myofibrillogenesis are even less clear. A comprehensive qRT-PCR analysis of formin expression in human tissues showed skeletal muscle expresses Fhod3 and mDia3 at high levels (Krainer et al. 2013), but no functional studies of formins in skeletal muscle have been reported. Studies using *Drosophila* and *C. elegans* have implicated formins in the development of

non-cardiac striated muscles in invertebrates, but again neither system exhibits a complete loss of thin filaments, leaving the precise function of their formins unknown (Mi-Mi et al. 2012; Molnár et al. 2014; Mi-Mi and Pruyne 2015). It is possible that the assembly of thin filaments in myofibrillogenesis might be an activity shared by multiple formins. Alternatively, formins may nucleate actin filaments for other functions in myofibrillogenesis, or modify thin filaments through non-ANF activities, such as bundling or severing.

Leiomodins (Lmods) make up a second class of myofibril-associated ANF. The three mammalian leiomodins, Lmod1, Lmod2, and Lmod3, are members of the tropomodulin (Tmod) superfamily, but where Tmods cap pointed ends, Lmods potently nucleate actin filaments (Chereau et al. 2008; Yuen et al. 2014; Boczkowska et al. 2015). Lmod1 is primarily expressed in smooth muscle, and Lmod2 and Lmod3 are both found in cardiac and skeletal muscles (Conley et al. 2001; Nanda and Miano 2012). Consistent with the ability of Lmods to bind actin filament sides but not to the pointed ends (Boczkowska et al. 2015), Lmod2 and Lmod3 are present along the distal portions of thin filaments in myofibrils but are not restricted to pointed ends like Tmod (Fig. 22b) (Tsukada et al. 2010; Skwarek-Maruszewska et al. 2010; Nworu et al. 2015; Yuen et al. 2014). Intriguingly, this distribution is similar to that of Fhod3 (Taniguchi et al. 2009; Kan-o et al. 2012b).

Lmods are important for normal myofibril structure. Mouse and human mutations of the *Lmod3* gene result in nemaline myopathy, with atrophic skeletal muscle fibers that contain disrupted sarcomeres, and the replacement of many Z-disks with nemaline bodies (Yuen et al. 2014; Cenik et al. 2015; Tian et al. 2015). Surprisingly, loss of *Lmod3* affects only a subset of skeletal muscle fibers, perhaps due to compensatory changes in Lmod1 or Lmod2 activity. In cultured neonatal rat cardiomyocytes, RNAi-mediated knockdown of Lmod2 results in loss of myofibril organization (Chereau et al. 2008). However, Lmod2 is expressed in chick cardiomyocytes in vivo only after myofibrils have assembled and cardiomyocytes have begun beating, arguing against the need for Lmod2 in de novo thin filament assembly or initial myofibril formation (Tsukada et al. 2010). Overexpression of Lmod2 and Tmod1 in cultured neonatal rat cardiomyocytes suggests that thin filaments are shortened by Tmod1-dependent pointed-end capping, while Lmod2 promotes thin filament elongation. Thus, Lmod2 may antagonize Tmod1 in regulating thin filament length (Tsukada et al. 2010). Additionally, *Lmod2*-null mutant mice produce cardiomyocyte myofibrils with shortened thin filaments and poor contractility (Pappas et al. 2015). However, an antagonistic relationship does not hold for all Lmod/Tmod combinations, as Lmod3 and Tmod4 can substitute for the loss of each other in skeletal muscle myofibrillogenesis in *Xenopus laevis* (Nworu et al. 2015). Biochemical studies have also alternatively found evidence for or against competition between Lmod and Tmod isoforms (Tsukada et al. 2010; Boczkowska et al. 2015). Understanding the biochemical relationships between these proteins may require direct observation of their behavior on individual actin filaments using such techniques as TIRF microscopy, while teasing out their in vivo functions in myofibrillogenesis may require simultaneous

manipulation of multiple isoforms to eliminate the confounding possibility of genetic redundancy.

A final protein involved in actin filament nucleation in association with myofibrillogenesis is N-WASP. Studies of adult mouse skeletal muscle demonstrated that IFG-1 signaling triggers the recruitment of N-WASP to myofibrils, where it stimulates incorporation of actin into filaments at the Z-line (Fig. 22) (Takano et al. 2010). While N-WASP is best known as a factor that activates the ANF activity of the Arp2/3 complex in nonmuscle cells, the ARP2C subunit of the complex was not detected in myofibrils. Instead, in vitro assays showed that N-WASP associates with the SH3 domain of the thin filament-binding protein, nebulin, and will significantly enhance a weak ANF activity of nebulin's actin-binding repeats (Takano et al. 2010). Based on a mouse mutant lacking the nebulin SH3 domain, it was concluded that N-WASP recruitment to myofibrils does not require nebulin (Yamamoto et al. 2013). Instead, evidence from knockdowns in cultured murine myotubes and in vivo implicates amphyphysin-2, another SH3 domain-containing protein, in N-WASP recruitment and activation (Falcone et al. 2014). Knockdown of N-WASP results in normal-appearing myofibrils, but the resultant skeletal muscle fibers had small cross-sectional areas, suggesting thin filament assembly does not strictly depend on N-WASP, but N-WASP-dependent F-actin contributes to thin filaments added to preexisting myofibrils (Takano et al. 2010; Falcone et al. 2014). Additionally, myotubes lacking N-WASP or amphiphysin-2 have the additional defects of failing to move their nuclei to peripheral positions and being unable to establish or maintain triads. Thus, N-WASP-generated actin filaments may also be important for membrane-organizing events that accompany myofibrillogenesis (Falcone et al. 2014).

While many ANFs have been shown to be important to myofibrillogenesis, it still remains unclear which of these contribute directly to the assembly of thin filaments. A major challenge to identifying such a link between a particular ANF and thin filament formation is the interplay between actin dynamics and the control of gene expression in muscle. Serum response factor (SRF) drives the expression of many cytoskeletal and muscle-related genes, but requires the presence of co-activators of the myocardin-related transcription factor (MRTF) family (Cen et al. 2004). In turn, MRTFs are regulated by actin, with unpolymerized monomeric actin binding and sequestering MRTFs in the cytoplasm (Miralles et al. 2003). Conversely, increased polymerization of actin lowers the unpolymerized actin pool and frees MRTF to stimulate SRF-dependent gene expression. Consistent with this, overexpression of Lmod3 or the formins mDia1, mDia2, or Fhod1 drives expression of smooth muscle cell-specific genes (Tominaga et al. 2000; Copeland and Treisman 2002; Staus et al. 2007, 2011; Cenik et al. 2015). It might therefore be expected that loss of a particular ANF activity would not only reduce actin polymerization but also suppress muscle gene expression and disrupt the formation of additional sarcomere components. This may explain why elimination of many formins or leiomodins generally disrupts myofibril organization.

In moving forward, attempts to identify ANF functions in myofibrillogenesis may require a more subtle approach. One possible line of action may be the

development of rapid-acting inhibitors specific to different ANFs. Currently, no leiomodin inhibitors have been described, and the only available small molecule formin inhibitor, SMIFH2, is potent but does not appear to target specific isoforms (Rizvi et al. 2009). An alternative approach could be the use of mutations to conditionally inactivate an ANF, particularly in genetically tractable model systems. Such mutants have been used to great effect to identify specific functions of formins in yeast and worms (Evangelista et al. 2002; Sagot et al. 2002b; Davies et al. 2014). Finally, the effects of an ANF on actin assembly might be teased apart from any effects on muscle gene expression by uncoupling SRF activity from actin polymerization. Mutants of MRTFs that are actin insensitive provide an attractive possibility for functional tests (Miralles et al. 2003). While it is clear that multiple ANFs are critical to myofibrillogenesis, much remains to be done in unraveling the details of their precise roles in this process.

10 Insights into Actin-Based Muscle Diseases

The ACTA1 gene in vertebrates encodes skeletal muscle actin that is composed of 377 amino acids. There are approximately 200 mutations in this key protein associated with human diseases leading to muscle weaknesses (Sparrow et al. 2003; Laing et al. 2009; Nowak et al. 2009, 2013; Duygu 2015; Selcen 2015). Five major categories have been described based primarily on histopathological properties: nemaline myopathy, intranuclear rod myopathy, actin filament aggregate myopathy, myopathy with core-like areas, and congenital-type disproportion (Laing et al. 2009). Single mutations can give rise to two or even three of these pathological designations. For example, the skeletal actin mutation Asp154Asn can give rise to an actin filament aggregate myopathy, nemaline myopathy, and an intranuclear rod myopathy (Laing et al. 2009). The properties of the actin mutations have been studied in biochemical assays (Rubinstein and Wen 2014), isolated mouse myofibers (Ochala et al. 2009), and more recently in living zebrafish (Sztal et al. 2015). We are still a long way from understanding the molecular basis of the effects these mutations have on human muscular diseases, some so severe that children die before their first full year of life, while others are less severe, with patients still alive in their 40s. Of particular interest is that in a number of patients with these actin mutations, high levels of cardiac α-actin, typical of embryological skeletal muscle cells, are still detected in their skeletal muscle cells (Laing et al. 2009). This observation suggests a long-term therapeutic approach to counter the effects of the skeletal actin mutations. Our recent unpublished FRAP analyses have revealed differences in the exchange rates of truncated and mutated sarcomeric proteins compared with control proteins. Thus, the dynamic exchange of mutated proteins can be used as a biomarker for the assembly, maintenance, and molecular insights into the causes of muscle diseases induced, not only by mutated actin molecules but by other sarcomeric mutated proteins as well.

11 Summary

Two papers in the literature raised challenges to be met in the studies to determine how myofibrils are assembled. First Goesta Haeggqvist in 1920 wrote"...in welcher Weise sich die Fibrillen entwickelt haben?. Diese Frage wiederum kann in drei getrennte Probleme zerfallen. In welcher Weise erscheinen die ersten Fibrillen? Wie entwickeln sich dieselben in der Folge? Auf welche Weise verbessert sich ihre Zahl?"

> ...in what way have the fibrils developed? This question, in turn, can be divided into three separate problems. How does the first fibril appear? How does the same process develop in succession? In what way does their number increase?

We suggest that the premyofibril model (Fig. 1) addresses all three of these problems: premyofibrils to nascent myofibrils to mature myofibrils. It is a model that guides us in digging deeper into all aspects of myofibrillogenesis, both in normal muscle development and in myopathies. The premyofibril model has also been used to document a three-step process for the formation of costameres or Z-Band attachments to the muscle cell membrane (Danowski et al. 1992), i.e., precostamere to nascent costamere to mature costamere (Quach and Rando 2006).

In the second publication Francis H. C. Crick published in The Encyclopedia of Ignorance in 1977 the following question and comment: "And how does a muscle fibre assemble all its components to produce a highly ordered contractile machine? The answer may come from studies of the fibrillar molecules themselves and how they interact or it may involve some other principle". These insights were published on the edge of the computer age, light sensitive cameras, and the development of microinjected fluorescent sarcomeric proteins, and then the Green Fluorescent Protein family of plasmids that would encode sarcomeric proteins usable for following the actual assembly of proteins into the different stages of myofibrillogenesis in living cells. These new methods and others led to the exciting discoveries of the dynamic exchanges of sarcomeric proteins in assembly and maintenance of myofibrillogenesis. Much has been accomplished, but much more remains to be explored in the study of myofibrillogenesis.

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Myosins: Domain Organisation, Motor Properties, Physiological Roles and Cellular Functions

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Abstract

Myosins are cytoskeletal motor proteins that use energy derived from ATP hydrolysis to generate force and movement along actin filaments. Humans express 38 myosin genes belonging to 12 classes that participate in a diverse range of crucial activities, including muscle contraction, intracellular trafficking, cell division, motility, actin cytoskeletal organisation and cell signalling. Myosin malfunction has been implicated a variety of disorders including deafness, hypertrophic cardiomyopathy, Usher syndrome, Griscelli syndrome and cancer. In this chapter, we will first discuss the key structural and kinetic features that are conserved across the myosin family. Thereafter, we summarise for each member in turn its unique functional and structural adaptations, cellular roles and associated pathologies. Finally, we address the broad therapeutic potential for pharmacological interventions that target myosin family members.

Keywords

Actin • Adaptor proteins • Cargo • Cytoskeleton • Deafness • Duty ratio • Intracellular transport • Molecular motors • Movement • Myosins

1 Introduction

The cellular cytoskeleton comprises a complex network of microtubules, intermediate filaments and actin filaments that maintain the shape and structural integrity of the cell. In addition, microtubules and actin filaments provide the tracks for intracellular transport. There are three classes of cytoskeletal molecular motors: the dyneins and kinesins that drive long-range movements along microtubules and the myosins that mediate slower short-range transport along actin filaments (Ross et al. 2008). In this review we will focus solely on the myosin motor proteins expressed in humans (classes I, II, III, V, VI, VII, IX, X, XV, XVI, XVIII, XIX) with an emphasis on their functions in non-muscle cells.

1.1 Myosin Motor Proteins

All myosins contain three domains. At the N-terminus there is a highly conserved catalytic motor domain (80–100 kDa) containing the actin binding and ATPase sites. This domain is followed by a central 'lever-arm' region with one to six IQ motifs (with amino acid sequence IQxxxRGxxxR). The lever arm binds to either light chains (regulatory or essential light chains) or calmodulin, which stabilise the lever arm and may also regulate motor function. The C-terminal region is termed the tail, and typically contains an α-helical coiled-coil region for dimerisation, and/or cargo binding domains that interact with specific adaptor proteins or lipids. The tails are the most hyper-variable regions of the myosins and are directly involved in specifying the cellular functions of the different myosin classes (Krendel and Mooseker 2005) (see Fig. 1).

Phylogenetic analysis of the myosin motor domain sequences identified in the human genome has revealed that they belong to 12 specific classes, which have been categorised with a Roman numeral-based numbering system (Foth et al. 2006; Goodson and Dawson 2006; Richards and Cavalier-Smith 2005). In addition, within each class distinct genes exist with different intracellular functions, denoted with the corresponding Arabic numerals and letters. A single gene can often be spliced producing a number of variants, each with distinctive expression profiles, intracellular localisation and function.

To provide a selective overview of the myosin superfamily, we will first discuss the general principles of myosin-driven movement. For each class of myosin, we will summarise their unique motor properties, myosin-cargo interactions and physiological functions and highlight several disease-causing mutations. Finally, we will briefly address the development of small molecules that affect myosin activity for potential therapeutic benefit.

1.2 Myosin-Driven Mechanochemical Movement

All myosins contain actin- and ATP-binding sites in their highly conserved N-terminal motor domain. Myosin movement along actin filaments is generated by the energy released from hydrolysis of ATP by the actin-activated Mg^{2+}-ATPase (Lymn and Taylor 1971). Net movement results from rectification of the cyclical motion (termed the 'cross-bridge' cycle) as myosin motors bind and unbind actin filaments, depending on their nucleotide-binding state (see Fig. 2). During the cross-bridge cycle, ATP binds to the ATPase site in the myosin motor domain, releasing the motor domain from actin. The ATP is rapidly hydrolysed to ADP with the cleaved phosphate (Pi) still associated with the head. The motor domain then binds to actin, and the subsequent release of Pi generates small conformational changes in the motor domain that are amplified and transmitted to the lever arm. In this way the structural rearrangement of the motor domain is further amplified into a directed stroke that moves the myosin along the actin filament. The motor domain will remain attached to actin until ADP is released and another ATP molecule is

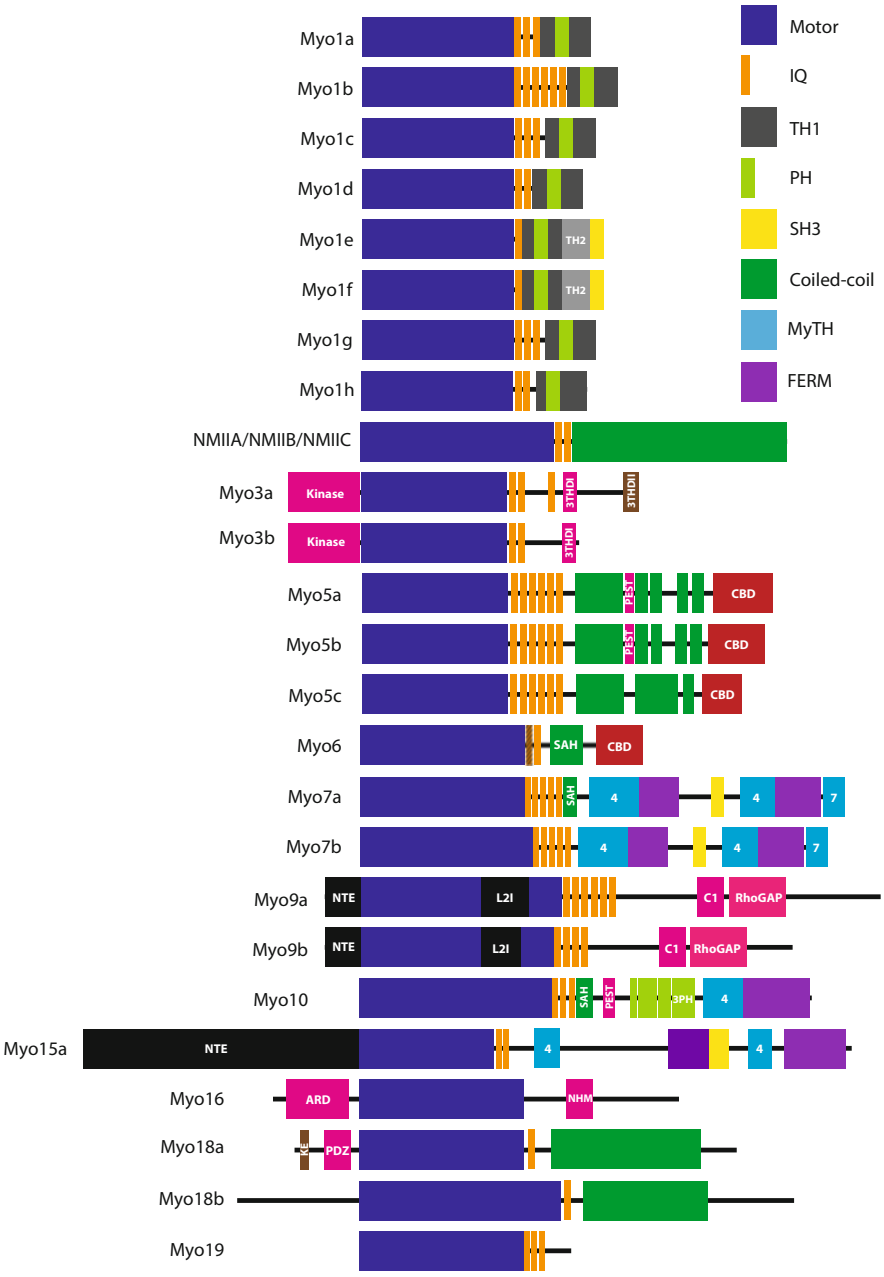


Fig. 1 Domain organisation of the myosin motors expressed in humans. The most common domains are also illustrated in the key in the *top right*. The 11 muscle-expressed myosins share domain homology with the three non-muscle myosin IIs Myh9/Myh10/Myh14. Full definitions of the domain labels are as follows: motor – actin-activated ATPase motor domain; IQ – IQ-binding motif; TH1 – tail homology 1; PH – pleckstrin homology domain; TH2 – tail homology 2; SH3 – Src homology 3; 3THDI – myosin 3 tail homology domain 1; 3THDII – myosin 3 tail homology domain 2 (contains an actin-binding motif); PEST – region rich in proline, glutamic acid, serine

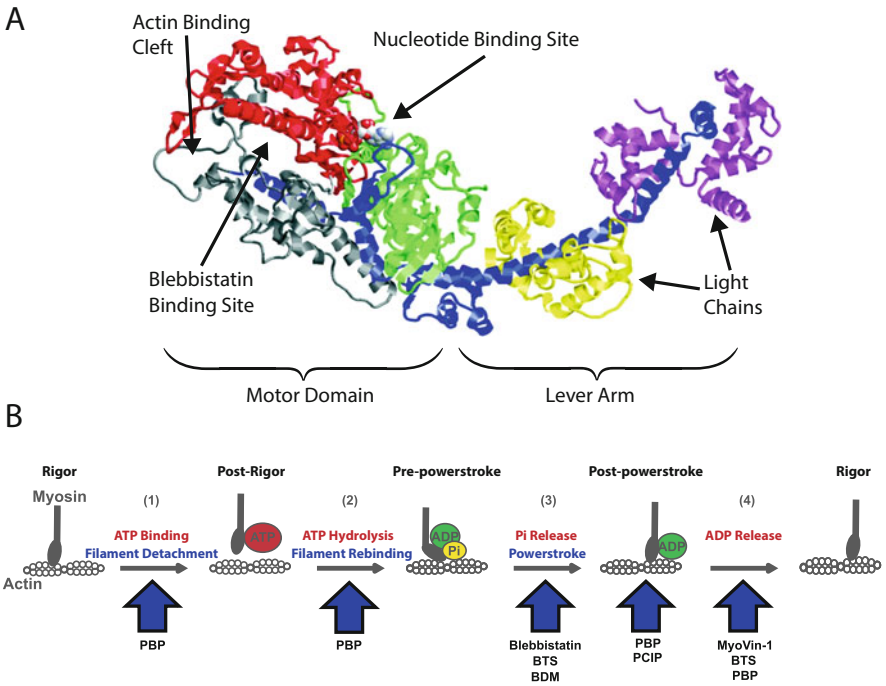


Fig. 2 Structure and activation cycle of myosins. (**a**) Structure of the S1 (head) fragment of chicken muscle myosin containing the motor domain and lever arm. Reproduced from Ruegg et al. (2002) based on published data Rayment et al. (1993). (**b**) The actin-activated ATPase cycle (or cross-bridge cycle) of a typical myosin motor. Initially, the motor is in the rigor state, bound to actin but not nucleotide. (*1*) ATP binding causes the motor domain to detach from actin. (*2*) Hydrolysis of ATP cocks the motor domain which rebinds to actin. (*3*) Phosphate release occurs, triggering execution of the working stroke. (*4*) ADP is released, completing the cycle. The indicated inhibitors act at different stages in the myosin kinetic cycle. Changes in nucleotide-binding state are labelled in *red*, structural changes in *blue*. Adapted from Bond et al. (2013). See Table 1 for full compound names

bound. This model of myosin movement, named the 'swinging lever-arm hypothesis', was originally based on studies using muscle myosin II (Holmes 1997). However, more recent studies on the other classes of myosin, especially Myo5a, have added their support for this model (Howard 1997). Thus, every myosin so far

Fig. 1 (continued) and threonine; CBD – cargo-binding domain; SAH – single alpha helical domains (lacking the dimerisation function of a coiled coil); 4 – MyTH4, myosin tail homology 4; 7 – MyTH7, myosin tail homology 7; NTE – N-terminal extension; L2I – loop 2 insert; RhoGAP – RhoGTPase-activating protein domain; C1 – protein kinase C conserved region 1; 3PH – 3 PH domains, including a split PH domain; ARD – ankyrin repeat domain; NHM – NYAP homology motif containing tyrosines and prolines; KE – region rich in lysine and glutamic acid; PDZ – PSD95-Dlg1-ZO1 protein domain; FERM – 4.1 protein-ezrin-radixin-moesin domain. The *hashed lines* on the first IQ domain of Myo6 indicates the reverse gear

studied in detail has the same sequence of steps in their ATPase cycles, whilst the differences between the myosin classes are due to variations in the rates and equilibrium constants of the steps in this cycle (De La Cruz and Ostap 2004).

As the kinetics of the different classes of myosins vary greatly, so do their duty ratios, which is the proportion of the time spent strongly attached to actin to the time taken to complete an ATPase cycle (El-Mezgueldi and Bagshaw 2008). Class II myosins have a relatively high ATPase rate and a low-duty ratio (spending only ~5% of the ATPase cycle time strongly attached to actin), whereas Myo5a and Myo6 have slow kinetics and a high duty ratio spending at least 50% of the time attached to actin during their ATPase cycle. Processivity is the ability of a myosin to take more than one step per encounter with actin. Myosin II (with a low-duty ratio) is non-processive, binding and releasing from actin in single steps. On the other hand, a single dimeric Myo5a molecule (with a high duty ratio) is processive and with its two motor domains is able to walk down an actin filament by a 'hand-over-hand' mechanism for multiple steps before detaching (Veigel et al. 2005). We will now discuss the main features and known functions of each class of myosin in turn.

2 Class I Myosins

2.1 Domain Organisation and Motor Properties

Myosins of class I are evolutionarily conserved, single-headed motors expressed in a large variety of tissues and organisms from ancestral eukaryotes to man. In humans a total of eight class I myosin genes have been identified that can be grouped into short tailed (Myo1a, Myo1b, Myo1c, Myo1d, Myo1g and Myo1h) and long tailed (Myo1e and Myo1f). All class I myosins are monomeric, nonfilamentous motors between 110 and 140 kDa sharing a very similar domain organisation. At the N-terminus is the motor domain, which is lacking the first 80 amino acids that is typically found in skeletal muscle myosin. An alternatively spliced nuclear form of myo1c has been identified, which contains a 16 amino acid N-terminal extension (Pestic-Dragovich et al. 2000). The lever arms of the different myosin Is can bind between one and six calmodulins. The class I myosin tails can be subdivided into three different tail homology domains (TH1, TH2 and TH3). Whereas the long-tailed Myo1e and Myo1f contain all three TH domains, the other six short-tailed myosin Is only have the TH1. This domain, common to all class I myosins, contains a pleckstrin homology (PH) domain (or similar sequences rich in positively charged, basic amino acids) enabling high affinity binding to membrane phospholipids (Feeser et al. 2010). The longer tails of Myo1e and Myo1f also contain protein-protein interaction domains such as the TH2 domain, a region enriched in proline, glycine and alanine/glutamine repeats, and the TH3 domain, a Src homology-3 (SH3) domain (McConnell and Tyska 2010).

For both the short- and long-tailed myosins of class I, the rate-limiting step of the cross-bridge cycle is the slow release of phosphate. Members of this class only spend a small fraction of their kinetic cycle attached to actin filaments and thus can be classified as low duty ratio motors (<0.5). Other parameters of the kinetic cycle such as the rate of ATP hydrolysis and also the response to force and load vary considerably amongst the different myosin Is. For example, Myo1b becomes a very high duty ratio motor whilst experiencing relatively low opposing forces (about 0.5 pN), acting as a tension-responsive anchor, whereas Myo1c only stalls at much higher forces (about 2.5 pN), and it can move under much larger loads, which is characteristic of a slow transporter (Greenberg and Ostap 2013).

Myosin I motor activity is allosterically inhibited by the binding of the motor domain to the tail region and is also regulated by calcium binding to the calmodulin light chains in the lever arm. However, the effect of calcium on the mechanical properties and ATPase activities of the different members of class I is very diverse (Stoffler and Bahler 1998).

2.2 Physiological Roles and Cellular Functions

All myosins of class I can directly associate with membranes and thereby provide a dynamic link between the actin cytoskeleton and the plasma membrane or intracellular organelles. Myo1a, Myo1b and Myo1c are the best characterised members of this class in humans. Myo1a is predominantly expressed in enterocytes in the intestinal tract, where it links the plasma membrane to the actin filament bundles in apical microvilli. In humans, mutations in Myo1a have been associated with the loss of epithelial polarity and gastric tumour formation and progression (Mazzolini et al. 2013). In mice, the loss of Myo1a affects brush border morphology causing microvilli fusion and membrane herniations (Tyska et al. 2005).

Myo1b is widely expressed, including in the lung, liver, brain, heart and intestine (Sherr et al. 1993). It is enriched at the plasma membrane in protrusions such as microvilli and lamellipodia and was shown to be required for directed cell migration in zebrafish embryo development (Diz-Munoz et al. 2010; Raposo et al. 1999; Tang and Ostap 2001). It has also been linked to cancer cell migration (Ohmura et al. 2015). In addition Myo1b was suggested to play a role in membrane tubule formation from endosomes and the trans-Golgi network (Almeida et al. 2011; Salas-Cortes et al. 2005; Yamada et al. 2014).

Myo1c is expressed in most eukaryotic cells, but is highly abundant in B lymphocytes and adipocytes (Sherr et al. 1993; Wagner et al. 1992). It is enriched in dynamic regions of the cell surface characterised by lamellipodia and filopodia and participates in a wide variety of cellular processes, for example, in the exocytosis of several lipid raft-associated cargoes such as GLUT4 (Bose et al. 2002, 2004; Chen and Saltiel 2007) and aquaporin 2 (Barile et al. 2005), in hair cell adaptation in the inner ear (Gillespie and Cyr 2004; Stauffer et al. 2005) and in the regulation of actin organisation during cell migration (Fan et al. 2012). Loss of Myo1c disturbs the intracellular distribution of cholesterol-enriched lipid raft

domains, which has a major impact on cell spreading, *Salmonella* infection and the autophagy pathway (Brandstaetter et al. 2012, 2014). A nuclear Myo1c isoform (NM1), containing a 16-amino-acid extension at the N-terminus, has also been described and may promote transcription by RNA polymerase I (Ye et al. 2008). How the interaction of NM1 with nuclear actin might regulate polymerase activity is currently unknown.

Myo1d is found in a wide variety of tissues with the highest expression in both the peripheral and central nervous system. During brain development, Myo1d is upregulated in myelinating oligodendrocytes and interacts with aspartoacylase, an important enzyme for myelination (Bahler et al. 1994; Benesh et al. 2012; Yamazaki et al. 2014). Interestingly, Myo1d is a potential candidate gene for autism spectrum disorders (ASD) (Stone et al. 2007). In addition Myo1d is involved in membrane trafficking to the apical domain in polarised epithelial cells (Huber et al. 2000) and in maintenance of rotational planar cell polarity in ciliated tracheal and ependymal epithelial cells (Hegan et al. 2015).

Myo1e is a long-tailed myosin I with a widespread expression pattern in many different tissues (Bement et al. 1994). This myosin localises to actin-rich regions at cell-cell contact sites (Stoffler et al. 1995) and to the phagocytic cup in macrophages (Diakonova et al. 2002). Myo1e is involved in clathrin-mediated endocytosis of the transferrin receptor by binding directly to synaptojanin-1 and dynamin, which both play important roles in receptor-mediated endocytosis (Krendel et al. 2007). Myo1e is required for kidney function as KO (knockout) mice show defects in podocyte function and renal filtration (Chase et al. 2012; Krendel et al. 2009), and mutations in the Myo1e gene cause glomerular disease in humans (Mele et al. 2011). In macrophages Myo1e regulates cell spreading, chemokine release and antigen presentation downstream of Toll-like receptor signalling (Wenzel et al. 2015).

The second long-tailed vertebrate myosin I, Myo1f, is predominantly expressed in human immune cells. Neutrophils from the Myo1f KO mouse show increased adhesion and reduced motility resulting in defects in innate immunity and protection against, for example, *Listeria* infection (Kim et al. 2006).

Myo1g is selectively expressed in haematopoietic cells (Olety et al. 2010; Patino-Lopez et al. 2010), predominantly localising to the plasma membrane, where it regulates membrane tension and is required for a variety of processes including FcγR-mediated phagocytosis (Dart et al. 2012), adhesion and a meandering motility specific for T cells to search for antigen-presenting cells (Gerard et al. 2014; Maravillas-Montero et al. 2014). Myo1h expression has been confirmed in humans; however, no information on tissue distribution, function or cellular localization is available at present.

3 Class II Myosins

3.1 Domain Organisation and Motor Properties

The class II myosins are hexamers consisting of two heavy chains (~205 kDa) and two pairs of light chains (essential and regulatory light chains, 16–20 kDa). The N-terminal globular motor domain is followed by the lever arm with two IQ sites for binding the light chains and then a long (160 nm) α-helical coiled-coil tail that assembles through charge-charge interactions to form bipolar filaments. In muscle, bipolar myosin filaments generate contractile force by interacting with actin filament arrays by the sliding filament mechanism (Holmes 1997; Huxley 1969). The interaction of class II myosin and actin also drives contraction/tension generation in non-muscle cells. However, the organisation of actin and myosin seen in non-muscle cell structures such as stress fibres, the cytokinetic contractile ring and epithelial cell-cell junctions shows far greater complexity and diversity.

Most of our understanding of the kinetics of the ATPase cycle has been gained by studies on skeletal muscle myosin II (Geeves and Holmes 2005). Early pioneering crystallographic work (Rayment et al. 1993) and later detailed analysis of the motor domain in the different nucleotide-binding states (reviewed in Holmes 2008) have confirmed many of the steps in the ATPase cross-bridge cycle (Fig. 2).

3.2 Physiological Roles and Cellular Functions

In humans the myosin IIs can be divided into two main groups: (1) the sarcomeric skeletal (Myh1, Myh2, Myh3, Myh4, Myh8, Myh13, Myh15) and cardiac (Myh6, Myh7, Myh7b) muscle myosin IIs and (2) the non-sarcomeric smooth muscle (Myh11, with two splice variants) and non-muscle myosin II (Myh9, 10 and 14) that self-assemble into bipolar/side-polar filaments and form dynamic flexible contractile structures in their respective cells (Berg et al. 2001; Korn 2000).

In skeletal muscle, about 280 myosin II molecules self-assemble to form bipolar thick filaments in the centre of the repeating contractile unit known as the sarcomere. These repeating sarcomeres give muscle a striated appearance. Within each sarcomere, there are also two sets of actin filaments attached by their plus ends to the Z-lines at opposite ends of the sarcomere. The central bipolar myosin filaments interdigitate with the actin filaments, and when they slide past each other driven by ATP-dependent repetitive interactions between the myosin motor domains and the actin filaments, it results in contraction of the sarcomere and so the whole muscle (Huxley 1969).

In human cardiac muscles, three myosin II isoforms are present. The α-isoform (Myh6) is preferentially expressed in the atrial myocardium and has a higher ATPase activity than the β-isoform (Myh7) that is predominantly expressed in the ventricular myocardium (Hoh et al. 1978; Pope et al. 1980). The third isoform, Myh7b, has only recently been identified and remains poorly characterised (Warkman et al. 2012). Mutations in cardiac myosin II lead to hereditary

hypertrophic cardiomyopathy, resulting in sudden cardiac death or congestive cardiac failure, with a prevalence of about 1 in 500 young adults.

The two human smooth muscle myosin II isoforms and the non-muscle myosin II isoforms (often termed NMIIA, NMIIB and NMIIC) differ from the skeletal muscle myosin IIs in that regulatory light-chain (RLC) phosphorylation regulates their motor activity and possibly their assembly into flexible bipolar filaments. In vitro, unphosphorylated smooth muscle and non-muscle myosin II are present in an inactive folded conformation with the tail interacting with the motor domains (possibly a storage form of the myosin) (Scholey et al. 1980; Sellers 1991; Trybus and Lowey 1984). RLC phosphorylation unfolds the myosin, activates its ATPase activity and strongly promotes its assembly into filaments. In vitro the basic ATPase mechanism in the smooth muscle myosin IIs is similar to that in the skeletal muscle myosins, but the product release steps are more than 20-fold slower (Somlyo and Somlyo 1994).

The human non-muscle myosin II isoforms (NMIIA, NMIIB and NMIIC) are encoded by three different genes (Myh9, Myh10 and Myh14, respectively) present on different chromosomes (Berg et al. 2001) and have many distinct and unique functions in controlling cell shape and migration. As described above, the ATPase activity of all three isoforms and their assembly into filaments is regulated by RLC phosphorylation on Ser 19 by a number of kinases under a range of activating signals (Vicente-Manzanares et al. 2009). However, major kinetic differences exist between these isoforms that have functional significance; for example, isoform IIA has the highest ATPase rate and moves on actin filaments the fastest, whereas IIB has the highest duty ratio (remaining bound to actin for longer, in a force-generating state with a low energy cost) and has a very high binding affinity for ADP (Vicente-Manzanares et al. 2009; Wang et al. 2003). Differences in their unique C-terminal non-helical tail regions are responsible for their different filament assembly properties and their distinct intracellular localisations. The wide spectrum of functions and the relative contributions of these isoforms in development, cell migration, adhesion, polarity and disease have recently been reviewed (Ma and Adelstein 2014).

4 Class III Myosins

4.1 Domain Organisation and Motor Properties

Class III myosin was first identified in *Drosophila* as a novel gene (NINA C) required for photoresponse and retinal maintenance (Montell and Rubin 1988). The class III myosins are unique because they contain an N-terminal kinase domain related to the human Ser/Thr kinases in the PAK (p21-activated kinase) superfamily (Komaba et al. 2003). In vertebrates two myosin III isoforms, Myo3a with a longer tail and Myo3b with a shorter tail, are expressed (Dose and Burnside 2000). Myo3a has a high affinity for actin (Kambara et al. 2006), but phosphorylation of the motor domain lowers actin affinity and the duty ratio in vitro (Komaba et al. 2010).

Intermolecular autophosphorylation mediated by the kinase domain reduces the accumulation of Myo3a at the tips of filopodia (Quintero et al. 2010) and microvilli (An et al. 2014). Phosphorylation of several threonine residues in the kinase domain is required for full kinase activity (An et al. 2014; Quintero et al. 2013). Autophosphorylation may regulate the biological function of Myo3a in protrusive actin structures such as stereocilia, although the details of this mechanism are unclear.

4.2 Physiological Roles and Cellular Functions

Most studies have focused on Myo3a, and although its precise physiological function in humans is still not known, mutations in Myo3a have been shown to be responsible for progressive non-syndromic hearing loss (DFNB30) (Walsh et al. 2002). Myo3a is expressed mainly in sensory cells in the retina and the inner ear, where it localises to the tips of the highly organised actin filament structures in the retinal photoreceptors and in the stereocilia in the inner ear hair cells (Dose et al. 2003; Schneider et al. 2006). It has been shown that Myo3a binds to espin 1 (ESPN1), a protein required for actin filament elongation, suggesting that Myo3a is a cargo transporter involved in ESPN1 translocation and in the maintenance of the structural integrity of the actin cytoskeleton in sensory cells (Salles et al. 2009). Myo3b requires the actin-binding domain present in ESPN1 to reach the tips of actin filaments (Merritt et al. 2012).

5 Class V Myosins

5.1 Domain Organisation and Motor Properties

Class V myosins are amongst the most evolutionarily conserved myosins, with three members, namely, Myo5a, Myo5b and Myo5c, expressed in humans. Extensive studies of Myo5 have made it a paradigm of myosin function, guiding the understanding of all other unconventional myosins (Hammer and Sellers 2012; Sellers and Veigel 2006; Trybus 2008; Vale 2003). Myo5a displays the archetypal domain organisation, an N-terminal motor domain, followed by a neck domain with six IQ motifs that acts as a lever arm, a coiled-coil domain that mediates constitutive dimerisation and a cargo-binding globular tail. Myo5a is plus end directed (Cheney et al. 1993) and, when attached to a solid substrate, can slide actin filaments at speeds up to 300 nm/s. In vitro, Myo5a displays processive movement, i.e. it can take multiple steps on a single actin filament before detaching (Mehta et al. 1999). The large working stroke of Myo5a has allowed direct observation of this processive stepping in vitro using video-rate atomic force microscopy (Kodera et al. 2010). Much effort has been directed at understanding the kinetics and structural dynamics that lead to processive motion.

Myo5a is a high duty ratio motor. This is because the rate of ADP release from the actin-bound head is rate limiting in the kinetic cycle, leading to extended periods of actin filament binding. The high duty ratio of Myo5a means typically at least one head of the constitutive Myo5a dimer is always attached to actin. The length of the rigid Myo5 lever arm (in combination with a Brownian search after the initial swing) results in a step size of 36 nm (Veigel et al. 2002). By labelling each head individually, rather than observing the net movement of the Myo5a dimer, 74 nm steps were observed (Yildiz et al. 2003). This experiment showed that Myo5a moves with a hand-over-hand mechanism, with each head of the dimer taking the role of lead head in turn.

As the duty ratio is around 0.7, if the two heads of a Myo5a dimer independently underwent their ATPase cycles, the combined molecule would most likely show little forward movement and rapidly detach, taking on average less than ten steps (Veigel et al. 2002). Therefore, there must be mechanisms that couple the two heads together, ensuring that the rear head detaches first and that the front head remains attached whilst the rear head moves to the front and rebinds. This was shown to be dependent on the effect of load on the chemical kinetics of the head (Purcell et al. 2005; Veigel et al. 2005). When both heads are bound to actin, the lead head experiences a mechanical load of 2 pN via intramolecular strain from the rear head. This load slows the release of ADP from the lead head, which thus remains bound to actin for an extended period (it can only detach from actin once ADP has been released and been replaced by ATP, see Fig. 2). This gives the rear head time to detach and rebind at the front, a cycle which can be repeated hundreds of times. In addition to the kinetics of each head being sensitive to intramolecular strain, the motile properties of Myo5a are sensitive to load on the molecule. Myo5a has a 'stall force' of 3 pN, above which it can no longer undertake forward steps and will eventually detach (Mehta et al. 1999). The loads that molecules of Myo5a are exposed to in vivo are currently unknown.

There are two established mechanisms that have been shown to regulate Myo5a motor activity; environmental calcium and cargo binding. Despite the fact that Ca^{2+} increases ATPase activity (Homma et al. 2000), movement of Myo5a along actin is inhibited by high calcium concentrations (Cheney et al. 1993), most likely because detachment of calmodulin from the first IQ domain destabilises the lever arm (Lu et al. 2012b). Whether calcium fluxes regulate Myo5a activity in cells has yet to be determined. When Myo5a is not bound to cargo, electron microscopy has revealed that it adopts a folded conformation, with the tail bound to allosteric sites on the two heads, inhibiting movement (Thirumurugan et al. 2006). Binding of cargo proteins such as melanophilin to the tail can relieve this inhibition (Yao et al. 2015), activating the motor. This mechanism ensures that Myo5a only undergoes movements that are 'useful', i.e. when it is bound to cargo and has something to transport.

In vitro studies of purified Myo5b indicate that like Myo5a, it is a high duty ratio processive motor although some of its kinetic rates differ significantly from those of Myo5a (Watanabe et al. 2006b). In contrast to Myo5a and Myo5b, Myo5c has been shown to be a non-processive, low-duty ratio motor (Takagi et al. 2008; Watanabe

et al. 2008). However, a recent study found that a processive molecular complex could be generated by coupling two non-processive Myo5c dimers together via a DNA linker (Gunther et al. 2014). It remains to be determined whether processive molecular complexes containing multiple Myo5c dimers exist in cells under physiological conditions.

5.2 Physiological Roles and Cellular Functions

Myo5a has a long established role in melanosome transport and loss of Myo5a function underlies Griscelli syndrome type 1 (see below). Myo5b is involved in polarised traffic and mutations in Myo5b cause microvillus inclusion disease. Myo5c is the least studied member of the class and until recently it was thought to lack significant transport functions. Extensive reviews have been written about the mechanochemistry and physiology of class V myosins (Hammer and Sellers 2012; Kneussel and Wagner 2013; Trybus 2008).

Myo5a is widely expressed in many tissues, in particular the brain, peripheral nervous system and pituitary gland (Rodriguez and Cheney 2002). Its major functions include the transport of melanosomes in melanocytes and of cargo-rich vesicles and endoplasmic reticulum in brain cells. Loss of the Myo5a *dilute* gene in mice leads to seizures followed by death a few weeks after birth (Silvers 1979). Myo5a knockout (*Dilute*) mice have a lightened coat as melanosomes are not transported from melanocytes to hair-producing keratinocytes (Silvers 1979). In humans, Myo5a is mutated in Griscelli syndrome type 1, characterised by pigment dilution in skin and hair and profound neurological dysfunction (Pastural et al. 1997).

To operate in cells, Myo5a-mediated transport on actin must coordinate with the other transport pathways, in particular microtubules. Given the extended length of microtubules and the high speed (microns per second) of kinesin motors, it was long thought that actin-based motors mediate only short peripheral movements. In the classical model of melanosome transport, termed cooperative capture, Myo5a is located in a dense actin meshwork at the terminus of microtubules and plays a tethering function, mediating only short-range transport (Wu et al. 1998). However, there is growing evidence that Myo5a plays a role in long-range transport in several systems. Using melanocytes grown on circular patterns, it was recently found that Myo5a can mediate long-range outward movement of melanosomes to the cell periphery over several micrometres, which was opposed by microtubule-based movement towards the cell centre (Evans et al. 2014). Stabilising actin filaments with jasplakinolide inhibited this transport as previously shown (Semenova et al. 2008). These results indicate that in contrast to systems reconstituted in vitro, Myo5a requires actin dynamics to mediate transport in cells. In neurons, transport of endoplasmic reticulum into Purkinje cell spines is mediated by actin-based movement of Myo5a over distances up to 2 μm (Wagner et al. 2011). This movement and tethering is required for long-term depression of neuronal synapses.

The best studied Myo5a cargo is the tripartite complex formed between Myo5a, its adaptor protein melanophilin and Rab27a on the surface of melanosomes (Strom et al. 2002). Cargo specificity is partly determined by alternative splicing. Melanocyte-expressed Myo5a contains exons D and F, which confer binding to Rab10 and melanophilin, respectively. In brain, Myo5a includes exon B, which binds to dynein light chain 2, although the function of this interaction is not yet clear (Hodi et al. 2006). In neurons, Myo5a has been implicated in the localisation of mRNA (Balasanyan and Arnold 2014), the transport and tethering of secretory granules (Rudolf et al. 2011) and the insertion of neurotransmitter receptors into the plasma membrane (Lewis et al. 2009). These functions give Myo5a a prominent role in synaptic plasticity.

Myo5b is predominantly expressed in the testis, kidney, liver, lung and heart (Zhao et al. 1996). The cellular functions of Myo5b are mainly related to the recycling of a variety of membrane receptors and vesicular compartments, processes required for apical-basolateral sorting in polarised epithelial cells and synaptic plasticity in neuronal cells (Wang et al. 2008). These functions are primarily mediated by the recruitment of Myo5b via the adaptor Rab11-FIP2 to Rab11a recycling endosomes (Hales et al. 2002). In oocytes, Myo5b-rich Rab11 vesicles organise an extended actin network (Schuh 2011) and undergo long-range movement towards the cell surface in a Myo5b-dependent manner. In neurons, Myo5b is required for the fission of Rab10 carriers from the trans-Golgi network, a process required for axon development (Liu et al. 2013). The Rab11a-Rab8a-Myo5b complex functions in stretch-regulated exocytosis in bladder umbrella cells (Khandelwal et al. 2013). Loss-of-function mutations in Myo5b underlie microvillus inclusion disease (Muller et al. 2008; Ruemmele et al. 2010), which is characterised by the absence of microvilli from the apical surface of the epithelial of the small intestine and leads to potentially fatal watery diarrhoea. Myo5b activity is reduced in gastric cancers, and Myo5b downregulation increases proliferation and invasion of cancer cells (Dong et al. 2012).

Myo5c is expressed in epithelial cells (Rodriguez and Cheney 2002) and has been shown to function in exocytosis (Marchelletta et al. 2008) and secretory granule traffic (Jacobs et al. 2009). A unique function (not overlapping with Myo5a) in the biogenesis and secretion of melanosomes has also been identified, dependent on interactions with Rab32 and Rab38 (Bultema et al. 2014).

6 Class VI Myosin

6.1 Domain Organisation and Motor Properties

Myo6 is the only myosin motor that is known to move towards the minus end of actin filaments (Wells et al. 1999), in the opposite direction to all other myosins so far characterised. Thus, Myo6 has a number of unusual structural and functional adaptations not found in other classes of myosins. In the motor domain, Myo6 contains a 22-amino-acid insertion next to the switch1 loop and the ATP-binding

pocket. This insert regulates ATPase activity by slowing down ATP binding in response to strain, thereby creating a mechanism that allows communication between the two heads to coordinate processive movement along the actin filament (Spudich and Sivaramakrishnan 2010; Sweeney and Houdusse 2010). Between the motor domain and the lever arm lies a unique 53 amino acid insert that forms an integral part of the converter region and can bind to a non-exchangeable calmodulin (Wells et al. 1999). This insert repositions the lever arm at the end of the power stroke by 120° and thus enables the reverse directionality of Myo6 (Menetrey et al. 2005). The lever arm itself is short and only binds one calmodulin through a canonical IQ motif. Despite the short 'conventional' lever arm, Myo6 has a large step size of around 36 nm, which seems at odds with the swinging lever-arm hypothesis. To account for this large step size, the three-helix bundle present in the proximal tail has been postulated to unfold to form a lever-arm extension. Alternatively, a single stable alpha helix (SAH) located in the distal tail before the cargo-binding region may act as part of the lever arm (Spudich and Sivaramakrishnan 2010; Sweeney and Houdusse 2010).

Myo6 exists as a monomer when purified from cells and tissues (Lister et al. 2004), but it may dimerise using stretches of the tail either before or after the SAH domain, induced by cargo adaptor binding (Mukherjea et al. 2014; Park et al. 2006). The C-terminal region of the tail enables attachment of Myo6 to a variety of vesicles and organelles through binding to compartment-specific cargo adaptor proteins. In addition, Myo6 can also directly bind to phospholipids such as $PI(4,5)P_2$ and to ubiquitin (Penengo et al. 2006; Spudich et al. 2007). Finally, the cargo-binding tail region of Myo6 is alternatively spliced, containing either a large (12–32 amino acids) or a small (9 amino acids) insert or both inserts or no inserts, generating four different Myo6 splice isoforms with different tissue-specific expression patterns (Buss et al. 2001; Dance et al. 2004).

6.2 Physiological Roles and Cellular Functions

In humans the Myo6 isoform containing the large insert is selectively expressed in polarised epithelial cells, where it is targeted to the microvilli-rich apical domain to facilitate clathrin-mediated uptake of cell surface receptors (Ameen and Apodaca 2007). The no-insert splice variant is widely expressed in most cell types and tissues and mediates the function(s) of this myosin in exocytosis, endocytosis as well as regulation of actin filament dynamics. The loss of Myo6 is associated with a large number of phenotypes in the Snell's Waltzer KO mouse including deafness and profound astrogliosis (Avraham et al. 1995; Osterweil et al. 2005). In humans, nonsense and missense mutations in Myo6 are also associated with deafness and an inherited form of hypertrophic cardiomyopathy (Mohiddin et al. 2004). In contrast, overexpression of Myo6 has been observed in both ovarian and prostate cancers and has been shown to correlate with metastatic aggressiveness and worsening of prognosis (Dunn et al. 2006). Inhibiting Myo6 expression reduces cancer cell motility in tissue culture models and invasion and spreading of ovarian cancer

cells in nude mice (Yoshida et al. 2004). The loss of Myo6 function in these pathologies may be linked to its role in trafficking and/or the regulation of cortical actin filament dynamics. Studies on the cellular level have shown that Myo6 is required during clathrin-mediated endocytosis from the apical domain of polarised epithelial cells (Ameen and Apodaca 2007; Buss et al. 2001), in biosynthetic exocytosis for maintenance of Golgi morphology and secretory vesicle fusion at the plasma membrane (Bond et al. 2011; Warner et al. 2003), for cargo sorting at early endosomes (Chibalina et al. 2007) and for delivery of endosomes to autophagosomes (Tumbarello et al. 2012). In addition Myo6 also plays an important role in anchoring plasma membrane domains to actin filaments, as found in the highly organised stereocilia on the hair cells of the inner ear (Self et al. 1999). These diverse cellular roles are mediated by a wide range of cargo adaptor proteins that bind to regions in the C-terminal cargo-binding tail via either the RRL (binding partners GIPC, TAX1BP1, NDP52 and optineurin) or WWY motifs (binding partners TOM1, LMTK2 and Dab2) (Sweeney and Houdusse 2007; Tumbarello et al. 2013). These adaptor proteins may not only target Myo6 to specific cargoes and cellular compartments but may also regulate the monomer-dimer state of the myosin and coordinate its cargo binding with motor activity. A monomeric Myo6 is able to exhibit load-depending anchoring functions (Altman et al. 2004), whereas a cargo-induced dimer is able to move processively as a transporter over short distances along actin filaments (Phichith et al. 2009).

7 Class VII Myosins

7.1 Domain Organisation and Motor Properties

Myo7a is expressed at significant levels in the cochlea, retina, testis, lung and kidney (Hasson et al. 1995). It has a large 250 kDa heavy chain with an N-terminal motor domain, five IQ domains (calmodulin or light-chain binding), a coiled coil and a tail containing two MyTH4 (myosin tail homology 4) domains, an SH3 domain and two talin-homology or FERM (four-point-one, ezrin, radixin, moesin) domains (Chen et al. 1996) (see Fig. 1). MyTH/FERM domains interact with microtubules and adhesion receptors and are also found in Myo10 and Myo15a. The domain organisations of Myo7a and b are very similar except Myo7a has an additional SAH domain in the proximal tail region. Human Myo7a has a high duty ratio (in the range 0.6–0.9), consistent with a function in cargo transport. The rate-limiting kinetic step for Myo7a is the slow release of ADP whilst bound to actin (Watanabe et al. 2006a). Of particular interest, the rate of ADP release for human Myo7a has been shown to be sensitive to Mg^{2+} concentration (Heissler and Manstein 2012). This is consistent with the rate of ADP release being sensitive to the strain on the molecule, as previously shown for Myo5a (Rosenfeld et al. 2005). As ADP release is generally the rate-limiting step in high duty ratio myosins, sensitivity to strain has a significant effect on motor function. Thus, as a slow, high duty ratio motor Myo7a can serve as a molecular transporter, whilst its strain-

sensitive kinetics may allow it to function as a tension sensor (Nyitrai and Geeves 2004). Forced dimerisation, by incorporation of a leucine zipper at the neck (Yang et al. 2006), or the overexpression of cargo proteins leads to the formation of processive complexes in cells (Sakai et al. 2011), but native Myo7a dimers have not yet been detected in cells.

Myo7b is reported to have a high duty ratio (Henn and De La Cruz 2005), with a very slow ADP dissociation rate. In particular, the ATPase is activated by very low actin concentrations, and since ADP and actin binding are uncoupled, Myo7b can remain bound to an actin filament for long periods. These properties indicate that Myo7b most likely functions as a tension generator or an anchor for membrane receptors or other binding partners. As with Myo5, the actin-activated ATPase activity of the head can be autoinhibited by binding to the tail (Umeki et al. 2009). This autoinhibition can be relieved either by binding to cargo (Sakai et al. 2015) or, in regions of high actin concentration, to actin (Yang et al. 2009).

7.2 Physiological Roles and Cellular Functions

In humans, impaired Myo7a function underlies both the autosomal recessive deafness condition DFNB2 and Usher 1B syndrome (Weil et al. 1997), in which patients present both deafness and blindness (Weil et al. 1995). The exact mechanisms by which loss of Myo7a leads to these defects are not known, but it has been shown that the shape of stereocilia is critically dependent on Myo7a (Boeda et al. 2002). Myo7a is localised to the retinal pigment epithelium (and to a lesser extent the photoreceptors), where it is required for functions such as melanosome positioning and the transport of phagosomes to lysosomes (Williams and Lopes 2011). Trials in the Myo7a KO mouse, the so-called *shaker-1* mouse, indicate that reintroducing Myo7a cDNA via gene therapy with adenoviral vectors can prevent Usher 1B-associated blindness (Lopes et al. 2013).

Myo7a forms numerous complexes through the array of interacting motifs in its tail (Wu et al. 2011). Its main functions are linked to receptors and adaptors regulating cell-cell and cell-matrix adhesion, with roles specified by its expression profile, in particular in the eyes, ears and testis. A general feature is the binding of the MyTH4-FERM domains to membrane receptors such as cadherins and integrins (Liu et al. 2014). This linkage of adhesion proteins to the cytoskeleton underlies an important function for Myo7a in dynamic membrane adhesive processes such as cell spreading, migration and phagocytosis (Tuxworth et al. 2001). Myo7a also binds to components of adherens junctions (Kussel-Andermann et al. 2000) via both its SH3 domain (Velichkova et al. 2002) and its Myth4-FERM domain (Etournay et al. 2007).

Of relevance to Usher 1B syndrome, Myo7a forms a complex with two other Usher proteins, Cadherin23 and harmonin, via its FERM domain and also interacts with phospholipids (Bahloul et al. 2010). Myo7a in hair cells binds to protocadherin15 (also an Usher protein) (Senften et al. 2006) and to SANS at the upper tip-link density (Grati and Kachar 2011) through its MyTH4-FERM domain

(Wu et al. 2011). It is also linked to the integral membrane protein PHR1 in inner ear sensory cells (Etournay et al. 2005). Myo7a functions in opsin transport through the connecting cilium of photoreceptor cells towards the outer segment (Liu et al. 1999). As directed movement in cilia is primarily mediated by kinesin, the function of Myo7a in opsin transport may be to maintain a selective diffusion barrier, controlling entry to, or exit from, the cilium (Williams and Lopes 2011). Myo7a also interacts via MyRIP (El-Amraoui et al. 2002) with Rab27a to position melanosomes in RPE cells (Lopes et al. 2007).

In contrast to Myo7a, almost nothing is known about the physiological and cellular functions of Myo7b. It is expressed mainly in the microvilli of the kidney and intestine (Chen et al. 2001). Consistent with this observation, a Myo7b polymorphism has been associated with kidney disease in a Japanese cohort (Yoshida et al. 2009). Myo7b has also been identified as a potential biomarker in patients with mesothelioma/metastatic carcinoma (Mundt et al. 2014).

8 Class IX Myosins

8.1 Domain Organisation and Motor Properties

In humans two genes encoding Class IX myosins have been identified, Myo9a (the orthologue of rat myr7) and Myo9b (rat myr5), which give rise to a number of splice variants. Class IX myosins are single-headed myosins with several unique features (Bement et al. 1994; Reinhard et al. 1995). They contain an N-terminal extension of unknown function that is structurally similar to a Ras-binding domain, without actually binding to G-proteins. Both Myo9a and Myo9b have a long insertion (>140 amino acids) within the motor domain in loop 2 at the 50/20 kDa junction (see Fig. 1). This insertion has been shown to function as an electrostatic actin tether that enables processive movement of the single-headed motor along the actin track (Elfrink et al. 2014). Interestingly, loop 2 can also bind to calmodulin, and Ca^{2+} binding to neck-associated calmodulin negatively regulates the ATPase activity and movement of this class of myosin (Liao et al. 2010; Struchholz et al. 2009). Myo9 displays some further unique motor properties as ATP hydrolysis and not ADP release is the rate-limiting step in the kinetic cycle. Myo9 therefore spends most of its time bound to both actin and ATP; in this state most other classes of myosins show a low affinity for actin (Kambara and Ikebe 2006; Nalavadi et al. 2005).

The neck region contains six IQ motifs in Myo9a and four IQ motifs in Myo9b. The C-terminal tail domain contains a C1 domain (protein kinase C conserved region 1) as well as a RhoGTPase-activating protein (RhoGAP) domain (Reinhard et al. 1995). A C1 domain can typically bind (in the presence of Zn^{2+}) to the second messenger diacylglycerol (DAG) and to phorbol esters such as PMA; however, the specificity and function of the C1 domain in Myo9 remain to be established. The C1 domain is followed by a GTPase-activating (GAP) domain, which is most similar to GAPs for the Rho family of small GTPases that control the dynamic organisation of the actin cytoskeleton. The RhoGAPs stimulate the hydrolysis of bound GTP,

thereby switching the RhoGTPase from the active GTP-bound to its inactive GDP-bound form.

8.2 Physiological Roles and Cellular Functions

Myo9a is most highly expressed in the brain, testis and kidney and to a lesser extent in lung and spleen (Gorman et al. 1999). In the brain Myo9a is required for ependymal epithelial cell maturation, and deletion of Myo9a leads to hydrocephalus formation due to stenosis in the ventricular system (Abouhamed et al. 2009). Myo9a KO mice also show signs of kidney disease with hydronephrosis and proteinuria. In contrast, Myo9b is most abundant in myeloid cells including dendritic cells and macrophages, but is also expressed at lower levels in the testis, lung and liver (Chieregatti et al. 1998; Wirth et al. 1996). Macrophages and dendritic cells from Myo9b-deficient mice show defects in chemotaxis and migration velocity (Hanley et al. 2010; Xu et al. 2014). Myo9b polymorphisms are associated with a variety of different autoimmune diseases (Sanchez et al. 2007; Santiago et al. 2008), and studies using experimental autoimmune encephalomyelitis revealed an important role for Myo9b in the propagation of the inflammatory response in the central nervous system (Liu et al. 2015b).

The best characterised and recognised cellular function of class IX myosins is linked to their RhoGAP domain and their role in negative feedback regulation of Rho signalling and actin filament organisation. No direct cargo adaptor proteins for this class of myosin have been identified so far, suggesting that they predominantly function as motorised signalling molecules to regulate localised actin turnover. In proximal kidney tubule epithelial cells, Myo9a is present at the apical brush border (Thelen et al. 2015), and it is present at cell-cell contact sites in brain ependymal epithelial cells and bronchial epithelial cells. In these cells Myo9a-regulated Rho activity is required to maintain cell-cell adhesions and the collective migration of epithelial cells (Omelchenko and Hall 2012). Myo9b is present in regions of the cell with high activity of actin polymerisation as seen in lamellipodia, filopodia and membrane ruffles at the leading edge of migratory cells. As a consequence, macrophages from the Myo9b KO mouse fail to spread, polarise, extend lamellipodia and perform directed migration towards a chemotactic gradient (Hanley et al. 2010).

9 Class X Myosin

9.1 Domain Organisation and Motor Properties

Myo10 has a 2058 amino acid (~240 kDa) heavy chain comprising a motor domain, a neck lever-arm region with three IQ motifs followed by a single alpha helix (SAH), three PEST motifs (rich in the amino acids proline, glutamic acid, serine and threonine) and a tail. The IQ domains bind to both calmodulin and calmodulin-

like protein (CLP), which has a much lower affinity for calcium. The PEST motifs are sites of proteolytic activity in vitro, but have no established function in vivo. The tail binds the membrane phospholipid PI(3,4,5)P_3 through three PH domains and also contains a single MyTH4-FERM domain (Berg et al. 2000).

As with many other unconventional myosins, Myo10 has a high duty ratio, spending most of its ATPase cycle complexed with ADP and bound to actin (Homma and Ikebe 2005). In vitro single-molecule and structural experiments from a variety of groups have produced a complex picture of Myo10 stepping behaviour (Kerber and Cheney 2011). Some reports indicate Myo10 exclusively walks on bundled actin, taking 18 nm steps on two parallel actin filaments 10 nm apart (Nagy et al. 2008; Nagy and Rock 2010). However, processive stepping on single actin filaments with 30–36 nm step sizes has also been observed (Bao et al. 2013; Sun et al. 2010; Takagi et al. 2014). Myo10 may also show a preference for fascin-bundled rather than alpha-actinin bundled actin (Bao et al. 2013). Whether and when full-length Myo10 walks on single actin filaments or bundles in the complex environment of the cell has yet to be established.

For Myo10, little gating (i.e. coordinated movement) of the heads has been observed, and processive movement occurs at relatively low loads (below 1 pN), above which the motors detach from actin (Takagi et al. 2014). It has been speculated that detachment at low load may promote navigation in crowded environments such as actin-rich cell protrusions termed filopodia. By virtue of its tendency to localise to filopodia (discussed further below), Myo10 is one of the few molecular motors for which single-molecule motility has been reported in intact cells (Kerber et al. 2009; Watanabe et al. 2010). Clusters of Myo10 were observed to move outwards at a speed of around 80 nm/s and inwards at 10–20 nm/s (consistent with the rate of retrograde actin treadmilling in filopodia). Much more rapid outward movements (600 nm/s) of faint particles were also observed indicating that single molecules or dimers of Myo10 can move processively in filopodia. These movements may serve to localise signalling proteins such as VASP to the tips of filopodia (Tokuo and Ikebe 2004).

Structural evidence indicates Myo10 may form an antiparallel coiled-coil dimer (Lu et al. 2012a). In addition it was shown that full-length Myo10 is monomeric with the tail bound to the head in an autoinhibitory conformation (Umeki et al. 2011). Binding of the membrane phospholipid PI(3,4,5)P_3 to the PH domains in the tail can relieve this autoinhibition leading to Myo10 dimerisation. However, the mechanisms underlying dimerisation of full-length Myo10 in cells, and the motor properties of such dimers, are unknown.

9.2 Physiological Roles and Cellular Functions

The biological functions of Myo10 have been reviewed in detail (Kerber and Cheney 2011). It is expressed in a broad array of tissues including the brain, kidney and liver (Berg et al. 2000) and is mainly found localised to the tips of actin-rich cellular protrusions termed filopodia. It has been linked to a broad array of

biological functions required for normal physiology including mitotic spindle assembly, angiogenesis, melanosome transfer and neuronal development.

In single cultured cells, Myo10 localises to the tips of filopodia and can move in both directions along them (Berg and Cheney 2002). Overexpression potently induces filopodia formation, and likewise knockdown of Myo10 by siRNA reduces the number of cellular filopodia (Bohil et al. 2006). The FERM domain of Myo10 interacts with integrins at filopodia tips (Zhang et al. 2004). The function and localisation of Myo10 in cells are regulated by binding of the three PH domains to the phospholipid $PI(3,4,5)P_3$ (Lu et al. 2011; Plantard et al. 2010), and this interaction is required for axon development and growth cone formation (Yu et al. 2012). The MyTH4 domain binds to microtubules, and Myo10 regulates spindle orientation in meiosis (Weber et al. 2004) and mitosis (Woolner et al. 2008). This is achieved by coupling of centrosomes to subcortical actin filaments (Kwon et al. 2015).

Myo10 regulates axonal pathfinding by interactions with the netrin receptors DCC and neogenin (Zhu et al. 2007). DCC appears to promote Myo10 movement on peripheral actin filaments, whilst neogenin regulates localisation to dorsal cell surfaces (Liu et al. 2012b). The structure of Myth4-FERM domain interacting with the cargo protein DCC (Hirano et al. 2011; Wei et al. 2011) indicates that binding to DCC, integrins and microtubules is all mutually exclusive. Myo10 localises to cell-cell contacts in polarised epithelia and functions in tight junction formation (Liu et al. 2012a). It has been shown to have numerous roles in cell migration (Hwang et al. 2009; Nie et al. 2009) and is required for phagocytosis downstream of PI3-Kinase (Cox et al. 2002). In addition to the full-length protein, a headless isoform is also expressed in brain cells (Sousa et al. 2006) that is required for their migration (Wang et al. 2009).

Dysregulation of Myo10 function may also be crucial for the progression of several diseases (Courson and Cheney 2015). Many cancer cells have high numbers of filopodia, and Myo10 expression is elevated in breast cancer and linked to metastasis (Arjonen et al. 2014). During breast tumour invasion triggered by mutations in p53, Myo10 facilitates integrin transport and localises to the tips of invadopodia, a structure that promotes invasion (Arjonen et al. 2014; Schoumacher et al. 2010). Depletion of Myo10 severely reduces matrix degradation (Schoumacher et al. 2010) and downregulates expression of invadopodia component genes (Cao et al. 2014b). In addition to cancer invasion, Myo10 also has functional roles during infection since it can associate with Marburg virus particles as they move along filopodia (Schudt et al. 2013) and facilitates the spread of *Shigella* between cells (Bishai et al. 2013).

10 Class XV Myosins

10.1 Domain Organisation and Motor Properties

Myo15a is the third member of the MyTH/FERM group of myosins expressed in humans and has the largest myosin heavy chain in the mammalian proteome (395 kDa). The full-length molecule contains an N-terminal extension, motor domain, three IQ domains, two MyTH4 domains, an SH3 domain and two FERM domains (see Fig. 1). An alternative isoform lacking the N-terminal extension is also expressed. Myo15b is most likely a transcribed but non-expressed pseudogene since its sequence lacks a functional motor domain and several frame shifts are observed in the cDNA in comparison to Myo15a (Boger et al. 2001).

Difficulty in expressing Myo15a prevented examination of its motor properties until recently, when it was found that co-expression with the molecular chaperone UNC45B significantly increases yield (Bird et al. 2014). Using purified Myo15a motor domain, it was found to be a plus end directed myosin with a high duty ratio, which moves actin filaments rapidly (400 nm/s) with a relatively short 8 nm power stroke (Bird et al. 2014). The Myo15a lever arm preferentially binds to light chains rather than calmodulin, similar to class II myosins. The motor properties of full-length Myo15a remain to be determined.

10.2 Physiological Roles and Cellular Functions

Myo15a has a limited expression profile, being mainly expressed in the inner ear and the pituitary gland (Liang et al. 1999). Loss of Myo15a function underlies the hereditary deafness DFNB3 and the genotype of the *shaker-2* mouse (Liang et al. 1999). Restoring expression of Myo15a can correct *shaker-2* deafness (Probst et al. 1998). Genetic polymorphisms of Myo15a are associated with deafness in several families and cohorts (Kalay et al. 2007; Liburd et al. 2001; Tabatabaiefar et al. 2011; Wang et al. 1998). A large N-terminal extension present in isoform 1 of Myo15a is essential for hearing, although some mutations leave residual hearing at low frequencies (Nal et al. 2007).

Myo15a plays a critical role in the hair cells of the inner ear (Anderson et al. 2000). It localises to the stereocilia tip (Belyantseva et al. 2003), where it recruits whirlin (Belyantseva et al. 2005) and Eps8 (Manor et al. 2011). Recruitment of Myo15a to stereocilia tips depends on stereocilia length, which is in turn regulated by Eps8 (Rzadzinska et al. 2004). Isoform 1, containing the large N-terminal extension, has recently been shown to localise specifically to shorter stereocilia (Fang et al. 2015), and without Myo15a, this subset of stereocilia retracts after birth leading to profound deafness.

11 Class XVI Myosin

11.1 Domain Organisation and Motor Properties

The only class XVI myosin, Myo16 (also termed Myo16b), is predominantly expressed during brain development (Patel et al. 2001). Myo16 contains an N-terminal extension, a motor domain, a single IQ and a tail domain with a proline-rich region (see Fig. 1). The N-terminal extension contains an ankyrin repeat domain, which interacts with protein phosphatase PP1c (Patel et al. 2001). Binding of actin to the motor domain is sensitive to ATP, indicating it has an active motor domain (Patel et al. 2001). However, its motor properties are poorly characterised. Sequence analysis of the Myo16 motor domain suggests that it may have a very low ATPase activity as many residues crucial for motor domain function are missing. The ankyrin repeat domain at the N-terminus of Myo16 binds skeletal muscle myosin in vitro, enhancing its ATPase activity (Kengyel et al. 2015).

11.2 Physiological Roles and Cellular Functions

Myo16 is expressed in hippocampal neurons and displays nuclear localisation (Cameron et al. 2007), most likely due to a 590 amino acid C-terminal extension with a non-canonical nuclear localisation sequence (Cameron et al. 2013; Patel et al. 2001). The expression level of Myo16 fluctuates during both the cell cycle and DNA replication stress, peaking at entry into S phase. Myo16 is subsequently targeted to the proteasome at the start of M phase and degraded. Myo16 is also known as NYAP3 (Yokoyama et al. 2011) and as such is a member of the NYAP phosphoprotein family. It interacts with the PI3-kinase (PI3K) regulatory subunit p85 leading to PI3K, Akt, Rac1 and WAVE activation and so regulates actin organisation (Yokoyama et al. 2011). PI3K signalling has been implicated in several brain disorders including autism and schizophrenia (Waite and Eickholt 2010) thus placing Myo16 in a crucial role for healthy brain function. During neuronal development, Myo16 interacts with the extracellular domain of KIRREL3, a synaptic immunoglobulin family cell adhesion molecule linked to autism (Liu et al. 2015a). A genetic variant of Myo16 is associated with schizophrenia (Rodriguez-Murillo et al. 2014), whereas deletion of Myo16 may result in intellectual disability (Tucker et al. 2011).

12 Class XVIII Myosins

12.1 Domain Organisation and Motor Properties

There are two members of the Class XVIII myosins expressed in humans, Myo18a and Myo18b, of which Myo18a has two isoforms, termed α and β. Myo18aα has a 233 kDa heavy chain and contains the following domains: an N-terminal extension

containing a KE-rich region and a PDZ domain, a motor domain, one IQ motif, a coiled coil and a C-terminal globular tail (Furusawa et al. 2000). The β isoform lacks the KE-rich regions and PDZ domain seen in the N-terminal extension of Myo18aα (see Fig. 1). Myo18a isoforms bind essential and regulatory light chains (Guzik-Lendrum et al. 2013) and are thus similar to class II myosins in this respect. Myo18a has two actin-binding sites. In addition to the expected site in the classical motor domain, an ATP-independent site is present in the N-terminal extension (Isogawa et al. 2005). The actin-binding properties of this region are regulated by binding of the PDZ domain to GOLPH3 (Taft et al. 2013). Actin binding in the active site is relatively weak, and significant ATPase activity is not observed in either the presence or absence of actin, indicating that mammalian Myo18a is a poor molecular motor (Guzik-Lendrum et al. 2013). Thus Myo18a most likely functions as an actin tether.

Myo18b has an N-terminal extension with unknown properties (Ajima et al. 2007; Nishioka et al. 2002), and no studies have explicitly reported the kinetic or actin-binding properties of this motor.

12.2 Physiological Roles and Cellular Functions

Myo18a is widely expressed, but detected at the highest levels in stromal and haematopoietic cells (Furusawa et al. 2000), cervix, testis and pancreas. It has been found to play diverse roles in health and disease. For instance, it has been found directly fused to membrane receptors in several myeloproliferative diseases (Ussowicz et al. 2012; Walz et al. 2005, 2009). Myo18a expression increases during macrophage maturation (Mori et al. 2003) and it modulates macrophage priming and activation (Yang et al. 2015). It also functions to maintain muscle integrity in the developing embryo (Cao et al. 2014a). The N-terminal PDZ domain localises Myo18aα to the ER/Golgi (Mori et al. 2003), where it has been shown to link Golgi membranes to F-actin through interactions with GOLPH3 (Dippold et al. 2009). In contrast, the distribution of the β isoform is not well characterised. Myo18a interacts with a PAK2/betaPIX/GIT1 complex (Hsu et al. 2010), and the interaction of Myo18a with betaPIX is required for epithelial cell migration (Hsu et al. 2014). Class XVIII myosins are evolutionarily related to class II myosins (Foth et al. 2006), and Myo18a co-assembles with non-muscle class II myosin into mixed bipolar filaments (Billington et al. 2015), although its precise function in this context is not clear.

Myo18b is mainly expressed in cardiac and skeletal muscle, with lesser amounts in the testis (Salamon et al. 2003). Deletion of Myo18b is embryonically lethal in mice (Ajima et al. 2008), and deletion, mutation or methylation of the Myo18b gene have been observed in many cancers including lung (Nishioka et al. 2002), colorectal (Nakano et al. 2005) and ovarian (Yanaihara et al. 2004). In lung cancers, expression has been shown to be regulated by histone acetylation (Tani et al. 2004). Furthermore, regulation of Myo18b expression by differential methylation has been linked to type-2 diabetes (Nitert et al. 2012). Myo18b has been

shown to bind HOMER2, an interacting partner of a diverse collection of membrane receptors such as glutamate, IP3 and TRP channels (Ajima et al. 2007). This interaction enhanced suppression of anchorage-independent growth by Myo18b in H1299 cells.

13 Class XIX Myosin

13.1 Domain Organisation and Motor Properties

Myo19 has a 990-amino-acid (109 kDa) heavy chain with a motor domain, three IQ motifs and a short (146 amino acid) basic (i.e. positively charged at neutral pH) tail (Quintero et al. 2009) (see Fig. 1). Little is known about the motor properties of Myo19, although it has been established to be a plus end directed (Lu et al. 2014), high duty ratio motor that binds to either calmodulin (Adikes et al. 2011) or regulatory light chain (Lu et al. 2014). Myo19 has a similar affinity for actin as Myo5a, but has a much reduced actin-activated ATPase activity and filament sliding velocity. In line with other high duty ratio myosins, ADP release is the rate-limiting step in the ATPase cycle (Lu et al. 2014).

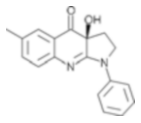
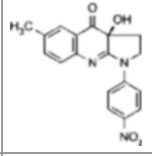
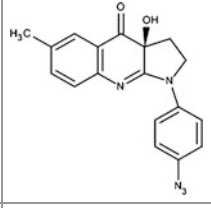
13.2 Physiological Roles and Cellular Functions

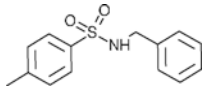
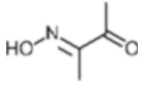
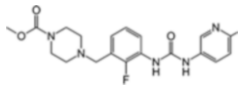
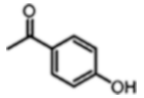
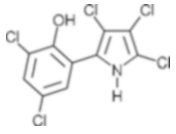
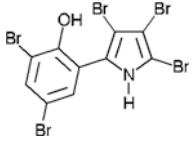
Myo19 is broadly expressed in vertebrate tissues (including the heart, liver, skeletal muscle and pancreas, but not the brain) and shares some sequence homology with Myo5a and Myo6. The most striking feature of Myo19 is its clear association with mitochondria (Quintero et al. 2009), and overexpression leads to continuous mitochondrial movement. Myo19 plays an important role in cell division by regulating symmetric partitioning of mitochondria during cytokinesis (Rohn et al. 2014). Loss of Myo19 frequently leads to a failure of division, with mitochondria asymmetrically distributed and aberrantly clumped around the spindle poles. The mechanism by which Myo19 organises the global distribution of mitochondria within cells throughout the cell cycle remains to be determined.

14 Conclusions and Future Perspectives

In this article, we have outlined some of the many crucial functions of the myosin family in human physiology. However, relatively few treatments are currently available to correct abnormal myosin activity. Thus, myosins represent an as yet underexploited set of targets for pharmacological and therapeutic intervention. A list of compounds established to alter myosin function or activity is given in Table 1. For discussion purposes, these small molecules can be divided into three groups: motor domain inhibitors, motor domain activators and cargo-binding regulators. The motor domain inhibitors are the largest group and may prove effective for

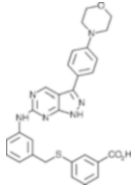
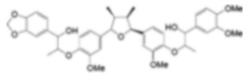
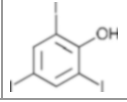
Table 1 An updated list of small molecules which affect myosin motor activity

Compound name	Molecular formula	Chemical structure	Primary target	Binding site	Mechanism	IC_{50}	References
Blebbistatin	$C_{18}H_{16}N_2O_2$		Inhibits myosin II (non-muscle, skeletal muscle)	Hydrophobic pocket at apex of 50 kDa cleft in motor domain	Noncompetitive: hinders inorganic phosphate (Pi) release	0.5–5 μM	Straight et al. (2003)
para-Nitroblebbistatin (photostable equivalent of blebbistatin)	$C_{18}H_{15}N_3O_4$		Inhibits myosin II (non-muscle, skeletal muscle)	Hydrophobic pocket at apex of 50 kDa cleft in motor domain	Noncompetitive: hinders inorganic phosphate (Pi) release	2 μM	Kepiro et al. (2014)
para-Azidoblebbistatin (photo-reactive equivalent of blebbistatin)	$C_{18}H_{15}N_5O_2$		Inhibits myosin II (non-muscle, skeletal muscle)	Hydrophobic pocket at apex of 50 kDa cleft in motor domain. On activation, irreversibly cross-links to binding site	Noncompetitive: hinders inorganic phosphate (Pi) release	5 μM	Kepiro et al. (2012)
CK-2018448/CK-448	–	Undisclosed	Inhibits smooth muscle myosin	–	–	–	Zhao et al. (2011)
CK-2018509/CK-509	–	Undisclosed	Inhibits smooth muscle myosin	–	–	–	Zhao et al. (2011)

N-benzyl-*p*-toluene sulphonamide (BTS)	$C_{14}H_{15}NSO_2$		Inhibits myosin II (fast-twitch skeletal muscle)	Predicted within 50 kDa cleft in motor domain	Noncompetitive: hinders inorganic phosphate (Pi) release	~5 μM	Cheung et al. (2002)
2,3-Butanedione monoxime (BDM)	$C_4H_7NO_2$		Inhibits myosin II (skeletal muscle)	Uncharacterized allosteric site in motor domain	Noncompetitive: hinders inorganic phosphate (Pi) release	~5 μM	Mckillop et al. (1994)
Omecamtiv mecarbil	$C_{20}H_{24}FN_5O_3$		Activates myosin II	–	Increases rate of actin-bound inorganic phosphate (Pi) release	0.6 μM (AC_{40})	Morgan et al. (2010)
4-Hydroxyacetophenone	$C_8H_8O_2$		Activates MYH10, MYH14	–	Enhances cortical localisation of class II myosin, increasing cell tension	~0.5 μM	Surcel et al. (2015)
Pentachloropseudilin (PCIP)	$C_{10}H_4Cl_5NO$		Inhibits myosin 1	Pocket near tip of 50 kDa cleft in motor domain (7.5 Å from blebbistatin-binding pocket)	Noncompetitive: reduces coupling between actin and nucleotide-binding sites	1–5 μM	Chinthalapudi et al. (2011)
Pentabromopseudilin (PBP)	$C_{10}H_4B4r_5NO$		Inhibits myosin V	Pocket near tip of 50 kDa cleft in motor domain (7.5 Å from blebbistatin-binding pocket)	Noncompetitive: reduces ADP dissociation, ATP binding/ hydrolysis and coupling between actin, nucleotide-binding sites	1.2 μM	Fedorov et al. (2009)

(continued)

Table 1 (continued)

Compound name	Molecular formula	Chemical structure	Primary target	Binding site	Mechanism	IC_{50}	References
MyoVin-1	$C_{29}H_{26}N_6SO_3$		Inhibits myosin V	Uncharacterized allosteric site in motor domain	Noncompetitive: hinders ADP release	~6 μM	Islam et al. (2010)
Manassantin B	$C_{41}H_{48}O_{11}$		Inhibits myosin V's transport function	Unknown	Disrupts myosin Va-melanophilin interaction	~30 nM	Chang et al. (2012)
2,4,6-Triiodophenol (TIP)	$C_6H_3I_3O$		Inhibits myosin VI	Predicted PCIP/PBP or blebbistatin-binding sites	Predicted noncompetitive	~2 μM	Heissler et al. (2012)

Adapted from Bond et al. (2013)

conditions such as certain cancers, where hyperactive myosins (such as Myo6 and Myo10) have been reported. However, many disease states (for instance, heart failure) result from the loss of myosin function, and so myosin activators such as omecamtiv mecarbil may have therapeutic benefit. If myosin function has been perturbed at the genetic level, this might best be treated by the introduction of a corrected myosin gene into affected tissues via gene therapy (Lopes et al. 2013). The final group of inhibitors, which specifically disrupt binding of myosins to their cargoes or regulatory proteins (such as manassantin B which may disrupt Myo5a binding to melanosomes), is still extremely small. Little is known about how cargo binding is regulated and how cargo directs myosin motors, either individually or in teams, to selectively transport them to specific compartments within cells. So the development of inhibitors targeting the cargo-binding domain may provide insight into these mechanisms, as well as the much more specific and targeted interventions required for particular conditions that are not possible with complete inhibition of motor activity. In summary, despite the enormous progress made over recent decades in understanding myosin function, a complete picture of the physiological mechanisms regulating these molecular motors is still a distant goal. Future progress will undoubtedly enable much greater therapeutic benefit to be derived from targeting such sophisticated machines.

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Actin Filament Structures in Migrating Cells

Jaakko Lehtimäki, Markku Hakala, and Pekka Lappalainen

Contents

Abstract

Cell migration is necessary for several developmental processes in multicellular organisms. Furthermore, many physiological processes such as wound healing and immunological events in adult animals are dependent on cell migration. Consequently, defects in cell migration are linked to various diseases including immunological disorders as well as cancer progression and metastasis formation. Cell migration is driven by specific protrusive and contractile actin filament structures, but the types and relative contributions of these actin filament arrays vary depending on the cell type and the environment of the cell. In this chapter, we introduce the most important actin filament structures that contribute to mesenchymal and amoeboid cell migration modes and discuss the mechanisms

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by which the assembly and turnover of these structures are controlled by various actin-binding proteins.

Keywords
Actin-binding proteins • Blebbing • Cell migration • Filopodia • Invadopodia • Lamellipodia • Myosin II • Podosome • Stress fibers

1 Introduction

Cell migration is a complex, dynamic process that has a fundamental role in the development and physiology of multicellular animals. Cell migration is also critical for the survival of many unicellular organisms. For example, unicellular protozoa migrate to hunt their prey, whereas immune cells travel in animal tissues to seek pathogens. Furthermore, antigen-presenting dendritic cells, which explore the tissues, migrate into the lymph nodes to present peripherally acquired antigens to T cells upon exposure to infection or inflammatory stimuli. On a higher level, cell migration is crucial for the majority of developmental processes, including gastrulation and organ formation (Nourshargh et al. 2010; Petrie and Yamada 2012; Doyle et al. 2013) and survival of animals, as, for example, wound healing depends on migration of different cell types to the injured area.

Due to its fundamental roles in developmental and physiological processes, it is not surprising that defects in cell migration are linked to a variety of human disorders, including problems in the function of the immune system. For example, phagocytic cells with a mutation in the actin-binding protein WASP, which is an important regulator of cell motility, have a poor chemotactic response toward inflammatory chemoattractants. WASP-deficient T cells display a marked migration deficiency (Moulding et al. 2013). Conversely, uncontrolled cell migration is linked to cancer invasion and metastasis. In the case of carcinomas, these processes initiate with a delamination of otherwise nonmigratory epithelial cells from epithelial sheets, followed by migration of carcinoma cells across basal lamina and underlying cell layers into blood or lymph vessels. Both normal and cancer cells use similar machineries for migration, but cancer cells appear to lack the "stop signals" important for their anchoring and adhesion. Also their ability to properly read the chemical and mechanical signals of the environment is typically lost (Friedl and Alexander 2011; Petrie and Yamada 2012).

For cells to move, they must extend their plasma membrane at the leading edge, subsequently move the cell body, and retract the tail of the cell (Ridley 2011). The force for these events is provided by actin, which is a ubiquitous protein found in all eukaryotes. In the cytoplasm, actin exists both in monomeric and filamentous forms. Actin filaments are polar structures that contain two biochemically and structurally distinct ends, named the *barbed* or *plus end* and the *pointed* or *minus end*. Under steady-state conditions, actin filament assembly takes place mainly at the barbed ends of existing filaments and strongly favors the addition of

ATP-charged actin monomers. The actin filament itself functions as an ATPase, which leads to enrichment of ADP-Pi and ADP-actin subunits toward the filament pointed end, where the net disassembly of actin filaments occurs. These features result in simultaneous ATP-dependent elongation of actin filaments at their barbed ends and shortening at the pointed ends. This process, named *actin filament treadmilling*, provides force for a number of cellular processes involving membrane dynamics. In addition to treadmilling, actin filaments generate force through a fundamentally different mechanism involving myosin-family motor proteins. Here, the actin filaments serve as tracks for myosin molecules that, depending on the type of myosin, travel either toward the barbed or pointed end of the actin filament and transport different types of cargo or form bipolar bundles that can induce the contraction of actin filament arrays (Pollard and Cooper 2009). To ensure the formation of desired actin filament arrays at the correct location inside the cell, the organization and dynamics of actin filaments in cells are controlled by a large array of actin-binding proteins. The activities of these proteins are linked to various intracellular and extracellular biochemical and mechanical signals through their regulation by phosphorylation, Rho family of small GTPases, and plasma membrane phospholipids (Heasman and Ridley 2008; Saarikangas et al. 2010).

In the following chapters, we will introduce the function and composition of the major three actin filament structures (lamellipodia, filopodia, and stress fibers) that contribute to cell migration in a two-dimensional environment. We will also introduce the types of actin structures that drive cell migration specifically in a complex three-dimensional tissue environment.

2 Actin-Rich Structures Involved in Adhesion-Dependent Cell Migration

Within the environment of the tissue, cells can use various different modes of individual migration, excluding the collective migration of entire epithelial sheets. Common to most of these is that cells require protrusion formation at the cell front, coupled to movement of cell body and retraction of the tail. Cell migration can either depend on adhesion to the extracellular matrix (ECM) or be independent of adhesion. In a two-dimensional environment such as on a tissue culture plate, most cell types display mainly the adhesion-dependent migration mode. Three different actin filament structures contribute to this type of cell migration. *Lamellipodial protrusions*, where branched actin filament arrays provide the force for the generation of wide plasma membrane protrusions that drive the advancement of the leading edge. Thin *filopodial* actin filament bundles form fingerlike plasma membrane protrusions that function as "sensory organs" at the leading edge. In addition to these actin treadmilling-dependent structures, also contractile myosin II-containing actin filament bundles, named *stress fibers*, contribute to adhesion-dependent cell migration. However, the function of stress fibers in cell migration varies depending on the cell type, ECM density, and stiffness of the matrix (Ridley 2011; Tojkander et al. 2012).

2.1 Structure and Function of the Lamellipodium

Lamellipodia are thin (100–160 nm), sheetlike plasma membrane protrusions at the leading edge of migrating cells. These dynamic, actin-rich structures extend for several micrometers behind the leading edge of cell. They tend to protrude and retract constantly creating membrane ruffles (Abercrombie et al. 1970a, b). Extracted lamellipodia and lamella of fish keratocytes lacking microtubules, nuclei, and most other organelles are still able to undergo directional motility (Euteneuer and Schliwa 1984), indicating that these structures harbor all necessary functions required for cellular movement.

In the three-dimensional environment of tissues and organs, lamellipodia are thought to serve as anchors for migrating cells to move through the tissue. They can extend over long distances in front of the cell body, attach to extracellular matrix, and pull the cell body through the tissue (Giannone et al. 2007). Although cells are able to migrate also without lamellipodia (Gupton et al. 2005), these structures are crucial for persistent directional migration, indicating that lamellipodia are responsible for sensing environmental cues for migration (Wu et al. 2012; Suraneni et al. 2012).

Actin filaments form a dense, branched network in lamellipodia with more than 100 filaments in one micrometer. Filament density is highest at the distal region of the leading edge and gradually decreases toward the rear of the lamellipodium (Svitkina et al. 1997; Abraham et al. 1999). All actin filament barbed ends in lamellipodia are facing toward the plasma membrane, forming brushlike structures. Practically only very few filament pointed ends are observed in the lamellipodium as they elongate from Y-shaped junctions formed by the Arp2/3 complex, an assembly of actin-binding proteins which nucleates the formation of new actin filaments from the sides of preexisting mother filaments (Mullins et al. 1998; Svitkina and Borisy 1999).

2.2 Regulation of Actin Dynamics in the Lamellipodium

The dendritic nucleation model proposes that an array of new daughter filaments is nucleated at the leading edge of cell close to the plasma membrane (Mullins et al. 1998; Pollard and Borisy 2003). On the other hand, actin monomers dissociate from filaments mainly at the proximal region of the lamellipodium through filament severing and depolymerization (Iwasa and Mullins 2007; Lai et al. 2008). Hence, assembly, dynamics, and disassembly of the complex meshwork of branched actin filaments are under tight regulation of a large number of actin-regulatory proteins. The activities of these proteins are precisely controlled by a variety of signaling proteins, including the small GTPase Rac1, which is the master regulator of lamellipodia formation in many animal cell types. By activating those proteins that induce the nucleation and polymerization of a branched actin filament network and inhibiting those proteins that drive filament disassembly close to the plasma membrane, Rac1 ensures the formation of a proper branched actin filament network at the leading edge of the lamellipodium (Ridley 2015) (Fig. 1).

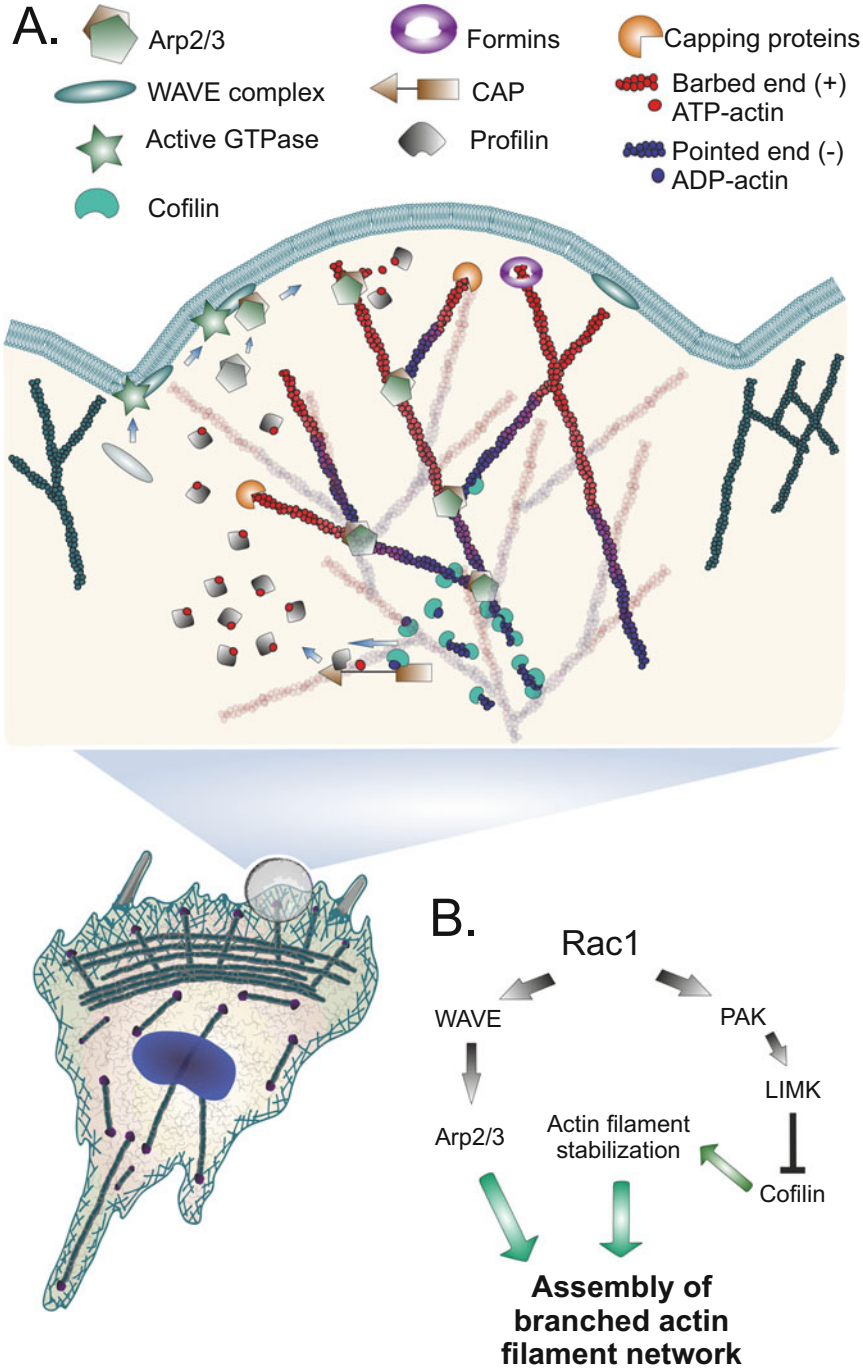

Fig. 1 Organization and assembly of the lamellipodial actin filament network. (**a**) The lamellipodium at the leading edge of a cell comprises a branched actin filament network, which is nucleated by the Arp2/3 complex. The rapidly growing actin filament barbed ends face the

2.2.1 Nucleation of New Filaments

The Arp2/3 complex, one of the key components in lamellipodial actin regulation, is composed of seven subunits, including two actin-related proteins, Arp2 and Arp3 (Machesky et al. 1994). It is present throughout the lamellipodium where it nucleates new actin filaments, stays associated at pointed ends of the newly formed filaments, and is released only after their dissociation (Lai et al. 2008). The initiation of new branched actin filaments occurs close to the plasma membrane, where the Arp2/3 complex binds to preexisting mother filaments and subsequently nucleates a new daughter filament. Daughter filaments elongate from the sides of mother filaments, forming characteristic $70^\circ \pm 7^\circ$ angles between the mother and daughter filaments (Mullins et al. 1998; Svitkina and Borisy 1999; Blanchoin et al. 2000). Formation of these branched filaments is critical for the generation of the dendritic lamellipodial network. This has also been addressed by Arp2/3 depletion and loss-of-function experiments, which resulted in the disappearance of lamellipodia and loss of directional cell migration (Rogers et al. 2003; Wu et al. 2012; Suraneni et al. 2012).

The Arp2/3 complex alone is not sufficient to initiate filament formation, because it needs both preexisting mother filaments and specific activators to be functional. Activation of the Arp2/3 complex takes place at the plasma membrane and is catalyzed by nucleation promoting factors (NPFs) such as Wiskott–Aldrich syndrome protein (WASP) family and SCAR/WAVE complexes. The activities of these NPFs are in turn controlled by Rho family of small GTPases and membrane phosphoinositides to ensure accurate spatial and temporal regulation of the Arp2/3-mediated assembly of lamellipodial actin filament meshworks (Bear et al. 1998; Machesky et al. 1999; Campellone and Welch 2010).

2.2.2 Regulation of Filament Elongation

There are several enhancers and suppressors controlling actin filament elongation following the Arp2/3-mediated nucleation. Heterodimeric capping protein localizes in the lamellipodia close to the plasma membrane where it prevents the assembly and disassembly of actin monomers to/from filaments by binding to filament barbed ends (Isenberg et al. 1980; Mejillano et al. 2004; Lai et al. 2008). Capping protein is vital for the Arp2/3-dependent cell motility and formation of proper lamellipodial

Fig. 1 (continued) plasma membrane and their elongation generates the force for advancement of the leading edge. In addition to the Arp2/3 complex, certain formins contribute to lamellipodial actin filament nucleation. Actin filament assembly and disassembly as well as monomer recycling in the lamellipodium are controlled by a large array of actin-binding proteins including capping protein, cofilin, profilin, and cyclase-associated protein (CAP). (**b**) The small GTPase Rac1 is the master regulator of lamellipodium assembly in many cell types. It controls actin filament assembly by activating Arp2/3 through the WAVE complex and prevents actin filament disassembly close to the plasma membrane by inhibiting cofilin through PAK and LIMK

protrusions. Depletion of capping protein in mammalian cells leads to the loss of lamellipodia and an explosive formation of filopodia (Mejillano et al. 2004). Capping protein may thus limit the amount of free actin filament barbed ends, thus funneling actin monomers to a smaller number of assembly-competent barbed ends in the lamellipodium (Loisel et al. 1999).

The counterparts for capping protein constitute from a family of formins and Ena/VASP proteins (Reinhard et al. 1992; Sagot et al. 2002; Pruyne et al. 2002). These proteins protect filament barbed ends from being capped, increase the rate of filament elongation, and reduce the amount of branches (Skoble et al. 2001; Kovar et al. 2003; Romero et al. 2004; Breitsprecher et al. 2011a). Fibroblasts lacking lamellipodial Ena/VASP proteins display abnormally short and branched actin filaments, whereas Ena/VASP overexpression leads to longer and less branched filaments (Bear et al. 2002). Unlike Ena/VASP proteins, formins are also able to nucleate linear actin filaments. Formins remain associated at the barbed ends after their nucleation, walking along the filaments as they elongate (Romero et al. 2004). Depletion of certain formins inhibits lamellipodial protrusions and consequently reduces cell migration (Yang et al. 2007; Block et al. 2012).

2.2.3 Actin Filament Disassembly and Monomer Recycling

Sustained actin polymerization and formation of new protrusions at the leading edge cannot continue for a long time without regeneration of the pool of polymerization-competent actin monomers. Therefore, it is necessary that cells can rapidly disassemble actin filaments and recycle monomers for the next elongation cycle. The members of the ADF/cofilin protein family are the most critical regulators of actin filament disassembly in cells. The majority of ADF/cofilins bind both monomeric and filamentous actin with a preference for ADP-charged actin subunits (Carlier et al. 1997). Importantly, ADF/cofilins promote rapid actin filament disassembly by severing actin filaments and thus increasing the number of filament pointed ends from where actin monomers can dissociate (Andrianantoandro and Pollard 2006). Depletion of ADF/cofilins indeed leads to problems in proper lamellipodium formation due to decreased filament disassembly and consequent depletion of the pool of assembly-competent actin monomers (Hotulainen et al. 2005; Kiuchi et al. 2007). Recently, it has been shown that ADF/cofilins do not work independently in cells but that at least three proteins, cyclase-associated protein (CAP), Aip1, and coronin, enhance the activity of ADF/cofilins to promote rapid disassembly of actin filaments (Chaudhry et al. 2013; Jansen et al. 2015; Gressin et al. 2015). Furthermore, even following severing by ADF/cofilins, spontaneous depolymerization of actin filaments at their pointed ends is slow compared to what is needed for efficient filament turnover in lamellipodia (Pollard 1986; Watanabe and Mitchison 2002). Twinfilin, another member of the ADF-H domain protein family, is able to enhance the depolymerization rate of actin filaments when cooperating with CAP. Therefore, cofilin, CAP, and twinfilin appear to work in concert to rapidly disassemble actin filaments in cells (Johnston et al. 2015).

In addition to proteins promoting filament severing and depolymerization, specific proteins evolved in eukaryotes that catalyze the dissociation of Arp2/3-stabilized filament branches. A most potent debranching factor is the glia-maturation factor (GMF), which is a small globular protein that, similarly to ADF/cofilins, is composed of a single ADF-H domain (Gandhi et al. 2010). However, unlike ADF/cofilins, GMF does not bind or sever actin filaments, but instead binds to and catalyzes the dissociation of Arp2/3-nucleated filament branches. In migrating cells, depletion of GMF leads to decreased lamellipodial dynamics and consequent defects in directional cell migration (Poukkula et al. 2014; Haynes et al. 2015).

Newly polymerized actin filaments consist of ATP-bound actin monomers, whereas monomers dissociating from the pointed ends predominantly contain ADP in the nucleotide-binding cleft. Thus, ADP in actin monomers has to be exchanged to ATP prior to a new round of filament assembly. CAPs and profilin are considered as the main cellular factors that promote the nucleotide exchange on actin monomers. From these, especially CAP is well suited for promoting nucleotide exchange in cells because it binds both ADP- and ATP-actin monomers with high affinity and efficiently catalyzes the nucleotide exchange on actin monomers (Mattila et al. 2004; Quintero-Monzon et al. 2009). Depletion of CAP leads to problems in lamellipodia formation and dynamics in migrating cells (Bertling et al. 2004). CAP also interacts with profilin and can potentially deliver ATP-actin monomers to this protein (Bertling et al. 2007). Profilin can promote the assembly of ATP-actin monomers to free filament barbed ends and localize ATP-actin monomers to Ena/VASP and formin family proteins. However, profilin inhibits the spontaneous nucleation of actin filaments and is thus well suited to function as a gatekeeper of actin filament nucleation and assembly of actin monomers to desired actin filament arrays (Pantaloni and Carlier 1993; Rotty et al. 2015; Suarez et al. 2015).

2.3 Structure and Functions of Filopodia

Filopodia are thin, dynamic plasma membrane protrusions that are filled with actin filament bundles. They are considered to function as sensory organs at the leading edge of motile cells by sensing, e.g., growth factors, chemokines, and the extracellular matrix (Mattila and Lappalainen 2008). Filopodia are also often oriented toward the gradient of chemoattractants in migrating cells (Bentley and Toroian-Raymond 1986). In addition to motility, filopodia are involved in cell-to-cell and cell-to-matrix adhesion and maturation of neurons (Vasioukhin et al. 2000; Gallo and Letourneau 2004; Galbraith et al. 2007). Furthermore, filopodia have an essential role in the activation of T lymphocytes in antigen-presenting cells, and they function as phagocytic tentacles in macrophages (Al-Alwan et al. 2001; Kress et al. 2007).

The diameter of filopodia is typically approximately 0.1–0.3 μm and their length can be over 10 μm as measured from the cell cortex. However, during sea urchin

embryo gastrulation, filopodia can extend even up to 80 μm from the cell cortex (Miller et al. 1995). In contrast, filopodial-like protrusions called microspikes are very short and often hardly visible by regular light microscopy approaches (Taylor and Robbins 1963). Thus, a huge variation exists between the morphological and dynamic parameters of different types of filopodial protrusions, and it is likely that membrane protrusions generally classified as "filopodia" consist of several functionally different groups of thin plasma membrane protrusions with distinct assembly pathways and molecular components (Mattila and Lappalainen 2008). Here, we focus on filopodia that are present at the leading edge of migrating vertebrate cells.

In filopodia of the leading edge, individual actin filaments extend from the cell cortex to the tip of filopodium, and they form unipolar bundles with rapidly growing barbed ends facing toward the tip. The core of a filopodium typically consists of 15–30 individual filaments, which are tightly packed and arranged in parallel to each other (Small and Celis 1978; Medalia et al. 2007). At least in mammalian cells, filopodial actin filaments can be generated either from the preexisting branched lamellipodial actin network (Svitkina et al. 2003) or through de novo nucleation of new actin filaments at specific foci at the plasma membrane (Small and Celis 1978; Medalia et al. 2007). The formation, morphology, and dynamics of filopodia are controlled by an array of actin-binding proteins. The activities of the actin-binding proteins are in turn controlled by various signaling proteins from which the small GTPase Cdc42 functions as the master regulator of filopodia formation in many vertebrate cells (Ridley 2015) (Fig. 2).

2.4 Actin Dynamics in Filopodia

Filopodia are not stable protrusive structures, but often undergo constant extension and retraction, especially in migrating cells and during neuronal growth cone pathfinding (Bentley and Toroian-Raymond 1986). Balance between extension and retraction is controlled by the rate of actin filament assembly at the tips of filaments (Mallavarapu and Mitchison 1999; Bornschlögl 2013) as well as by filament disassembly through ADF/cofilin-mediated severing at the base of filopodia (Breitsprecher et al. 2011b).

2.4.1 Nucleation and Elongation of Filaments

Based on electron microscopy and genetic studies (Svitkina et al. 2003; Schirenbeck et al. 2005; Medalia et al. 2007), two alternative models for filament nucleation in filopodia have been suggested. *The convergent elongation model* proposes that actin filaments in filopodia arise from the lamellipodial actin network, whereas *the tip nucleation model* suggests that short individual filaments in a "terminal cone" of filopodia act as a nucleation site for continuous filament bundles (Mattila and Lappalainen 2008; Yang and Svitkina 2011).

Depending on the model, either the Arp2/3 complex or formins are the best candidates for nucleating actin filaments for filopodia. From the tip nucleation point of view, formin mDia2 is the primary candidate to nucleate, elongate, and protect

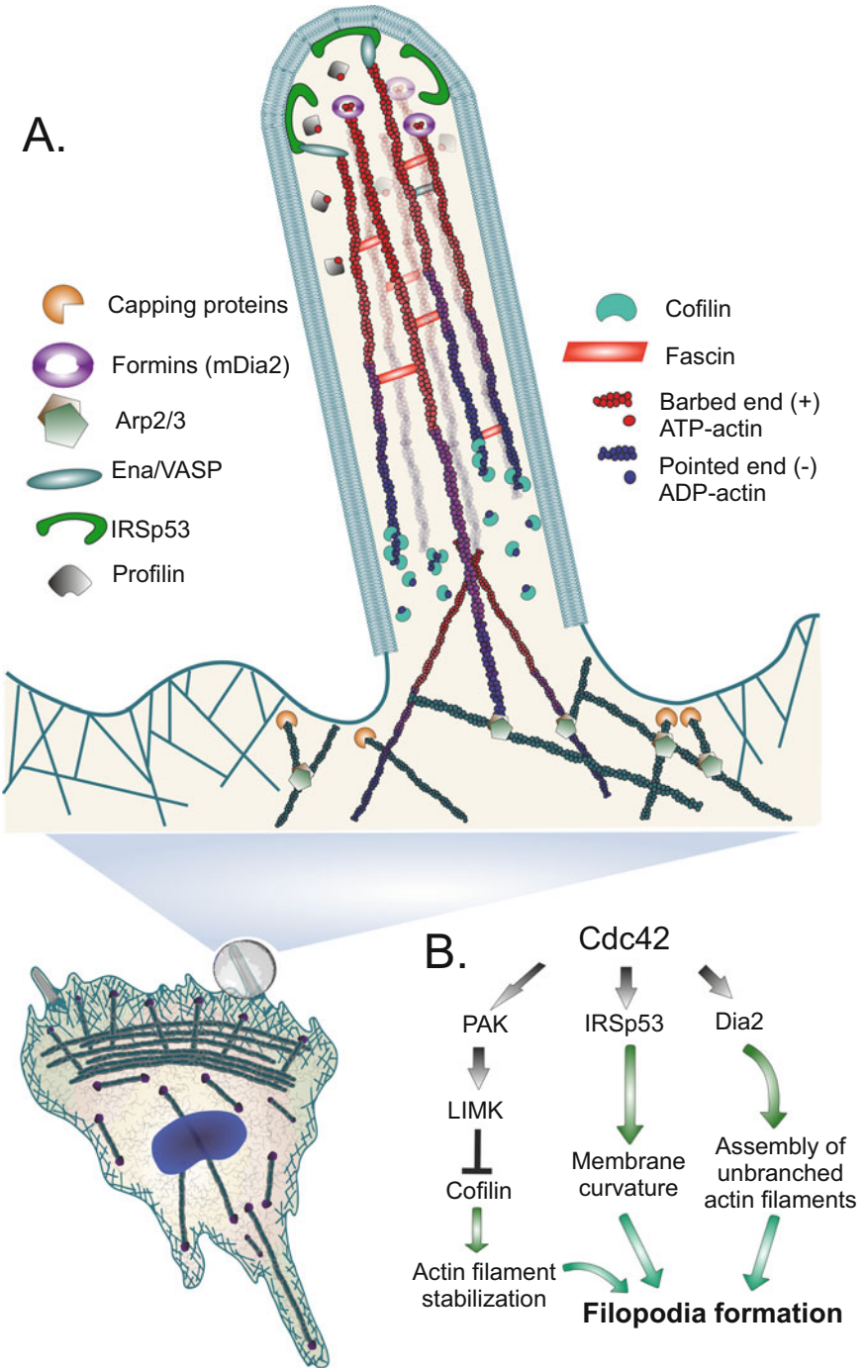

Fig. 2 Organization and architecture of filopodial actin filament arrays. (**a**) Filopodia contain a bundle of unipolar actin filaments with their rapidly growing barbed ends facing the tip of the filopodium. Fascin is the main actin filament cross-linking/bundling protein in filopodia. Actin

filament assembly in filopodia (Peng et al. 2003; Schirenbeck et al. 2005; Yang et al. 2007). Also other formins such as DAAM1 have been linked to filopodia formation (Jaiswal et al. 2013). However, defects in Arp2/3 expression, function, or localization lead to significant problems in filopodial formation in cultured neurons, HeLa cells, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Machesky and Insall 1998; Korobova and Svitkina 2008; Norris et al. 2009), supporting the convergent elongation model. Since cells contain different types of filopodia with distinct molecular compositions and dynamics, it is likely that both the convergent elongation and the tip nucleation model are relevant and that their relative contributions for filament nucleation vary depending on the cell and the filopodium type.

How then could linear, parallel actin filaments of filopodia be nucleated by the Arp2/3 complex? This is possible if filament barbed ends are uncapped and protected from capping by formins or Ena/VASP proteins (Svitkina et al. 2003; Yang et al. 2007). Interestingly, heterodimeric capping protein and Ena/VASP operate on the lamellipodial and filopodial actin networks in concert. Capping protein silencing leads to loss of lamellipodia and increased formation of filopodia, but when capping protein is depleted simultaneously with Ena/VASP, ruffles are formed at the leading edge (Mejillano et al. 2004). Capping protein is present also in filopodia in low levels but its role in the regulation of the filopodial actin network remains to be elucidated (Sinnar et al. 2014).

2.4.2 Filament Cross-Linking Proteins

In filopodia, actin filaments form tight, unipolar bundles. Bundling is necessary to overcome the resistance of the plasma membrane because individual filaments are too flexible to push the membrane forward. It has been mathematically estimated that a bundle of over 10 filaments has sufficient rigidity to overcome the membrane resistance and that the optimal number of filaments in a bundle is around 30 (Mogilner and Rubinstein 2005). Fascin is an actin-bundling protein essential for generation of actin filament bundles in filopodia. It decorates the entire length of the filopodium, and its depletion reduces the number of filopodia in cells. Interaction of fascin with actin filaments is dynamic, which allows efficient coordination of filament elongation and bundling during filopodia protrusion (DeRosier and Edds

Fig. 2 (continued) filaments in filopodia can be either nucleated de novo by formins or be generated from the preexisting lamellipodial Arp2/3-nucleated network through Ena/VASP-mediated filament elongation and protection of filament barbed ends from capping protein. Ena/VASP also interacts with the I-BAR domain protein IRSp53, which senses or generates the negative membrane curvature at the tip of the filopodium. Cofilin-promoted filament severing is inhibited toward the tip of the filopodium to stabilize the actin filament bundle. (**b**) The small GTPase Cdc42 is the master regulator of filopodial actin filament dynamics in many cell types. Cdc42 activates Dia2 formin to promote actin filament assembly. It inactivates cofilin through LIMK and PAK, thus inhibiting actin filament disassembly, and interacts with IRSp53, which generates membrane curvature and functions as a scaffolding protein for other regulators of actin dynamics

1980; Vignjevic et al. 2006). In addition to fascin, also fimbrin, α-actinin, and filamin are able to support actin-bundling-dependent motility (Brieher et al. 2004). Interestingly, also in the amoeboid cells of *Dictyostelium discoideum*, an Ena/VASP protein seems to be involved in the filament bundling in filopodia (Schirenbeck et al. 2006).

2.4.3 Actin–Plasma Membrane Interactions and Membrane Curvature

Members of the Bin–amphiphysin–Rvs (BAR) protein family are important regulators of membrane curvature and can additionally link the actin cytoskeleton to the plasma membrane. N-BAR and most F-BAR domain proteins generate a positive membrane curvature and induce plasma membrane invaginations, whereas the I-BAR domain proteins generate a negative membrane curvature and can thus induce the formation of plasma membrane protrusions (Peter et al. 2004; Suetsugu et al. 2006; Mattila et al. 2007; Shimada et al. 2007). Interestingly, the diameter of membrane tubules induced by I-BAR domains is similar to the diameter of filopodia in cells, and overexpression of I-BAR domain proteins or their isolated I-BAR domains can induce filopodia-like protrusions at the plasma membrane (Saarikangas et al. 2009).

From the I-BAR domain proteins, the insulin receptor substrate protein of 53 kDa (IRSp53) has been intimately linked to filopodia formation. In addition to sensing or generating negative membrane curvature, IRSp53 functions as a scaffolding protein for several actin-regulating proteins, such as Rho GTPases, Arp2/3 complex activator WAVE2, Ena/VASP, and actin filament-bundling/capping protein Eps8. Thus, IRSp53 can either sense or generate membrane curvature at the tip of the filopodium through its membrane-binding I-BAR domain and additionally promote actin filament assembly at this region by interacting with actin-binding proteins through its other domains (Krugmann et al. 2001; Disanza et al. 2006; Lim et al. 2008; Prévost et al. 2015).

2.5 Organization and Functions of Stress Fibers

Stress fibers were observed over 90 years ago under plain bright-field microscope and were initially thought to be fibers that reversibly build up in response to cytoplasmic tension or stress (Lewis and Lewis 1924). However, it is now well established that stress fibers do not form due to internal stress, but rather reflect and respond to the physical rigidity of the extracellular matrix (Riveline et al. 2001; Costa et al. 2002; Discher et al. 2005; Tojkander et al. 2015). In cell types that experience strong external shearing forces, such as the endothelial cells of vasculature, stress fibers are distinctively prominent (Franke et al. 1984). Moreover, stationary cells display thicker and more stable actomyosin bundles, i.e., stress fibers, compared to highly motile cells to better resist and respond to the external mechanical forces or to remodel the surrounding tissue (Hinz et al. 2001; Pellegrin and Mellor 2007). The role of stress fibers in cell migration varies depending on the cell type and the extracellular environment of the cell. In two-dimensional

environment, stress fibers are believed to be involved in regulating cell polarity and promoting retraction of the tail of migrating cell, whereas in cells migrating in a three-dimensional extracellular matrix (ECM), stress fiberlike actomyosin bundles may be more directly involved in adhesion to the matrix and advancement of the leading edge (Tojkander et al. 2012).

The core of contractile stress fibers is built up from short actin filaments that are organized in a bipolar manner. This resembles the sarcomeric units in muscles and enables the movement of bipolar myosin II filaments along the actin bundles to create contractility. However, whereas muscle myofibrils are entirely composed of bipolar arrays of actin filaments, stress fibers display often a mixed polarity and are composed of both unipolar and bipolar actin filament arrays (Sanger et al. 1983; Cramer et al. 1997; Svitkina et al. 1997).

Stress fibers are often coupled to the extracellular matrix via focal adhesions. These are large, multi-protein structures that enable cells to communicate with the extracellular matrix as they migrate (Geiger et al. 2009). Diverse members of the large family of integrins and other focal adhesion components anchor the extracellular matrix to the cytoplasmic stress fibers (Geiger and Yamada 2011). The assembly and dynamics of stress fibers are controlled both at focal adhesions and along the stress fibers by a large array of actin- and myosin-binding proteins. The activities of these proteins are in turn controlled by mechanical forces and various signaling proteins, from which the RhoA family of small GTPase is often considered as the master regulator of stress fiber assembly (Ridley and Hall 1992) (Fig. 3).

2.5.1 Different Stress Fiber Subtypes

Stress fibers can be generally divided into three categories: dorsal stress fibers, transverse arcs, and ventral stress fibers (Heath 1983; Small et al. 1998). *Dorsal stress fibers* are noncontractile actin bundles that associate with a focal adhesion at their distal end and extend toward the cell center through actin polymerization at focal adhesions. Dorsal stress fibers display uniform polarity near the focal adhesion anchorage site, but appear to contain mixed polarity actin filaments toward the proximal part of the fiber (Cramer et al. 1997). Although dorsal stress fibers do not contain myosin II and are unable to contract, they can connect the other stress fiber types to focal adhesions. Dorsal stress fibers appear also important for cell migration at least in a two-dimensional tissue culture environment (Hotulainen and Lappalainen 2006; Tojkander et al. 2011; Kovac et al. 2013).

Transverse arcs are contractile, myosin II-containing actin bundles that are generated from the lamellipodial actin filament network. Transverse arcs are not directly associated with focal adhesions, but are linked to these contact sites through dorsal stress fibers. As the transverse arcs contract, they flow toward the cell center with a characteristic speed of ~0.3 μm/min (Hotulainen and Lappalainen 2006; Tee et al. 2015). Although transverse arcs have not been directly linked to cell migration, they are important for the formation of the flat lamellum at the leading edge of motile cells due to their association with the plasma membrane at the dorsal side of the cell (Burnette et al. 2014; Jiu et al. 2015).

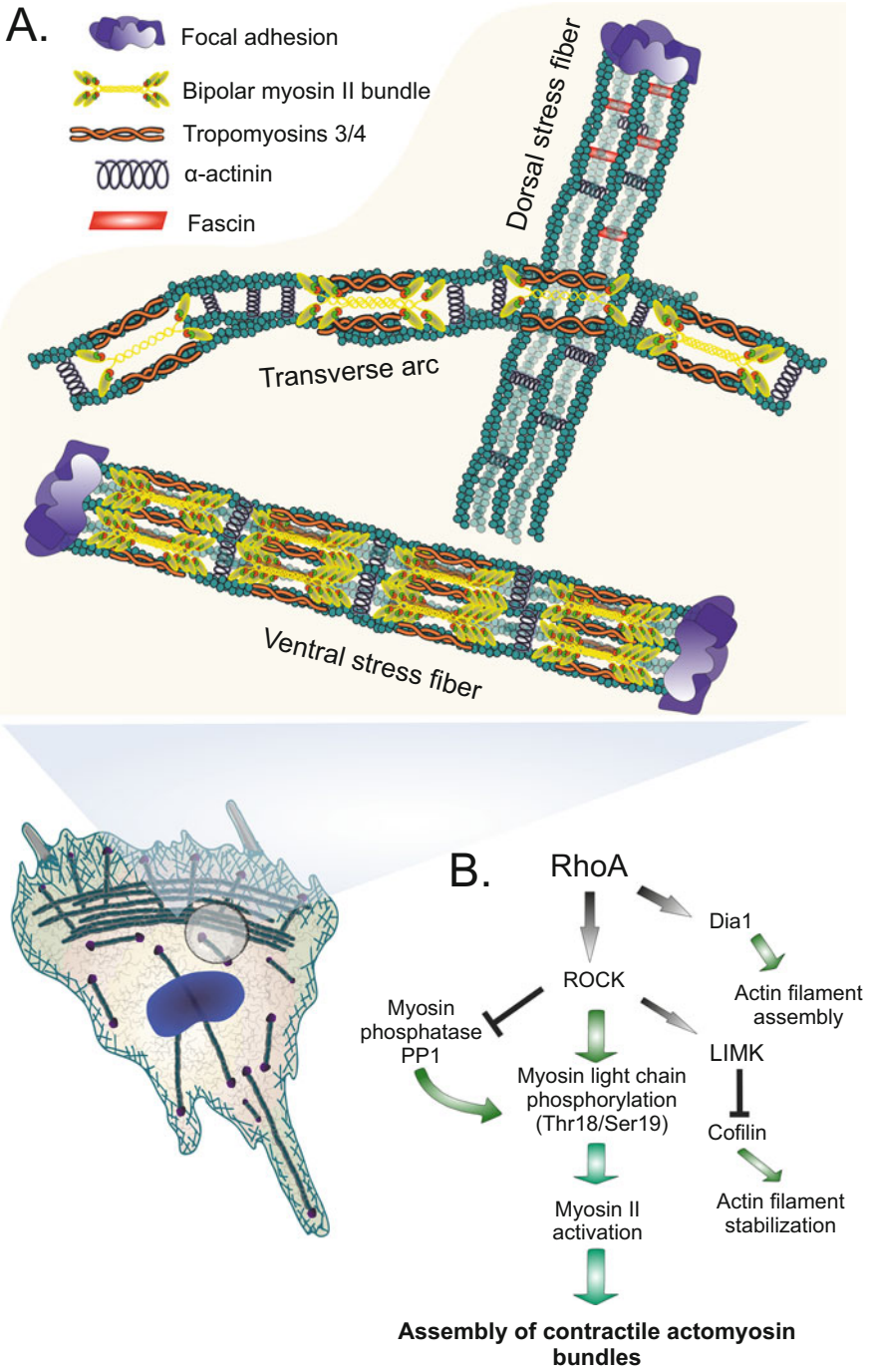

Fig. 3 Assembly and organization of actin stress fibers. (**a**) Stress fibers are thick bundles of actin filaments, which are decorated by tropomyosins and cross-linked mainly by α-actinin. Stress fibers can be further divided into three different categories. *Dorsal stress fibers* are noncontractile actin

Dorsal stress fibers and transverse arcs serve as precursors of *ventral stress fibers*, which are usually oriented perpendicular to the direction of migration and are connected to focal adhesions with both their ends (Small et al. 1998; Tojkander et al. 2012). Ventral stress fibers contain a sarcomeric array of bipolar myosin II and actin filament bundles and are thus able to contract and apply tensile force to focal adhesions located at the ends of the bundle. Therefore, ventral stress fibers are important for cell adhesion, morphogenesis, and mechanosensing (Tojkander et al. 2015). Ventral stress fibers are also responsible for retraction of the tail in many migrating cell types (Kolega 2003).

2.6 Mechanisms of Stress Fiber Assembly

Compared to the lamellipodial and filopodial actin filament arrays, assembly of contractile stress fibers is a complex process and involves many actin nucleation mechanisms and several different actin filament populations. The assembly of stress fibers is regulated mainly by Rho family of GTPases, whose activation promotes stress fiber formation/stabilization through a number of actin-binding proteins. RhoA also enhances stress fiber contractility through activation of myosin II by myosin light chain (MLC) and ROCK (Heasman and Ridley 2008).

In stress fiber assembly, lamellipodial actin filaments serve as building blocks for the generation of transverse arcs (Hotulainen and Lappalainen 2006). During this process, Arp2/3 complex-nucleated lamellipodial actin filaments assemble with formin-nucleated, myosin II-decorated actin filaments at the proximal end of the lamellipodium to generate transverse arcs (Hotulainen and Lappalainen 2006; Tojkander et al. 2011). Lamellipodia undergo cycles of protrusion and retraction in migrating cells. The appearance of transverse arc precursors coincides with the retraction phase, when actin filaments condense from the more criss-crossed meshwork into bundled filament precursors (Burnette et al. 2011). Additionally, material from filopodial filaments can be recycled for generating stress fibers (Nemethova et al. 2008; Anderson et al. 2008). Dorsal stress fibers, on the other hand, are nucleated and elongated by different formins (including at least Dia1 and INF2) and Ena/VASP family proteins at focal adhesions (Hotulainen and Lappalainen 2006;

Fig. 3 (continued) filament bundles that associate with, and elongate through, actin polymerization at focal adhesions located at their distal ends. *Transverse arcs* are myosin II-containing contractile actin bundles that associate with dorsal stress fibers, but are not directly linked to focal adhesions. Transverse arcs are generated from the lamellipodial actin filament arrays. *Ventral stress fibers* are contractile, myosin II-containing actin filament bundles that can be generated from the preexisting network of dorsal stress fibers and transverse arcs. Ventral stress fibers communicate with the extracellular matrix through focal adhesions located at both their ends. (**b**) The RhoA family of small GTPase is the master regulator of stress fiber assembly and contractility in many animal cell types. RhoA promotes actin filament assembly through Dia1 formin and inhibits actin filament disassembly through ROCK- and LIMK-mediated inactivation of cofilin. Additionally, RhoA promotes myosin II activation through ROCK

Gateva et al. 2014; Skau et al. 2015; Tojkander et al. 2015). As the dorsal stress fibers elongate, they associate with transverse arcs through a mechanism that is currently unknown. Thus, dorsal fibers and transverse arcs create an entwined network in the lamellum, where individual arcs can be connected to several different dorsal stress fibers (Tojkander et al. 2011). Ventral stress fibers can be subsequently generated from the network of dorsal stress fibers and transverse arcs through a complex mechanosensitive process if the cell is on a rigid matrix or if external forces are applied to the cell (Tojkander et al. 2015). Finally, it is important to note that stress fibers are dynamic structures that undergo continuous assembly and disassembly as well as organization of the network (Tojkander et al. 2012).

2.6.1 Actin Filament Cross-Linking Proteins in Stress Fibers

To date over 20 different protein classes have been reported to possess actin cross-linking activity. Most actin cross-linking proteins exist either as dimers or contain two actin-binding domains. α-Actinin and fascin are the most well-characterized, abundant cross-linking proteins of the contractile fibers. Homodimeric α-actinin, with the subunits arranged in antiparallel fashion, links adjacent actin fibers through a bivalent binding mechanism with its two actin-binding sites (Puius et al. 1998; Edlund et al. 2001; Türmer et al. 2015). Monomeric fascin links actin filaments in a much more compact manner, forming tightly packed parallel actin bundles that have been recently shown to be important in the termini of mature stress fibers, close to focal adhesions (Otto et al. 1979; Elkhatib et al. 2014). Cooperative cross-linking by α-actinin and fascin was shown to produce more elastic bundles than either of the two proteins could generate individually (Tseng et al. 2001). α-Actinin cross-links align periodically in transverse arcs and ventral fibers, interposing with the two other central components of the contractile fibers, myosin II and tropomyosin, which also display a periodical localization pattern along stress fibers. Thus, the two types of contractile stress fibers display a similar periodic α-actinin–myosin II pattern that is characteristic for muscle myofibrils (Tojkander et al. 2012).

2.6.2 Myosins and Tropomyosins

Tropomyosins and myosin II bundles are important components of stress fiber function and contractility. Class II myosins are the major contractile proteins of the cardiac and skeletal muscle tissues, but have specific isoforms also in the non-muscle cells that associate with stress fibers (Vicente-Manzanares et al. 2009). Mammalian non-muscle cells are able to utilize up to three different non-muscle myosin II (NMII) isoforms (myosin IIA, IIB, and IIC) each displaying distinct characteristics in terms of catalytic activity, localization, and role in various cellular functions (Katsuragawa et al. 1989; Kawamoto and Adelstein 1991; Golomb et al. 2004). Recent work demonstrated that these isoforms can also co-assemble with each other into bipolar bundles in different stress fiber subtypes (Beach et al. 2014; Shutova et al. 2014). In a polarized cell, NMIIA and NMIIB are both central components of stress fibers, but are however enriched in the peripheral lamella and posterior contractile units, respectively (Maupin et al. 1994; Sandquist and Means 2008). The differential distribution reflects differences in the three

isoforms regarding their rate of hydrolyzing ATP. The motor domain of NMIIB is able to hold the tension longer than the other isoforms, via its high affinity for ADP. Long upkeep of tension is well suited for more stable contractile fibers in the cortical and posterior part of the cell (Wang et al. 2000). In the more rapidly progressing cell front, NMIIA, with markedly higher ATP hydrolysis rate and lower affinity for ADP, is able to exert tension more rapidly, e.g., in response to extracellular signals (Wang et al. 2003).

Tropomyosins are a family of actin-binding proteins that function as coiled-coil dimers forming continuous polymers located in the grooves of filamentous actin. The canonical mechanism of function for tropomyosins is to regulate skeletal muscle contraction by steric blocking of the myosin binding to actin filaments prior to Ca^{2+} influx (Parry and Squire 1973). Tropomyosins also efficiently stabilize actin filaments and may functionally specify different actin filament populations (Hitchcock-DeGregori et al. 1988; Broschat et al. 1989; Gunning et al. 2015). Over 40 different tropomyosins can be generated by alternative splicing from four mammalian *tropomyosin* genes (Gunning et al. 2008). Interestingly, recent studies revealed that in migrating osteosarcoma cells, at least five functionally nonredundant tropomyosin isoforms localize to stress fibers and are important for their assembly and/or stability. Furthermore, some tropomyosin isoforms appear to be essential for myosin II recruitment to stress fibers (Tojkander et al. 2011). Thus, tropomyosins are central components of stress fibers, where they seem to specify functionally distinct actin filament populations and recruit myosin II molecules to stress fiber precursors.

3 Cell Migration in a Three-Dimensional Environment

In tissues of multicellular animals, cells migrate predominantly in a complex three-dimensional environment. Here the cells can crawl on or through extracellular matrix as well as migrate on top of each other. In some cases, such as during transcellular migration of leukocytes from blood vessels to the underlying tissues, cells can even move through other cells (Ridley 2011; Muller 2015). In tissue environment, migration of cells can also be guided by variable biochemical or mechanical signals. Chemical signals include growth factor gradients that can either attract or repel migrating cells during developmental processes and angiogenesis as well as chemokine-induced migration of lymphocytes. On the other hand, fibroblasts can read the mechanical properties of the environment and move toward the regions of highest substrate stiffness in a process called durotaxis (Majumdar et al. 2014; Haeger et al. 2015).

Compared to migration on a two-dimensional tissue culture plate, cells in a three-dimensional tissue environment display more variation in their migration modes. The most widely studied migration types are *mesenchymal* and *amoeboid* migration modes. In the mesenchymal, adhesion-dependent migration mode, the cells moving along ECM are typically elongated and employ similar lamellipodial and filopodial structures for movement as used in a two-dimensional environment.

In contrast, *amoeboid* migration does not require the formation of adhesions or lamellipodial-like protrusive structures, but instead employs the myosin-dependent contractile actin cortex to form membrane blebs toward the direction of migration. Importantly, the same cell can switch between different types of migration modes, depending on the mechanical and chemical properties of the environment (Petrie and Yamada 2012; Paluch and Raz 2013). Cell migration in a three-dimensional tissue environment also employs other specific actin-dependent processes, such as invadopodia and podosomes, which are involved in matrix degradation, thus permitting cells to move through the dense extracellular matrix (Linder and Wiesner 2015).

3.1 Podosomes and Invadopodia

Cell movement in two-dimensional matrices is limited to a planar level, whereas in a tissue environment, all three dimensions are available. In a three-dimensional environment, however, cells frequently need to degrade the extracellular matrix in order to crawl. Special structures termed podosomes and invadopodia can then be formed and applied to degrade the extracellular matrix during mesenchymal migration (Hoshino et al. 2013). Both structures initially appear as punctae on the plasma membrane and utilize the polymerization of actin to breach the substratum perpendicularly. Podosome architecture consists of an actin core where the Arp2/3-initiated branched actin network is surrounded by myosin II-containing unbranched actin filaments (Kaverina et al. 2003; Osiak et al. 2005; Bhuwania et al. 2012). A third structural component, surrounding the actin–myosin core, is composed of clusters of focal adhesion-associated proteins such as paxillin or vinculin (Cox et al. 2012; van den Dries et al. 2013). Also integrins co-localize with maturing podosomal structures and establish a linkage between the cell and the extracellular matrix (Pfaff and Jurdic 2001).

In contrast to the compact, column-like podosomes, invadopodia are extended membrane protrusions invading the extracellular matrix (Linder et al. 2011). While podosomes display rapid actin dynamics and a short life span with a typical turnover time of one minute, invadopodia are able to persist over an hour (Destaing et al. 2003; Li et al. 2010). The tip of an invadopodium inhabits parallely aligned actin filament bundles that precede the branched actin meshwork at the base. Arp2/3 and several filament-polymerizing formins are important for establishing nascent invadopodia that form mature structures after recruitment of matrix metalloproteases (Lizárraga et al. 2009; Wiesner et al. 2010). Interestingly, invadopodia do not extensively recruit integrins and it is still unknown whether they have adhesive contacts with the extracellular matrix (Mueller and Chen 1991; Deryugina et al. 2001).

3.2 Blebbing-Based Cell Migration

Typically, animal cells contain a contractile cell cortex under their plasma membrane. Although this structure is not considered to be a subtype of the stress fibers, it similarly constitutes from myosin II-decorated actin filaments with mixed or random polarity, associated with other actin-binding proteins (Bray and White 1988; Charras et al. 2006). Similarly to stress fibers, the formation of the actin cortex requires both formins (Dia1) and the Arp2/3 complex (Bovellan et al. 2014). The cortical actin filament network is linked to the plasma membrane at least through ezrin/radixin/moesin (ERM) family proteins (Charras et al. 2006).

The contractile actin cortex has an important role in amoeboid cell migration, where propulsive blebs drive the migration, and adhesion points with the extracellular matrix are not utilized (Wolf et al. 2003; Pinner and Sahai 2008). Especially cancer cells are often highly contractile, making them prone to blebbing (Bergert et al. 2012). Furthermore, at least in mouse tissues, melanoma and breast cancer cells migrate by blebbing, suggesting that this is an important migration mode of many cancer cell types in vivo (Tozluoğlu et al. 2013).

A bleb forms when the actin cortex detaches from the plasma membrane or when the cortex itself is disrupted due to cytoplasmic buildup of pressure and tension caused by actomyosin contraction. Due to the contraction of the remaining actomyosin cortex, cytosol flows into the newly formed bleb lacking the cortex. Actin filaments then begin to reform under the membrane of the bleb (Fig. 4). This is followed by a retraction phase, driven by myosin II-promoted contraction of the newly formed actomyosin cortex underlying the plasma membrane of the bleb (Cunningham 1995; Paluch et al. 2005; Charras et al. 2006; Charras and Paluch 2008). Cells confined in a nonadhesive environment often migrate in the direction of a very large "leader bleb," whose formation requires the presence of the actin filament-bundling protein Eps8 (Logue et al. 2015).

Three distinct models for the role of blebbing in cell migration have been presented. The first model proposes that blebbing plays a critical role in forward migration by transporting the cytoplasm and thereby moving the center point of mass of the cell forward. Actin is linked to the cell–cell contacts formed by E-cadherins, which anchor the migrating cell to the extracellular matrix (Kardash et al. 2010). "The chimneying" model, named after a rock climbing technique, proposes a migration mechanism in the total absence of adhesion with the environment. Either actin polymerization against the sides of a cell or a backward flow of the cell cortex could generate force that is strong enough to permit the cell to move forward. In either case, movement is supported by specific or nonspecific friction between the cell and the substrate (Lämmermann et al. 2008; Hawkins et al. 2009; Poincloux et al. 2011). Finally, "the swimming model" suggests that cells could use blebbing in a swimming fashion, whereby the cell body translocates after cell shape changes during blebbing (Lim et al. 2013).

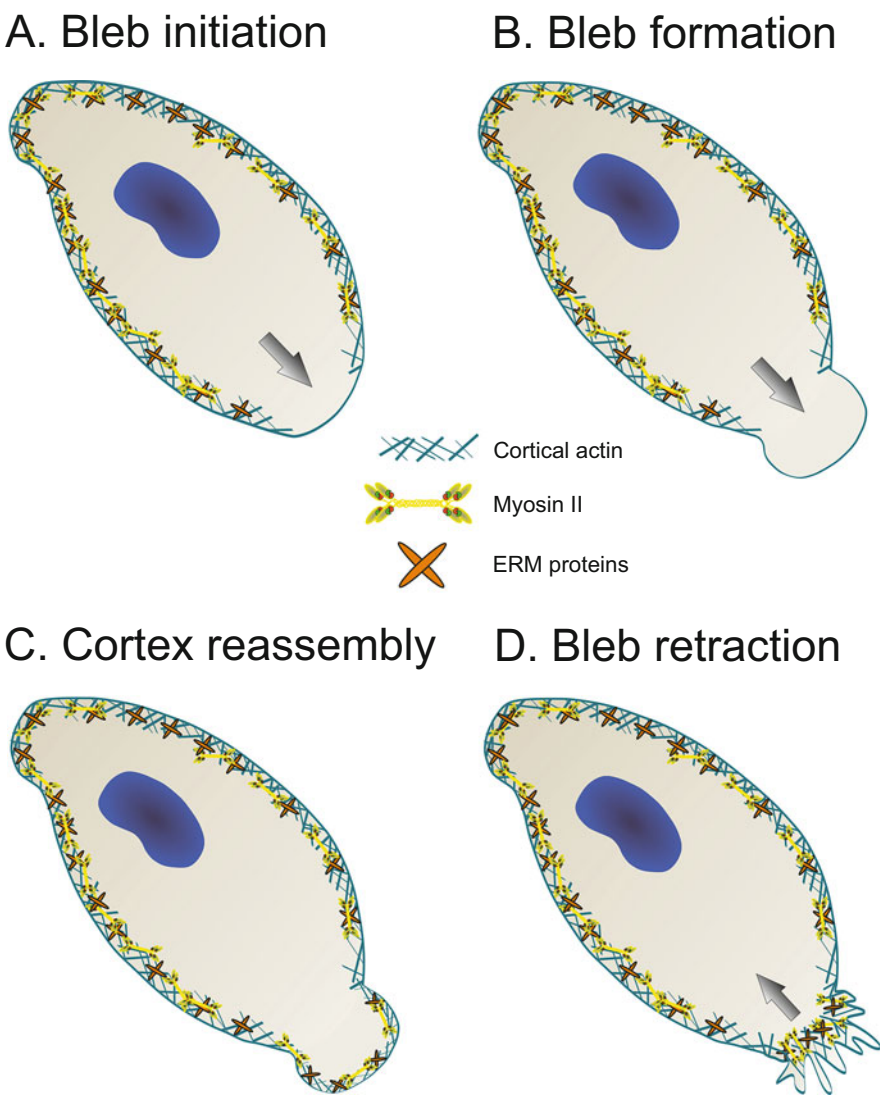

Fig. 4 The life cycle of a bleb. (**a**) Many cells contain an actomyosin cortex, which can be linked with the plasma membrane via ezrin/radixin/moesin (ERM) proteins. The initiation of a bleb can result from a local disassembly of the actomyosin cortex. (b) Hydrostatic pressure of the cytoplasm drives the expansion of the bleb. (**c**) A new actin cortex assembles inside the bleb via Arp2/3 and formin-mediated actin filament nucleation and subsequent recruitment of myosin II. (**d**) Assembly of the actomyosin cortex is followed by myosin II-driven bleb retraction (modified from Charras and Paluch 2008)

4 Future Perspectives

Cell migration requires several different actin-based structures and is finely tuned by a large amount of regulatory proteins. Although actin dynamics and its role in cell migration have been intensively studied over several decades, many unanswered questions concerning the mechanisms underlying cell migration still remain. For example, although the principles of actin filament assembly and disassembly in lamellipodia are beginning to be relatively well established, we still do not have a complete picture about how filopodia and contractile actomyosin bundles, such as stress fibers, are assembled in cells. Furthermore, cell migration is not only controlled by chemical cues, but the mechanical environment of the cell is also important in regulating the assembly and turnover of actin filament structures involved in cell migration. However, the principles by which different actin filament arrays respond to mechanical cues remain largely elusive. Finally, the organization, dynamics, and regulation of actin filament structures in cells migrating in a three-dimensional tissue environment are poorly understood. However, rapid evolution of light and electron microscopy methods, together with the revolution of CRISPR/Cas9-based genome editing approaches, have now made it more feasible to examine actin dynamics also in normal as well as in cancer cells migrating in their "native" three-dimensional tissue environment.

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Signalling Pathways Controlling Cellular Actin Organization

Anika Steffen, Theresia E.B. Stradal, and Klemens Rottner

Contents

Abstract

The actin cytoskeleton is essential for morphogenesis and virtually all types of cell shape changes. Reorganization is per definition driven by continuous disassembly and re-assembly of actin filaments, controlled by major, ubiquitously operating machines. These are specifically employed by the cell to tune its activities in accordance with respective environmental conditions or to satisfy specific needs.

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Here we sketch some fundamental signalling pathways established to contribute to the reorganization of specific actin structures at the plasma membrane. Rho-family GTPases are at the core of these pathways, and dissection of their precise contributions to actin reorganization in different cell types and tissues will thus continue to improve our understanding of these important signalling nodes. Furthermore, we will draw your attention to the emerging theme of actin reorganization on intracellular membranes, its functional relation to Rho-GTPase signalling, and its relevance for the exciting phenomenon autophagy.

Keywords
Actin turnover • Autophagy • Leading edge • Migration • Protrusion • Rho-GTPase • Trafficking

1 Introduction

The actin cytoskeleton is outstandingly dynamic and capable of mediating a huge range of specific functions in distinct tissues and cell types. Even structures that have long been thought to be static and stable, such as skeletal muscle, were more recently found to display dynamic filament turnover, mediated by various distinct mechanisms (Gurniak et al. 2014; Littlefield et al. 2001; Skwarek-Maruszewska et al. 2009).

Traditionally, however, actin reorganization phenomena are most evident and studied in highly motile cells, in processes involving cell migration or phagocytosis of foreign particles or during actin-dependent invasion and spreading of bacterial and viral pathogens (Pollard and Borisy 2003; Rottner et al. 2005; Welch and Way 2013). The latter have also emerged as valuable tools for establishing the relevance of specific actin regulators for actin reorganization (Abella et al. 2016; Benanti et al. 2015; Egile et al. 1999; Niebuhr et al. 1997; Welch and Way 2013).

In principle, actin reorganization in motile cells concerns structures that are composed of bundles of actin filaments or actin networks or both. When studying signalling to actin reorganization in distinct cell types or tissues, specific actin reorganizations or "output" responses are usually triggered by extracellular ligands such as growth factors or extracellular matrix components. Well-studied structures that mostly harbour networks of actin filaments are the lamellipodium (Small et al. 2002) or sites of endocytosis and actin assemblies at intracellular membranes, whereas the most prominent structures built by actin filament bundles include the finger-shaped filopodia (Aspenstrom 2014; Mattila and Lappalainen 2008), bundles embedded into lamellipodia networks called microspikes (Adams 2004), microvilli and stereocilia (Burianek and Soderling 2013; Feng et al. 2014), and of course actin stress fibres anchored in focal adhesions (Vallenius 2013).

Although reasonably well understood, the complexity of contributions of various cellular factors to initiation and turnover of a specific structure, such as the

lamellipodium, is still overwhelming (Krause and Gautreau 2014; Russo et al. 2016). More specifically, we can now pinpoint essential components at least of some specific, well-studied actin structures in cells, but we are far from drafting complete signalling pathways starting from signal ignition, e.g., upon receptor occupation at the plasma membrane through to the mechanistic formation of the actin structure of interest.

Nevertheless, we will try here to briefly sketch signalling pathways generally agreed upon to drive the formation of selected, well-studied actin assemblies in mammalian cells such as the lamellipodium of migrating cells. In addition, we will discuss progress in less well-advanced fields, such as signalling and actin assembly on different types of endomembranes, or last but not least, the regulation of actin accumulation during the increasingly recognized process of autophagy.

2 The "Make and Break" of Filamentous Actin

All types of cell migration, adhesion to extracellular substrata and to other cells, engulfment of particles, secretion of signalling molecules as well as endo- and exocytosis depend on dynamic rearrangements of the actin cytoskeleton. Single filaments that build the actin cytoskeleton are constantly turned over by catalysed cycles of polymerization and depolymerization with strongly varying dynamics. Actin filaments are born by nucleation, a process which is tightly regulated and catalysed by molecular machines such as the Arp2/3 complex, members of the formin family of proteins and other less well-studied factors (Fig. 1). These generators of F-actin are in turn regulated by additional factors, foremost Rho-GTPases (see below). In case of the Arp2/3 complex, the so-called nucleation promoting factors (NPFs) are essential intermediates for the integration of incoming signals resulting in localized generation of branched actin structures. The WASP superfamily of NPFs recently gained 2 new subfamilies with 3 new members, now comprising 4 subfamilies with 8(+) members (Fig. 2). Activation of the Arp2/3 complex by any of these members leads to the formation of branched actin networks that are believed to be crucial for the proper shaping of membranes, including the plasma membrane during shape changes of the cell such as phagocytosis or protrusion (see below). Our current knowledge about different Arp2/3 complex-mediated actin structures will be the focus of the text below.

The formin family consists of 15 genes in mammals that have been implicated in the generation of long, unbranched filaments. There are multiple cellular functions that were reported to involve formin-mediated processive actin polymerization (recently reviewed in Kuhn and Geyer 2014). Their differential contributions to and their intricate interplay with Arp2/3-mediated structures are far from being completely understood. However, certain mammalian formins have so far been found prominently associated with the formation of finger-shaped cell protrusions termed filopodia or of actin-rich stress fibres (reviewed in Faix et al. 2009; Kuhn and Geyer 2014).

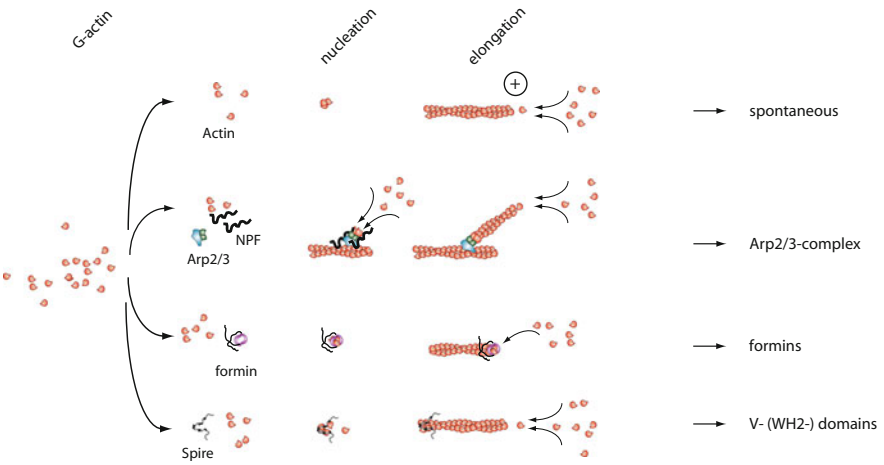

Fig. 1 Molecular mechanisms of actin polymerization. Globular actin monomers (G-actin) can spontaneously polymerize into non-covalent filaments to form filamentous actin (F-actin). The initial step of this process is termed nucleation, generating polar mini-filaments with growing plus ends capable of rapid elongation. In vivo, nucleation is strictly controlled by diverse molecular machines: Upon activation by NPFs, *Arp2/3-complex* binds to a mother filament and mimics a plus end that can effectively elongate, leading to branching of a new daughter filament off the mother filament. Arp2/3 complex remains attached to the minus end of the daughter filament until de-branching takes place (not shown). In contrast, *formins* can nucleate and elongate actin filaments while staying attached to their growing plus ends, operating therefore as so called processive cappers. Processive elongation requires profilin-associated actin monomers (not shown), whereas in the absence of profilin, formins act as barbed end capping proteins, inhibiting barbed end elongation and/or protecting them from depolymerization. Finally, a growing group of proteins can nucleate actin filaments by bringing monomers together, using, e.g., an array of G-actin-binding, *WH2-domains*. The first protein shown to act in this fashion was Spire, but the family now includes additional prominent and physiologically relevant members, including Cobl and Leiomodin (Campellone and Welch 2010; Chesarone et al. 2010; Chesarone and Goode 2009; Goley and Welch 2006; Le Clainche and Carlier 2008)

Finally, filament generation by several, consecutive copies of WH2 (WASP homology 2) domains, prevalent actin-binding modules, is a common feature of a class of relatively novel actin nucleators, the founding member of which is termed Spire (Quinlan et al. 2005). Spire proteins localize at distinct cytoplasmic spots and can induce F-actin at intracellular membranes. Spire can also co-operate with formins and profilin (Kerkhoff 2011; Montaville et al. 2014; Quinlan 2013). Yet another prominent member of this family constitutes the vertebrate protein Cobl (Cordon-bleu), which has originally been implicated in actin dynamics and cell morphogenesis in the nervous system (Ahuja et al. 2007). However, recent evidence suggests a key function for Cobl in actin nucleation in the microvilli at the base of intestinal epithelial cells (Grega-Larson et al. 2015).

On top of this complexity, actin polymerization alone is not sufficient to promote the formation of defined structures: the so-called turn-over of filaments is tightly tuned by additional factors. Examples include the ADF/cofilin family of

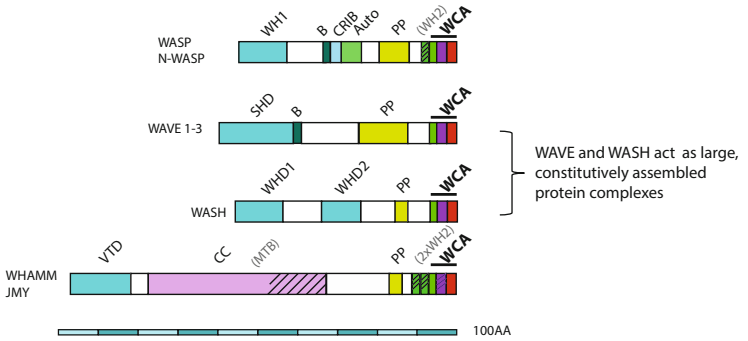

Fig. 2 Class I nucleation promoting factors (NPFs). The WASP superfamily of proteins embodies the canonical activators of the actin branching activity of Arp2/3 complex and comprises eight members in mammals subdivided into four subfamilies. They all have in common the conserved C-terminus mediating Arp2/3-complex activation, comprising the Arp2/3-interacting connector region (C) and acidic motif (A) plus the G-actin-binding WH2-motifs (W), which are preceded by a proline-rich region (PP). All NPFs comprise at least one WH2 domain, while N-WASP and WHAMM harbour two and JMY even three. The entire N-terminal parts of these proteins are dedicated to localize the actin assembly complex, to bind accessory factors and/or to integrate activation signals. Respective family specific domains are depicted in turquoise: WASP homology 1 (*WH1*), scar-homology domain (*SHD*), WASH homology domain1 (*WHD1*), Wash homology domain 2 (*WHD2*), Vesicle targeting domain (*VTD*). Other domains and motifs are: basic stretch for phosphoinositide binding (B), CRIB (Cdc42 and Rac interactive) motif for GTPase binding, auto inhibitory domain (Auto) and coiled-coil domains (CC) with microtubule binding feature in case of WHAMM (Campellone and Welch 2010; Pollitt and Insall 2009; Rottner et al. 2010; Seaman et al. 2013; Stradal and Scita 2006)

depolymerizing factors and capping protein, both of which modulate different actin filament ends. Both are essential constituents of actin-based motility by affecting shrinkage and growth by different mechanisms, respectively (Blanchoin et al. 2014). Interestingly, capping protein was found to directly associate with certain actin polymerizing machines (Derivery et al. 2009; Harbour et al. 2010). Finally, filamentous networks are decorated, stabilized and compartmentalized by a huge number of F-actin-binding and bundling proteins, which determine function, fate and longevity of the given structure. These mechanisms add further levels of regulation and complexity mentioned in specific cases below.

3 Classical Protrusion Types and How They Compare

A prominent and obvious consequence of actin remodelling events constitutes the protrusion of networks or bundles of actin filaments or of structures that essentially contain both to various relative extents at the plasma membrane (Fig. 3). All of these structures per definition formed at the cell periphery are built by the polymerization of actin filaments, which are associated with a myriad of factors regulating their turnover (Blanchoin et al. 2014). However, they cannot be viewed as isolated

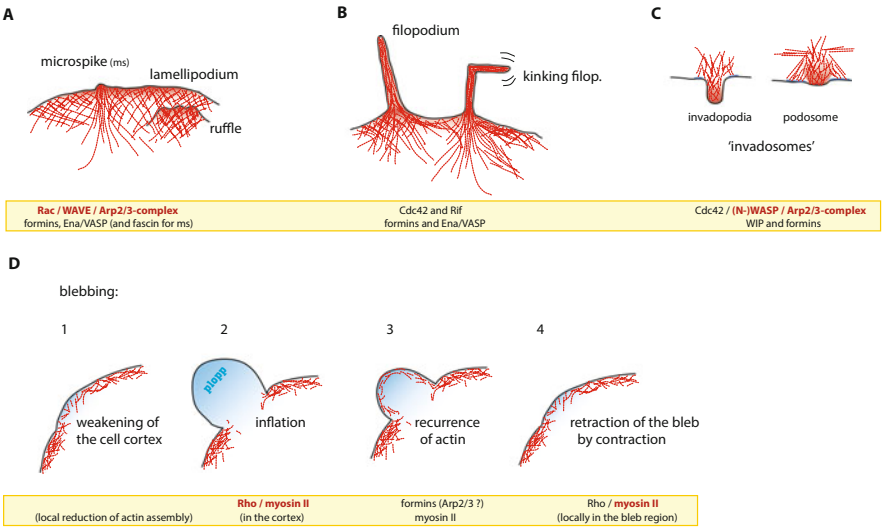

Fig. 3 Signalling proteins associated with actin-based membrane protrusions. Lamellipodia (**A**) are wide, thin sheets of plasma membrane filled with a dense meshwork of actin filaments that abut the membrane with their fast growing plus ends. *Lamellipodia* protrude as the plus ends grow and push the membrane forward. Generation of filaments for lamellipodium formation not only depends on the Rac/WAVE-/Arp2/3-complex signalling axis, but also involves the activities of formins such as FMNL2 or of Ena/VASP proteins. Lamellipodia can lift up from, fold backwards and/or travel over the cell body and are then termed *ruffles*. F-actin bundling proteins such as fascin stabilize structures called *microspikes*, which are embedded into the lamellipodial meshwork. *Filopodia* (**B**) are made of parallel bundles of actin filaments. Microspikes can serve as precursors of filopodia, which, however, can also form and protrude independently of Arp2/3-complex and thus lamellipodia. Filopodia can also kink and fold back into the cell body, although the mechanisms underlying this behaviour are still poorly defined. *Invadosomes* (**C**) include canonical *podosomes*, which occur in cells of the immune system as well as endothelial and smooth muscle cells, and *invadopodia*, which are a hallmark of invasive cancer cells. Invadosomes involve both, (N-)WASP-dependent Arp2/3 complex activation and integrin-mediated cell adhesion and contractility. *Blebbing* (**D**) stands out among actin-regulated plasma membrane protrusions driving cell migration, because the protrusion of a bleb (*1* and *2*) occurs passively, through hydrostatic pressure built up by global, myosin II-based contractility, whereas its retraction depends on local actin filament polymerization (*3*) followed by contraction ([*4*]; for comprehensive reviews on different types of membrane protrusions, see Charras and Paluch 2008; Faix and Rottner 2006; Gimona et al. 2008; Lammermann and Sixt 2009; Small et al. 2002). Note that signalling and/or actin regulatory proteins central to the processes depicted in A-D are highlighted below respective illustrations. It should be kept in mind that we consider the Rac/WAVE/Arp2/3-complex signalling pathway in case of lamellipodia formation, (N-)WASP/Arp2/3-complex in case of invadosomes and myosin II in case of blebbing highly likely to be absolutely required for respective process (marked *red*), while remaining factors may solely contribute to a certain extent or remain formally proven to be essential

entities, because they are anchored in proximal parts of the underlying cytoskeleton, and they are filled with cytosol undergoing nearly unlimited diffusion and thus exchange with the cytosol in the main cell body.

One of the best-studied networks of actin filaments belonging to a classical type of protrusion is the lamellipodium (Fig. 3a). This term is common to a flat sheet of cytoplasm, the protrusion of which is studied best with cells spreading out on flat and rigid surfaces, which enhances protrusion of these networks (Small et al. 2002). Lamellipodia are frequently connected to the capability of cells not only to change shape and migrate, but also to engulf material if formed in not substratum-attached areas of the cell, e.g., during phagocytosis or macropinocytosis (see below). Some motile cells, in particular fibroblasts, epithelial or endothelial cells, migrate by using cycles of protrusion and retraction within a relatively narrow region of the cell front, commonly termed the leading edge of the cell (Ridley 2011). This activity enables motile cells to explore new space, but at the same time to scan the substratum for appropriate specific sites of adhesion, the formation of which allows to pull themselves forward. A related activity frequently observed during the forward advancement of migrating cells is the up- and backwards folding of these lamellipodia, which are then termed membrane ruffles, and the process is called membrane ruffling (Small et al. 1999). There is currently little reason to believe that lamellipodia and ruffles at the cell periphery display any differences in molecular organization, at least concerning the actin turnover machinery that builds and maintains them. However, it is entirely elusive at present what triggers the lift of the flat sheet of cytoplasm (of 100–200 nm thickness) that usually occurs at its base, and whether the folding backwards requires filament severing and force generating activities, for instance, through interaction of actin filaments or bundles with myosin motors. Of note, individual cells can form multiple lamellipodia and ruffles simultaneously, if they are expressed in different subcellular locations, and they can vary significantly in size. In migrating fibroblasts or murine B16-F1 melanoma cells, a common lab model for migrating, metastasizing tumour cells, lamellipodia are usually 1–3 μm deep, but their lateral size along the cell periphery usually is multiple of this figure. As an exception, epidermal fish keratocytes migrate at extreme rates using one single, fan-shaped lamellipodium at their front, which is followed by a rolling cell body and nucleus (Anderson et al. 1996).

Aside from driving migration in isolated cells or cell sheets, lamellipodia and ruffles have also been implicated, e.g., in the initiation of adherens junctions of epithelial or endothelial cells (Collins and Nelson 2015), where they have recently been termed junction-associated intermittent lamellipodia (JAIL) (Schnittler et al. 2014). Lamellipodia and ruffles can contain variable numbers of actin filament bundles, which are known as microspikes, and the polymerization of which is coordinated with the surrounding filaments of the lamellipodium network (Small et al. 2002). The physiological function of microspike bundles is not entirely clear, but it is tempting to speculate that their relative abundance in lamellipodia and ruffles might correlate with the mechanical stability of these structures.

Aside from microspikes, the second major type of bundled protrusion is the filopodium (Fig. 3b) (Faix and Rottner 2006; Mattila and Lappalainen 2008). As opposed to microspikes and in contrast to some models of their formation, filopodia can protrude independently from the presence of lamellipodia (Steffen et al. 2006, 2013; Young et al. 2015). Although they are frequently formed together with

lamellipodia and can accompany migration, their excess formation was observed to reduce migration and lamellipodia formation rather than increasing them (Block et al. 2008; Schirenbeck et al. 2005). Although they are commonly characterized as parallel, stiff bundles that can harbour dozens of actin filaments, they can frequently kink and move along the cell periphery or dorsal cell surface, reminiscent of the folding and ruffling behaviour of lamellipodial actin networks (Fig. 3).

It is quite commonly assumed in the field that microvilli such as those found at the apical surface of epithelial cells or the stereocilia on hair cells of the inner ear are structures that are highly related to filopodia, but this view might change with increasing our understanding of the molecular players forming all these distinct structures (Delacour et al. 2016; Faix and Rottner 2006; Grega-Larson et al. 2015).

In addition to classical protrusions like lamellipodia and bundled structures such as filopodia or microvilli-like structures, non-apoptotic blebbing (Fig. 3d) has increasingly received attention as a protrusion mechanism. It is employed by cells in addition or alternatively to the classical protrusions described above, in particular in 3D environments and in tumour cell migration (Charras and Paluch 2008). Bleb formation also depends on a functional actin cytoskeleton, but distinct from the aforementioned protrusions that are driven by continuous, site-directed actin polymerization, blebs initially are actin-free plasma membrane bulges inflated by hydrostatic pressure built by cell contraction (Charras et al. 2005). Actin filaments are then polymerized subsequently at the inner bleb membrane surface, eventually driving bleb retraction (Charras et al. 2006). More details are summarized in excellent, recent reviews (Charras 2008; Lammermann and Sixt 2009; Paluch and Raz 2013). It should be noted though that cell plasticity during migration is, for instance, reflected by their capability to rapidly switch between lamellipodium- and bleb-based modes of migration, which constitutes a robust mechanism of adjusting to rapid, environmental changes (Bergert et al. 2012).

Last but not least, there are specific types of protrusions or pseudopodia that exert specialized functions during adhesion and/or matrix degradation (Fig. 3c). The most prominent examples of those types of protrusions are the so-called podosomes in haematopoietic cells, such as macrophages or dendritic cells and the related invadopodia of tumour cells (Gimona et al. 2008; Hoshino et al. 2013; Linder and Wiesner 2015). Podosomes and invadopodia harbour prominent networks of actin filaments, which are currently viewed to be composed of Arp2/3-dependent structures at the central core and mechanosensitive lateral fibres and connecting cables surrounding and interconnecting them, respectively. The properties and actin regulatory proteins orchestrating these specialized cell-matrix contacts have been excellently reviewed recently (Linder and Wiesner 2015).

4 Rho-GTPases and Effectors Forming Actin-Based Protrusions

Since the seminal studies by Alan Hall and his colleagues Anne Ridley and Kate Nobes in the early and mid-1990s, which have been cited thousands of times (Nobes and Hall 1995; Ridley and Hall 1992; Ridley et al. 1992), Rho-GTPases emerged as the core signalling nodes regulating actin biochemical processes affecting the formation of various distinct actin structures, including the different types of protrusions mentioned above (Fig. 4). Like the related small GTPases such as Ras

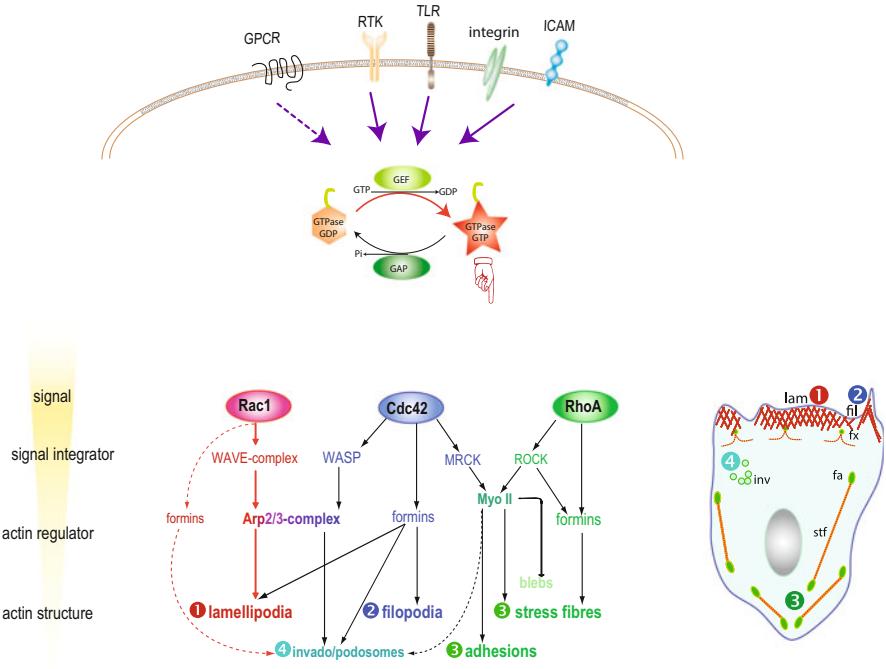

Fig. 4 Signalling pathways leading to actin-based motility. Different types of receptors receive extracellular signals and ignite signalling that aside from phosphorylation cascades culminate in activation of small GTPases of the Rho family. Activated Rho-GTPases in turn lead to the formation of actin-based protrusions and to adhesion and contraction. In concert, these activities result in cell locomotion. Paradigmatic are the induction of lamellipodia through the Rac1/WAVE/Arp2/3-complex signalling axis, induction of filopodia downstream of Cdc42 as well as contraction and adhesion induced downstream of the GTPase RhoA. Note that there is considerable crosstalk between the Rho-GTPases (omitted in the figure for the sake of simplicity), such as activation of Rac1 by Cdc42 signalling or mutually antagonistic relations between RhoA and Rac1. In spite of the canonical top-down signalling from receptor engagement to actin remodelling, accumulating evidence suggests positive and negative feedback loops arising from induced actin structures and influencing upstream signalling events (not shown). Schematic steps of signal propagation from active Rho-GTPases to respective output structures are shown in the middle, and a schematic, mesenchymal cell using the structures depicted (*1–4*) during migration is shown on the right (see also Ridley 2015; Rottner and Stradal 2011)

or Rab, they operate as signalling switches interacting with their effector proteins in their active, GTP-bound form. In case of Rho-GTPases directly regulating actin-based processes, these effectors are actin-binding proteins or regulate factors directly or indirectly affecting actin dynamics (Raftopoulou and Hall 2004).

The family of Rho-GTPases now comprises 20 members in mammals, with RhoA, Rac1 and Cdc42 being by far the best characterized ones, in particular concerning their specific functions in actin remodelling. Yet, additional family members are receiving increasing attention in actin dynamics and cell migration, as illustrated in excellent recent reviews (Ridley 2015; Sadok and Marshall 2014). Here we will focus on established effector pathways operating in various cell types and experimental conditions that drive the classical protrusions mentioned above. Remarkably, in spite of the wealth of publications and information available on the topic, we are still far from a comprehensive picture of the multitude of components co-operating in the regulation of these protrusions, and we are generally suffering from a clear distinction between Rho-GTPase-effector interactions relevant for a wide range of cell types and tissues versus cell-type specific interactions and/or outcomes of functional interference experiments. Moreover, it is still difficult to bridge in vitro observations of fundamental biochemical regulatory pathways, as, for instance, the biochemical regulation of N-WASP by Cdc42, with the formation of a given subcellular structure in cells (see below). Many of these aspects have been previously extensively discussed (Ladwein and Rottner 2008).

Nevertheless, there is an increasing consensus in the field on the following statements:

The formation of actin networks such as lamellipodia or membrane ruffles in mammals requires one of the three Rac subfamily GTPases, Rac1, Rac2 or Rac3. The presence of either one of the three Rac family GTPases generates lamellipodia and membrane ruffles, and no differences are known concerning their interaction with effectors mediating this pathway (Steffen et al. 2013). The formation of microspikes embedded into lamellipodia has not been associated with a particular Rho-GTPase pathway, except that their existence per definition requires the presence of a lamellipodium. We speculate that the relative abundance of these structures will likely depend on the bundling activity present in a given lamellipodium, as mediated, for instance, by fascin, which is prominently enriched in microspikes (Adams 2004), and the knockdown of which significantly reduces efficiency of their formation (Vignjevic et al. 2006). Curiously, fascin-mediated bundling may be supported by the formin Daam1 (Jaiswal et al. 2013), an activity less common than the conventional actin assembly functions exerted by formins (Fig. 3a).

Apart from the essential requirement of Rac, lamellipodia formation is suppressed upon functional interference with the Arp2/3 complex (see also Figs. 1 and 4) (Steffen et al. 2006; Suraneni et al. 2012; Wu et al. 2012), the seven subunit protein complex mentioned above, harbouring actin-related proteins 2 (Arp2) and -3 (Arp3) and 5 additional proteins today commonly called ArpC1-ArpC5 (Campellone and Welch 2010).

Depletion of Arp2/3 complex expression by RNAi or genetic removal of the ArpC3 subunit of the complex appeared to consistently suppress lamellipodia formation (Nicholson-Dykstra and Higgs 2008; Steffen et al. 2006; Suraneni et al. 2012; Wu et al. 2012), suggesting a crucial function at least in the initiation of the formation of these networks. Moreover, inhibition of cellular Arp2/3 complex function by abrupt, potent sequestration of the complex resulted in retraction and collapse of already existing lamellipodial actin networks within minutes (Koestler et al. 2013), indicating that continuous, Arp2/3-dependent actin filament assembly is crucial also for the maintenance of these networks. So if Rac induces lamellipodia and membrane ruffles, which factor then connects Rac signalling to the Arp2/3 complex in vivo? Indeed, a large body of biochemical, structural and functional interference studies in different model systems ranging from human cells down to evolutionarily distant organisms such as *Dictyostelium* amoeba now suggests that this is achieved through direct interaction of Rac with a pentameric complex, in mammals now generally termed WAVE-complex (Chen et al. 2010; Krause and Gautreau 2014; Steffen et al. 2004; Stradal and Scita 2006). The WAVE-complex is composed of five subunits: Sra-1/CyFIP1, Nap1, Abi1/2, WAVE1/2/3 and HSPC300. The necessity for several isoforms of each subunit, such as three WAVE isoforms in mammals (WAVE1, WAVE2 and WAVE3) is curious, and the specific function of each of them within the WAVE-complex is unclear. However, one variant of each individual subunit of the heteropentameric complex is required for its respective integrity and function, and thus thought to be essential for lamellipodia formation and membrane ruffling (Stradal and Scita 2006).

So if Rac signalling through WAVE and Arp2/3 complex activation is essential for lamellipodia formation and membrane ruffling, what about additional effectors or signalling intermediates? In our view, such factors including serine/threonine kinases such as PAK kinase, a prominent Rac-effector, or ERK, which operates downstream of Ras, are more likely to exert modulatory roles, as exemplified, for instance, by the recently discovered regulation of WAVE-complex by ERK-mediated phosphorylation (Mendoza et al. 2011). The contribution of Cdc42 to lamellipodium protrusion and membrane ruffling is also not essential (Czuchra et al. 2005), but includes several separable pathways, as, for instance, activation of Rac (Baird et al. 2005; Nobes and Hall 1995), or promotion of actin assembly by the Cdc42-effector FMNL2 (Block et al. 2012).

Last but not least, effective continuous lamellipodial protrusion requires actin turnover and recycling pathways, including filament disassembly, as mediated, for instance, by gelsolin or members of the ADF/cofilin family. Genetic removal or RNAi-mediated depletion of these factors indeed caused problems with membrane ruffling and protrusion, but the mechanistic reasons for these defects in each case and the precise interplay between their activities and Rac signalling remain to be fully uncovered (Azuma et al. 1998; Bernstein and Bamburg 2010; Bravo-Cordero et al. 2013; Hotulainen et al. 2005).

In addition to contributing to Rac-mediated lamellipodia formation, Cdc42 is best known to stimulate the formation of filopodia (Nobes and Hall 1995), although

the mechanisms of formation of these rod-like protrusions are highly controversial (Faix et al. 2009; Yang and Svitkina 2011). The controversy mainly has to do with the origin of the filaments that initiate protrusion of these rods. Do they derive from lamellipodia, in analogy to what can be assumed for microspikes, or are filopodial filaments initiated by de novo nucleation? The latter hypothesis is clearly supported by data showing filopodia formation upon Rac or Arp2/3 complex inhibition (Koestler et al. 2013; Steffen et al. 2006, 2013; Wu et al. 2012) and by strong stimulation of formation of these structures upon activation of Arp2/3 complex-independent nucleation, e.g., through formins (Block et al. 2008; Schirenbeck et al. 2005). However, additional inquiries into this topic appear to increase complexity rather than simplify models on filopodia formation (Young et al. 2015). Hence, ultimate clarification of this question will depend on the ability to completely erase these structures experimentally, which is not yet possible, at least not in mammalian cells (Fig. 3b). Candidates of actin filament nucleators and polymerases operating in filopodia formation, perhaps in a redundant fashion, certainly include formins such as mDia2 (Yang et al. 2007) or FMNL2 and -3 (Block et al. 2012; Gauvin et al. 2015; Wakayama et al. 2015) as well as Ena/VASP family members (Breitsprecher et al. 2008; Dent et al. 2007; Svitkina et al. 2003; Winkelman et al. 2014).

In vitro, Cdc42 strongly binds and activates the Arp2/3 complex activator N-WASP, synergistically in part with additional signals such as the phosphoinositide PIP2 (Rohatgi et al. 1999). This explains why protrusion of filopodia-like structures observed upon co-overexpression of Cdc42 and the Arp2/3 activator N-WASP was initially interpreted to represent a *bona fide* mechanism of formation of these structures (Miki et al. 1998). However, almost two decades of research have now shown both N-WASP and Arp2/3 complex to be dispensable for filopodia formation (Lommel et al. 2001; Steffen et al. 2006; Suraneni et al. 2012), and N-WASP to operate in endocytosis, vesicle trafficking or invadopodia formation (Fig. 3c) rather than filopodia protrusion (Benesch et al. 2005; Merrifield et al. 2004; Stradal and Scita 2006; Yamaguchi et al. 2005). The redundancy in the system is illustrated even further by the observation that Cdc42 is also not essential for the process in various cell types (Czuchra et al. 2005; Pleines et al. 2010), and indeed, another small Rho-GTPase termed Rif (Rho in filopodia) drives formation of particularly prominent versions of these structures (Ellis and Mellor 2000), likely through its effector mDia2 (Pellegrin and Mellor 2005). In conclusion, a comprehensive picture of the diversities and redundancies in the system will, in our view, only arise from systematic, individual as well as combined gene removal efforts of potential GTPase-effector couples potentially involved in the formation of these fascinating actin filament bundles.

The potential regulation of actin polymerization in membrane blebs by Rho-GTPase signalling and connected actin-binding proteins is still understudied and thus not well understood, except that it is clear today that an essential prerequisite of non-apoptotic blebbing represents the actin/myosin II-based contractility downstream of Rho (Charras and Paluch 2008; Charras et al. 2005; Ridley 2015;

Ruprecht et al. 2015). Concerning bleb initiation, the actin/myosin II cortex is thought to be locally weakened, although it has long been unclear which mechanisms allow for this localized cortex modifications. However, recent evidence indicates local reduction of actin assembly (for instance, through focal reduction of formin or Arp2/3 complex activity) to be at play rather than active depolymerization (Bovellan et al. 2014; Wyse et al. 2012). Upon protrusion, actin filaments re-populate the bleb by mechanisms just starting to be characterized (Bovellan et al. 2014), followed by retraction, likely in a Rho/myosin II-dependent fashion (Charras et al. 2006) (Fig. 3d).

5 Shaping and Trafficking of Intracellular Membranes

While it is clear that long-range movements of vesicles are facilitated by motor proteins walking on microtubules, dynamic actin rearrangements play a role during endocytosis, vesicle trafficking, tubulation of endomembranes, autophagy and of course phagocytosis. Signalling to the actin cytoskeleton during phagocytosis will not be covered in this chapter (see excellent review by Freeman and Grinstein 2014). Instead, we will focus below on types of actin rearrangements driving the protrusion of tubules and membrane surfaces through actin polymerization. Endocytosis, phagocytosis and autophagy deliver material that is destined for degradation to lysosomes. Lysosomal proteins are important for various processes, such as cholesterol homeostasis, plasma membrane (PM) repair, pathogen defence, cell death and signalling (Saftig and Klumperman 2009). Therefore, it is not surprising that genetic defects in proteins involved in lysosomal function can lead to diseases with highly divergent severity (Parenti et al. 2015; Xu and Ren 2015). Before reaching the lysosomes, soluble as well as membrane bound components must be trafficked to this organelle. All these trafficking steps are regulated by a plethora of signalling events, including GTPases, phosphoinositides and Ca^{2+}, as excellently reviewed recently by Li et al. (2013 and see also the references herein).

6 Dorsal Ruffling Leading to Macropinocytosis

Distribution of membrane or membrane-enclosed content such as proteins requires partitioning of sub-fractions of membrane bilayers and their trafficking to new destinations. This process involves membrane budding and can be observed at most if not all membraneous surfaces, but is actually most often observed at the plasma membrane and called endocytosis here. A specialized type of endocytosis is called micropinocytosis, accompanied by cell surface ruffling (Swanson and Watts 1995). Ligands that induce micropinocytosis, such as hepatocyte growth factor/scatter factor (HGF/SF) (Dowrick et al. 1993), activate Rho-GTPases through binding to their plasma membrane-bound receptors (Bosse et al. 2007; Chianale et al. 2007; see Fig. 4). It is tempting to speculate that ruffling during macropinocytosis is mediated by the Rac/WAVE complex pathway (see also

above) driving protrusion of the membrane. However, more work will be required to clarify whether and to what extent the molecular regulation of dorsal ruffling diversifies from the regulation of membrane ruffling and lamellipodia formation driving migration. Mechanistically, solute macromolecules are engulfed in a cave-like invagination culminating in formation of a macropinosome upon ruffle closure (Ladwein and Rottner 2008; Swanson 2008). Recently, a certain type of cancer cells was shown to utilize this process to receive amino acids from extracellular proteins. Human pancreatic and urinary bladder cancer cell lines harbouring oncogenic Ras mutations were found to support their own metabolic needs by forcing nutrient uptake into the tumours (Commisso et al. 2013). Despite being established as oncogenic proteins since decades – mutated Ras genes were the first genes being discovered in human cancers – Ras is still unsusceptible to pharmacological inhibition in clinical terms (Cox et al. 2014). This might be due, at least in part, to the tight connection of Ras signalling to fundamental actin reorganization pathways, such as induction of protrusions mediated through Rac (Scita et al. 2000). It remains to be seen whether such fundamental pathways and connected phenomena such as macropinocytosis will serve as useful targets for potential cancer treatment.

7 Endocytosis

In the classical view, endocytosis is referred to as invagination of the plasma membrane without prior protrusion. Endocytic vesicles, as well as most intracellular vesicles, are formed through a budding process, which is characterized by (1) membrane invagination, (2) elongation of the vesicle neck, (3) fission and (4) movement away from the plasma membrane (Kirchhausen et al. 2014). While actin may in fact participate in all of these consecutive steps, it is only well documented to drive membrane scission that is accompanied by a burst of actin polymerization. Multiple studies in yeast showed that endocytosis is not completed when actin polymerization is disturbed. However, not all of the multiple pathways of endocytosis in mammalian cells seem to strictly rely on actin polymerization, although frequently accompanied by it (Merrifield et al. 2002). At the molecular level, actin assembly events during endocytosis in mammals are most prominently regulated by the Arp2/3 complex and its NPF WASP/N-WASP, generating a dendritic actin network that can help to drive the emerging endocytic vesicle away from the membrane (Benesch et al. 2005; Kaksonen et al. 2005; Mooren et al. 2012; Skau and Waterman 2015).

8 Vesicle Trafficking

All vesicle trafficking processes in cells have the purpose to distribute material, which can be either soluble cargo inside the vesicle, proteins bound to or embedded in the vesicular membrane or the membrane itself. Trafficking normally ends with

fusion of the vesicle with its target membrane. The purposes can be as different as degradation of the material in lysosomes, cytokine secretion, synaptic vesicle release or receptor recycling to name only a few. The different steps on these multiple paths are regulated by a plethora of regulating proteins or protein families such as Rab small GTPases, SNAREs, sorting adapter proteins, coat proteins and tethering factors (for details, see reviews by Di Fiore and von Zastrow 2014; Lu and Hong 2014). Recent research has shed light on the role of actin not only during vesicle propulsion, which is somehow phenocopied by invasive, intracellular pathogens (Gouin et al. 2005; Rottner et al. 2005; Welch and Way 2013), but also during deformation of the membranes leading to establishment and segregation of subdomains (see below).

Actin polymerization is thought to generate the force necessary to deform membranes or to propel them over short distances, and the respective actin generator is believed to provide spatiotemporal control over the actin assembly required. In fact, Arp2/3 complex with its different NPFs appears to be of utmost importance in these processes. So far, the class I NPFs N-WASP, WASH and WHAMM, as well as JMY (junction-mediating and regulatory protein) were shown to be involved in different membrane trafficking processes (Burianek and Soderling 2013; Rottner et al. 2010). Aside from Arp2/3-complex and its activators, additional factors were described to participate in endocytic trafficking, including Spire proteins and associated formins, although the precise mechanistic functions of Spire/formin-generated filaments operating in these processes remained ill defined (Dietrich et al. 2013).

One of the first vesicular processes shown to be actin polymerization-dependent was vesicle rocketing (Rozelle et al. 2000). Elevation of PIP2 (phosphatidylinositol-4,5-bisphosphate) levels, e.g., by overexpression of phosphatidylinositol-4-phosphate 5-kinase (PIP5K) stimulated the formation of actin comet tails tipped by N-WASP (or WASP) and associated factors, driving activation of the Arp2/3 complex and actin polymerization (Benesch et al. 2002; Taunton et al. 2000). Although the precise physiological function of this process is under debate, the observed propulsion of such vesicles by N-WASP-dependent actin assembly likely represents an exaggeration of actin polymerization accompanying endocytosis (Benesch et al. 2005; Merrifield et al. 2004), fitting the notion that the PM is enriched for this phospholipid. Here, PIP2 likely constitutes one module contributing to N-WASP activation, unleashing Arp2/3-dependent actin nucleation (Prehoda et al. 2000; Rohatgi et al. 2000).

WASH is an NPF that is – similar to the WAVE-complex – assembled in a multimeric protein complex (Jia et al. 2010) and was identified to function in retrograde transport from endosomes "back" to the transgolgi network, TGN (Derivery et al. 2009; Gomez and Billadeau 2009). Knockout of WASH in mice leads to early embryonic lethality (Xia et al. 2013 and see also below), and knockout of WASH in cells caused enlarged and collapsed endosomes lacking F-actin and Arp2/3 complex (Gomez et al. 2012; King et al. 2013). Of note, while N-WASP and the WAVE complex are essentially regulated by Rho-GTPases, no such regulation is known for the WASH complex (Rottner et al. 2010).

Interestingly, while WASP/N-WASP and WAVE-complex bind directly to phosphatidyl-inositol-containing membranes, WASH complex requires an additional, multimeric protein complex, the retromer complex (Trousdale and Kim 2015), to bind to the endosomal membrane (Harbour et al. 2010, 2012; Jia et al. 2012). Strikingly, mutations of a subunit of the retromer complex occurring in Parkinson's disease (PD) were found to perturb binding to the WASH complex (McGough et al. 2014; Zavodszky et al. 2014a; see also below), indicating that loss of WASH-mediated actin assembly is causally connected to the development of PD.

A unique feature among the group of Arp2/3 activators was ascribed to the WASP-related NPF WHAMM: In addition to the C-terminal WCA domain common to all NPFs driving Arp2/3-mediated branching and nucleation (see above), a central domain of WHAMM harbours a microtubule binding module. Upon association of WHAMM with microtubules, the N-terminus can bind to and remodel membranes. Depletion of WHAMM was observed to perturb transport processes in the so-called ERGIC region (ER-Golgi intermediate compartment), also termed tubulo-vesicular cluster (Campellone et al. 2008; Rottner et al. 2010; Shen et al. 2012). How exactly both features of WHAMM, actin assembly and microtubule binding, are contributing to ERGIC trafficking awaits further investigation. WHAMM has recently been found to be regulated and recruited to tubulovesicular structures by the small GTPase Rab1, but in contrast to other GTPase-nucleation factor interactions, Rab1 appears to inhibit WHAMM-mediated Arp2/3 activation (Russo et al. 2016). Hence, a potential activation mechanism of WHAMM-mediated, Arp2/3-dependent actin assembly remains to be determined.

JMY was initially found as a DNA damage-responsive p53 cofactor (Coutts et al. 2007; Shikama et al. 1999) and later shown to be a WH2 domain-containing actin nucleator (Zuchero et al. 2009). Surprisingly, JMY seems to be endowed with canonical class I NPF activity to drive Arp2/3-dependent actin assembly as well as with direct and Arp2/3-independent nucleation capacities. The latter function is achieved through a modular organization of three adjacent WH2 domains that might bind actin monomers and stimulate the generation of filaments in a fashion similar to Spire (see above) (Zuchero et al. 2009). JMY was observed to accumulate both in the nucleus and cytosol. In the cytosol, JMY is localized to dynamic TGN-derived tubular vesicles, co-incident with Arp2/3 complex and F-actin accumulation. Interference with JMY function led to defects in anterograde transport from the trans-Golgi network (TGN) to the PM (Schluter et al. 2014), which may also causally link to the observed defects in migration (Coutts et al. 2009, 2010; Zuchero et al. 2009). The precise function of Arp2/3-independent relative to Arp2/3-dependent actin assembly driven by JMY remains to be established.

9 Autophagy

Autophagy is a degradative process through which cells turn over their own constituents and can be understood as a recycling pathway. Autophagy serves to maintain cellular homeostasis, but is strongly induced under stress conditions. This is most apparent during starvation, which is accompanied by break down of cytoplasmic material and organelles (Boya et al. 2013). Perturbation of autophagy is associated with multiple diseases falling into age-related, cardiovascular, infectious, neoplastic, neurodegenerative and metabolic categories. For instance, polymorphisms in genes playing an exclusive role in autophagy, called **au**toph**a**g**y**-related genes (ATG), are associated with diseases like breast cancer and hereditary neurologic disorders (Kroemer 2015).

As opposed to vesicle budding that manifests itself as formation of a single membrane (see also above), autophagy is induced by the formation of the so-called isolation membrane/phagophore, a cup-shaped double membrane (depicted in Fig. 5). Structures targeted for degradation are sequestered into the nascent double-membrane autophagosome (Boya et al. 2013). Such autophagosomes are accumulated in neurological disorders including PD, Alzheimer's disease (AD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). Clarifying function and interplay of the molecular machineries involved might help to identify putative pharmaceutical targets to treat these diseases (Kroemer 2015; Zhang et al. 2013). While most of the research on autophagy is concentrated on the mechanism of action of ATG proteins and upstream signalling events, different laboratories recently reported interesting findings on how actin is involved in this process.

Actin was first implicated in autophagy through work published by the Klionsky lab in 2008. Genetic analysis in the yeast *Saccharomyces cerevisiae* pointed towards a role of the Arp2/3 complex in selective autophagy. Mutant strains defective in the Arp2/3 complex subunit Arp2 were shown to have partial defects in autophagy through impaired transport of a component of the machinery called ATG9 (Monastyrska et al. 2008). To date, as many as three NPFs have been reported to play a role during autophagy (see below). However, while it becomes apparent that actin is central in this process, it is still uncertain whether Arp2/3 complex- or NPF-activities are directly required.

The WASH complex is involved in retrograde transport (see above) and was surprisingly reported to inhibit autophagy via suppression of an upstream regulatory complex called the PI3K (Phosphatidylinositol 3-phosphate kinase) complex I. Beclin 1 is a central component of this complex and WASH was shown to directly interact with it. Depletion of WASH led to augmented PI3P formation, which is a key to the initiation of phagophore formation. Strikingly, WASH knockout in mice causes early embryonic lethality (E7.5–E9.5) accompanied by massive non-apoptotic cell death and accumulation of autophagic structures. This led to the conclusion that WASH is an inhibitor of autophagy through negative regulation of Beclin 1. Interestingly, this inhibitory function of WASH was suggested to be independent of Arp2/3 complex (Dupont and Codogno 2013; Xia et al. 2013).

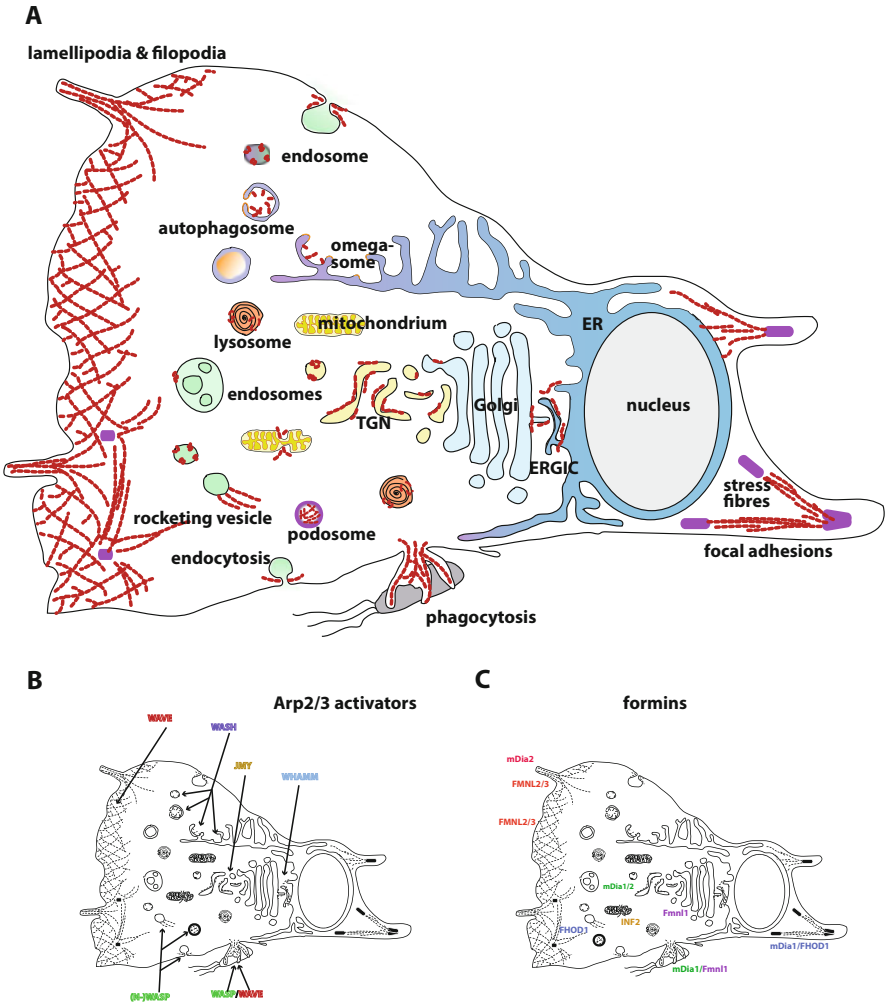

Fig. 5 Actin assemblies on cellular membranes. (**A**) A schematic cell with inner structures and organelles as detailed in the text. According to our current view, cell surface shape changes or maintenance of a given shape of membranes all involve actin reorganization events – at least at certain, specific steps. This applies to the plasma membrane as well as to large cell-spanning organelles like the ER, to single vesicles and also to double-membrane encircled organelles like mitochondria. (**B**) Same cell with those NPFs highlighted that are known to spatiotemporally control Arp2/3 complex activation (Campellone and Welch 2010; Rottner et al. 2010). (**C**) Same cell with local formin-activities highlighted (Kuhn and Geyer 2014)

These data are currently difficult to reconcile with recent observations on WASH function in PD (see also above). These reports describe that a defect in binding of the WASH complex to the retromer complex results in impaired autophagy (McGough et al. 2014; Zavodszky et al. 2014a, b). The latter data thus collectively

suggest a supporting role of the WASH complex in autophagy. Future studies are clearly required to sort out the precise role of the WASH complex, and furthermore, its NPF function in the process.

Recently, a second NPF was reported to act in autophagy, namely JMY, through its WH2 domains. JMY harbours an LIR (LC3-interacting region) motif mediating localization of JMY to autophagosomes (Coutts and La Thangue 2015). Support of JMY functions during autophagy came from contemporaneous findings, also localizing JMY to LC3-positive structures (Kast et al. 2015). Moreover, the same authors also implicated WHAMM and its nucleation promoting function in autophagy (Kast et al. 2015). However, a colocalization of WHAMM with nascent phagophores is not entirely surprising, given that the ER membrane has frequently been observed to be the origin of the isolation membrane (Feng et al. 2014). In any case, the relevance of WHAMM in the autophagic process remains unsolved, since interfering with WHAMM expression or applying small-molecule Arp2/3 inhibitors had no effect when performing functional assays, such as measurement of autophagic flux (Kast et al. 2015). Finally, actin was found to be localized inside the central cavity of isolation membranes and to be important for the shape of this structure (Mi et al. 2015). The nucleator of this portion of filamentous actin, however, needs to be unambiguously identified. Taken together, actin is dynamically orchestrated in autophagy through several regulatory proteins and protein complexes, but how these are coordinated still awaits future investigations.

10 Concluding Remarks

It is becoming increasingly clear that polymerizing and/or contractile actin filaments exert forces of various kinds on the plasma membrane and virtually every intracellular membrane (Fig. 5a). Interestingly, generation of actin filaments appears to occur in a highly specific fashion, likely due to their specific needs and physiological functions, and implemented through recruitment and activation of distinct actin assembly machines. Thus, Arp2/3 activation on intracellular versus plasma membrane is executed by the various distinct NPFs (Fig. 5b), and likewise, if we inspect subcellular locations so far discovered to be populated by a specific formin, a similar, highly divergent pattern of formin involvement emerges (Fig. 5c). In conclusion, virtually any movement of membrane, either at the plasma membrane or inside cells, will be powered and/or regulated to a certain extent by specific assembly and turnover of actin filaments. Although much remains to be learned, in particular concerning mutual dependence and crosstalk between these diverse populations of actin filament structures, recent years have been highly successful in identifying and functionally characterizing novel actin filament regulators and pathways. Now we have to establish how they all work together, which might ultimately lead to identification of novel pharmaceutical targets.

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ADP-Ribosylation and Cross-Linking of Actin by Bacterial Protein Toxins

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Abstract

Actin and the actin cytoskeleton play fundamental roles in host–pathogen interactions. Proper function of the actin cytoskeleton is crucial for innate and acquired immune defense. Bacterial toxins attack the actin cytoskeleton by targeting regulators of actin. Moreover, actin is directly modified by various bacterial protein toxins and effectors, which cause ADP-ribosylation or cross-linking of actin. Modification of actin can result in inhibition or stimulation of actin polymerization. Toxins, acting directly on actin, are reviewed.

Keywords

Actin • Actin cross-linking • ADP-ribosylation • Formins • Host–pathogen interaction • Thymosin-β4 • Toxins

1 Introduction

Actin is an essential constituent of the cytoskeleton and one of the most abundant cellular proteins. The actin cytoskeleton has many crucial functions in host–pathogen interactions. This is especially true not only for immune cells but also for all types of cells targeted by pathogens (Higley and Way 1997; Dramsi and Cossart 1998; Haglund and Welch 2011). The actin cytoskeleton secures epithelium barrier functions and is involved in bacterial adhesion, invasion, endocytosis, and phagocytosis. Actin is crucial for all types of motility and migration and essential for signaling at the immunological synapse. Accordingly, numerous bacterial pathogens target and manipulate the actin cytoskeleton of host cells (Gruenheid and Finlay 2003; Barbieri et al. 2002; Aktories et al. 2011; Popoff 2000). This happens by protein toxins (exotoxins), which are released by bacteria in the environment and are able to enter target cells in the absence of the producing pathogen and by the so-called bacterial effectors, which act on target cells after delivery by injection machines, which depend on the direct contact of the pathogen with host cells. Evolution of host–pathogen interaction allowed the development of different strategies to attack the actin cytoskeleton (Aktories et al. 2011).

1.1 Regulators of the Actin Cytoskeleton Are Targeted by Bacterial Toxins

Many bacterial toxins or effectors[1] act on proteins that are regulators of the actin cytoskeleton or they mimic these regulators and hijack their functions (Barbieri et al. 2002; Aktories and Barbieri 2005; Popoff 2014). The GTP-binding Rho proteins, which act as molecular switches, are master regulators of the actin cytoskeleton and are preferred targets of toxins (Aktories 2011; Popoff 2014;

[1]®In the following both types of bacterial virulence factors will be assigned as “toxins.”

Lemichez and Aktories 2013). Rho proteins, which favor actin polymerization via different mechanisms, including activation of actin nucleators like formins or WH2-domain containing proteins, are inactivated or activated by bacterial toxins. Inactivation occurs by ADP-ribosylation (Aktories et al. 1989, 2004; Paterson et al. 1990), AMPylation (Yarbrough et al. 2009), glycosylation (Just et al. 1995; Jank et al. 2013, 2015), or proteolytic cleavage (Shao et al. 2002). Rho proteins are activated by ADP-ribosylation (Lang et al. 2010) and deamidation (Schmidt et al. 1997; Flatau et al. 1997). Moreover, bacterial toxins manipulate the Rho-dependent control of the actin cytoskeleton by mimicking regulators of Rho proteins. Numerous bacterial toxins have GEF (guanine nucleotide exchange factors)-like function and activate Rho proteins, eventually leading to enhanced polymerization of actin. This group of bacterial toxins includes, for example, *Salmonella enterica* SOP proteins (Hardt et al. 1998), *Burkholderia pseudomallei* BopE (Upadhyay et al. 2008), *Shigella flexneri* IpgB proteins (Klink et al. 2010; Ohya et al. 2005), *E. coli* Map protein (Huang et al. 2009), and others. On the other hand, bacterial toxins switch off the active state of Rho proteins by mimicking GTPase-activating proteins (GAPs), resulting in inhibition of polymerization of G-actin and/or depolymerization of F-actin. These GAP mimics include, for example, *Yersinia* ssp. YopE (von Pawel-Rammingen et al. 2000), *Pseudomonas aeruginosa* ExoS (Goehring et al. 1999), Exo T (Krall et al. 2000), and *S. Typhimurium* SptP (Fu and Galan 1999).

Other bacterial toxins (effectors) interfere with actin polymerization not via Rho proteins but by using actin nucleators like *Listeria monocytogene*s protein ActA (Pistor et al. 1994), which activate Arp2/3, or *Shigella flexneri* surface protein IcsA (Suzuki et al. 1998), which also activate Arp2/3, however, via N-WASP. Still another group of bacterial toxins directly hijacks actin nucleator functions like *Vibrio cholerae* and *Vibrio parahaemolyticus* proteins VopF (Pernier et al. 2013) and VopL (Tam et al. 2007), respectively, or *Rickettsia* ssp. protein Sca2 (Haglund et al. 2010) and others.

However, actin is also directly targeted by bacterial toxins. These toxins modify the actin molecule by covalent modification, including ADP-ribosylation and protein cross-linking (Satchell 2009). In the following, these toxins will be described in more detail.

2 Inhibition of Polymerization by Toxin-Catalyzed ADP-Ribosylation of Actin

ADP-ribosylation is a very common mechanism by which bacterial toxins modify eukaryotic targets. Well-known substrates are heterotrimeric G proteins (e.g., cholera toxin and pertussis toxin), elongation factor 2 (e.g., diphtheria toxin), small GTPases like Rho proteins (*Clostridium botulinum* C3 toxin), and actin. In all these cases the toxins split NAD^+ and transfer the ADP-ribose moiety onto the protein target and release nicotinamide. Two major groups of actin-ADP-ribosylating toxins, which differ in their target amino acid of actin have been

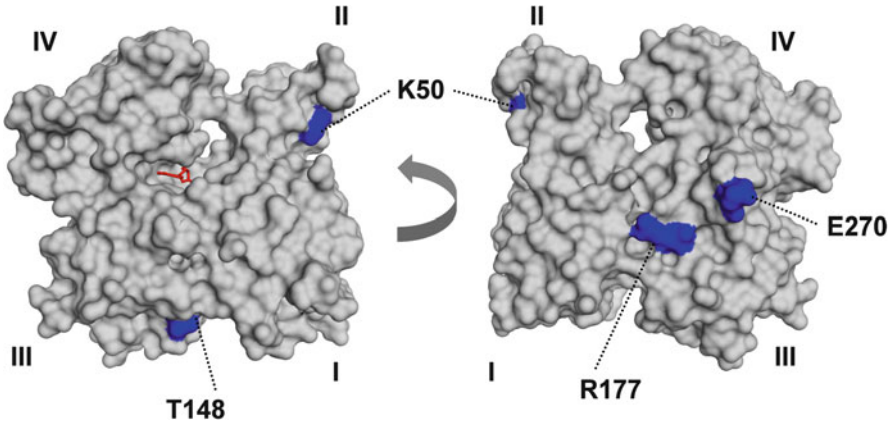

Fig. 1 Space-filling model of actin (PDB 1ATN). The four subdomains of actin are indicated (I-IV). Amino acids, which are modified by bacterial protein toxins, are marked in blue. Whereas arginine177 (R177) is ADP-ribosylated by several toxins like C2 toxin from *Clostridium botulinum* to prevent polymerization and to induce depolymerization of actin, threonine148 (T148) is exclusively ADP-ribosylated by *Photorhabdus luminescens* toxin (TccC3), which causes polymerization of actin. Various other toxins like VgrG1 of *Vibrio cholera* catalyze actin cross-linking between lysine50 (K50) and glutamate270 (E270). For details, see text

described. One group of the ADP-ribosylating toxins modifies actin at arginine177 and the second group ADP-ribosylates actin at threonine148 (Fig. 1). The functional consequence is exactly the opposite. While toxin-induced ADP-ribosylation of arginine177 of actin inhibits actin polymerization, modification of threonine148 increases polymerization. Prototypes of the toxins, which modify actin in arginine177, are the binary actin-ADP-ribosylating toxins.

2.1 Binary Actin-ADP-Ribosylating Toxins

Members of the family of binary actin-ADP-ribosylating toxins are *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin (ST), *C. difficile* transferase CDT toxin, and *Bacillus cereus* vegetative insecticidal proteins (Aktories et al. 1986b; Barth et al. 2004; Stiles et al. 2014). All these toxins are comprised of the components A and B. The B component is involved in receptor-binding and toxin uptake, while the A component possesses the ADP-ribosyltransferase activity (Fig. 2). Both components are separately released from the bacteria. Excellent groundbreaking studies were first performed with *C. botulinum* C2 toxin (Ohishi et al. 1980, 1981; Ohishi 1983; Ohishi and DasGupta 1987).

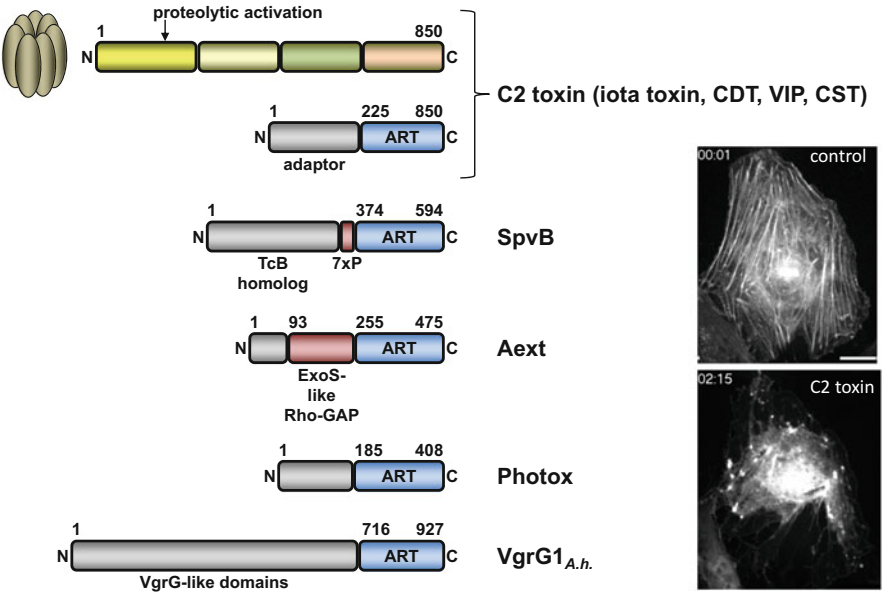

Fig. 2 Scheme of the structures of actin-ADP-ribosylating toxins/effectors, which all modify actin at arginine177. The family of binary toxins consists of *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. difficile* transferase (CDT), *Bacillus cereus* vegetative insecticidal toxin (VIP), and *C. spiroforme* toxin (CST). These toxins are binary in structure, because they consist of a four-domain binding/translocation component and the separated enzyme component. After proteolytic activation, the binding/translocation component forms heptamers. The enzymatic component consists of a C-terminal ADP-ribosyltransferase (ART) domain and an N-terminal adaptor domain, which interacts with the binding/translocation component. Numbers given are from *C. botulinum* C2 toxin. The other toxins/effectors are not binary in structure but all possess a C-terminal actin-ADP-ribosylating domain (ART). These toxins are translocated into host cells by type-III secretion systems (SpvB, AexT) or by unknown mechanisms. The effector SpvB from *Salmonella enterica* possesses a C-terminal actin-ADP-ribosylating domain. AexT is produced by *Aeromonas salmonicida* and possesses in addition to the actin-ADP-ribosyltransferase domain (ART), a domain with Rho GTPase-activating activity (GAP), which is related to *Pseudomonas* ExoS protein. Photox is an effector, which is produced by *P. luminescens*. VgrG1 from *Aeromonas hydrophila* possesses an actin-ADP-ribosyltransferase domain at its C-terminus. The right panel shows the effect of the binary *Clostridium botulinum* C2 toxin on HeLa cells after 2.15 h. Actin-staining is shown (bar 10 μm)

2.2 Binding Components of Binary Toxins

The binding and translocation components of all these toxins have molecular masses of 80 to 100 kDa, and share sequence identity between 36 and 85%. Notably, they all share sequence similarity with the binding component of anthrax toxin, the protective antigen PA (Barth et al. 2004; Young and Collier 2007; Stiles et al. 2011). All components (including PA) are produced as precursor proteins and are activated by proteolytic cleavage at the N-terminus (Ohishi 1987). Thereby, an ~20 kDa peptide is released, a process that initiates oligomerization of the rest of

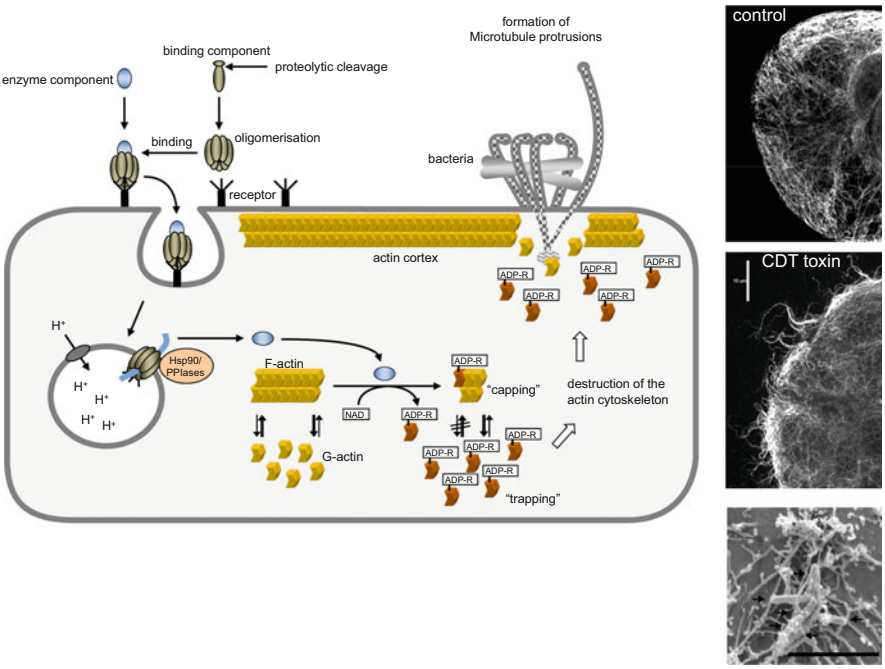

Fig. 3 Effects of binary actin-ADP-ribosylating toxins. Binary actin-ADP-ribosylating toxins consist of a binding component and an enzyme component, which are separate proteins. The binding component is proteolytically activated and forms heptamers, which bind to the cell surface receptor of the toxins. After binding of the enzyme component, the toxin complex is endocytosed. At low pH of endosomes the heptamer inserts into the endosomal membrane and forms a pore. Through the pore the enzyme component is translocated into the cytosol, where G-actin is ADP-ribosylated at arginine177. Thereby actin polymerization is blocked. ADP-ribosylated actin acts like a barbed-end capping protein and prevents further F-actin elongation. Partial destruction of cortical actin induces formation of microtubule based cell protrusions. The cell protrusions are involved in increased adherence of bacteria. Right panel (upper and middle pictures). Effects of the binary *Clostridium difficile* toxin CDT (CDTa 20 ng/ml, CDTb 40 ng/ml) on human colon adenocarcinoma Caco-2 cells. CDT causes formation of microtubule-based cell protrusions. Tubulin is stained (bar 10 μm). Right panel (lower picture). Scanning electron microscopy of Caco-2 cells. Cells were treated as above. After 1 h, *Clostridium difficile* bacteria were added. After 90 min, cells were washed and fixed. Bacteria were caught in toxin-induced protrusions

the protein to form heptamers (Barth et al. 2000). According to PA, they consist of four functional domains (Young and Collier 2007; Schleberger et al. 2006). The N-terminal domain I, which is proteolytically cleaved, is involved in activation and in initial binding of component A. While domain II of the binding domain is responsible for membrane insertion, domain III is essential for oligomerization of the binding component. Finally, domain IV is the receptor-binding domain. Domains I-III exhibit highest sequence similarity among the toxins. Whereas the receptor-binding domains of C2 toxin (C2II) and PA are completely unrelated

Table 1 Typical R, STS, and ExE motifs of bacterial actin-ADP-ribosyltransferases

Toxin	Bacterium	R	STS	ExE
SpvB	*Salmonella enterica*	RVVY**R**GLK^{474}	FM**STS**PD^{505}	FKG**E**A**E**ML^{540}
iota a	*Clostridium perfringens*	LIVY**R**RSG^{339}	FI**STS**IG^{383}	YAG**E**Y**E**VL^{423}
VgrG1	*Aeromonas hydrophila*	DFVY**R**GLA^{812}	FM**STS**PD^{843}	FKG**E**A**E**ML^{878}
Photox	*Photorhabdus luminescens*	KKVY**R**GLK^{291}	FL**STS**PD^{322}	FKG**E**A**E**ML^{357}
VIP2	*Bacillus cereus*	ITVY**R**WCG^{338}	YI**STS**LS^{376}	FAN**E**K**E**IL^{416}
AexT	*Aeromonas salmonicida*	QHLN**R**SLR^{306}	YL**STS**RD^{368}	EGD**E**Q**E**IL^{405}
C2I	*Clostridium botulinum*	LIAY**R**RVD^{302}	FS**STS**LK^{352}	FQD**E**Q**E**IL^{391}

Sequence alignment of highly conserved residues of bacterial ADP-ribosyltransferases. The NAD-interacting arginine residue (R), the first serine residues from the STS motif (S), and the catalytic glutamate (second E) from the ExE motif, which form the RSE-motif of ADP-ribosyltransferases are highlighted (Fieldhouse and Merrill 2008). Alignments were made with the following sequences: SpvB (Acc. No. D0ZHS9), iota a (Acc. No. F7J0A4), VgrG1 (Acc. No. A0KHA9), Photox (Acc. No. Q7N8B1), VIP2 (Acc. No. G8FSA8), AexT (Acc. No. Q93Q17), and C2I toxin (Acc. No. D4N871)

between all toxins, the binding domains of iota toxin (Ib), *C. spiroforme* toxin (STb) and CDTb, are highly similar and share the same membrane receptor (see below). These latter three toxins are combined in the iota toxin-family, because the binding components of the toxins are interchangeable and they are able to transport the A components of each other toxin of this family (Stiles and Wilkens 1986; Stiles and Wilkins 1986; Simpson et al. 1989).

The A components of the toxins have molecular masses of ~44–52 kDa and possess ADP-ribosyltransferase activity (Figs. 2 and 3). All toxins ADP-ribosylate actin at arginine177 (Vandekerckhove et al. 1987, 1988). The crystal structures of the enzyme components of *B. cereus* VIP toxin (VIP2), C2 toxin (C2I), and iota toxin (Ia) have been determined. Because iota toxin was crystallized in complex with actin, we now know a lot about the actin–toxin interaction and the ADP–ribosylation reaction. The toxins share the typical folding of ADP-ribosyltransferases. Interestingly, each toxin component consists of two domains with typical ADP-ribosyltransferase folding, which most likely developed by gene duplication (Han et al. 1999). The N-terminal ADP-ribosyltransferase domain is inactive and involved in interaction with the binding domain, while the C-terminal ADP-ribosyltransferase domain harbors the enzyme activity. This domain possesses the highly conserved RSE-motif of ADP-ribosyltransferase, which is involved in NAD^+ binding and catalysis (Table 1).

2.3 Toxin Binding and Uptake

Figure 3 shows a scheme of the binding and uptake of actin-ADP-ribosylating binary toxins. After tryptic activation (e.g., in the gut) the toxin forms heptamers and interacts with cell surface receptors. It appears that also the monomer can bind to receptors. While the functionally important part of the receptor for C2 toxin

seems to be a carbohydrate structure (Eckhardt et al. 2000), the membrane receptor for CDT, iota toxin, and ST has been identified as the lipolysis stimulated lipoprotein receptor (LSR) (Papatheodorou et al. 2011, 2012). This receptor was initially suggested to be involved in lipid metabolism (Yen et al. 1994), later it turned out that it is essential for triangular tight junctions (Masuda et al. 2011).

After receptor-binding, the toxins are endocytosed (Nagahama et al. 2002, 2004), a process that probably involves interaction with lipid rafts (Nagahama et al. 2004; Papatheodorou et al. 2013). The low pH of endosomes induces conformational changes of the binding component and facilitates the membrane insertion of the beta-barrel-like structure of the binding component (Barth et al. 2000) (Stiles et al. 2002; Nagahama et al. 2002; Blöcker et al. 2001). Thereby, pores are formed, which allow transport of the enzyme components into the cytosol (Schmid et al. 1994; Knapp et al. 2002). Because the diameter of the pores is small, partial unfolding of the enzyme component is necessary. Translocation of the enzyme component is facilitated by cytosolic proteins including chaperons like heat shock protein90 (Haug et al. 2003, 2004), and protein-folding helpers like peptidyl-prolyl cis/trans isomerases cyclophilin A, cyclophilin 40 and FK506 binding protein (FKBP) (Kaiser et al. 2011, 2012; Dmochewitz et al. 2011).

2.4 Toxin-Induced ADP-Ribosylation of Actin in Arginine177

After translocation of the enzyme component into the cytosol, the toxin ADP-ribosylates monomeric G-actin at arginine177 (Vandekerckhove et al. 1987, 1988) (Fig. 1). Polymerized F-actin is a very poor substrate (Aktories et al. 1986a, 1987; Schering et al. 1988), and phalloidin, a fungal toxin that binds to F-actin, blocks ADP-ribosylation of actin (Aktories et al. 1986a). This is reasoned in the localization of arginine177 in F-actin. Arginine177 is located near the axes between the long two-start helices. The voluminous ADP-ribose moiety cannot be accommodated between the strands (Holmes et al. 1990; Margarit et al. 2006) (Fig. 4). Therefore, F-actin has to be depolymerized first to be subsequently modified by the toxin. ADP-ribosylation of G-actin at arginine177 causes only minor structural changes (Margarit et al. 2006). Nevertheless, the attachment of ADP-ribose at this position has functional consequences for G-actin. For example, the ATPase activity of actin is blocked. This is observed not only for basal ATPase activity (Geipel et al. 1989; Margarit et al. 2006) but also for cytochalasin-stimulated ATPase activity (Geipel et al. 1990). Moreover, the exchange of ATP bound to actin is enhanced (Geipel et al. 1989). Because the nucleotide is important for actin stabilization, ADP-ribosylation results in a decrease in thermal stability (Perieteanu et al. 2010). Although arginine177 is conserved in all actin isoforms, the toxins differ in their substrate specificity. While C2 toxin exhibits high preference for β/γ-non-muscle actin, the other toxins (so far studied) accept non-muscle as well as muscle actin as protein substrates (Mauss et al. 1990; Schering et al. 1988).

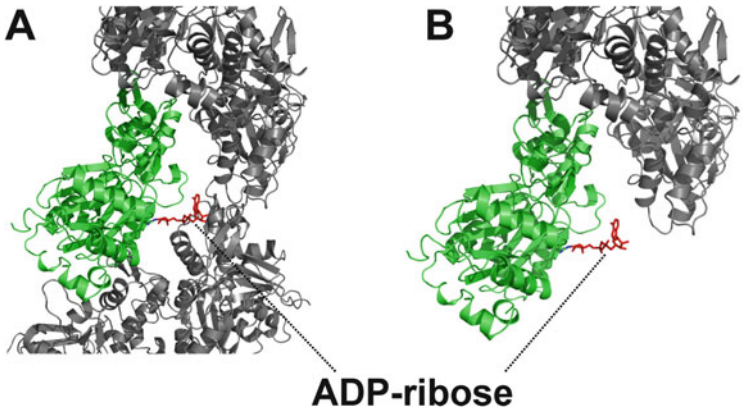

Fig. 4 Effect of ADP-ribosylation of arginine177 on actin–actin interaction. (**a**) Ribbon presentation (PDB 1ATN) of ADP-ribosylated actin (*green*) within the F-actin filament (*grey*); ADP-ribose is marked in *red*. The steric hindrance induced by ADP-ribosylation of arginine177 is shown. (**b**) Binding of ADP-ribosylated actin to the plus end of F-actin. Figure is from Aktories et al. (2011)

2.5 The Physiological Role of Arginine177

Arginine177 is highly conserved in various actin isoforms from different species including yeast actin. It is located in the nucleotide-binding cleft of actin and of major importance for actin functions. Karlsson and coworkers changed arginine177 to aspartate in chicken β-actin (Schüler et al. 2000). They found a 10-fold increase in critical concentration, lowered thermostability, and an increased nucleotide exchange rate. Arginine177 is the pivotal residue in the *cardiofunk* actin mutation in zebrafish. Here, the mutation of arginine177 to histidine causes lack of endocardial cushion formation, defects in cardiac morphogenesis and embryonic lethality (Bartman et al. 2004). The *cardiofunk* mutation (R177H) was biochemically studied in the yeast actin model showing an increase in the critical concentration of actin polymerization, a prolonged nucleation phase and a rapid elongation process of actin polymerization, indicating that this mutation causes increased fragmentation of actin filaments (Wen and Rubenstein 2003).

2.6 Functional Consequences of ADP-Ribosylation of Arginine177

Most important for the functions of actin is its property to reversibly polymerize from G-actin to F-actin and its interaction with actin-binding proteins. What are the functional consequences of the ADP-ribosylation of actin at arginine177 concerning these properties? Early studies showed that C2 toxin and iota toxin inhibit actin polymerization (Aktories et al. 1986b; Schering et al. 1988). As stated above this is explained by steric hindrance of ADP-ribosylated actin to form F-actin

(Holmes et al. 1990) (Fig. 4). However, ADP-ribosylated actin can bind to barbed ends of F-actin filaments (Fig. 4). Here, it acts like a capping protein and blocks F-actin elongation by inhibiting attachment of non-ADP-ribosylated actin (Wegner and Aktories 1988; Weigt et al. 1989; Aktories and Wegner 1989; Perieteanu et al. 2010). By contrast, ADP-ribosylated actin does not interact with pointed ends of actin filaments (Wegner and Aktories 1988; Perieteanu et al. 2010). Thus, depolymerization of F-actin, which preferentially occurs at the pointed ends is still possible. On the other hand, pointed end polymerization can occur, when the concentration of non-modified actin is higher than the critical actin concentration for pointed end polymerization (Wegner and Aktories 1988; Perieteanu et al. 2010). Under in vitro conditions, ADP-ribosylation of actin is reversible in the presence of excessive amounts of nicotinamide (Just et al. 1990; Perieteanu et al. 2010).

The interaction of actin with various actin-binding proteins is crucial for its functions. Toxin-induced ADP-ribosylation affects the interaction of actin with actin-binding proteins. From >100 actin-binding proteins known, only the interaction of gelsolin with ADP-ribosylated actin was studied in some detail. Gelsolin consists of 6 homologous domains (G1-G6), only three of which (G1,G2 and G4) interact with actin resulting in multiple effects. Depending on the Ca^{2+} concentration, gelsolin causes actin sequestering, capping, severing, and nucleation. Actin in complex with gelsolin is modified by toxin-induced ADP-ribosylation in vitro (Wille et al. 1992) and in intact cells (Just et al. 1993). At least four different types of actin-gelsolin complexes (G-Ar, G-Ar-A, G-A-Ar, and G-Ar-Ar) were identified (Wegner et al. 1994; Wille et al. 1992). In vitro studies showed that the G-Ar and G-Ar-A complexes are able to nucleate actin polymerization, while this was not the case for the G-A-Ar and G-Ar-Ar complexes. These data are in line with the finding that ADP-ribosylated actin binds to the barbed ends of actin filaments (see Fig. 4). Moreover, actin dimers and trimers are also ADP-ribosylated by the toxins. As expected, modified oligomers are still able to interact with DNaseI and gelsolin (Perieteanu et al. 2010).

2.7 ADP-Ribosylation of Actin as Tool to Investigate Physiological Actin Functions

Inhibition of actin polymerization by toxin-induced ADP-ribosylation of arginine177 has typical cytotoxic effects in cell culture (Wiegers et al. 1991). The actin cytoskeleton depolymerizes with rounding up of cells (Fig. 2) and, eventually, loss of cell adherence occurs followed by apoptosis (Heine et al. 2008). In numerous previous studies, toxin-induced depolymerization of actin was used to analyze the role of the actin cytoskeleton on various cellular functions. It was shown that toxin-induced depolymerization of the actin cytoskeleton inhibits migration of chemoattractant-evoked human neutrophils but enhances superoxide anion production and secretion of lipid mediators and inflammatory factors (Norgauer et al. 1988, 1989; Al-Mohanna et al. 1987; Grimminger et al. 1991a, b). Effects of actin-ADP-depolymerizing toxins on degranulation of mast cells (Prepens

et al. 1997, 1998; Wex et al. 1997), release of neurotransmitters from PC12 cells (Matter et al. 1989), insulin release from endocrine cells (Li et al. 1994) and on endothelial cell functions (Ermert et al. 1995, 1997; Schnittler et al. 2001; Suttorp et al. 1991) have been reported. Most of these studies were performed with rather high toxin concentrations, which clearly affected the F-actin and the actin cytoskeleton. However, not only the actin cytoskeleton but also the microtubule system is affected by binary actin-depolymerizing toxins.

2.8 Actin-Depolymerizing Toxins Induce Microtubule-Based Protrusions

At rather low concentrations, the binary actin-ADP-ribosylating toxins induce formation of cell membrane protrusions that form a network of up to 150 μm long tentacle-like structures on the surface of epithelial cells (Schwan et al. 2009) (Fig. 3). These protrusions contain microtubules. Mainly 1–2 microtubule filaments but no actin filaments are detected in the protrusions. The plus ends of the microtubules are at the distal ends of protrusions and typically decorated with EB1-3 proteins. The microtubule-based structures are highly dynamic, grow and retract quite rapidly at the beginning of the intoxication process (Schwan et al. 2009). Later (>4 h) they are more stable. Precise mechanisms of the development of the protrusions are still enigmatic. However, it has been suggested that capture proteins like ACF7 and Clasp2, which are usually involved in stabilization of growing microtubules at the actin cell cortex, are mislocalized into the cytosol after toxin treatment, thereby losing the ability to capture growing microtubules (Schwan et al. 2009). Changes in structure of microtubules induced by binary actin-depolymerizing toxins have been also reported for leukemia cells (Uematsu et al. 2007) but in this case, protrusion formation was not prominent. Role and functions of the protrusions, which form after partial depolymerization of F-actin, are not clear. Since toxins play a crucial role in host–pathogen interaction, it was a surprising but plausible finding that the toxin-induced protrusions are involved in bacterial adhesion (Fig. 3). The protrusions form a network on the cell surface where *C. difficile* bacteria are caught.

Furthermore, it is suggested that the protrusions are involved in signaling between host cells and pathogens. Electron microscopic studies of human adenocarcinoma Caco-2 cells revealed that the toxin-induced microtubule-based protrusions contain vesicles showing antero- and -retrograde trafficking (Schwan et al. 2014). Moreover, tubes of endoplasmic reticulum (ER) are in the protrusions. The ER membranes are connected to the distal tips of microtubules via the ER-membrane protein Stim1, which is a well-known regulatory protein of the calcium channel protein Orai (Fahrner et al. 2013). Notably, in the protrusions, Stim1 couples to Orai proteins, which function as channels in store-operated calcium entry (SOCE) (Taylor 2006). Thus, vesicle movements, Stim1–Orai interaction, and toxin-induced calcium signaling suggest that the protrusions are capable of specific signaling in a cilia-like fashion.

Another aspect of toxin-induced actin depolymerization is related to actin-integrin interaction, which plays a pivotal role in cell migration and interaction with the extracellular matrix (ECM). ECM proteins (e.g., fibronectin) interact with integrin receptors at the basolateral side of epithelial cells. After partial cleavage by proteases, ECM proteins are taken up via their integrin receptors and reach Rab5- and Rab11-associated vesicles (Pellinen and Ivaska 2006). Usually the vesicles recycle with the integrins in an actin-dependent manner to the basolateral membrane (Sheff et al. 2002; Durrbach et al. 2000; Powelka et al. 2004). These cycling processes are suggested to be essential for cell movement and motility. However, toxin-induced partial depolymerization of actin disturbs re-cycling of vesicles (Schwan et al. 2014). Moreover, using microtubules as tracks, the vesicles are re-routed to the apical membrane where the microtubules form protrusions. Here, fibronectin and other ECM proteins are released and function as receptor for bacterial surface proteins, thereby bacterial adhesion is increased (Schwan et al. 2014).

2.9 ADP-Ribosylation of Actin by Single Chain Toxins

Various bacterial single chain protein toxins and effectors, which do not belong to the family of binary toxins, modify actin also at arginine177 (Fig. 2). These toxins include *Salmonella Typhimurium* effector SpvB, *Aeromonas salmonicida* and hydrophila toxin AexT, *Photorhabdus luminescens* toxin Photox and *Aeromonas hydrophila* effector VgrG1. These toxins are introduced into target cells by type III (e.g., SpvB and AexT) or type VI secretion systems (e.g., VgrG1) and, thus, depend on the direct contact of bacteria with target cells. The uptake mechanism of Photox is not clear so far but it may also use type III secretion. By sequence comparison SpvB, Photox, and VgrG1 share maximal sequence similarity. AexT appears to be more distantly related. As shown in Table 1, the typical features of ADP-ribosyltransferases like the RSE motif are highly conserved.

2.10 *Salmonella typhimurium* Effector SpvB

SpvB is an ~65 kDa virulence factor of *Salmonella typhimurium* (Otto et al. 2000; Tezcan-Merdol et al. 2001; Lesnick et al. 2001). It is a two-domain protein (Fig. 2). The N-terminus part exhibits sequence similarity with parts of the linker (TcB) of the tripartite *Photorhabdus luminescens* Tc toxin (see below). The C-terminus harbors the ADP-ribosyltransferase activity that modifies actin. After invasion of host cells and formation of the so-called Salmonella containing vacuole, where the pathogens proliferate, SpvB is microinjected by Salmonella into the cytosol of host cells. SpvB ADP-ribosylates actin at arginine177 (Hochmann et al. 2006). This is responsible for cytotoxicity and apoptosis of macrophages and crucial for *Salmonella* proliferation and infection.

2.11 Photorhabdus Luminescens Photox Toxin

Photox is an ~46 kDa two-domain protein produced by *P. luminescens*, which shares 39% sequence identity overall with SpvB (Visschedyk et al. 2010) (Fig. 2). However, the C-terminal 200 amino acids exhibit 61% sequence identity with the ADP-ribosyltransferase domain of SpvB. While binary actin-targeting ADP-ribosyltransferases possess significant NAD-glycohydrolase activity and are able to split NAD^+ in the absence of their substrate actin, Photox like SpvB does not possess NAD-glycohydrolase activity, again showing the high similarity of the ADP-ribosyltransferase domain of Photox with the Salmonella effector SpvB. However, the N-terminus of Photox is largely different to SpvB. It consists of a domain that is also found by other type-III secretion effectors and might be involved in chaperone and membrane interaction. As SpvB, Photox ADP-ribosylates skeletal and non-muscle actin at arginine177. This was confirmed by mass spectrometry, also showing that arginine177 is the only modification site (Visschedyk et al. 2010). A detailed analysis of cross-linked actin-ADP-ribosylated by Photox has been performed showing no significant differences to actin-ADP-ribosylated by binary toxins with respect to actin polymerization and interaction with actin binding proteins like gelsolin (Perieteanu et al. 2010).

2.12 AexT, an ADP-Ribosylating Toxin with GAP Activity

AexT (~50 kDa) is a type III secretion effector, which is produced by *Aeromonas salmonicida* (Fehr et al. 2007) and *A. hydrophila* (Vilches et al. 2008). Also *A. salmonicida* AexT is a two-domain protein (Fig. 2). The C-terminus has ADP-ribosyltransferase activity and modifies actin at arginine177 (Fehr et al. 2007). In addition, AexT contains an N-terminal Rho-GAP domain, which possesses high enzyme activity and efficiently stimulates the GTP-hydrolysis of Rho proteins thereby it inactivates these master regulators of actin. Accordingly, the cytopathic effects of AexT, which is characterized by destruction of the cytoskeleton and rounding up of cells, depend on both direct modification of actin and inactivation of Rho proteins by its GAP activity (Fehr et al. 2007).

2.13 *Aeromonas hydrophila* VgrG1

Aeromonas hydrophila effector VgrG1 proteins possess an ADP-ribosyltransferase domain at the C-terminus of the 102 kDa protein, which exhibits high sequence similarity with SpvB and Photox (Suarez et al. 2010) (Fig. 2). VgrG (Valine-glycine repeat G) proteins are components of the bacterial type VI secretion system, which forms a syringe-like structure and is involved in cell puncturing and effector delivery into target cells (Pukatzki et al. 2009) (see below). Some of these proteins have not only puncture functions but possess also domains with effector functions. In case of *A. hydrophila* VgrG1, this domain ADP-ribosylates actin (Suarez

et al. 2010). So far, the site of actin modification has not been determined. However, VgrG1 of *A. hydrophila* causes redistribution of the actin cytoskeleton with rounding-up of cells and increase of G-actin and depolymerization of F-actin, suggesting a similar action on actin as SpvB, Photox, and binary toxins.

3 Actin Polymerization by *P. luminescens* Toxins

Bacterial protein toxins, which covalently modify actin, induce not only depolymerization of actin but also polymerization. This is true for TccC3, the active component of the tripartite Tc toxins of *P. luminescens*. *P. luminescens* are motile Gram-negative enterobacteria, which possess insecticidal activity. They live symbiotically in the intestine of entomopathogenic nematodes of the family *Heterorhabditidae* (Forst et al. 1997; Joyce et al. 2006; Waterfield et al. 2009). The nematodes invade insect larvae, where the bacteria are released by the worms by regurgitation to produce toxins, which kill the insects (Waterfield et al. 2001a, b). Thereby, an enormous source of nutrients is generated for proliferation of nematodes and bacteria. Eventually, the nematodes take up bacteria again to find and invade new insect preys.

Among a large array of insecticidal toxins produced by *P. luminescens* (see also Photox), the tripartite Tc (toxin complex) toxins have been shown to target the actin cytoskeleton (Fig. 5). Tc toxins consist of 3 components TcA, TcB, and TcC (ffrench-Constant and Waterfield 2006) and form a large complex of >1.5 MDa. TcA is the binding component, which interacts with the cell membrane of target cells (e.g., enterocytes of insect larvae) and initiates toxin uptake (Fig. 5). TcC possesses catalytic activity and TcB acts as a structural linker between TcA and TcC. Several homologs of Tc toxins exist not only in *Photorhabdus* bacteria but also in other species like *Yersinia* (Waterfield et al. 2007). Recently, the crystal structure of the TcA, TcB, and TcC complex has been obtained, exhibiting a novel type of a bacterial injection machine for translocating the toxin into target cells (Gatsogiannis et al. 2013; Meusch et al. 2014). According to these data, TcA forms pentamers of >1.5 MDa, which combine with TcB to form a cage or cocoon-like structure of TcB and TcC. The Tc component, which consists of a highly conserved N-terminal region and a C-terminal variable part, participates in cage formation with its N-terminal region, while the variable region is suggested to be inside the cage in an unfolded conformation. By gross conformational changes of the syringe-like translocation machine, the variable part of TcC, which is probably cleaved by an inbuilt protease activity, is injected into host cells. Two of the various TcC isoforms known, namely TccC3 and TccC5 possess ADP-ribosyltransferase activities. TccC5 affects the actin cytoskeleton via Rho proteins. It ADP-ribosylates Rho proteins at glutamine61/63. Thereby, Rho proteins are persistently activated and induce strong formation of stress fibers and lamellipodia.

The TcC isoform TccC3 is an ADP-ribosyltransferase that directly induces polymerization of actin. The toxin ADP-ribosylates actin in threonine148 (Figs. 1 and 6). This residue is part of the binding site of thymosin-β4. Thymosins are

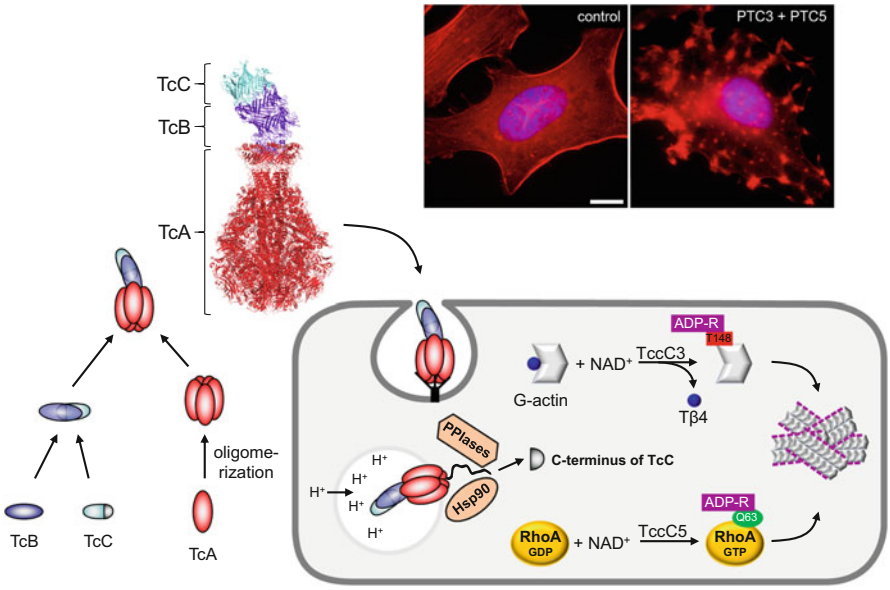

Fig. 5 Model of the structure and uptake of *Photorhabdus* toxin complexes. *P. luminescens* toxin complexes consist of three different types of proteins called TcA, TcB, and TcC. TcA forms pentamers and is the receptor-binding component, which interacts with the cell membrane of target cells and initiates uptake of the biologically active component TcC. TcB and the N-terminal region of TcC form together a large cocoon-like structure while the C-terminal part of TcC is suggested to be inside the cage in an unfolded conformation. This C-terminal domain of TcC is cleaved in an autoproteolytic process and enters the translocation channel of TcA after holotoxin formation. After receptor-mediated endocytosis and endosomal acidification, the translocation channel of TcA penetrates endosomal membranes and the C-terminal part of TcC is injected into the cytosol of host cells. The TcC isoforms TccC3 and TccC5 possess ADP-ribosyltransferase activities. TccC5 affects the actin cytoskeleton via Rho proteins. It ADP-ribosylates Rho proteins at glutamine61/63. Thereby, Rho proteins are persistently activated and induce strong formation of stress fibers and lamellipodia. TccC3 ADP-ribosylates actin at threonine148, thereby preventing the binding of the actin sequestering protein thymosin-β4 to actin and favoring actin polymerization. Together, TccC3 and TccC5 cause clustering of F-actin. The upper panel shows intoxication of HeLa cells with *Photorhabdus luminescens* ADP-ribosylating toxins PTC3 (TcA + TcB-TccC3) and PTC5 (TcA + TcB-TccC5). Cells were fixed and stained with TRITC-conjugated phalloidin and DAPI (scale bar, 10 μm). Crystal structure of TcA is from PDB 4O9Y and of TcB-TccC3 is from PDB 4O9X

~5 kDa peptides of 42–45 amino acids (Mannherz and Hannappel 2009). They bind to G-actin in an extended conformation, which sequesters actin in its monomeric form. Therefore, thymosin-β4 can inhibit salt-induced actin polymerization. ADP-ribosylation by TccC3 inhibits thymosin-β4-actin interaction, and this might increase G-actin availability for polymerization. Notably, in contrast to actin-ADP-ribosylation at arginine177, toxin-induced attachment of ADP-ribose to actin at threonine148 also occurs with polymerized actin (Lang et al. 2010). This is in line with the localization of threonine148 in F-actin (Fig. 6).

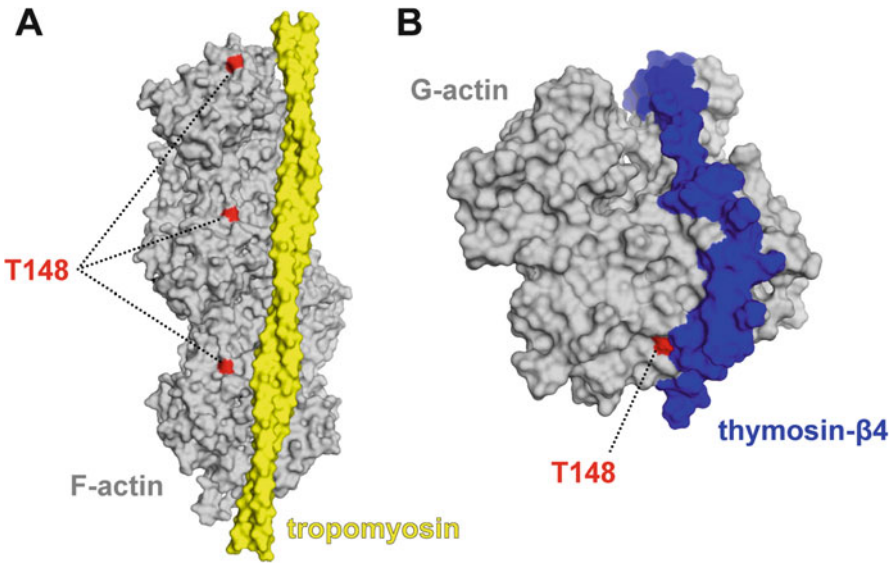

Fig. 6 Localization of threonine148 (T148) in actin. (**a**) Space-filling model of F-actin (*grey*) in complex with tropomyosin (*yellow*). Data are from PDB 3J8A. In F-actin, T148 (*red*) is accessible for modification by *Photorhabdus luminescens* toxins TccC3. (**b**) Interaction of thymosin-β4 with actin in a space-filling model. The ~5 kDa thymosin-β4 (*blue*) interacts with actin (*grey*) in an extended conformation partially covering residue T148 (*red*) of actin. Data are from PDB 1UY5

4 Cross-Linking of Actin by Bacterial Toxins

Cross-linking is another type of toxin-induced covalent modification of actin. Various bacterial pathogens produce toxins that possess a conserved actin cross-linking domain (ACD). At first, the actin cross-linking activity was identified in a *Vibrio cholerae* toxin called MARTX (multifunction-autoprocessing repeats-in-toxin) (Fullner and Mekalanos 2000; Satchell 2015). *Vibrio cholerae* MARTX ($MARTX_{V.c.}$) is a multimodular single chain toxin that contains glycine rich repeats at the N and C-terminus, which are suggested to be involved in pore formation and translocation into the cytosol (Satchell 2015) (Fig. 7). $MARTX_{V.c.}$ possesses three effector domains, which are located in the middle part of the toxin. In addition, it carries an autoprocessing protease domain. The effector domains are the Rho-inhibitory domain (RID) that inactivates Rho proteins at the cell membrane, the alpha/beta hydrolase (ABH), which was shown to activate Cdc42 (Dolores et al. 2015) and the actin cross-linking domain ACD. All three domains are translocated into the cytosol where they are released by activity of the protease domain. This protease is related to the autoprocessing protease of large clostridial glycosylating toxins and is similarly activated by cytosolic inositol hexakisphosphate.

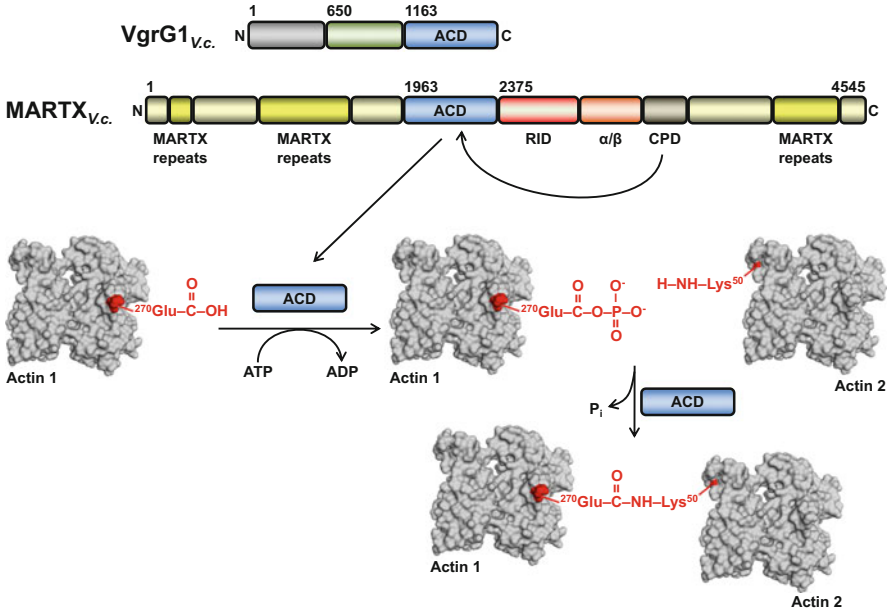

Fig. 7 Structure and function of actin-cross-linking toxins. VgrG1 from *Vibrio cholerae* harbors an actin cross-linking domain (ACD) at its C-terminus. VgrG1 proteins are part of the type VI secretion system, which is present in many Gram-negative pathogens. The N-terminal and middle part of VgrG1 harbors domains with similarity to bacteriophage tail spike complex like proteins, which might function as a translocon. MARTX (Multifunctional, autoprocessing RTX toxin) of *Vibrio cholerae* is a large multimodular protein, which consists of several conserved glycine-rich RTX repeats (MARTX repeats), a Rho GTPase inactivating domain (RID), a α/β hydrolase (α/β), a cysteine protease domain (CPD), and an actin cross-linking region (ACD). The CPD is involved in mobilization and release of the ACD (arrow), which then catalyzes cross-linking of G-actin. Cross-linking is caused by bond formation between glutamate270 and lysine50 of two actin molecules. ATP is required for formation of the glutamyl phosphate intermediate. Structural data are from PDB 1ATN

ACD catalyzes the cross-linking of G-actin to form dimers, trimers, and higher order oligomers. Thereby, the formation of actin filaments is inhibited and the physiological dynamics of actin polymerization/depolymerization are blocked. In cell culture, this results in rounding up of cells.

ACD domains are also present in MARTX toxins produced by *A. hydrophilia* (Suarez et al. 2012) and *Vibrio vulnificus*, which are both aquatic pathogens and involved in intestinal and extra-intestinal infections in humans (Satchell 2015). Similarly, as for $MARTX_{V.c.}$, the toxin from *A. hydrophilia* causes actin cross-linking in vitro and disrupts the actin cytoskeleton in vivo which results in apoptosis of target cells (Suarez et al. 2012).

4.1 Functional Consequences of Actin Cross-Linking by ACDs

ACDs cause cross-linking of actin between lysine50 and glutamate270 (Kudryashov et al. 2008) (Fig. 7). The reaction is highly specific. Exchange of lysine50 or glutamate270 blocks cross-linking. Lysine50 is located in the DNase-I binding loop or D-loop of subdomain 2 of actin (Kabsch et al. 1990). This region is often disordered in G-actin crystal structures, can adopt different kinds of conformation, and is flexible. The D-loop is involved in intersubunit contacts also explaining why cross-linked actin cannot polymerize. Glutamate270 is located in subdomain 3 and is part of a loop, which is the connection to subdomain 4. In the F-actin structure, lysine50 and glutamate270 are not in close contact. Therefore, cross-linking is not compatible with the F-actin structure. The functional consequence is the inhibition of actin polymerization and blockade of actin functions. For host–pathogen interactions, this means inhibition of migration of immune cells or blockade of phagocytosis.

At least in vitro, the rate of actin cross-linking induced by ACD is rather low (Heisler et al. 2015). Considering the large pool of actin in cells, the rapid effects of ACD on cell morphology are difficult to explain. Moreover, only a small fraction of actin modification by ACD appears to be sufficient to induce major cellular effects. The answer to these questions may be the recent finding of the role of cross-linked actin on formin-regulated actin polymerization (Heisler et al. 2015) (Fig. 8). Formins are crucial regulators of actin nucleation and elongation. Humans possess 15 different formins, which can be listed in 7 subfamilies. Typical for formins are three highly conserved formin homology regions (FH1, FH2, and FH3). The proline rich FH1 region, which plays an important role in enhancing the velocity of formin-induced F-actin elongation (Kovar et al. 2006) binds profilin-actin. The FH2 domain forms dimers, which arrange in a donut-like fashion by head to tail assembly and causes nucleation of actin polymerization and elongation of actin filaments. The N-terminal FH3 domain forms an auto-inhibitory connection with the C-terminal Dia-Autoregulatory-Domain (DAD). Inhibition is released by binding of Rho proteins to the GTPase-binding domain (GBD), which allows FH2 dimer formation and nucleation and elongation of actin filaments. Heisler et al. found that ACD-cross-linked actin binds with high affinity (low nanomolar range) to formins (e.g., mDia1/mDia2), thereby inhibiting nucleation as well as elongation of actin. The inhibitory effects were less prominent with ACD-induced actin dimers than with oligomers, suggesting multivalent interaction. Thus, ACD induces the formation of toxic products (cross-linking products) that block actin regulation with high potency, although only a small fraction of actin is modified.

4.2 Structure of ACD and Mechanism of Actin Cross-Linking by VgrG1

ACD is not only a toxin component of multimodular MARTX but is also a bacterial effector of type-VI secretion systems. Type-VI-secretion systems are encoded by

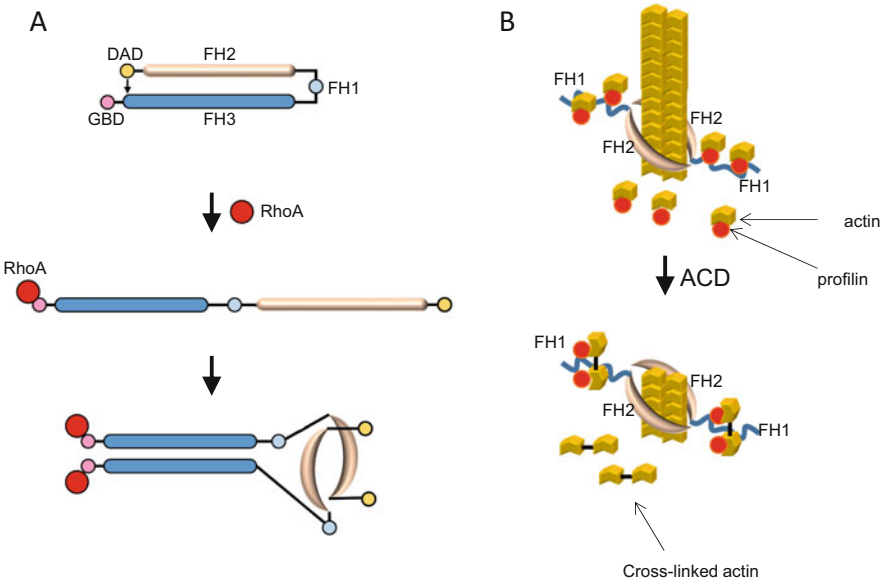

Fig. 8 Cross-linked actin inhibits formin-dependent actin elongation. (**a**) Activation of formins. Formins possess a proline rich FH1 domain, which interacts with profilin-actin. The FH2 region is involved in actin nucleation and elongation. The FH3 domain is involved in autoinhibition by the DAD (DIA autoregulatory domain). Binding of Rho proteins (RhoA) to the GTPase-binding domain (GBD) disinhibited formins and the FH2 region forms a doughnut like structure, which is important for actin nucleation. (**b**) Under normal conditions, profilin-actin binds to FH1 domain of formins to accelerate actin filament nucleation and elongation. Cross-linked actin interacts with profilin and binds with high affinity to FH1 but blocks actin elongation. Modified from (Baarlink et al. 2010)

conserved clusters of 15–25 genes, which are found in many Gram-negative bacteria (Pukatzki et al. 2009; Ho et al. 2014). Many essential proteins of this secretion system are related to the tail spike of T4 bacteriophage components. In many cases, Hcp (hemolysin-coregulated protein) proteins are the structural and functional basis of this system. Hcps form hexameric rings with a central lumen of ~40 Å. Around this tail tube is a sheath of contractile elements. Contraction of the sheath causes extension or injection of the tail tube into target cells, which can be bacterial or eukaryotic cells. On top of the Hcp tail is the VgrG protein, which is involved in piercing the target cell. The VgrG1 protein of *V. cholerae* contains a C-terminal extension with an effector domain that is ~60% identical to ACD of $MARTX_{V.c.}$ and able to cross-link actin (Pukatzki et al. 2007, 2009). The crystal structure of VgrG1 ACD has an overall V-shape, exhibiting the proposed catalytic site in a cleft between the two arms of the three-dimensional structure. The left arm of the V is mainly formed by β-strands, while the right arm is formed by seven α-helices and does not contain any β-strand. Although the fold of ACD is unique, the protein exhibits some similarity with glutamine ligase/γ-glutamyl-cysteine

synthetase (GSC ligase, PDB 1VA6) (Durand et al. 2012). Importantly, most of the catalytic residues of GSC ligase are shared with ACD. Cross-linking of actin by ACD depends on divalent cations and ATP (Fig. 7). Here, ATP is required for ACD activation and not for actin. Without ATP the N-terminal part of ACD is located in the catalytic cleft. Upon ATP binding this N-terminal part is dislodged from the catalytic cleft and catalysis can occur. Moreover, ATP is essential for catalysis, because glutamine270 interacts with the γ-phosphate of ATP to form a glutamyl phosphate intermediate, which could be directly detected. Subsequent hydrolysis of the intermediate allows cross-linking with lysine50 of a second actin molecule (Kudryashova et al. 2012; Durand et al. 2012; Satchell 2009). Similar mechanisms have been proposed for all ACDs including MARTX toxins.

5 Conclusions

Actin is a highly conserved protein, which plays a crucial role in numerous essential cellular functions. So far three different types of bacterial protein toxins have been identified, which affect the physiological functions of actin. One large toxin group causes depolymerization of F-actin by ADP-ribosylation of arginine177. So far, only one toxin is able to enhance actin polymerization by ADP-ribosylation of threonine148. A third group of toxins causes cross-linking of actin between glutamate270 and lysine50, thereby inhibiting actin polymerization. Thus, it appears that the spectrum of toxin mechanisms directly targeting actin is rather limited. By contrast, a large number of toxins and effectors, target regulators of actin functions like Rho proteins or their GEF and GAP proteins. One can speculate that bacteria favor the manipulation of the actin cytoskeleton by attacking actin regulators compared to covalent modifications of the actin itself, especially when a reversible manipulation is more appropriate than a permanent modification of actin.

Although the progress in research on actin-targeting toxins has been highly successful during recent years, many important questions remain. In many cases, the precise pathophysiological role of toxin-induced actin modification is not well understood. What are the consequences of actin modification for the infection process and what are the consequences of the attack of the actin cytoskeleton for the inflammatory responses of the host. Because many studies with toxins have been performed in cell culture, the answers to these intriguing questions remain elusive. Therefore, it is crucial to get further insights into pathophysiological consequences of toxin-induced actin modification in whole tissues and, better, in intact organisms.

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Common Themes in Cytoskeletal Remodeling by Intracellular Bacterial Effectors

Guy Tran Van Nhieu and Stéphane Romero

Contents

Abstract

Bacterial pathogens interact with various types of tissues to promote infection. Because it controls the formation of membrane extensions, adhesive processes, or the junction integrity, the actin cytoskeleton is a key target of pathogens

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during infection. We will highlight common and specific functions of the actin cytoskeleton during bacterial infections, by first reviewing the mechanisms of intracellular motility of invasive *Shigella*, *Listeria*, and *Rickettsia*. Through the models of EPEC/EHEC, *Shigella*, *Salmonella*, and *Chlamydia* spp., we will illustrate various strategies of diversion of actin cytoskeletal processes used by these bacteria to colonize or breach epithelial/endothelial barriers.

Keywords

Actin-based motility • *Chlamydia* • Cytoskeleton • EPEC/EHEC • Invasion • *Listeria* • *Rickettsia* • *Salmonella* • *Shigella* • Type III effectors

1 Introduction: Cytoskeletal Remodeling During Bacterial Infections

The ability of the pathogens to promote disease depends critically on their ability to adhere to the target cells. For this, bacteria have developed a variety of adhesins, surface appendages such as fimbriae or pili, often acting as lectins, binding to sugars that are present at the intestinal mucosal surface of the host cells. For these extracellular pathogens, the disease symptoms may be associated with the secretion of toxins or cytotoxins, leading to the destabilization of tissue integrity and loss of homeostasis (Aktories et al. 2011; Lemichez and Aktories 2013). In some instances, however, the loss of tissue homeostasis is not linked to the production of secreted toxins, but to the pathogen's ability to reorganize the actin cytoskeleton of host cells.

Invasive bacterial pathogens have evolved a diversity of specialized secretion systems allowing the specific injection of effectors into target cells which, as opposed to diffusely acting toxins, function at the close to precisely divert or reprogram processes of the infected cell. The pathogenic bacteria discussed here are Gram negative and express the T3SS protein complex that serves to inject effectors into host cells (for details, see below). For all infectious processes, one can distinguish three phases: at the onset of infection, bacterial pathogens promote their adherence or internalization beyond epithelial or endothelial barriers (Fig. 1, 1). Adhesins or invasins expressed by these bacterial pathogens may show specificities for receptors reflecting tissue tropism. In a second phase and depending on the pathological strategies, disease progression depends on the bacterial ability to multiply within the epithelial/endothelial tissue, to spread from cell to cell using actin-based motility, or to disseminate to deeper tissues (Fig. 1, 2). Specifically for pathogens responsible for chronic infection, persistence may be determined by the bacterial ability to survive within macrophages and reach deeper tissues. Finally, acute phases are associated with the loss of tissue integrity corresponding to the pathogen's replicative niche, associated with uncontrolled bacterial replication and inflammation (Fig. 1, 3). These phases can be accompanied with the release of bacteria from lysed infected cells, the shedding of bacteria-loaded cells, or cell buds

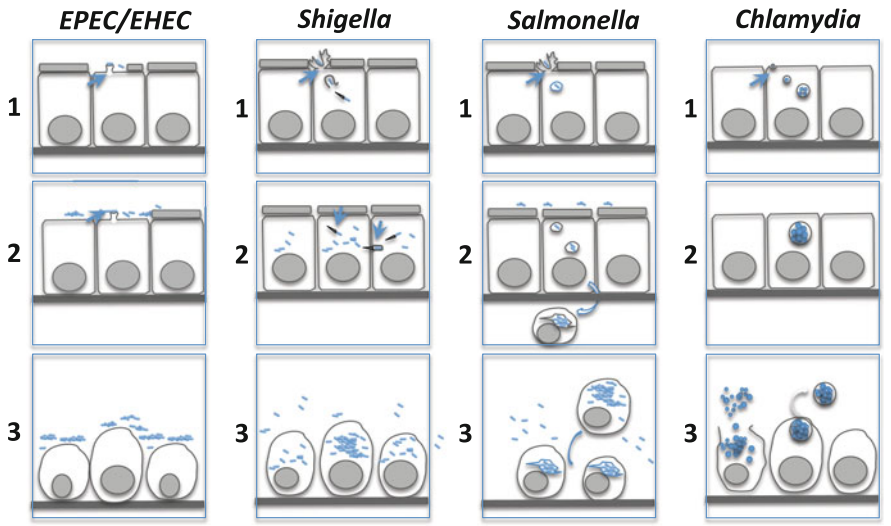

Fig. 1 Colonization and breaching of epithelial/endothelial tissues by T3SS-expressing pathogens. Epithelial/endothelial interaction models of various T3SS-expressing bacteria are depicted. Three stages implicating cytoskeletal reorganization can be distinguished: (**1**) an initial interaction stage, leading to the injection of T3 effectors inducing adhesion and pedestal formation for EPEC/EHEC, or invasion for *Shigella*, *Salmonella*, and *Chlamydia* (*arrow*). Following invasion, *Shigella* rapidly lyses the phagocytic vacuole, while *Salmonella* and *Chlamydia* reside and multiply within vacuoles. For *Chlamydia*, invasion and intracellular replication occur through the EB (*small blue circle*) and RB (*bigger blue circle*) forms, respectively; (**2**) a replication/dissemination stage, where EPEC may surf at the surface of infected epithelial cells, *Shigella* uses actin-based motility to spread from cell to cell, and *Salmonella* may infect macrophages and disseminate to other tissues; (**3**) an acute phase, where bacterial replication is not controlled by host responses, leading to loss of tissue integrity. This stage is usually associated with mounting inflammation and in the case of invasive bacteria, release of bacteria in the extracellular milieu. Such release may correspond to lysis of infected cells or an egress strategy in the case of cells infected with bacteria that freely replicate in the cytosol for *Salmonella* or the budding of *Chlamydia*-containing vesicles

from infected tissues (Fig. 1, 3). In this chapter, we will mostly focus on the initial phases of bacterial infection corresponding to the interactions with epithelial or endothelial tissues.

2 Bacterial Actin-Based Motility (ABM)

2.1 The ABM Input on Fundamental Principles of Actin Dynamics

A number of invasive bacterial pathogens such as *Shigella* sp., *Listeria monocytogenes*, *Burkholderia pseudomallei*, *Mycobacterium marinum*, and *Rickettsia* sp. induce actin comet tails to move intracellularly and spread from cell to cell (Kuehl et al. 2015; Stevens et al. 2006; Truong et al. 2014; Welch and Way 2013; Willcocks et al. 2016).

Actin-based motility of intracellular pathogens has provided key insights into the mechanism regulating actin polymerization. Intracellular motile bacteria were initially observed as inducing the polymerization of long actin comet tails associated with the spreading in epithelial or endothelial cells (Kuehl et al. 2015; Welch and Way 2013). Intracellular motility is promoted by the bacterial surface proteins IcsA/VirG for *Shigella* and ActA for *Listeria* (Bernardini et al. 1989; Kocks et al. 1992), the latter being key to the identification of the first cellular endogenous actin nucleator, the Arp2/3 complex from platelet extracts (Welch et al. 1997). ActA and IcsA, localized at one bacterial pole, induce the formation of actin comet tails by directly or indirectly activating the Arp2/3 complex, respectively (Egile et al. 1999; Suzuki et al. 1998; Welch et al. 1998).

Major advances were subsequently achieved using purified proteins to reconstitute bacterial motility in vitro (Loisel et al. 1999). ActA-mediated actin comet tail formation was shown to only require at minima, the Arp2/3-mediated nucleation of filaments, ADF/cofilin, and capping proteins to maintain a high pool of actin monomers at steady state (Loisel et al. 1999). Profilin and the actin-bundling protein alpha-actinin render bacterial motility more effective. (1) ADF/cofilin enhances the depolymerization rate of actin filaments, resulting in an increase in the pool of monomeric ADP-G-actin; (2) profilin binding to ADP-G-actin accelerates nucleotide exchange to regenerate ATP-G-actin and catalyzes its exclusive assembly at barbed ends; (3) by preventing barbed end growth, capping proteins funnel the polymerization flux to newly created uncapped filaments and increase the Arp2/3 branching frequency through the capping of newly created filaments (Akin and Mullins 2008; Pantaloni et al. 2001; Wiesner et al. 2003). Actin filaments polymerizing against the bacteria create a propelling pushing force (Loisel et al. 1999; Theriot et al. 1994) that has been modeled at different scales. At the molecular scale, in the "elastic Brownian ratchet" model, the incorporation of monomers at the filament's end is rendered possible by the undulations of the actin fiber, the growing filament providing an elastic force pushing the bacteria forward (Mogilner and Oster 1996, 2003). At the mesoscopic scale, the actin meshwork growing from the bacterial surface is considered as a viscoelastic gel, responsible for elastic forces in addition to those mediated by the pushing actin comet tail and accounting for the saltatory intracellular motility observed for some bacteria (Boukellal et al. 2004; Gerbal et al. 2000; Jasnin et al. 2013; Soo and Theriot 2005).

2.2 Bacterial Models of Actin-Based Motility

Shigella flexneri and *S. dysenteriae*, the causative agents of bacillary dysentery, invade the colonic mucosa where they elicit an intense inflammation responsible for the tissue destruction. Following uptake by intestinal epithelial cells, *Shigella* lyses the phagocytic vacuole to replicate in the cytosol. During this replication phase,

Shigella uses actin-based motility to spread from cell to cell (Fig. 2). *Shigella* also uses a T3SS to invade and to disseminate within intestinal epithelial cells. To activate Arp2/3, the *Shigella* IcsA/VirG protein mimics the activated form of Cdc42 by binding to the N-terminal autoregulatory domain of the N-WASP NPF (Fig. 2a). This interaction releases the C-terminal VCA domain of N-WASP, which binds to and activates Arp2/3 (Egile et al. 1999; Rohatgi et al. 1999; Suzuki et al. 1998, 2002). In addition, the Btk and Abl kinases that phosphorylate

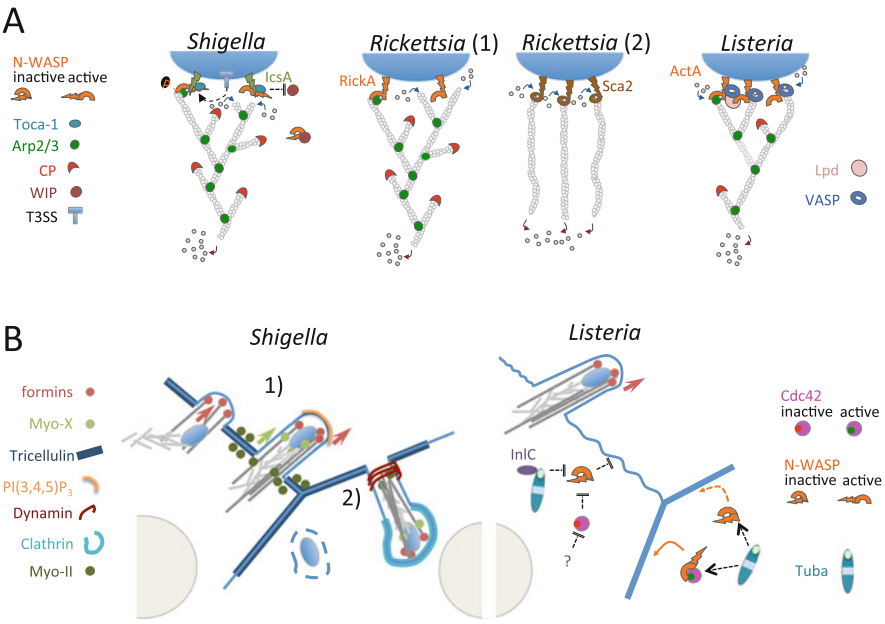

Fig. 2 Bacterial actin-based motility and intercellular spreading. (**a**) Bacterial actin-based motility. The *Shigella* autotransporter surface protein IcsA binds to N-WASP and activates Arp2/3-dependent actin polymerization to generate an actin comet tail composed of branched filaments. T3SS also triggers the recruitment of Toca-1 that relieves the WIP-mediated inhibition of N-WASP. The *Rickettsia* uses two modes of actin-based motility via distinct bacterial surface proteins: (*1*) RickA directly binds to Arp2/3 to induce actin polymerization; (*2*) at later stages of infection, Sca2 induces the polymerization of unbranched actin filaments, in a formin-like manner. The *Listeria* ActA protein activates Arp2/3 and binds to VASP. Lamellipodin (Lpd) is also recruited at the bacterial surface to enhance VASP-dependent bacterial motility and directionality. (**b**) The formation of *Shigella* protrusion is favored by the formin Dia1 and by tricellulin at multi-junctions (*1*). Protrusion elongation is facilitated by Myosin-X. $PI(3,4,5)P_3$ at the protrusion membrane may trigger the activation of PI3K and the phagocytosis of the protrusion by the recipient cell in a noncanonical clathrin and dynamin-dependent pathway (*2*). *Listeria*-containing protrusion formation is favored by the formins Dia1, Dia2, and Dia3. The InlC protein releases cell-cell junctions' tension by interfering with tuba-mediated activation of N-WASP, thus favoring protrusion formation. Tuba contributes to cell-cell junctions' tension trough its GEF activity for Cdc42 or by allowing the recruitment of N-WASP at cell junctions. The tuba GEF (*blue inset*) and SH3 (*pale green*) domains are depicted. An unidentified *Listeria* factor (?) also contributes to cortical tension release by inhibiting Cdc42

N-WASP (Burton et al. 2005; Dragoi et al. 2013), as well as Toca-1, which prevents N-WASP inhibition by WIP, are also major components required for *Shigella* actin-based motility (Ho et al. 2004; Leung et al. 2008).

Rickettsia spp. are obligate intracellular bacteria that include *R. parkeri*, *R. rickettsii*, and *R. conorii*, the causative agents of spotted fever. *Rickettsia* also uses actin-based motility to disseminate within tissues. *Rickettsia*-mediated actin comet tails consist of long and parallel actin filaments (Gouin et al. 1999; Van Kirk et al. 2000). The discovery of RickA, a surface protein of *Rickettsia* that acts as a NPF for Arp2/3 through a canonical VCA domain, was puzzling, since this protein induced a branched actin network (Gouin et al. 1999; Harlander et al. 2003; Jeng et al. 2004). However, recent studies showed that *Rickettsia* actin-based motility involves two distinct phases (Fig. 2a). The first phase based on RickA activity occurs within the first 2 h following bacterial internalization, during which short Arp2/3-containing actin comet tails propel bacteria in the cytosol at a slow speed. The second phase occurring after 12–24 h post internalization is based on Sca2, another *Rickettsia* surface protein (Reed et al. 2014). Sca2 nucleates unbranched actin filaments while remaining attached to the barbed end of elongating actin filaments, thus accelerating actin assembly in a formin-like manner, but using a different molecular mechanism (Haglund et al. 2010; Reed et al. 2014) (Fig. 2a). This formin-like nucleation and accelerated processive elongation of actin filaments explain the structure of actin comet tails associated with bacteria and the enhanced velocity of bacterial propulsion (Gouin et al. 1999; Reed et al. 2014).

Listeria monocytogenes is an enteroinvasive bacterium responsible for septicemia, encephalomeningitis, and abortion. Following ingestion and invasion of the intestinal mucosa, *Listeria* uses actin-based motility to disseminate within infected tissues. The *Listeria* ActA protein acts as a NPF, which, through a VCA domain located at its N-terminal part, directly activates Arp2/3 (Egile et al. 1999; Welch et al. 1998). In addition to Arp2/3, profilin and VASP have been involved in the formation of *Listeria* actin comet tails (Smith et al. 2010) (Fig. 2a). The proline-rich domain of ActA interacts with the EVH1 domain of VASP, and profilin is recruited through the proline-rich domain of VASP (Niebuhr et al. 1997; Reinhard et al. 1995). VASP tetramers in cooperation with profilin can processively accelerate barbed end elongation in a formin-like manner (Breitsprecher et al. 2011). Thus, ActA may nucleate new actin filaments through Arp2/3 activation and processively elongate these filaments by recruiting the VASP/profilin (Fig. 2a). This model is consistent with the role of VASP in the shape of *Listeria* actin comet tails and the increased bacterial velocity, as well as in the elastic modulus of the actin comet tails (Samarin et al. 2003; Auerbuch et al. 2003; Diakonova et al. 2007; Suei et al. 2011). Lamellipodin, a cytoskeletal protein involved in cell migration, is also recruited at *Listeria* surface, via its Ena-VASP homology 1 (EVH1) and plekstrin homology (PH) domains interacting, respectively, with VASP and $PI(3,4)P_2$ present at the surface of intracellular bacteria in an ActA-dependent manner (Wang et al. 2015). As for VASP depletion, depletion of Lamellipodin also affects *Listeria* intracellular

velocity and directionality, suggesting a role for Lamellipodin as a scaffold protein organizing ActA and VASP at the surface of bacteria.

Propulsion of bacteria against the plasma membrane induces filopodia-like protrusions that are internalized by the neighboring cells allowing bacterial spreading. However, recent studies showed that other cellular factors facilitate the formation of bacteria-containing protrusions. For *Shigella*, the formin nucleator Dia might contribute to force generation required to form protrusion at cell-cell junctions (Heindl et al. 2010). Myosin-X could also contribute to protrusion formation by carrying membranes or host cell components at the protrusions' tip (Bishai et al. 2013) (Fig. 2b). Phagocytosis of the protrusions also requires active signaling to neighboring cells, such as a clathrin noncanonical pathway implicating the multicellular junction protein tricellulin, PI3K, and dynamin and myosin II (Fukumatsu et al. 2012; Lum and Morona 2014; Rathman et al. 2000) (Fig. 2b).

In the case of *Listeria*, the 3 Dia formins may also play a similar role in facilitating protrusion formation (Fattouh et al. 2015). In addition, the high tension at cell-cell contacts was shown to limit the formation of protrusions and cell-to-cell spread (Fig. 2b). By acting on tuba, an activator of N-WASP and GEF for Cdc42, the *Listeria*-secreted protein InlC releases junctional tension (Rajabian et al. 2009). Clearly, beyond actin-based motility, much is yet to be learned about cytoskeletal processes that involved bacterial dissemination in tissues.

3 Induction and Hijacking of Filopodia by EPEC/EHEC and *Shigella*

Filopodia are fingerlike thin cell extensions present on the surface of a variety of cell types, including epithelial and endothelial cells. Filopodia are generally considered as sensing organelles that cells use to probe their environment to establish adhesion structures. An increasing number of viral and bacterial pathogens such as HIV, or MLV, *Yersinia*, *Shigella*, or *Borrelia* have been reported to divert filopodial function to adhere to or invade host cells (Hoffmann et al. 2014; Lehmann et al. 2005; Romero et al. 2011; Young et al. 1992).

Enteropathogenic *E. coli* (EPEC) is an important cause of infantile diarrhea in developing countries. Upon interaction with enterocytes, EPEC induces the disappearance of microvilli in a process referred to attaching-effacing (A/E) lesions. The A/E lesions induced by EPEC/EHEC strains are closely linked to virulence, with the loss of electrolytes from infected enterocytes and epithelial integrity. EPEC/EHEC A/E lesions are associated with the formation of F-actin-rich structures supporting bacteria coined "pedestals" (Fig. 3a). The physiological relevance of EPEC/EHEC pedestals has been a matter of debate.

During early phase of adhesion, EPEC and EHEC induce filopodia-like structures at the sites of bacterial contact with epithelial cells (Kenny et al. 2002). These filopodia only form transiently and disappear as actin pedestals are formed. EPEC-/EHEC-induced filopodia are triggered by the type III effector MAP, acting as a GEF for the Cdc42 GTPase (Huang et al. 2009). In addition to its GEF domain,

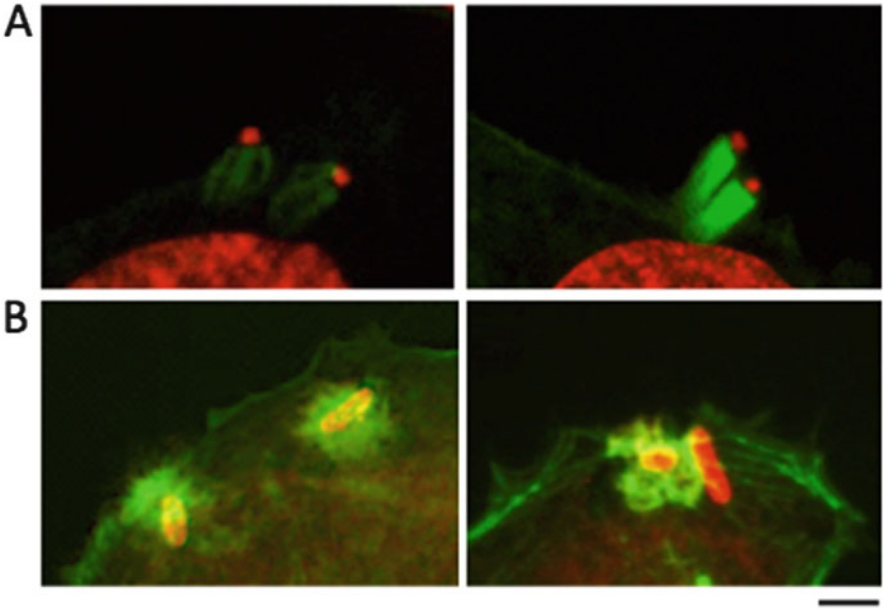

Fig. 3 Immunofluorescence micrographs of EPEC- and *Shigella*-induced cytoskeletal reorganization at the surface of epithelial cells. HeLa cells were infected for 45 min (**a**) or 15 min (**b**) with bacteria. Samples were fixed and processed for F-actin (*green*) and bacterial (*red*) staining. Various F-actin-containing structures are observed. EPEC induces the formation of pedestals composed of several actin shafts, reminiscent of the "medusa" phenotype (**a**, *left*; (Smith et al. 2010)) and pedestal-like structures (**a**, *right*). *Shigella* induces the formation of actin foci-forming filopodia (**b**, *left*) and ruffles (**b**, *right*). Scale bar = 5 μm

MAP contains a carboxyterminal PDZ (PSD-95/Disc Large/ZO-1) domain that interacts with the ezrin-binding protein Epb50. Binding of MAP to Ebp50 and ezrin promotes the formation of MAP scaffolds at the plasma membrane, which determine the local activation of Cdc42 and actin polymerization (Alto et al. 2006). The role of MAP and filopodia during EPEC/EHEC infection remains unclear. MAP was shown to synergize with the Tir-translocated effector to promote EPEC/EHEC invasion of epithelial cells (Jepson et al. 2003). An EPEC mutant lacking MAP induces the formation of pedestal structures, which do not appear to support the stable adhesion of bacteria (Shaw et al. 2005). This evidence suggests that, rather than performing antagonistic functions, MAP and other type III effectors contribute to the "maturation" of pedestals through the activation of Cdc42 and actin polymerization. MAP-induced filopodia may increase the surface of contact between bacteria and the plasma membrane, thereby stabilizing EPEC/EHEC adhesion upon pedestal formation.

To invade host cells, viruses glide along the side of filopodia in a myosin-II-dependent process. In contrast, prior to invasion of epithelial cells, *Shigella* interacts with the tip of filopodia, presumably via the T3SS tip complex. Following

contact, filopodia retract and drag bound bacteria into contact with the cell body (Fig. 3b). This retraction process depends on the activity of MAP kinase Erk, which controls the retrograde flow of actin filament bundles in filopodia in epithelial cells (Romero et al. 2011). This retrograde flow is driven by the polymerization of actin in the cortical actin network that constantly pulls on filopodial actin filaments. Upon bacterial interaction, filopodia retract because actin polymerization at filopodia tips fails to compensate the pulling of filopodia actin filaments by the retrograde flow (Bornschlogl et al. 2013). The mechanism underlying the decrease of actin polymerization at the filopodia tip following bacterial contact has not been elucidated, but is likely to involve cell receptors of the T3SS tip complex and barbed end capping proteins. Filopodial capture may represent an explanation for the paradoxical cell invasion properties of *Shigella* in the absence of constitutive cell-binding activity.

4 Intracellular Delivery of Bacterial Effectors via "Injectisomes"

To date, up to nine types of secretion systems have been described in bacteria, three of which enable the delivery of effectors into host target cells, illustrating the striking capacity of pathogens to adapt and improve molecular machines. Historically the type III secretion system (T3SS) was the first one described to allow the injection of bacterial effectors into host cells. T3SSs are widely spread among Gram-negative bacterial pathogens and are expressed in a wide variety of enteropathogens, such as EPEC/EHEC, *Salmonella enterica*, *Yersinia*, or *Shigella* spp. The structure of these macromolecular machines is related to the bacterial flagella, with the notable difference that the basal body is prolonged by a needle and a so-called tip complex. This tip complex negatively regulates secretion and allows sensing of host cell membranes (for review see Galan et al. 2014). While there may be variations between bacterial models, it is generally admitted that the mechanism of type III secretion is conserved. Upon host cell contact, the T3SS activity is triggered, leading to the insertion of two hydrophobic proteins, called the translocon, into the host cell plasma membrane. The translocon forms a ring connecting to the T3SS needle through which type III effectors are channeled directly into the host cell cytosol without extracellular steps. In the case of *Yersinia*, however, it has been shown that type III substrates can also follow an alternate, AB5-toxin-like route of delivery into host cells (Akopyan et al. 2011). Because of the estimated inner diameter of the T3SS needle or the T4SS secretion conduit (1.5–3 nm), it is thought that substrates are secreted in a fully or partially unfolded state, to adopt their final conformation upon translocation into host cells. These considerations are important, because post-secretion folding may potentially provide means to control the effector activity, for example, by posttranslational modification or association with host cell cofactors (Cui and Shao 2011).

4.1 WXXXE Effectors

A variety of EPEC, *Shigella*, and *Salmonella* type III effectors contain the "WXXXE" sequence (Orchard and Alto 2012). Structural and in vitro assays confirmed that WXXXE effectors are GEFs for Rho GTPases. Strikingly, structural analysis of WXXXE effectors revealed a highly conserved fold, with two helical bundles forming a V-shaped structure connected by a "catalytic" loop. The conservation of the WXXXE motif, located at the interface between the two bundles, is explained by its key structural role. All WXXXE effectors promote their GEF activity by interacting via key residues located in their alpha-2 helix and catalytic loop, with the Rho GTPase Switch1 and Switch2 domains (Huang et al. 2009). Sequence comparisons, supported by modeling and mutagenesis studies, indicate that the specificity of interaction is determined by contacts between residues of the alpha-2 and alpha-6 helixes of the WXXXE effector that pair to the beta-2–3 inter-switch strand residues of the GTPase (Huang et al. 2009).

5 Cytoskeletal Remodeling at the Plasma Membrane by T3SS-Expressing Bacteria

5.1 EPEC/EHEC Pedestals

Through actin treadmilling, EPEC-mediated pedestal formation is associated with the "surfing" motility of bacteria on the cell surface and has been proposed to enable bacterial dissemination over the intestinal mucosa (Lai et al. 2013; Wong et al. 2011) (Fig. 3). Alternatively, EPEC-mediated pedestal formation on epithelial cells (Fig. 3a) may reflect the diversion of cytoskeletal processes involved in the inhibition of phagocytosis by macrophages (Fallman et al. 2002; Westermark et al. 2014).

EPEC pedestal formation is dependent on the injection of effectors mediated by the T3SS. Upon cell contact, EPEC injects the Tir protein that inserts in host cell plasma membrane (Fig. 4). Folding of Tir in the plasma membrane results in the cell surface exposure of a loop, which is recognized by the EPEC/EHEC intimin, a bacterial surface adhesin containing a cell-binding domain with structural similarities with the *Yersinia* invasin protein. As commonly observed during cell outside-in receptor-mediated signaling, clustering of Tir molecules by multimers of intimin induces the tyrosyl phosphorylation of Tyr474 of Tir. Host cell tyrosine kinases involved in Tir phosphorylation include members of the Src, Arg/Abl, or Tec family kinases (Bommarius et al. 2007; Swimm et al. 2004) (Fig. 4). Tir phosphorylation probably involves loops of amplification through the recruitment and activation of various tyrosine kinases. Tir phosphorylation at Y474 mediates the recruitment of the Nck adaptor through its SH2 domain. Nck SH3 domains, in turn, allow the recruitment and activation of N-WASP, itself responsible for the activation of the Arp2/3 complex leading to pedestal formation (Campellone and Leong 2003) (Fig. 4). This view, however, is probably oversimplistic and has been

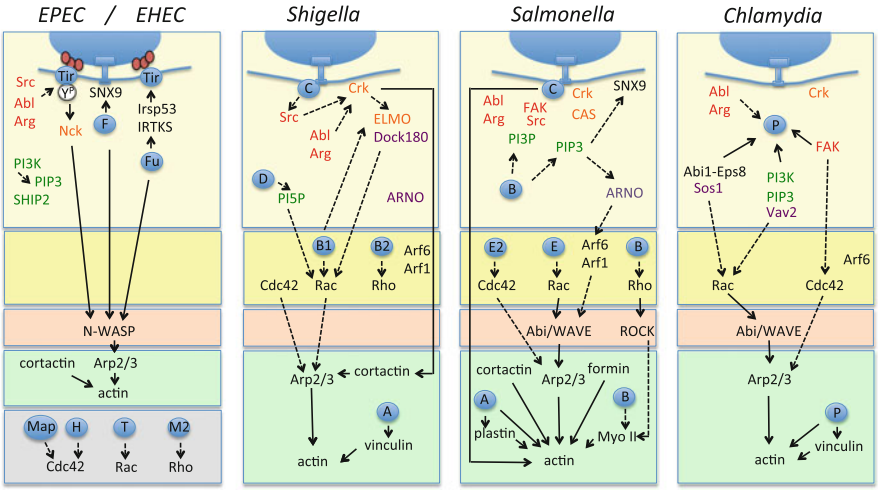

Fig. 4 Common and specific processes targeted by T3SS-expressing bacteria to induce cytoskeletal reorganization at the host cell plasma membrane. Components implicated in cytoskeletal reorganization are indicated. Boxes correspond to *pale yellow*, tyrosine kinases (*red*), adaptor (*orange*), phosphoinositides (*green*), BAR-domain-containing proteins, and GEFs (*purple*); *dark yellow*, Rho GTPases; *orange*, WAVE-family proteins; *green*, cytoskeletal proteins; and *blue circles*, type III effectors. *Solid arrows* indicate direct interaction/activation. *Dashed arrows* indicate activation. See main text for description. (EPEC/EHEC), *gray box*: type III effectors contributing to cytoskeletal reorganization but not critical for pedestal formation. *F* EspF, *Fu* EspFu/Tccp, *H* EspH, *T* EspT, *M2* EspM2. (*Shigella*), *A* IpaA, *B1* IpgB1, *B2* IpgB2, *D* IpgD. (*Salmonella*), *C* SipC, *B* SopB, *E* SopE, *E2* SopE2, *A* SipA. (*Chlamydia*), *P* TarP

challenged by the observations that N-WASP is required for the recruitment of Nck and that the Nck-binding region of N-WASP is dispensable for its localization at pedestal (Garber et al. 2012). While Tir Y474 phosphorylation is the major route for actin pedestal formation, the existence of Nck-independent pathways involving two other phosphorylated tyrosine residues of Tir could account for N-WASP recruitment in the absence of direct interaction with Nck (Lai et al. 2013).

In EHEC, tyrosine phosphorylation at Y458 (the EPEC equivalent of Y454) does not appear to play a prominent role in actin polymerization and pedestal formation (Fig. 4). Instead, the EHEC NPY458 motif was shown to bind to the I-BAR containing proteins IRTKS and Irsp53. In addition, EHEC injects the type III effector EspFu/Tccp that binds to the carboxyterminal SH3 domains of IRTKS/Irsp53 through a proline-rich repeated sequence and to N-WASP through an N-terminal helix. The EspFu N-terminal helix acts as a super-mimic that outcompetes the N-WASP inhibitory helix and relieves its intramolecular interaction with the Arp2/3-activating domain VCA (Lai et al. 2013). While this Tir "NPY"-dependent actin polymerization pathway appears to function with low efficiency in EPEC, the presence of EspFu/Tccp renders it predominant in EHEC (Campellone and Leong 2003). Other minor pathways include the EPEC Tir-dependent Nck-independent and EHEC EspFu-/Tccp-dependent N-WASP-independent

pathways (Wong et al. 2011). Interestingly, some EPEC strains also express EspF, which shares homology and can functionally substitute for EHEC EspFu/Tccp. Through its proline-rich repeats, however, EspF was shown to bind and activate Snx9, a BAR-domain protein, leading to the formation of tubular membrane structures (Alto et al. 2007). The interaction of EspF with Snx9 appears to determine its localization at the plasma membrane and N-WASP-dependent actin polymerization, although its role during bacterial infection is not entirely clear.

While MAP and Tir are present in all EPEC/EHEC strains, other EPEC/EHEC type III effectors that are strain specific might control cytoskeletal reorganization. Because the functions of these effectors are potentially antagonistic, their expression is likely to be coordinated during the course of the infection. The type III effector EspH binds the Dbl homology/pleckstrin homology domains present in endogenous GEFs, thereby inhibiting the GTPase activation (Wong et al. 2012). EspM1/2 is a WXXXE effector acting as a RhoA GEF. Expression of EspM1/2 accelerates the disappearance of pedestals and perturbs the distribution of tight junction components in polarized epithelial cells, while decreasing the epithelial barrier permeability (Simovitch et al. 2010). The function of EspM1/2 during infection is not clear; as postulated for the CNF1/2 toxins, EspM1/2 may serve to limit inflammation associated with leakage from tight junctions (Arbeloa et al. 2010). EspT, another WXXXE effector expressed only in a minority of EPEC strains, is a GEF for Cdc42 and Rac, associated with membrane ruffling and with higher invasive properties (Bulgin et al. 2009). The EspH inhibitory activity on GTPases has been linked to the ability of EPEC strains to prevent their phagocytosis by macrophages and to inhibition of MAP-induced filopodia. While inhibiting Rho GTPase activation, EspH stimulates actin polymerization and the formation of EPEC pedestals in a Tir-WIP- and N-WASP-dependent but Nck- and Rac-independent manner. These seemingly paradoxical functions of EspH have led to speculations that EPEC/EHEC strains "reprogram" signaling downstream of Rho GTPases (Cui and Shao 2011). In this view, "reprogrammed" cytoskeletal reorganization would ensue from the capacity of EspH to inhibit the activation of endogenous Rho GTPases, without affecting that of WXXXE effectors (Cui and Shao 2011).

5.2 *Shigella* Invasion

Upon host cell contact, activation of the *Shigella* T3SS leads to the insertion of the IpaB and IpaC translocon components, required for the injection of type III effectors (Picking and Picking 2016). In addition to being involved in type III effector translocation, IpaC also triggers the recruitment and activation of the Src tyrosine kinase through its carboxyterminal moiety, by a process that remains ill defined (Fig. 4). Src-dependent tyrosyl phosphorylation of cortactin allows its interaction with the Crk adaptor at the plasma membrane and actin polymerization via the cortactin Arp2/3-binding domain (Valencia-Gallardo et al. 2015). Inhibition of Src- and cortactin-mediated signaling results in actin-rich smaller structures and

in a partial defect in bacterial internalization, suggesting that rather than triggering, Src signaling amplifies actin polymerization at bacterial invasion sites (Bougneres et al. 2004). Cytoskeletal reorganization also results from the activity of injected type III effectors acting at different levels to control dynamics of actin polymerization/depolymerization. IpgB1 and IpgB2 are WXXXE effectors acting as GEFs for Rac and Rho, respectively (Alto et al. 2006). While the activation of Rac and Rho is generally considered antagonistic, both IpgB1 and IpgB2 are required for efficient *Shigella* invasion in polarized intestinal epithelial cells for reasons that are still unclear (Hachani et al. 2008). Through its GEF activity for Rac, IpgB1 stimulates actin polymerization and membrane ruffling at invasion sites, thereby amplifying *Shigella* invasion. IpgB1 has also been involved in the hijacking of the ELMO/Dock180 pathway, by acting as a mimic of the RhoG GTPase (Handa et al. 2007). In light of compelling evidence for the role of IpgB1 as a Rac GEF, how this WXXXE effector could also mimic the function of RhoG deserves clarification. Interestingly, other studies have shown that Arg/Abl are involved in actin polymerization at *Shigella* invasion sites and in bacterial entry (Burton et al. 2003; Wessler and Backert 2011). Abl/Arg are tyrosine kinases implicated in cytoskeletal reorganization downstream of growth hormone receptor signaling. Transactivation of Abl/Arg may occur upon binding of their proline-rich domain to the Crk adaptor, but the activity of these kinases is also regulated by direct interaction with cortactin. Abl/Arg can also act upstream of the Rac GTPase by stabilizing a Crk/ELMO/Dock180 complex (Wessler and Backert 2011). It is therefore likely that actin polymerization during *Shigella* invasion is subjected to loops of amplification dependent on tyrosine kinase signaling and Rac GTPase, making the deciphering of pathways downstream of *Shigella* effectors a complex task.

5.3 Salmonella Invasion

Salmonella enterica enterica regroups various serovars predominantly responsible for food-borne gastroenteritis and enteric fever. The vast majority of studies have been performed on *Salmonella enterica* serovar Typhimurium. *Salmonella* contains two pathogenicity islands SPI-1 and SPI-2, each encoding a T3SS critical for virulence. While the SPI-2 locus is implicated in intracellular survival in macrophages and bacterial dissemination to various organs, the SPI-1 T3SS is involved in the crossing of the intestinal barrier and cytoskeletal reorganization during *Salmonella* invasion (Valdez et al. 2009). Interestingly, similar actors involved in tyrosine kinase signaling have also been implicated during *Salmonella* invasion (Ly and Casanova 2009; Shi and Casanova 2006). Consistent with the notion that T3SSs may bypass receptor-mediated signaling, *Salmonella* invasion implicates many components involved in cell adhesion, with the exception of beta 1 integrins (Fig. 4). During cell adhesion, tyrosyl phosphorylation of the focal adhesion kinase FAK determines the recruitment of the Src kinase and subsequent maturation of adhesion structures through the scaffolding activity of adaptor proteins such as p130Cas (Shi and Casanova 2006) (Fig. 4). FAK and p130Cas

are also involved in *Salmonella* invasion, in a process that does appear to depend on the FAK kinase but through its scaffolding activity. Although their inactivation leads to a reduction of bacterial-induced actin foci of invasion, FAK and Cas play distinct roles during *Salmonella* invasion, since, as opposed to FAK −/− cells, remaining foci in Cas −/− cells also present a defect in phagocytic cup formation suggesting a defect in actin organization (Shi and Casanova 2006). Also, *Salmonella* invasion does not require the focal adhesion protein paxillin, suggesting that bacterial invasion implicates different levels of cytoskeletal tethering than those associated with the maturation of cell adhesion structures. Like for *Shigella*, the CrkII adaptor, as well as the Arg/Abl kinases, participates in *Salmonella* invasion suggesting common invasion mechanisms between these bacterial pathogens (Ly and Casanova 2009).

The type III *Salmonella* effector SopE is a WXXXE effector acting as a GEF for Cdc42 and Rac, implicated in actin polymerization and Arp2/3-dependent membrane ruffling during bacterial invasion (Hardt et al. 1998; Orchard and Alto 2012). SopE activates Cdc42 and Rac during bacterial invasion. The role of Cdc42 during invasion, however, requires clarification. Targeted RNAi inhibition indicated that Cdc42 was dispensable and Rac1 was essential for actin polymerization and membrane ruffling at invasion sites (Patel and Galan 2006).

The *sopE* gene is not present in all pathogenic *Salmonella enterica* serovars, as opposed to *sopE2* sharing 64% identity with *sopE*, and showing a broader specificity. As opposed to SopE, SopE2 shows GEF activity specific to Cdc42 (Friebel et al. 2001). Inactivation of *sopE* or *sopE2*, as opposed to double mutants, leads to partial inhibition of membrane ruffling and bacterial invasion, indicating a joint action of these type III effectors (Zhou et al. 2001). While actin polymerization determines membrane ruffling during the early stages of *Salmonella* contact with host cells, actin depolymerization is required to complete the internalization process. To this end, *Salmonella* injects another type III effector, SptP, into host cells, which has a dual tyrosine phosphatase and GAP activity toward Cdc42 and Rac (Stebbins and Galan 2001). The dual activity of SptP depends on two distinct domains: an N-terminal GAP domain and a C-terminal tyrosine phosphatase domain. The SptP GAP domain shares sequence and structural homologies with the *Pseudomonas* ExoS and *Yersinia* YopE type III effectors, while the tyrosine phosphatase domain shows some homology with the *Yersinia* YopH phosphatase (Stebbins and Galan 2001). The SptP GAP domain is the smallest characterized RhoGAP and is structurally distinct from endogenous GAPs. Various structural SptP features argue in favor of convergent evolution, where minimal domains of various eukaryotic GAPs appear to have been hijacked to compose a particularly effective bacterial GAP (Stebbins and Galan 2001). In particular, the catalytic mechanism of SptP is similar to that of endogenous GAPs and involves a so-called arginine finger, with an arginine residue exposed in the GTPase active site and neutralizing the negative charge at the β-phosphate during the GTPase transition state. In the case of SptP, however, the arginine residue is exposed on a helical bundle, as opposed to the loop structure of endogenous "arginine fingers." The regulation of potentially antagonistic activities of SopE/SopE2 and SptP was

proposed to be mediated by a differential stability of these type III effectors following injection into host cells, following their ubiquitination and proteasomal degradation (Kubori and Galan 2003). Imaging of the kinetics of delivery of these effectors into host cells also argues that SopE is injected faster than SptP, providing another levels of temporal control of their activity during invasion (Van Engelenburg and Palmer 2008).

T3SS-mediated invasion by *Salmonella* was shown to involve two distinct signaling pathways. In addition to the SopE-Rac-/Cdc42-dependent pathway leading to Arp2/3 complex-dependent actin polymerization and membrane ruffle formation, the type III effector SopB induced the activation of the RhoA GTPase leading to Rho kinase (ROCK) activation and bacterial uptake in a myosin-II-dependent process (Hanisch et al. 2011). In independent studies, SopB was shown to mediate the activation of the FHOD-1 formin downstream of ROCKI (Truong et al. 2013). In these aspects, *Salmonella* invasion presents similarities to CR3-mediated phagocytosis in macrophages, which involves a Rac-WAVE-Arp2/3 and a RhoA-formin-/myosin-II-dependent pathway (Sarantis and Grinstein 2012). However, it is unclear how independent the SopE- and SopB-mediated pathways are, since SopE is also involved in FHOD-1 phosphorylation, and as described earlier, SopB synergizes with SopE to mediate activation of the Abi1-WAVE complex and Arp2/3-dependent actin polymerization (Humphreys et al. 2012; Truong et al. 2013).

5.4 Chlamydia Invasion

Chlamydia trachomatis is a member of the obligate intracellular bacterial pathogens *Chlamydiae*, responsible for infectious keratoconjunctivitis, a predominant cause of blindness in developing countries, and for sexually transmitted diseases, which can lead to infertility (Bastidas et al. 2013; Mehlitz and Rudel 2013). *Chlamydia* has a biphasic cycle with elementary bodies (EBs) able to resist in the extracellular environment and invade host cells and reticulate bodies (RBs) associated with intracellular replication (Fig. 1). While the genetic manipulation of *Chlamydiae* is still at its infancy, the genomic analysis and use of orthologous systems have allowed significant advance in the study of bacterial invasion mechanisms. Various adhesins triggering host cell signaling during initial interactions with the host have been described and reviewed elsewhere (Mehlitz and Rudel 2013). Through screening using RNA interference, tyrosine kinase signaling implicating the Arg/Abl family was shown to be required for *C. trachomatis* invasion (Elwell et al. 2008; Mital and Hackstadt 2011). Remarkably, *Chlamydia* also possesses a T3SS that has been involved in the cytoskeletal reorganization during invasion, in particular, through the injection of the T3 effector TarP (Clifton et al. 2005) (Fig. 4). TarP is phosphorylated by Arg/Abl and Src kinases at the level of tyrosine-rich tandem repeats triggering the recruitment of the Rac GEFs Sos1 and Vav2 (Jewett et al. 2008; Lane et al. 2008). Phosphopeptide pulldown experiments showed that Sos1 associates with the TarP

tyrosyl-phosphorylated domain through Abi1 and Eps8. Vav2, on the other hand, requires PI(3,4,5)P_3 synthesized by class I PI3Ks also recruited at bacterial cell interaction sites, through its interaction with the TarP phosphorylated domain (Lane et al. 2008). Thus, following Arg/Abl-mediated tyrosyl phosphorylation, TarP permits the recruitment of the Sos1 and Vav2 GEFs, Rac, and the Abi1-WAVE2. The recruitment of the Abi1-WAVE2 complex downstream of Rac activation is potentiated by the concurrent activation of Arf6 and Arf1 to trigger Arp2/3 complex-dependent actin polymerization (Krause and Gautreau 2014). While the precise link with TarP-mediated activation of Rac has not been established, former studies have shown that Arf6 is required for *Chlamydia*-mediated cytoskeletal rearrangements suggesting the induction of pathways similar to those observed during *Shigella* and *Salmonella* invasion (Balana et al. 2005). Downstream of the tyrosine-rich domain, TarP contains an LD motif similar to that found in the focal adhesion protein paxillin, involved in the recruitment of the focal adhesion kinase (FAK) (Thwaites et al. 2014). TarP-mediated FAK recruitment is required for the downstream activation of Cdc42 and Arp2/3 complex-dependent actin polymerization. The molecular links between FAK and Cdc42 leading to the activation of the GTPase and actin polymerization during *Chlamydia* invasion are not as yet identified and could potentially include the GEFs from the DOCK family (Thwaites et al. 2014). Hence, through various modules, TarP may trigger actin polymerization through tyrosine kinase signaling and activation of the Cdc42 and Rac GTPases.

6 The Role of Phosphoinositides During T3SS-Mediated Cytoskeletal Reorganization

Phosphatidyl inositol phosphates (PIPs) are implicated in fundamental cell processes, including vesicular trafficking and cytoskeletal remodeling (Sarantis and Grinstein 2012; Viaud et al. 2016). The phosphatidyl moiety of PIPs mediates their association with membranes, and various kinases and phosphatases phosphorylate/dephosphorylate the inositol ring at different positions, generating a variety of second messengers (Viaud et al. 2016). Among these, PI(4,5)P_2 regulates the localization of cytoskeletal linkers at the plasma membrane by allowing their recruitment through so-called pleckstrin homology (PH) domains. PI(3,4,5)P_3 is upregulated following activation of class I PI3Ks downstream of receptor-mediated signaling and is pivotal in the recruitment of GEFs for Rho GTPases or actin nucleating-promoting factors (Viaud et al. 2016).

The *Shigella* type III effector IpgD was found to harbor a PI(4,5)P_2-5 phosphatase activity leading to the synthesis of PI5P (Niebuhr et al. 2002). IpgD has been implicated in various processes during *Shigella* infection, which are linked to PI(4,5)P_2 hydrolysis or PI(5)P synthesis. Through its PI(4,5)P_2 hydrolysis, IpgD was shown to disconnect cortical actin from the plasma membrane as shown by the membrane-pulling experiments using beads manipulated by magnetic tweezers in IpgD-transfected cells (Niebuhr et al. 2002). In epithelial cells, disconnection of

cortical actin by IpgD would facilitate actin polymerization at bacterial invasion sites. In T lymphocytes, however, hydrolysis of $P(4,5)P_2$ at the plasma membrane by injected IpgD prevents cell migration via the phosphorylation of ERM proteins, thereby impeding cell migration and function (Konradt et al. 2011). These results indicate that through $PI(4,5)P_2$ hydrolysis, *Shigella* not only locally regulates the dynamics of actin polymerization but also may interfere with the global cytoskeletal machinery. PI(5)P synthesized by IpgD may also directly bind to the PH domain of Tiam 1 and stimulate its exchange factor for Cdc42 and Rac (Viaud et al. 2014). While activation of Tiam 1 leading to Rac-dependent actin polymerization and ruffle formation was demonstrated in cells transfected with IpgD, it remains unclear whether this pathway is triggered during *Shigella* invasion. IpgD upregulates the levels of $PI(3,4,5)P_3$ at entry sites, presumably through an interplay between PIPs phosphatases and kinases, and the activation of PI3K (Garza-Mayers et al. 2015). PI3K has been involved in the regulation of Rac activity through the activation of various GEFs. In the case of *Shigella*, PI3K activation is required for the recruitment of ARN0, a GEF for the Arf6, a GTPase involved in endocytic process at the plasma membrane and endosome recycling (Garza-Mayers et al. 2015). Arf6 has also been implicated in the activation of the Rac GEF ELMO/Dock180 (Santy et al. 2005). Because Arf6 also contributes to actin polymerization at *Shigella* invasion sites in an IpgD-dependent manner, it is proposed that IpgD triggers a positive feedback loop implicating PI3K-ARNO-Arf6 that amplifies Rac-dependent actin polymerization (Garza-Mayers et al. 2015).

Interestingly, prior to the *Shigella* study, it was shown that the *Salmonella* type III effector SopB triggers a similar pathway involved in actin polymerization during bacterial invasion (Humphreys et al. 2012). SopB was first characterized as an inositol-polyphosphate (InsPPs) phosphatase leading to the production of $InsP_6$, involved in *Salmonella* invasion of epithelial cells (Zhou et al. 2001). It became clear that SopB also harbored PIPs dual 4- and 5-phosphatase activity and that both activities were required for bacterial-induced cytoskeletal remodeling (Piscatelli et al. 2016). SopB leads to higher levels of PI(3)P, either through its direct 4-phosphatase activity toward $PI(3,4)P_2$ or via the Rab5- and Vps34-dependent recruitment of PI(3)P-enriched vesicles, at *Salmonella* invasion sites (Mallo et al. 2008). In a process that is not fully understood, SopB is also responsible for the increases in $PI(3,4)P_2$ and $PI(3,4,5)P_3$ at *Salmonella* invasion sites in a wortmannin-insensitive manner, suggesting that it is independent of PI3K (Mallo et al. 2008).

In response to the $PI(3,4,5)P_3$ increase induced by SopB, ARNO was shown to recruit and activate Arf6 (Humphreys et al. 2012). It is not known, however, whether Arf6 recruitment stimulates the activation of a Rac GEF such as DOCK/ELMO180 during *Salmonella* invasion. Instead, Arf6 leads to the recruitment of Arf1, a GTPase involved in the trans-Golgi network trafficking. Arf1, in turn, is proposed to stimulate the activation of the Abi/WAVE complex downstream of Rac and actin polymerization at *Salmonella* invasion sites, in a scheme similar to that proposed for lamellipodia formation upon growth factor receptor stimulation (Humphreys et al. 2012; Krause and Gautreau 2014). Consistent with a cooperative

action of injected type III effectors, *Salmonella sopB* and *sopE* double mutants are severely impaired in bacterial invasion (Zhou et al. 2001).

PIPs also regulate EPEC pedestal formation. PI(4,5)P_2 is enriched in microdomains at the site of interaction of EPEC/EHEC with the host cell plasma membranes (Sason et al. 2009). Tyrosine phosphorylation of Tir at Y454 was proposed to mediate the recruitment and activation of PI3K through its SH2 domain, which phosphorylates PI(4,5)P_2 to generate PI(3,4,5)P_3 proposed to participate in actin polymerization during the formation of bacterial pedestals (Sason et al. 2009). How the recruitment of IRTKS/Irsp53 and that of PI3K are regulated by EPEC Tir Y454 is not known. Tir also contains tyrosine-based motifs at its C terminus, similar to ITIM motifs found in immunoreceptors, involved in the recruitment of lipid phosphatases via their SH2 domains (Smith et al. 2010). These motifs, including EPEC Tir Y483 and Y511, serve as docking motifs for the SHIP2 phosphatase that hydrolyzes PI(3,4,5)P_3 into PI(3,4)P2. PI(3,4)P_2 was shown to permit the recruitment at EPEC pedestal of Lamellipodin through its PH domain, an adaptor protein regulating the dynamics of lamellipodia formation (Smith et al. 2010). SHIP2 was also shown to act as a scaffold by recruiting the cytoskeletal adaptor SHC. Strikingly, upon inhibition of SHIP2, EPEC induces the formation of longer pedestal containing multiple stalks reminiscent of "medusa" tentacles (Fig. 3a) (Smith et al. 2010). Thus, while PI3K kinase, through PI(3,4,5)P_3, stimulates Tir-mediated actin polymerization and pedestal elongation, the recruitment of SHIP2, and of Lamellipodin through the synthesis of PI(3,4)P2, is required for the formation of a dense actin network in EPEC pedestals. Interestingly, despite the presence of putative ITIMs motifs in EHEC Tir, SHIP2 does not appear to control the dynamics of EHEC pedestals (Smith et al. 2010). It is possible that the role of SHIP2 in the regulation of actin polymerization in pedestal is restricted to signaling implicating the tyrosyl phosphorylation of Tir Y474 and Nck, as observed for EPEC but not EHEC.

7 Type III Effectors as Actin-Binding Proteins and Cytoskeletal Linkers

Bacterial pathogens may also inject effectors into host cells, which directly affect the dynamics of actin polymerization/depolymerization, or cytoskeletal anchorage. In addition to injected effectors, the *Salmonella* translocon component SipC was shown to directly nucleate actin polymerization (Hayward and Koronakis 1999). During *Salmonella* invasion, SipC may contribute to actin polymerization induced by the injected SopE and SopB type III effectors. The *Salmonella* type III effector SipA was shown to bind to and stabilize actin filaments, acting in concert with SipC to promote *Salmonella* invasion (McGhie et al. 2001). The crystal structure of the SipA carboxyterminal fourth region shows a packed globular structure with a large basic patch likely involved in the positioning of SipA onto actin filaments, extended at opposite ends by non-globular arms that "tether" opposite actin strands (Lilic et al. 2003). By stabilizing actin filaments, SipA decreases the critical concentration

of G-actin monomers, thereby enhancing actin polymerization (Lilic et al. 2003). SipA was also shown to protect actin filaments from disassembly mediated by ADF/cofilin and gelsolin. Both activities, together with the decrease in critical concentration, can account for increased actin polymerization and membrane ruffling at *Salmonella* invasion sites (Lilic et al. 2003). Remarkably, following bacterial invasion, SipA remains associated with the cytoplasmic side of the *Salmonella*-containing vacuole (SCV) (Brawn et al. 2007). A precise balance of SCV-associated SipA levels appears to be critical for the stabilization of SCVs and their perinuclear positioning (Brawn et al. 2007). In this context, SipA is required for the proper localization on the SCVs of the *Salmonella* type SPI-2 effector SifA, another WXXXE effector described to antagonize the function of the Rab9 small GTPase and involved in the membrane tubulation of SCVs (Brawn et al. 2007; Jackson et al. 2008).

The *Salmonella sip* and *Shigella ipa* operons encode the T3SS translocator components and the SipA and IpaA orthologs. While SipA and IpaA share significant homology in the amino-terminal two-thirds of their primary sequence, the carboxyterminal region of SipA, corresponding to the F-actin-binding domain, differs from that of IpaA. Instead, the IpaA carboxyterminal region contains three binding sites for vinculin (Park et al. 2011). Vinculin is a cytoskeletal linker, which, upon activation, bridges integrin and cadherin receptors to the cytoskeleton (Atherton et al. 2016). Vinculin is composed of a globular head domain and a carboxyterminal tail domain containing an F-actin-binding site. Under its inactive form, intramolecular interactions between the vinculin head and tail domains maintain vinculin in a folded conformation where most ligand-binding sites are masked (Bakolitsa et al. 2004). Endogenous activators of vinculin, such as the focal adhesion protein talin, often contain several vinculin-binding sites (VBSs). For all VBSs described to date, vinculin activation occurs through binding to the amino-terminal region of the vinculin head domain, promoting major conformational changes that disrupt the head-tail intramolecular interactions, freeing the vinculin F-actin-binding region and enabling its binding to actin filaments (Izard et al. 2004). The IpaA carboxyterminals VBS1 and VBS2 act together as a super-mimic of endogenous VBSs. By binding to vinculin's amino-terminal domain with an extremely high affinity, they promote vinculin activation (Nhieu and Izard 2007). The more proximal IpaA VBS3 functionally cooperates with IpaA VBS1 and VBS2 to trigger the recruitment of vinculin at bacterial invasion sites (Park et al. 2011). Through its VBSs, IpaA triggers the formation of an adhesion structure, stabilizing the bacteria at sites of invasion and potentially bypassing receptor-mediated anchorage (Valencia-Gallardo et al. 2015).

Recently, a domain in the *Chlamydia* type III effector TarP homologous to the vinculin-binding domain of IpaA was reported (Thwaites et al. 2015). As for IpaA, the *Chlamydia* vinculin-binding carboxyterminal domain contains three VBSs, with a similar organization and that appear to play similar roles as IpaA VBSs in triggering vinculin recruitment and bacterial invasion (Thwaites et al. 2015). TarP was also shown to interact in vitro with G- and F-actin via its carboxyterminal domain. Two carboxyterminal F-actin-binding TarP domains termed FAB1 and

FAB2 were shown to mediate the bundling of actin filaments, an activity which may account for the formation of actin bundles underneath bound bacteria during the *Chlamydia* invasion process (Jiwani et al. 2013). TarP was initially reported as an actin nucleator, with the actin nucleation being mediated by a ≈200-residue proline-rich domain containing the G-actin-binding site with some similarity to the actin-binding site of WH2-family proteins (Jewett et al. 2006). Interestingly, the TarP proline-rich domain is also involved in TarP oligomerization, the latter being inseparable from the actin-nucleating activity. In vitro, TarP induces the formation of long unbranched filaments, through a mechanism that probably differs from those reported for the Arp2/3 complex or the endogenous actin nucleators formins and Spire (Jewett et al. 2006). The TarP proline-rich domain is essential for actin polymerization, but the precise role of its nucleating activity during *Chlamydia* invasion is not entirely clear, because, as discussed above, TarP-mediated actin polymerization requires Rac and Arp2/3 complex activation (Jewett et al. 2010). As for the *Salmonella* SipC translocon component, it has been postulated that following injection by the T3SS into host cells, the TarP actin-nucleating activity may serve to build an initial actin scaffold onto which Arp2/3-dependent actin nucleation would further expand. Interestingly, the number of tyrosine-rich repeats varies between *C. trachomatis* isolates, and these repeats are even absent from many *Chlamydia* species. The number of actin-binding domains also varies between species. These observations suggested that TarP is among the few known genes to play a role in *C. trachomatis* adaptations to specific niches within the host (Lutter et al. 2010). TarP was the first T3 effector of infectious *Chlamydia* to be identified and remains by far the best characterized one. Several other effectors are translocated together with TarP, and it is likely that they also contribute to the actin and membrane remodeling needed for invasion. For instance the effector CT694 interacts with the cytoskeletal organizing protein AHNAK, but how this contributes to bacterial uptake is not known (Hower et al. 2009).

8 Type III Effectors Interfering with Tyrosine Kinase Signaling

Pathogenic bacteria inject effectors into host cells that act as toxins, interfere with actin polymerization or actin filament organization. Such activities may serve to prevent phagocytosis by immune cells, downregulation of inflammatory signals, and adaptive Th1 immunity or to alter the endothelial or epithelial junctional integrity (Sarantis and Grinstein 2012). As for effectors involved in actin polymerization, these inhibitory effectors can act at the level of Rho GTPases or directly at the level of actin. Both these processes have been described in several excellent reviews (Aktories et al. 2011; Lemichez and Aktories 2013; Aktories et al, this issue). Tyrosine kinase signaling required for cytoskeletal reorganization is also targeted by type III effectors. *Yersinia enterocolitica* and *Y. pseudotuberculosis* are invasive enteropathogens that multiply extracellularly, following the crossing of the intestinal epithelial layer. *Yersinia* injects various type III effectors to prevent

cytoskeletal reorganization required for phagocytosis at different levels of the signaling cascade. Among them, YopH is a highly efficient tyrosine phosphatase that appears to specifically target signaling downstream of β1-integrins, such as FAK, the Fak homolog Pyk, Cas, and paxillin (Viboud and Bliska 2005). In addition to the canonical tyrosine phosphatase motif C(X)5R(S/T) present on the P-loop, the substrate specificity of YopH is determined by two additional domains allowing its localization to cell adhesion structures and binding to tyrosyl-phosphorylated proteins (Ivanov et al. 2005). YopH-mediated inhibition of tyrosine kinase signaling thus prevents the activation of Rac and actin polymerization downstream of β1-integrin (Viboud and Bliska 2005). The targeting of β1-integrins by *Yersinia* via the invasin surface protein suggests a sophisticated pathogen-host cell interplay, whereby *Yersinia* hijacks β1-integrins to invade M cells and to prevent its phagocytosis by macrophages through the additional action of Yop proteins.

As discussed earlier, the *Salmonella* SptP also possesses a tyrosine phosphatase activity in addition to its RhoGAP function (Stebbins and Galan 2001). The substrate specificity of SptP is not clear at present. Structural characterization of SptP indicates that the tyrosine phosphatase domain is not affected by conformational changes in the carboxyterminal GAP domain (Stebbins and Galan 2001). This had led to speculations that, following targeting of Cdc42 or Rac via its GAP domain, SptP may specifically dephosphorylate substrates in complex with these GTPases.

EPEC also inhibits opsonized phagocytosis by macrophages via FcRgRIIa receptors through the action of the type III effector EspJ (Young et al. 2014). EspJ harbors a unique dual amidase-ADP-ribosyltransferase activity that modifies key residues in the catalytic domain of Src family kinases, required for FcRgRIIa phosphorylation and actin polymerization (Young et al. 2014). While the precise mechanism underlying the dual modification or residues remains undefined, mass spectroscopy analysis argues in favor of coupled, rather than sequential, amidation and ADP-ribosylation mediated by EspJ (Young et al. 2014). As for EspH, in addition to inhibiting bacterial phagocytosis by macrophages, EspJ may also be involved in the downregulation of pedestal formation during late phases of EPEC/EHEC infection (Wong et al. 2012; Young et al. 2014).

9 Concluding Remarks

In this chapter, we have tried to illustrate the wide diversity of strategies employed by bacterial pathogens to move intracellularly or to divert cytoskeletal processes using injected type III effectors. While being different, the comparison of the various strategies point to the targeting of converging hubs corresponding to tyrosine kinase signaling, RhoGTPases, phosphoinositides, and cytoskeletal proteins. Further investigation on other injected effectors reorganizing the actin cytoskeleton, as well as on their mechanisms of action, should allow to further deconvolve specific and fundamental features of each system. Because of the

functional versatility of the actin cytoskeleton, much is probably yet to be learned about the manipulation of cytoskeletal processes by bacterial pathogens, beyond the initial epithelial/endothelial interactions. For example, relevant but less studied aspects include processes linked to the dissemination or egress of bacteria from infected tissue. Also, within mucosal surfaces and in addition to epithelial cells, pathogens also interact with immune cells, such as professional phagocytes, dendritic cells, macrophages, or neutrophils. Various pathogens have been reported to impair cytoskeletal responses involved in phagocytosis or lymphocyte activation. Because of their role in bacterial clearance or mounting of the inflammation, these interactions dictate the outcome of infection. Clearly, throughout the course of the infectious process, disease progression will critically depend on the pathogen's ability to counter innate immunity.

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Viruses That Exploit Actin-Based Motility for Their Replication and Spread

N. Bishara Marzook and Timothy P. Newsome

Contents

Abstract

The actin cytoskeleton is a crucial part of the eukaryotic cell. Viruses depend on host cells for their replication, and, as a result, many have developed ways of manipulating the actin network to promote their spread. This chapter reviews the various ways in which viruses utilize the actin cytoskeleton at discrete steps in their life cycle, from entry into the host cell, replication, and assembly of new progeny to virus release. Various actin inhibitors that function in different ways to affect proper actin dynamics can be used to parse the role of actin at these steps.

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Actin • Budding • Clathrin • Cofilin • Colocalization • Cytochalasin • Filopodia • Jasplakinolide • Latrunculin • Macropinocytosis • Myosin • Viruses

1 Introduction

As is apparent from accompanying chapters, actin plays an essential role in the function of eukaryotic cells. For example, the cortical actin network forms a structural and protective barrier to extracellular stresses. In addition, force generation by actin polymerization promotes a variety of processes from vesicle motility to the deformation of membranes as macromolecule complexes are passed between the cytoplasm and the outside of the cell. Viruses require host cells for their replication, and hence the ability to interface with actin is an opportunity to facilitate this process. Many pathogens have developed both unique and sometimes convergent mechanisms of manipulating the host actin cytoskeleton and associated machinery (Bear et al. 2001; Favoreel et al. 2007; Harries et al. 2009; Humphries et al. 2012; Leite and Way 2015; Naghavi and Goff 2007). This chapter will highlight different stages in the replication cycle of several viruses that utilize the actin network to promote infection, replication, and spread.

Briefly, actin exists as globular actin (G-actin), or soluble actin monomers, which can undergo polymerization, promoted by accessory factors, to form filamentous actin (F-actin), or the insoluble polymer form of actin (Carlier et al. 2015; Disanza et al. 2005). Proteins or protein complexes that increase the number of actin polymers are called actin nucleators, which in turn promote overall polymerization (more filaments are available to extend). Examples of actin nucleators include the Arp2/3 complex and formins, which initiate polymerization of branched and unbranched actin filaments, respectively (Disanza et al. 2005; Pollard et al. 2000; Pollard and Borisy 2003). The Arp2/3 complex is activated by nucleation-promoting factors (NPFs) such as the WASP or WAVE family of proteins, which localize the Arp2/3 complex and G-actin to the site of actin branch formation (Stradal et al. 2004). These protein families are sensitive to signaling molecules involved in actin remodeling, such as the Rho family of GTPases (including Rac, Cdc42, and RhoA) (Aspenstrom et al. 2004; Humphries et al. 2014; Raftopoulou and Hall 2004). Myosins are a class of motor proteins that associate with actin filaments and mediate transport along them (Hartman and Spudich 2012; Spudich 1989). There are 18 different classes of myosins known to date, and their functions range from intracellular transport and endocytosis to cell adhesion and migration (Berg et al. 2001; Hasson 2003; Vicente-Manzanares et al. 2009). Other players in the actin polymerization process include actin depolymerizers, actin bundlers, and filament-severing and filament-capping proteins (Pollard and Borisy 2003). Therefore, many classes of protein interact with, or are implicated in, actin-based motility.

The various ways viruses utilize actin in its many forms can be understood by studying the effects of inhibitors of actin dynamics on virus replication. Such inhibitors are small compounds synthesized in marine sponges, fungi, and mushrooms and act as natural toxins on the actin cytoskeleton. Actin polymerization can be affected at different stages: actin monomers can be sequestered by the drug latrunculin A (LatA), preventing the formation of actin filaments by binding G-actin in a 1:1 ratio (Coue et al. 1987; Spector et al. 1983), or growth can be halted by capping the growing end of actin filaments using cytochalasins (A–E and H), which prevent both the addition of new monomers and the disassembly of the actin filament at that end (Braet et al. 1996; Cooper 1987). Additionally, drugs such as jasplakinolide specifically block actin-filament disassembly, essentially fixing existing filaments within a cell by halting actin treadmilling (Cramer 1999).

While some viruses require interactions with actin for a particular stage of their replication cycle, others rely on actin for multiple events including entry, intracellular transport, and exit. For example, HIV-1 subverts actin remodeling at the cell surface prior to entry, which concentrates co-receptors CD4 and CXCR4 that are required for virus entry, while treatment of cells with cytochalasin D prevents the same (Iyengar et al. 1998; Liu et al. 2009). Binding of viral gp120 receptors induces localized F-actin rearrangements through a RhoA-, Rac1-, Arp2/3-, and moesin (a protein that links the plasma membrane to the actin cytoskeleton)-dependent mechanism (Barrero-Villar et al. 2009; Jimenez-Baranda et al. 2007; Thomas et al. 2015). While transport of internalized virus particles toward the nucleus is microtubule based, this switches to an actin-based mechanism at the perinuclear region, prior to nuclear entry (Arhel et al. 2006). Treatment of cells with latrunculin prior to infection reduces virus cytoplasmic transport leading to an accumulation of particles in proximity to the plasma membrane. On the other hand, treatment 1 hour postinfection (hpi) results in an accumulation of particles adjacent to the nucleus (Arhel et al. 2006). This indicates a requirement for actin in both cell and nuclear entry. Other HIV proteins including Gag and Nef also interact with the actin cytoskeleton during later stages of infection, which is important for viral assembly and/or budding (Fackler et al. 1997; Gaudin et al. 2013; Rey et al. 1996). Finally, cell-to-cell transmission of HIV is facilitated by the actin-dependent formation of virological synapses and/or filopodia (Lehmann et al. 2011; Sherer et al. 2007). High-resolution imaging of budding HIV particles by cryoelectron tomography reveals a directed arrangement of cortical actin filaments around budding sites, half of which are associated with F-actin-rich filopodia (Carlson et al. 2010). This use of filopodia for viral transport can be followed by the live imaging of HIV-infected dendritic cells, where virus particles hijack the dendritic–CD4 T cell contacts. As illustrated in Fig. 1, newly formed virus particles are moved along filopodial trajectories that are pivoted from the dendritic cell surface toward T cells (Aggarwal et al. 2012).

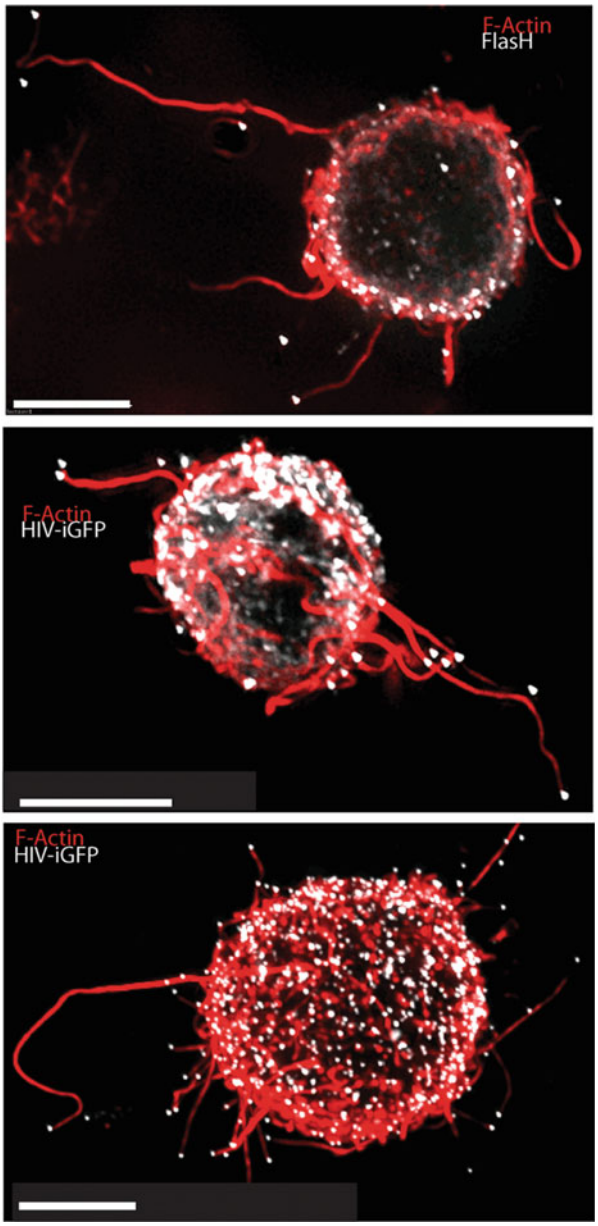

Fig. 1 HIV particles (in *white*) are present on the tips of filopodia (F-actin in *red*) produced by infected dendritic cells. Scale bars are 5 μm. Adapted from Aggarwal et al. (2012)

2 Membrane Entry

2.1 Virus Cell Surfing

Actin-rich protrusions called filopodia, which are structures used by cells to interact with their environment, are exploited by viruses to infect cells (Mattila and Lappalainen 2008). Filopodia exhibit retrograde actin flow (Chhabra and Higgs 2007; Mitchison and Kirschner 1988) that can be harnessed by viruses to traverse or "surf" the cell surface prior to internalization to seek endocytic hotspots (Lehmann et al. 2005). Herpes simplex virus-1 (HSV-1) induces dendritic filopodia formation in neuronal cells upon infection, which virus particles bind to and traverse to reach the cell body, as observed in Fig. 2 (Akhtar and Shukla 2009; Clement et al. 2006; Dixit et al. 2008). This process is actin dependent, and virus infection induces RhoA and Cdc42 activation (Clement et al. 2006; Oh et al. 2010). In addition, treatment of cells with cytochalasin D prior to infection leads to a reduction in cell entry (Dixit et al. 2008), highlighting the importance of underlying actin dynamics for this process. Similarly, the murine leukemia virus (MLV), the avian leukosis virus (ALV), and the human papillomavirus type 16 all show similar filopodial "surfing" prior to internalization (Lehmann et al. 2005; Schelhaas et al. 2008). Therefore, for many viruses, this is their first encounter with the actin cytoskeleton, and engaging with filopodia aids in their movement toward the cell body and favorable centers of endocytosis. Here viruses face further challenges before they access the intracellular space. These subsequent steps may also be actin dependent and are outlined below.

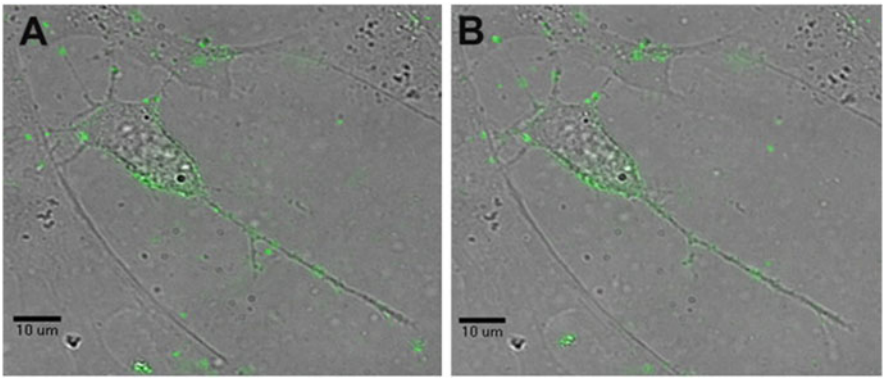

Fig. 2 HSV-1 virus particles (*green*) attach to a neuron and its dendrites at 5 min postinfection (**a**) and surf along dendrites toward the cell body (**b**). Still frames were captured from live imaging of a GFP-tagged HSV-1 virus infection of P19 neuronal cells. Adapted from Dixit et al. (2008)

2.2 Clathrin-Mediated Entry

Clathrin-mediated endocytosis (CME) occurs via clathrin-coated pits (CCP), specialized plasma membrane invaginations typically up to 0.2 μm in size (Ehrlich et al. 2004; McMahon and Boucrot 2011). This process is mediated by adaptor proteins such as AP-2, allowing the CCP to pinch off from the plasma membrane into the cytosol with the aid of dynamin (Praefcke and McMahon 2004). Dynamin in turn can interact with the actin cytoskeleton through its ability to recruit cortactin, a promoter of actin nucleation and an actin bundler (McNiven et al. 2000). CME is a major pathway by which the cell shuttles molecular cargo across the membrane and a site targeted by many viral (and some bacterial) pathogens (Humphries and Way 2013). Movement of clathrin-coated structures toward the cytosol is accompanied by the recruitment of actin at the site of budding, and actin polymerization may provide the mechanical force required to detach and propel these structures away from the membrane (Merrifield et al. 2002; Taylor et al. 2012). Myosin VI, an actin-based molecular motor, localizes to CCPs further supporting a role of actin in this process (Hasson 2003). Although analysis of CCP formation in the presence of cytochalasin D or latrunculin A reveals that an intact actin cytoskeleton is required for the sustained assembly of new CCPs (Boucrot et al. 2006; Yarar et al. 2005), it does not divulge a direct role in the specific events leading to regular CCP creation, such as their initiation or subsequent endocytosis (Boucrot et al. 2006). However, actin polymerization is required for the formation and internalization of what are known as "clathrin-coated plaques," or more stable clathrin-coated structures, which may carry viruses or bacterial particles (Cureton et al. 2009; Saffarian et al. 2009). Therefore, actin may only be recruited when the size of the CCP needs to accommodate large objects (greater than 0.2 μm), and the force-generating properties of actin polymerization are then required for vesicle budding and scission (McMahon and Boucrot 2011).

Some viruses such as the non-enveloped dsRNA reovirus associate with, and enter through, randomly generated CCPs on the cell surface (Ehrlich et al. 2004). A member of the *Reoviridae* family, the bluetongue virus (BTV), possesses a complex capsid structure, of which VP2 binds to a cellular receptor (most likely a sialic acid-containing glycoprotein (Hassan and Roy 1999)) prior to internalization by CME. siRNA knockdown of the clathrin-AP-2 adaptor complex results in reduced BTV entry and replication in cells (Forzan et al. 2007).

Other viruses including influenza A (Rust et al. 2004) and vesicular stomatitis virus (VSV) (Cureton et al. 2009) induce CCP formation following virus-receptor binding. Single-particle tracking of lipophilic dye-labeled influenza viruses and enhanced yellow fluorescent protein (EYFP)-labeled clathrin enabled the visualization of clathrin-mediated endocytosis of 65% of internalized influenza virus particles. The appearance of EYFP-clathrin on the cell surface after viral binding suggests the de novo formation of CCP at influenza virus particles (Rust et al. 2004). A functioning and dynamic actin cytoskeleton is required for the entry of influenza virus into polarized cells, but not in the case of polarized cells, suggesting the existence of distinct, cell type-dependent mechanisms of entry

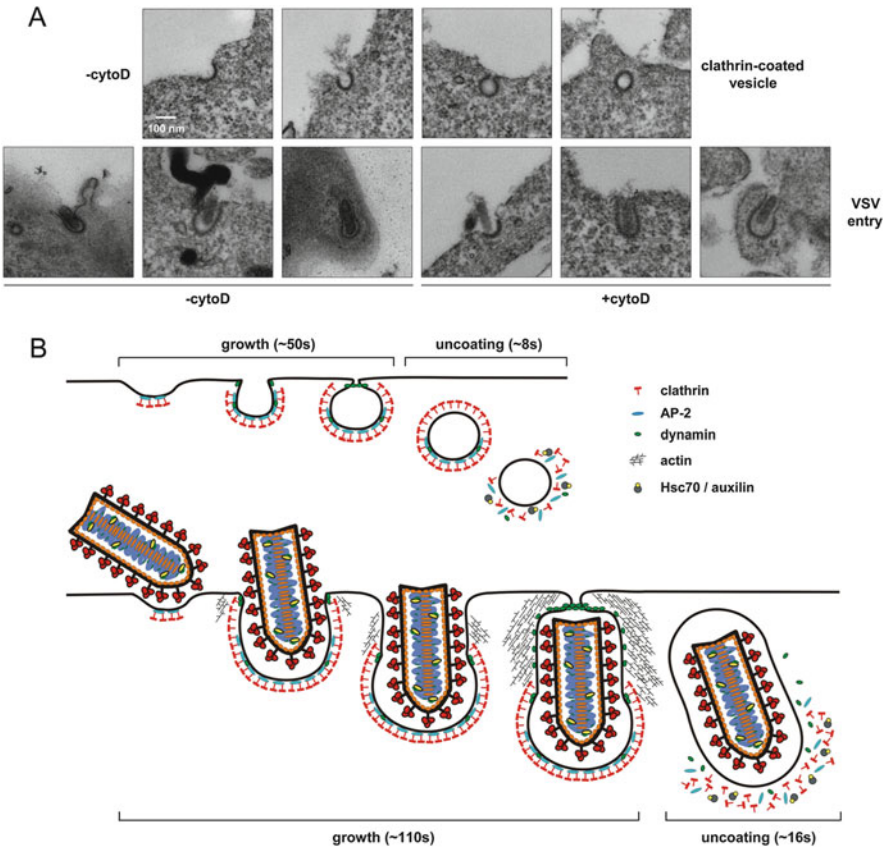

Fig. 3 Comparison of clathrin pit formation with and without VSV entry. (**a**) Electron micrographs of successive stages of clathrin-coated vesicle formation. Conventional pits possess a characteristic "U" shape, while pits containing virus particles are more elongated. The treatment of cells with cytochalasin D (cytoD) prior to infection freezes the assembly of virus-containing clathrin pits. (**b**) Schematic representation of the assembly of clathrin-coated pits (CCP) with the help of the actin machinery. Conventional pits only require dynamin for CCP scission; however, virus-containing pits recruit the actin cytoskeleton to aid in severance. Obtained from Cureton et al. (2009)

(Sun and Whittaker 2007). Physical forces exerted by the actomyosin and microtubule dynamics are required for the uncoating of influenza A virus postentry (Banerjee et al. 2014) highlighting the importance of both cytoskeletal systems in this process. Live-cell imaging of single fluorescently labeled VSV particles and clathrin reveals a clathrin-mediated endocytic entry pathway for this virus. siRNA-mediated depletion of AP-2 and small molecule inhibitors of dynamin support their respective roles in virus internalization. Finally, eGFP (green fluorescent protein)-tagged actin, Arp3, and cortactin were found to localize to virus-containing CCPs, and the inhibition of actin polymerization results in reduced internalization of VSV (Cureton et al. 2009). Figure 3 depicts the various stages in the CME of VSV particles.

Kaposi's sarcoma-associated herpesvirus (KSHV), African swine fever virus (ASFV), and dengue virus (DENV-1) utilize a dynamin-dependent, clathrin-mediated cell entry pathway, as inhibitors of CCP assembly such as dextrose and chlorpromazine reduce virus entry and trafficking (Acosta et al. 2011; Galindo et al. 2015; Greene and Gao 2009; Hernaez and Alonso 2010; van der Schaar et al. 2008). While several studies suggested that KSHV enters via CME in fibroblasts and endothelial cells (Akula et al. 2003; Greene and Gao 2009), another study found that KSHV uses an actin-dependent macropinocytic pathway to enter human umbilical vein endothelial cells (Raghu et al. 2009). This highlights the observation that viral entry mechanisms can be highly cell type specific. KSHV also induces a rearrangement of the actin cytoskeleton almost immediately following infection, with distinct actin filaments or spikes appearing on the cell surface at 15 minutes postinfection (mpi) in association with the majority of KSHV particles. In addition, chemically disrupting the actin cytoskeleton or interfering with regulators of actin nucleation, like Rho GTPases, N-WASP, and Arp2/3, reduces the entry and trafficking of virus particles to the nucleus, supporting the importance of de novo actin nucleation in this process (Greene and Gao 2009).

2.3 Macropinocytosis

Macropinocytosis is an actin-dependent, growth factor-induced endocytic process that enables the uptake of extracellular macromolecules and fluid (Lim and Gleeson 2011; Swanson and Watts 1995). Unlike CME, macropinocytosis requires actin cytoskeleton remodeling, as treatment with cytochalasin D reduces membrane ruffling (Araki et al. 1996). Actin-mediated cell surface projections such as lamellipodia- and filopodia-related membrane ruffling initiate macropinocytosis, although they do not always result in an endocytic event. In addition, PI3-kinase activity (Amyere et al. 2000), Na+/H+ exchange pumps, and Rac1 and Cdc42 signaling (Koivusalo et al. 2010) are all involved in macropinocytosis. Macropinocytosis is able to nonselectively accommodate endocytosis of large macromolecular complexes (0.2–5 μm) and fluids (Mercer and Helenius 2009). As a result, many larger pathogens exploit this non-receptor-mediated process to enter host cells.

Orthopoxviruses such as vaccinia and variola viruses are large enveloped DNA viruses that exploit macropinocytosis to gain access to the host cytoplasm. Following a replication cycle, vaccinia virus (VACV) produces two morphological distinct infective forms: intracellular mature virus and extracellular enveloped virus, both of which enter cells in a macropinocytic-, actin-, PAK1-, and Na+/H+ exchange-dependent manner (Mercer and Helenius 2008; Schmidt et al. 2011). Both forms of the virus induce the formation of cell-wide membrane blebs (containing Rac1, RhoA, ezrin, and cortactin) during entry, which, in the case of mature VACV entry, is triggered by exposed phosphatidylserine in the virus envelope (Schmidt et al. 2011). Uptake by cells of extracellular fluid marked by Alexa 488-labeled

dextran following exposure to virus is indicative of induction of macropinocytosis activity in infected cells.

Similarly, Ad35, a member of the B2 species of adenovirus, undergoes actin-, PAK1-, PKC-, and sodium/proton exchanger-dependent macropinocytosis to mediate entry following virus association with CD46 receptors on the cell surface (Kalin et al. 2010). While adenoviruses 2 and 5 enter cells in a clathrin-dependent mechanism, macropinocytosis is also initiated on entry, which results in the release of viral contents into the cytosol (Meier et al. 2002; Meier and Greber 2004). Filoviruses such as *Zaire ebolavirus* enter cells in a clathrin-independent mechanism, but entry does require actin polymerization and glycoprotein-triggered PAK1 activity. Infected cells also display increased uptake of extracellular fluid (Saeed et al. 2010). Fluorescently labeled ebolavirus was found to colocalize with sorting nexin (SNX) 5, a protein that associates with newly formed macropinosomes (Nanbo et al. 2010).

Viruses may engage multiple cell entry pathways, possibly to widen their host range or cell-type tropism. A novel marine iridovirus, the Singapore grouper iridovirus (SGIV), uses both clathrin-mediated endocytosis and macropinocytosis to enter cells, as inhibitors of both are capable of reducing the entry of fluorescently labeled virus particles (Wang et al. 2014). Interestingly, virus particles were also observed associated with actin-rich protrusions on the cell surface during the early stages of viral entry (Fig. 4). Similarly, as mentioned earlier, while ASFV was

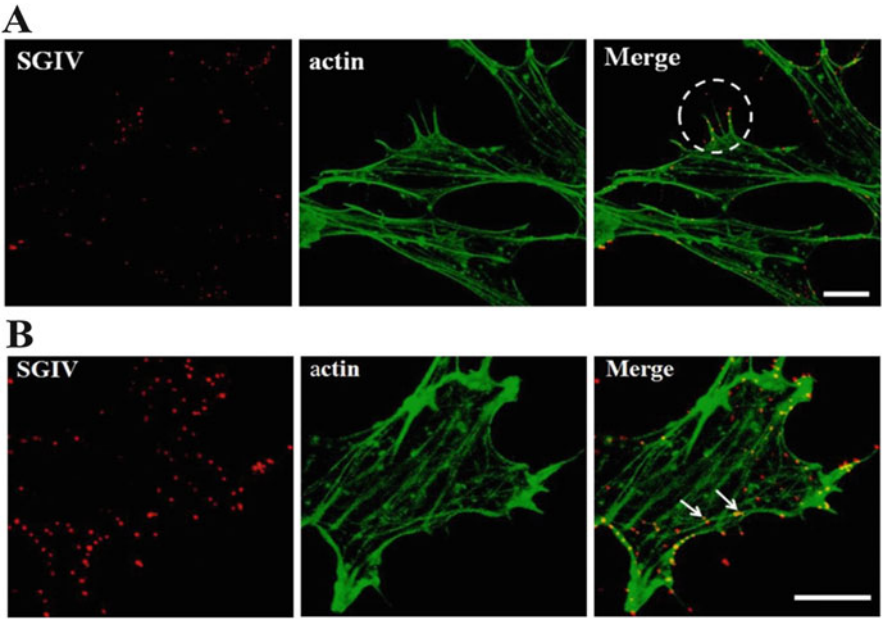

Fig. 4 Cy5-labeled SGIV (in *red*) colocalizes with actin protrusions (in *green*) on entry (**a**) and with actin filaments early in infection (**b**). Adapted from Wang et al. (2014)

thought to enter cells by CME, another study has found evidence that points to an actin-dependent macropinocytic entry mechanism utilized by this virus (Sanchez et al. 2012). However it is yet to be determined whether both CME and macropinocytosis occur independently of each other or whether activation of actin polymerization by macropinocytosis may aid in the clathrin-mediated uptake of ASFV (Alonso et al. 2013).

2.4 Clathrin-Independent Entry

There are several non-clathrin-mediated, non-macropinocytic pathways of viral entry. Caveolin-mediated entry is one of the best characterized clathrin-independent pathways and involves cholesterol-rich cup-shaped invaginations of the plasma membrane in the presence of proteins from the caveolin family (Bastiani and Parton 2010). A dynamic actin cytoskeleton regulated by dynamin, Src kinase, and PKC is required for caveolin-mediated endocytosis (Mayor and Pagano 2007). Simian virus 40 (SV40) and hepatitis B virus (HBV) engage in caveolin-mediated entry for the infection of host cells (Hao et al. 2011; Pelkmans et al. 2002). Infection of CV-1 cells with SV40 reduces the presence of actin stress fibers but induces the formation of tail-like actin patches 20–120 min postinfection. This was revealed using fluorescently tagged β-actin and a caveolin protein-1 fusion that both localize to SV40-bound caveolae on the cell surface (Fig. 5) (Pelkmans et al. 2002).

Non-clathrin-, non-caveolin-dependent pathways of viral entry also exist, and many of these also require dynamic regulation of the actin cytoskeleton. While the majority of influenza A virus-infecting BSC-1 cells employ CPPs, a minority utilize an equally efficient non-clathrin, non-caveolin-mediated entry pathway in parallel; however, there does not seem to be any dependence on actin (Rust et al. 2004).

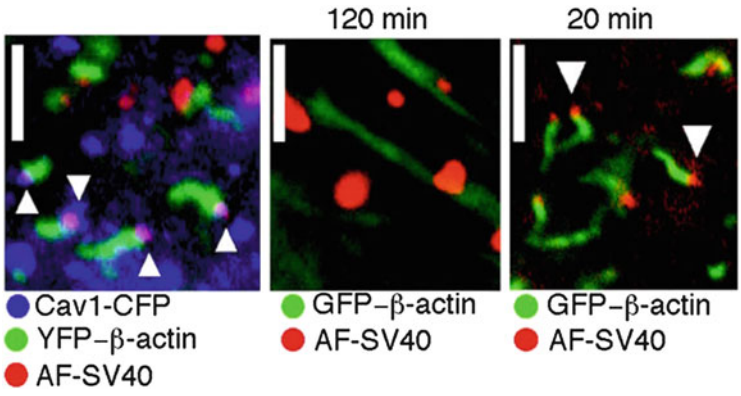

Fig. 5 Beta-actin tails (in *green*) colocalize with both SV40 particles (in *red*) and SV40 containing caveolin domains (in *blue*) in infected cells. Scale bar is 3 μm. Adapted from Pelkmans et al. (2002)

On the other hand, the clathrin-, caveolin-, and microtubule-independent cell entry mechanism of poliovirus (PV) does depend on actin polymerization as treatment of cells with cytochalasin D inhibits PV internalization and RNA release (Brandenburg et al. 2007). Dual labeling of the capsid and RNA of PV enables tracking of its entry and genome release process, indicating a reduction in virus internalization following actin depolymerization by treatment with cytochalasin D.

As previously discussed, the mode of viral entry can differ based on cell type. The Japanese encephalitis virus (JEV) utilizes CME in fibroblasts and a clathrin-independent, dynamin-dependent, and caveolin-dependent pathway in neuronal cells, which sets JEV apart from other flaviviruses that solely use CME for cell entry (Kalia et al. 2013). Virus binding induces filopodia formation on the cell surface 5 mpi and induces RhoA activation on entry. HPV-16 entry, while also being clathrin and caveolin independent, does not rely on signaling by Rho-like GTPases as it is unaffected by dominant negative mutants of RhoA, Cdc42, and Rac1, despite being dependent on actin polymerization dynamics (Schelhaas et al. 2012).

3 Intracellular Actin Dynamics

In addition to microtubules, actin also plays a role in the transport of endocytosed vesicles away from the cell periphery (Merrifield et al. 1999; Taunton 2001; Welch and Mullins 2002). There are two forms of actin-based transport within cells. One is based on the actomyosin network where cargo travels along actin microfilaments aided by the myosin motor proteins. The other form is based on highly localized actin polymerization occurring on the surface of the cargo itself (Khaitlina 2014). Following entry via endocytosis, many pathogens, both bacterial and viral, exploit the force-generating reaction of actin polymerization to aid movement within host cells (Gouin et al. 2005). Actin-myosin dynamics can also influence various stages of the viral replication cycle, not only from its movement away from sites of entry but to (and the creation of) regions of genome replication, progeny assembly, and subsequent return to the plasma membrane for release. Here we highlight several viruses that exploit both mechanisms for the completion of their intracellular life cycles.

3.1 Intracellular Transport

Influenza virus displays actin-dependent transport of virus following endocytosis in the cell periphery (distances within 2 μm from the point of initial virus binding); however, this is superseded by a burst of microtubule-based movement toward the nucleus (the site of viral RNA synthesis) (Lakadamyali et al. 2003). On the other hand, intracellular movement of HBV as imaged by single-particle tracking of labeled surface antigen HBsAg reveals rapidly moving virus particles that rely on actin- but not on microtubule-based motility (Hao et al. 2011). This was revealed by

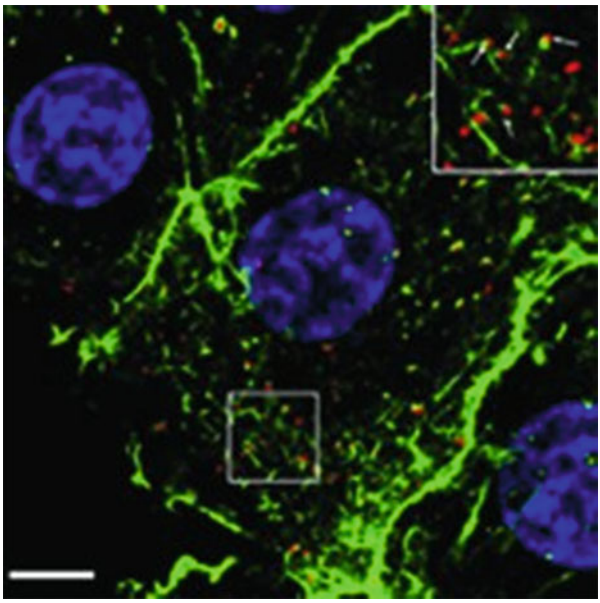

Fig. 6 Cy5-labeled HBsAg particles (in *red*) colocalize with GFP-tagged actin fibers (in *green*) 30 mpi of COS-7 cells. Nuclei (*blue*) are stained with Hoechst 33342. Scale bar is 10 μm. Obtained from Hao et al. (2011)

comparing virus movement in cells treated with either cytochalasin D or nocodazole, inhibitors of the actin or microtubule network, respectively. In addition, labeled HBsAg-infected cells transfected with GFP-tagged actin revealed their colocalization (Fig. 6), confirming the intracellular association of HBV and actin.

Only one example of intracellular transport mediated by virally stimulated actin nucleation has been characterized. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is a baculovirus of lepidopterans that initiates actin polymerization 5–30 mpi after endocytosis of virus particles (Charlton and Volkman 1993; Ohkawa et al. 2010). Viral nucleocapsid protein P78/83 is a viral NPF located on one end of the viral particle and activates the Arp2/3 complex, inducing localized actin nucleation at the virus surface (Goley et al. 2006; Rohrmann et al. 2013). On entering a host cell, AcMNPV particles use their actin-driven motility to either navigate to the nucleus (as seen in Fig. 7), where uncoating and gene expression can occur, or to proceed to neighboring uninfected cells via cell surface spikes that appear 2 hpi (prior to the creation of virus progeny) (Ohkawa et al. 2010). The addition of a myosin inhibitor such as butanedione monoxime reduces transport of AcMNPV to the nucleus, suggesting a role for the actin-myosin network in complementing intracellular transport of the virus (Lanier and Volkman 1998).

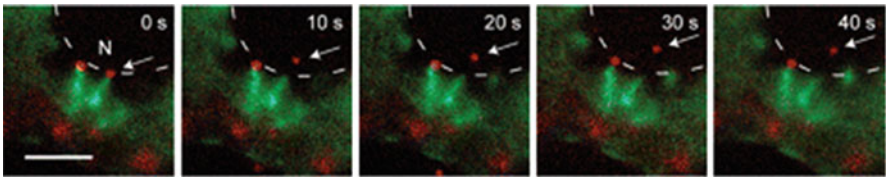

Fig. 7 Time-lapse images of AcMNPV nucleocapsids (*red*) approaching the nuclear envelope on actin tails (*green*) while one separates from the actin corkscrew and enters the nucleus (N-nuclear periphery marked by *dashed lines*). Scale bar is 5 μm. Adapted from Ohkawa et al. (2010)

3.2 Intracellular Replication

Following the delivery of incoming virus to their site of replication, engagement with the actin cytoskeleton can be used to promote the replication and assembly of progeny virus. Respiratory syncytial virus (RSV) relies on both actin and profilin (an actin monomer-binding protein) to stimulate the transcriptional activity of RSV polymerase (Burke et al. 2000). During a measles virus (MV) infection, the creation of viral replication centers close to the nucleus is dependent on cofilin, an actin-severing factor (Koga et al. 2015). RNA-mediated knockdown of cofilin decreases ribonucleoprotein (RNP) complex formation and MV RNA synthesis. Interestingly, the phosphorylation of cofilin, which renders it inactive (Arber et al. 1998), increases during MV infection suggesting a tight temporal regulation of activity. The role of cofilin in actin dynamics is a multifactorial one, as the severing of actin filaments can both suppress the elongation of existing F-actin structures and also create sites for branching of new actin filaments via the Arp2/3 complex (Bravo-Cordero et al. 2013). Actin severing increases the G-actin pool, and cofilin also possesses actin-nucleating activity. HSV-1 replication in neuronal cells relies on F-actin dynamics, although this occurs via a biphasic process: the cofilin-1-mediated assembly of F-actin during early stages of entry, followed by the disassembly of F-actin during later stages of replication (Xiang et al. 2012). HIV-1 also induces cofilin-mediated actin dynamics to aid in entry and nuclear localization of the virus (Yoder et al. 2008). Therefore, cofilin may act as a sensitive regulator of F-actin dynamics that is targeted by several viruses to aid in various stages of their replication and hence shows potential as a novel antiviral target.

Many viruses replicate, transcribe their genomes, and assemble progeny in the nucleus of host cells. Several viruses engage with actin in the nucleus for successful replication (Cibulka et al. 2012). In addition to AcMNPV being able to manipulate intracellular actin for its own motility in the cytoplasm, nuclear F-actin is also essential for AcMNPV nucleocapsid morphogenesis (Ohkawa and Volkman 1999). P78/83 is a viral WASP-like protein that interacts with Arp2/3, which translocates into the nucleus following infection (Goley et al. 2006), along with monomeric G-actin (Ohkawa et al. 2002), to induce nuclear actin polymerization. P78/83 is stabilized by a further AcMNPV nucleocapsid protein C42, which is essential for viral-induced actin polymerization in the nucleus (Wang et al. 2015). AcMNPV

VP80 also interacts with actin in the nucleus and may play a myosin-like role in transport of nucleocapsids to the nuclear surface (Marek et al. 2011). The linear dsDNA of adenoviruses replicates in the nucleus and recruits actin as well as myosins I, V, and VI to sites of viral DNA replication (Fuchsova et al. 2015). While nuclear actin and myosin I have been shown to colocalize with viral DNA accumulations, recent analysis with structured illumination microscopy (SIM) revealed that myosins V and VI were also recruited to replication centers during the early and intermediate stages of Ad5 infection, respectively. Treatment with latrunculin B reduces viral replication, as does the expression of actin mutants that resist polymerization in infected cells, supporting a requirement for actin polymerization in this process.

3.3 Post-replicative Transport and Particle Assembly

Transport of retroviral RNA such as HIV-1 Gag mRNA out of the nucleus is actin dependent (Hofmann et al. 2001; Kimura et al. 2000), and beta-actin colocalizes with nuclear viral RNA "tracks" (curvilinear structures observed by fluorescence microscopy) (Kimura et al. 2000). Marburg virus (MARV) nucleocapsids also travel along, and between, F-actin filaments through the cytosol from viral replication centers to the plasma membrane (Schudt et al. 2013). This is facilitated by an actin cytoskeletal regulator IQGAP1, whose suppression reduces MARV release (Dolnik et al. 2014). Actin-dependent host motor protein myosin 10 is also co-transported along with mature MARV nucleocapsids to filopodia, which serve as sites of MARV budding and release (Schudt et al. 2013).

While alpha-herpesviruses such as pseudorabies virus (PRV) and HSV-1 were thought to rely on nuclear F-actin for transport of nucleocapsids (Feierbach et al. 2006), more recent studies refute this hypothesis (Bosse et al. 2014). While it is clear that treatment of neuronal or mouse embryonic fibroblast (MEF) cells with latrunculin A reduces intranuclear capsid motility, Bosse et al. discovered that treating cells with other actin inhibitors such as jasplakinolide (stabilizes actin and stops actin treadmilling) had a rather unexpected effect on nuclear capsid transport (Bosse et al. 2014). Infecting MEFs that stably express Lifeact, a live F-actin-binding probe bound to GFP, with capsid-tagged PRV in the presence of LatA revealed the formation of thick actin rods that also bound to nucleocapsids in an immunoprecipitation assay, thus preventing capsid motility. This finding calls into question the use of broad-acting drugs that disturb actin dynamics to understand the role of actin in viral replication, as it appears that the modes of viral manipulation of host actin may be more nuanced (both spatially and temporally controlled and/or dependent on delicate actin homeostasis) than initially thought.

The assembly of influenza virus particles takes place beneath the plasma membrane, and this can be disrupted by blebbistatin (Kumakura et al. 2015), an inhibitor of non-muscle myosin II, an actin cross-linking protein crucial for actin-myosin dynamics (Conti and Adelstein 2008). Indeed, clustering of the viral transmembrane glycoprotein HA (hemagglutinin) at the cell surface is mediated by actin, as

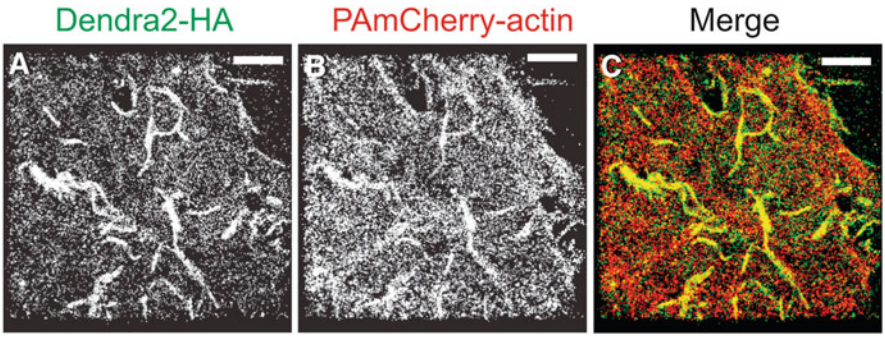

Fig. 8 Live-cell fluorescence photoactivation localization microscopy (FPALM) imaging reveals colocalization (**c**) of labeled HA (**a**) and actin (**b**) in nonpolarized NIH3T3-HAb2 cells transfected with the designated plasmids. Adapted from Gudheti et al. (2013)

revealed by super-resolution microscopy (Fig. 8) (Gudheti et al. 2013). Assembly of filamentous influenza particles, a morphological variant, is also affected when the normal actin cytoskeleton is interfered with by either cytochalasin D, latrunculin A, or jasplakinolide, all of which also disrupt clustering of cell-surface HA, despite their varying modes of action (Simpson-Holley et al. 2002).

4 Viral Exit Actin Dynamics

The final stage in the viral replication cycle is release from the infected host cell. As with entry, viral egress requires a reckoning of the many barriers to cell exit, particularly in the case of non-lytic viruses. Several viruses have developed methods of both manipulating existing actin polymers and promoting nucleation for this process, and VACV is one such virus that is capable of both (Leite and Way 2015). Newly formed enveloped VACV particles move from a perinuclear site of wrapping to the cell periphery via microtubule-based transport driven by the kinesin-1 motor complex. Here, virus particles switch to actin-based transport at the cell periphery (Hollinshead et al. 2001; Rietdorf et al. 2001; Ward and Moss 2001). While treatment of cells with a low concentration of latrunculin B stimulates virus movement to the cell periphery and does not affect virus release, latrunculin B at higher concentrations and cytochalasin D reduce virus release, indicating the importance of actin dynamics for VACV exit (Arakawa et al. 2007a). It was discovered that RhoA, a regulator of the actin cytoskeleton, normally inhibits VACV release and that the viral protein F11 overcomes this action by inhibiting RhoA and its downstream signaling (Arakawa et al. 2007b; Handa et al. 2013). This occurs via a PDZ domain (a commonly occurring protein-binding domain, although the first to be discovered in a viral protein) in F11, which binds to myosin-9A, an inhibitor of RhoA signaling (Handa et al. 2013). F11 also promotes the migration of infected cells by inhibiting RhoA activity (Cordeiro et al. 2009; Valderrama

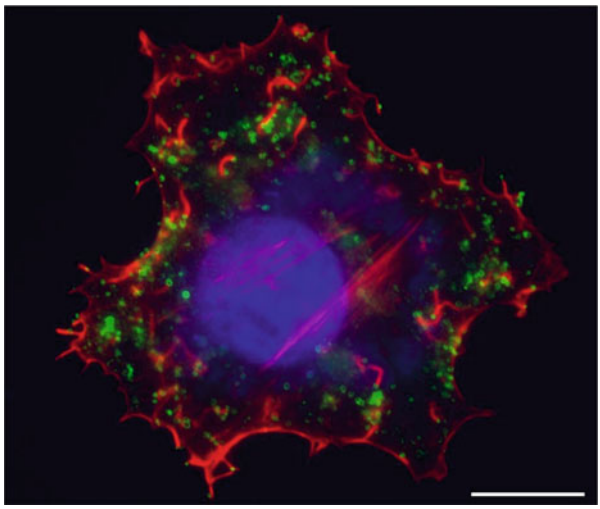

Fig. 9 Vaccinia virus particles (envelope protein labeled in *green*) exit infected HeLa cells on actin tails (visualized by phalloidin in *red*) at 8 hpi. The nucleus is stained with DAPI (in *blue*). Scale bar is 10 μm

et al. 2006). Therefore, VACV has developed a method to manipulate the regular cortical actin cytoskeleton, which obstructs virus access to the cell surface, to facilitate its release.

After traversing the cortical cytoskeleton, VACV particles that reach the cell periphery fuse with the plasma membrane but remain attached to it. Here, VACV triggers localized actin polymerization beneath cell-associated extracellular virus to facilitate its spread to adjacent cells (Fig. 9) (Cudmore et al. 1995, 1996; Doceul et al. 2010; Horsington et al. 2013). Activating actin nucleation in this manner is unique to the *Orthopoxviridae* genus but bears remarkable similarity to F-actin pedestals induced by enteropathogenic *Escherichia coli* (EPEC) (Stevens et al. 2006). The viral transmembrane protein A36 is phosphorylated by host Src and Abl kinases (Frischknecht et al. 1999; Newsome et al. 2004, 2006; Reeves et al. 2005), which then recruits various factors of the host actin polymerization cascade, including N-WASP (Moreau et al. 2000; Snapper et al. 2001). N-WASP is a cellular NPF, a signaling molecule that activates the Arp2/3 complex, which results in comets of F-actin or "tails" adjacent to extracellular VACV particles (Frischknecht et al. 1999; Goley et al. 2006; Moreau et al. 2000; Weisswange et al. 2009). Interestingly, clathrin and its adaptor protein AP-2 are also recruited to the site of actin polymerization, stabilizing A36 and N-WASP and promoting actin comet formation (Humphries et al. 2012; Schmidt and Mercer 2012). ASFV also induces actin projections at the plasma membrane on exit; however, the mechanism appears to be distinct to that employed by VACV and remains to be characterized at the molecular level (Jouvenet et al. 2006).

Actin is also necessary for the budding of measles virus (MV) and respiratory syncytial virus (RSV) particles as the inhibition of actin dynamics reduces cell-free virus titers, although viral protein synthesis is unaffected (Berghall et al. 2004; Kallewaard et al. 2005). The role of actin in MV release was determined by the use

of different actin inhibitors; cytochalasin D reduced transport of viral capsids (complexes of the MV M protein and newly formed nucleocapsids) from the nucleus to the plasma membrane, confirming the requirement for intact actin filaments for this process. Jasplakinolide treatment reduced virus release but not viral synthesis, supporting a role for actin dynamics in MV particle budding and release (Dietzel et al. 2013). Here the authors propose an interaction between the M protein of the measles virus and F-actin, which was subsequently confirmed by Wakimoto et al. by immunoprecipitation of the viral M protein and actin following MV infection (Wakimoto et al. 2013). Interactions between the M protein of other *Paramyxoviruses* such as Sendai and Newcastle disease viruses and actin have also been observed (Giuffre et al. 1982).

The budding of MV particles is not the sole mechanism for MV spread; infected cells are able to fuse with uninfected cells to form syncytia. This process is mediated by MV H and F glycoproteins that are incorporated into the MV envelope and the cell membrane of infected cells (Wild et al. 1991). Furthermore, this interaction can be modulated by point substitutions on the M protein, which affect its interaction with the H protein (Tahara et al. 2007). A recent study using a clinically isolated strain of MV identified a variant form of this strain that exhibits greater MV release and reduced syncytia formation on infection, due to a single-point mutation in its M protein (F50P) that attenuates its binding to F-actin (Wakimoto et al. 2013). It was proposed that F-actin binding to the M protein in this strain might normally promote syncytia formation, and hence abrogation of this binding leads to an increase in MV budding. Interestingly, cytochalasin D treatment of cells infected with this clinical isolate and the F50P mutant MV strain stimulated virus release, contrary to expectations (Dietzel et al. 2013). A comparison of the amino acid sequences of the M proteins from the two strains revealed four amino acid substitutions, which are currently being investigated to dissect the role of this protein in F-actin-mediated control of MV release (Wakimoto et al. 2013).

In addition to syncytia formation, virus infection can also induce the creation of intercellular connections that facilitate virus spread. Infectious influenza A virus cores can travel along actin-containing connections between cells in the absence of budding or release of cell-free virions (Roberts et al. 2015). Retroviruses such as MLV and HIV-1 also spread by establishing cell–cell filopodial bridges or conduits, which can be inhibited by disrupting actin dynamics (Kadiu and Gendelman 2011; Sherer et al. 2007); however, the role of actin in this process is distinct from that involved in budding or entry (Sherer et al. 2007). Interestingly, prions have also been shown to utilize this actin-dependent method for spread in neuronal cells (Gousset et al. 2009).

5 Conclusion

It is quite clear that actin is targeted for manipulation by a number of viruses as a result of the fundamental roles it plays in a cell. While many of their techniques may be unique, trends in how the actin cytoskeleton is harnessed during virus

replication can be observed. Viruses require a rearrangement of the cortical actin cytoskeleton to gain entry to cells; however, the size of the virus plays a role here. While smaller viruses such as HIV and DENV-1 can enter by actin-assisted clathrin-mediated pathways, larger adenoviruses and orthopoxviruses harness the more flexible macropinocytic entry mechanism. Following entry, actin-mediated cellular transport pathways present an efficient means for invading pathogens to travel to sites of replication and/or exit. More complex viruses with larger genomes such as VACV and AcMNPV have evolved to encode proteins that specifically interact with actin cytoskeleton signaling pathways to initiate their movement.

While the use of various actin-destabilizing drugs to study the role of actin in virus infection has been invaluable, care must be taken in their interpretation as these drugs often induce broad or off-target effects in a cell. A more precise understanding of the specific function of these drugs and their use in combination may be useful to narrow down the roles of actin at various stages of the virus replication cycle. Additionally, caution must be observed when using different viral strains to answer broad questions on viral-actin interactions as we have seen that different viral strains have evolved different relationships to actin depending on their specific host cell targets in vivo.

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Multiscale View of Cytoskeletal Mechanoregulation of Cell and Tissue Polarity

Chen Luxenburg and Benjamin Geiger

Contents

Abstract

The ability of cells to generate, maintain, and repair tissues with complex architecture, in which distinct cells function as coherent units, relies on polarity

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cues. Polarity can be described as an asymmetry along a defined axis, manifested at the molecular, structural, and functional levels. Several types of cell and tissue polarities were described in the literature, including front-back, apical-basal, anterior–posterior, and left-right polarity. Extensive research provided insights into the specific regulators of each polarization process, as well as into generic elements that affect all types of polarities. The actin cytoskeleton and the associated adhesion structures are major regulators of most, if not all, known forms of polarity. Actin filaments exhibit intrinsic polarity and their ability to bind many proteins including the mechanosensitive adhesion and motor proteins, such as myosins, play key roles in cell polarization. The actin cytoskeleton can generate mechanical forces and together with the associated adhesions, probe the mechanical, structural, and chemical properties of the environment, and transmit signals that impact numerous biological processes, including cell polarity. In this article we highlight novel mechanisms whereby the mechanical forces and actin-adhesion complexes regulate cell and tissue polarity in a variety of natural and experimental systems.

Keywords
Actin • Adhesion • Mechanobiology • Myosin • Polarity • Tension

1 Introduction

1.1 The Metazoan Revolution and the Emergence of Multicellular Morphogenesis

The evolution of multicellular forms of life ("metazoa") was a long and gradual process, which is commonly believed to have taken place between 1,300 and 600 million years ago (Conway Morris 1998). During that period, unicellular organisms started to develop colonial forms of life, which led, over time, to the development of independent, "individualized" metazoan organisms, most likely resembling today's sponges (Muller and Muller 2003). This transition from relatively simple unicellular organisms (protozoa) to the considerably more complex multicellular life was apparently associated with major changes in the cells' survival strategy; while in protozoan organisms, all life processes (e.g., reproduction, motility, feeding, and protection) are independently executed by all individual cells. In metazoans these missions are performed by specialized sub-populations of cells. With the advent of metazoan evolution, mainly during the "Cambrian explosion," an increasing order in body plan was observed (mainly, through detailed fossil records), with a common form, characterized by tissue polarization along a single longitudinal axis [anterior (front)–posterior (back)], which is often associated with the direction of locomotion (Martindale 2005) (Fig. 1). The cellular and molecular processes underlying this tissue polarization will be discussed below.

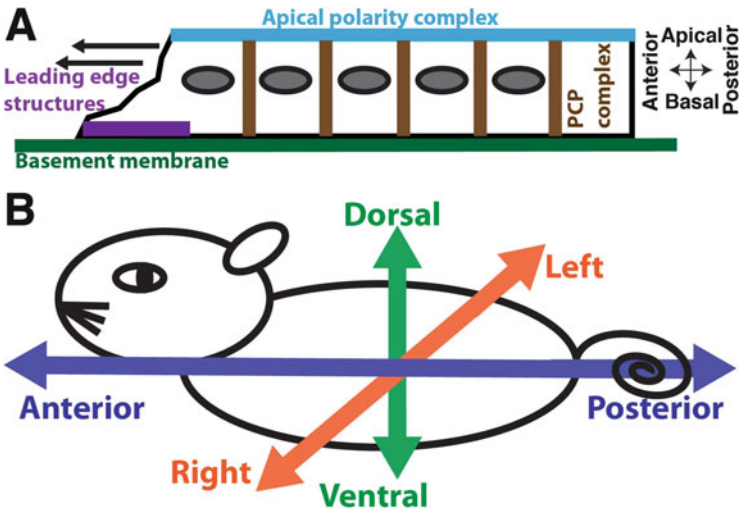

Fig. 1 Schematic overview of polarity from the cell and tissue level to the whole organism level. The example shown here is of simple epithelia that exhibit front-back polarity, apico-basal polarity, and anterior–posterior polarity (**a**). At the whole organism level (bilaterians) three distinct axes can be evident: anterior–posterior, dorsal-ventral, and left-right (**b**)

Interestingly, many studies, initially based on biochemical information (e.g., Burger and Jumblatt 1977; Muller et al. 1982; Wiens et al. 2003) and later on molecular genetic data (Wiens et al. 2003), revealed diverse adhesion molecules in sponges ("aggregation factors," Burger and Jumblatt 1977), which promote species-specific clustering of sponge cells. Moreover, the inter-cellular space in sponges contains galectin (Wagner-Hulsmann et al. 1996), as well as additional components related to the extracellular matrix (ECM) of higher metazoans, including collagen, and fibronectin-like protein (Labat-Robert et al. 1981). Naturally, in higher metazoa the repertoire of ECM proteins is considerably larger than that of sponges, reflecting the increasing complexity of tissue and organ architecture.

1.2 Cell-to-Tissue Polarity

Asymmetric distribution of molecules, sub-cellular structures, and organelles is a hallmark of many cell types. These basic asymmetries can lead to local changes in cell shape and/or function, transiently or constitutively, and are largely known as cell polarity. In fact, polarity is a fundamental property of nearly all living cells; it can be detected in vitro and in vivo, in a wide variety of cell types from those forming complex metazoans to simpler unicellular eukaryotes such as amoeba and yeast and also in some bacteria.

Polarity plays a critical role in cells' life. The fate of a new daughter cell may be determined by polarity cues that orient the mitotic spindle and assign the daughter

cells into a specific niche (Williams and Fuchs 2013; Vorhagen and Niessen 2014). Polarity also plays a key role in cells' ability to execute many biological functions, such as migrating directionally as individuals or collectively (Vicente-Manzanares et al. 2009; Devreotes and Horwitz 2015). It is also essential for cell ability to maintain properly its architecture and function by regulating cytoskeletal organization, trafficking, and signaling events (Guyer and Macara 2015; Narimatsu et al. 2015; Sasaki 2015). Morphogenesis, a process that shapes tissues and organisms, rely on complex polarity cues, and once a tissue takes shape, polarity allows it to maintain homeostasis and function as a coherent and, largely, independent unit (Roignot et al. 2013; Tellkamp et al. 2014). Moreover, upon injury, polarity cues allow cells to repair the tissue effectively and resume normal function.

With so many vital processes that take place throughout the life of an organism, it is not surprising that defects in cell polarity may have dire consequences, such as severe neural tube closure defects (Juriloff and Harris 2012), deafness and kidney diseases, and severe homeostasis defects associated with the development of cancer (Fischer et al. 2006; Martin-Belmonte and Perez-Moreno 2012).

While most cells exhibit some sort of asymmetry, polarity can be quite diverse in space and time (Fig. 1). Some cell types maintain a highly polarized architecture throughout life, while other cells transiently polarize to execute a specific biological function (e.g., immune cells during inflammation).

As briefly mentioned above, polarity can be classified, according to the axis that defines the asymmetry. The most common types of polarity are

Front-back polarity, found in many types of migratory cells; it can be recognized by the assembly of motility related actin-rich protrusions at the front of the cell (the leading edge), while at the back of the cell is enriched with contractile structures that facilitate the retraction of the trailing edge of the cell (Devreotes and Horwitz 2015).

Apical-basal polarity is commonly found in simple, single-layered epithelia (Figs. 1, 2, and 3) where it relates to the following sub-cellular domains: (a) The apical domain – the cell's part that faces the lumen of an organ or the environment; (b) The lateral domain – the cell's part that faces the neighboring cells in the monolayer; and (c) The basal domain – the part of the cell that faces and interacts with the ECM. In some simple epithelia apico-basal polarity can be recognized by the appearance of actin-rich microvilli at the apical region, cell–cell adhesion structures at the lateral domain, and cell–ECM adhesions at the basal aspect (Roignot et al. 2013; Rodriguez-Boulan and Macara 2014).

Planar cell polarity (PCP) refers to the collective polarization of cells along the plain of a tissue, orthogonal to apical-basal polarity (Figs. 1 and 2). This polarity allows long-range communication between cells, affecting their internal organization relative to the anterior–posterior/proximal-distal axes and supports their functional coordination as coherent units (McNeill 2010; Devenport 2014; Sebbagh and Borg 2014; Sokol 2015) (for more details, see Chapter 2).

Left-right polarity (also known as "chirality" or "handedness") refers to bilateral asymmetry that can be detected in many organisms. It can be evident at the cell

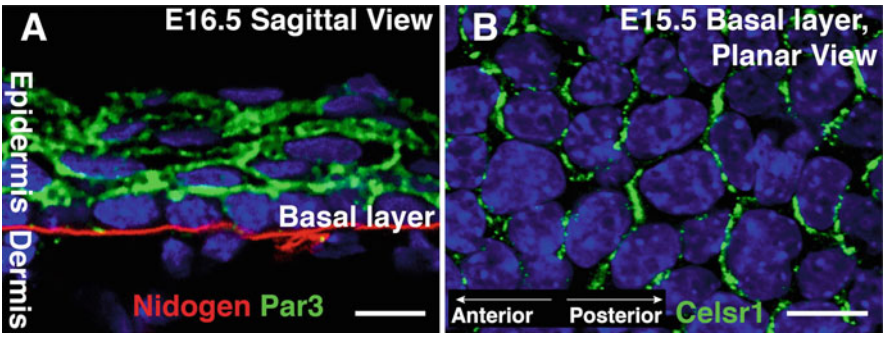

Fig. 2 Apico-basal and planar cell polarity in the skin epidermis. The apico-basal polarity protein PAR3 (*green*) labels the apical domain of basal layer cells; the basement membrane protein Nidogen (*red*) labels the dermal-epidermis boundary (**a**). The core PCP protein Celsr1 (*green*) labels the anterior and posterior faces of basal layer cells (**b**). Scale bar = 10 μm

level as many cultured cell types establish asymmetry with consistent handedness. At the tissue/organ level left-right polarity cues shape our organs, such as heart and viscera that exhibit clear asymmetry (Coutelis et al. 2014; Hamada and Tam 2014; Yoshiba and Hamada 2014) (for more details, see Chapter 3).

While every type of polarity has unique regulators, some proteins and cellular structures are essential for most, if not all, types of polarity. The actin cytoskeleton and its associated adhesion structures are the primary drivers of polarization at the cell's level (see Figs. 3 and 4 for actin/adhesion complexes in epithelial cells and fibroblasts). Below, we discuss the roles of the actin cytoskeleton and its adhesions in cell and tissue polarity. Given that cellular and trans-cellular mechanical forces are major regulators of both the actin cytoskeleton and its associated adhesions, we will also briefly address here classical examples (Chapter 1) and novel mechanisms that play a role in the bio-mechanical regulation of planar, and left-right polarities (Chapters 2 and 3).

1.3 The Roles of the Actin Cytoskeleton in the Acquisition and Maintenance of Cell Polarity

The cytoskeleton is made of three major types of filaments: actin filaments (F-actin), microtubule, and intermediate filaments. The first two exhibit an intrinsic asymmetry and play a major role in polarity (Li and Gundersen 2008; Mullins 2010). The actin filament is a polymer composed of globular subunits made of the monomeric G-actin, which polymerizes in a uniform orientation, giving rise to a polarized linear polymer with two distinct ends that exhibit different structural and dynamic properties. The two ends of F-actin are the "barbed" (+) and "pointed" (−) ends. At steady state, G-actin associates with the polymer primarily at the barbed end and dissociates at the pointed end, a process that leads to a "treadmilling"

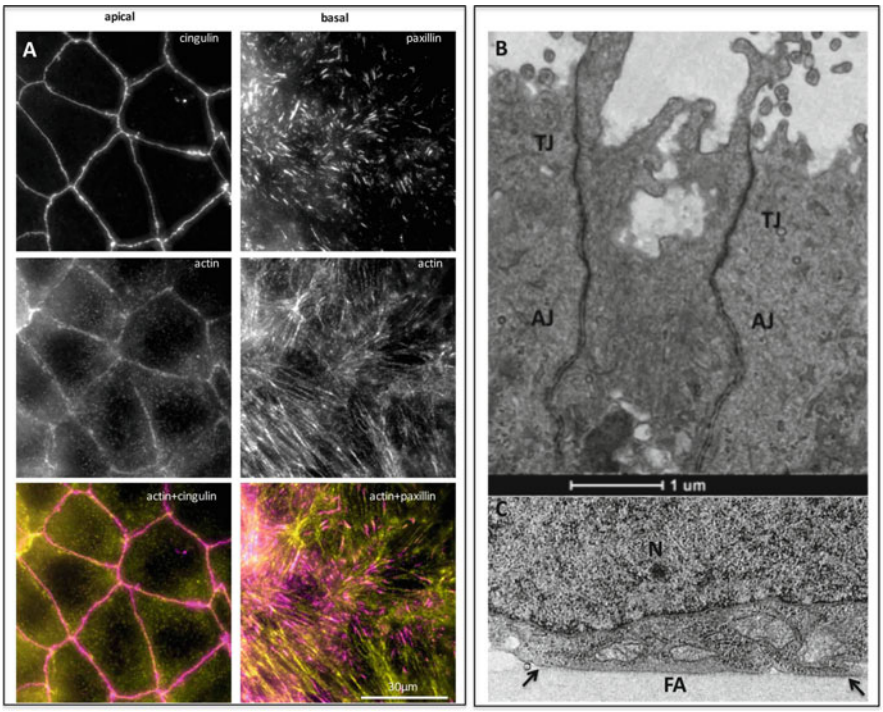

Fig. 3 Organization of inter-cellular tight junctions and cell–ECM adhesions formed by cultured intestinal epithelial (CaCo2) cells. (**a**) A panel showing immunofluorescently labeled CaCo2 cells visualizing cell adhesions formed at the apical (*left*) and basal (*right*) areas of the cells. The cells were triple-labeled for the tight junction component cingulin (*top*, *left*), actin, which is associated with both cell–cell adherens junctions and cell–ECM focal adhesions (*middle*) and paxillin (*top*, *right*), associated with cell–ECM FAs. Two-color (*pink-green*) super-positions of cingulin/actin and paxillin-actin are depicted in the images shown at the bottom (courtesy of Inna Grosheva). (**b**) Transmission electron microscopy, showing (in a grazing section) the apical tight junction (*TJ*) and the sub-apical adherens junction (*AJ*). (**c**) Transmission electron microscopy, showing (in a cross section) a basal focal adhesion (marked by the *two arrows*); *N* nucleus (electron micrographs were provided by Ilana Sabanay)

process that is characteristic of the internal dynamics of the actin filament in cells. These dynamic properties also involve chemical energy: new G-actin that undergoes polymerization binds ATP (G-actin-ATP) that will be hydrolyzed soon after polymerization (G-actin-ADP-Pi) followed by a slower dissociation of the phosphate (G-actin-ADP) eventually leading to the dissociation of G-actin from the pointed end of the filament. ATP hydrolysis alters the local conformation of the polymer and therefore allows actin binding proteins such as cofilin to distinguish between "new" and "old" parts of the actin filaments. Beyond cofilin, more than 100 different proteins bind actin filaments. Some of these can differentiate between the barbed and the pointed ends (e.g., capZ and tropomodulin, respectively), others can alter the stability of the filament and modify its dynamic properties (e.g.,

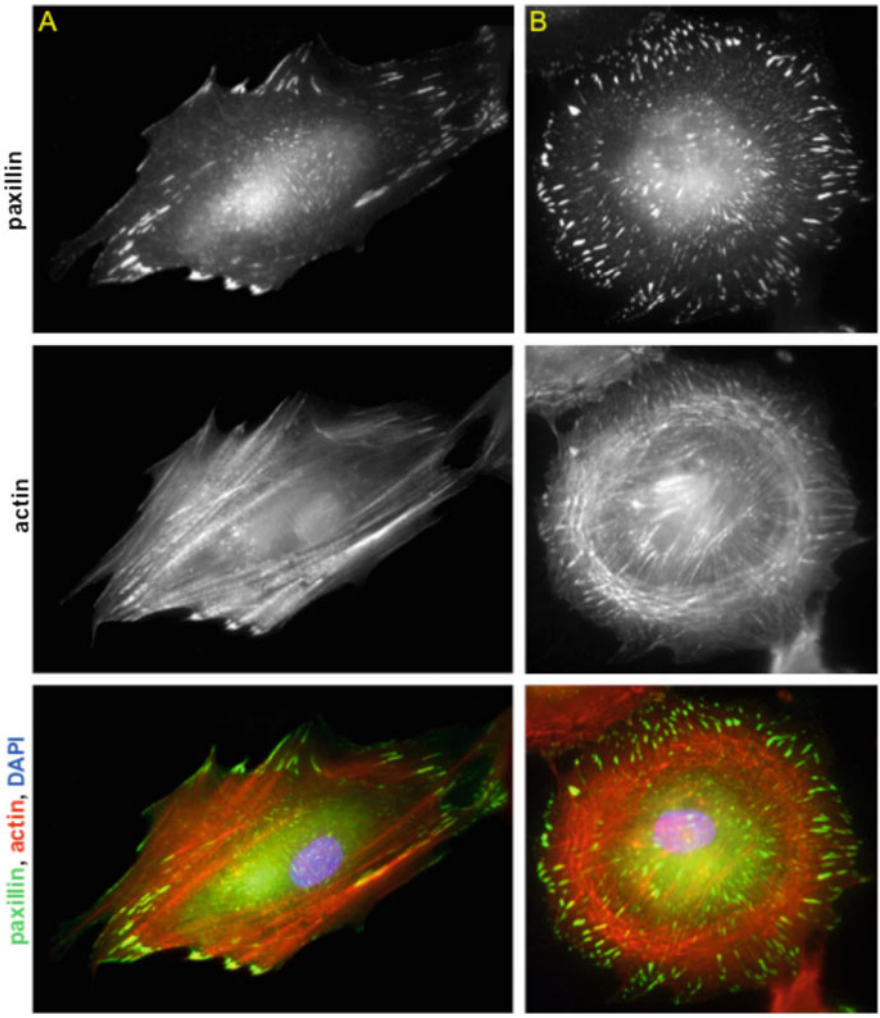

Fig. 4 Effect of matrix rigidity on cell polarization, focal adhesion formation, and actin organization. Fibroblasts, stably expressing YFP-paxillin (*green*), were plated on elastomeric PDMS substrates with different stiffness ("rigid" = 2 MPa (**A**); "soft" = 5kPa (**B**)), incubated for 6 h, and then fixed-permeabilized and labeled with phalloidin to visualize actin (*red*), and DAPI (*blue*), to visualize the nucleus. Notice the polarization of the cell adhering to the rigid surface, and the radial spreading of the cell adhering to compliant surface (courtesy of Alexandra Lichtenstein)

tropomyosins), create bundles with different orientation/polarity (e.g., fimbrin that forms parallel bundles, α-actinin that is associated with anti-parallel bundles, and filamins that support the formation of F-actin networks). This extraordinary variety of actin modulators allows the actin cytoskeleton to function as a highly versatile platform that regulates many processes including cell polarity (Pollard 2007; Pollard and Cooper 2009; De La Cruz and Gardel 2015).

Special players in actin-based mechanics are myosins that are actin binding molecular motors that translate chemical energy (ATP) into mechanical work (transport or contraction). Myosins' ability to "walk" unidirectionally along actin fibers and rotate the actin filament with constant handedness (Sase et al. 1997; Beausang et al. 2008) renders them key players in the generation and maintenance of cell polarity. The myosin superfamily is large and versatile; however, it can be divided into two groups: conventional and unconventional myosins. Conventional myosin, also known as myosin II, assembles into bipolar filaments that together with actin filaments generate tension forces. Myosin II plays a key role in many processes that require the generation of tension within and between cells, such as muscle contraction, cell migration, cytokinesis, and cell shape regulation (Zaidel-Bar et al. 2015). Unconventional myosins that do not form filaments play a role in many processes including transport of cargo and functional assembly of actin-rich structures such as stereocilia in the inner ear, epithelial microvilli, and filopodia (Hartman and Spudich 2012; Vicente-Manzanares et al. 2009). Although the two groups of myosins have distinct structures, enzymatic properties, and functions, members of both groups play key roles in the establishment of polarity.

In the *C. elegans* zygote myosin II activity plays a key role in the polarization of *PAR* ("partitioning defective") proteins and the establishment of anterior/posterior polarity (Munro et al. 2004). Sperm entry into *C. elegans* egg alters cortical actomyosin activity ("actomyosin" refers to a contractile complex of F-actin and myosin II). Specifically, it induces flow of actin and myosin II from the sperm entry site that will become the posterior of the embryo, to the opposite pole, that will become the embryo's head.

A role for non-conventional myosin was demonstrated in the budding yeast *S. cerevisiae*. Polarization of non-polar yeast (G1 arrested) can be induced by expressing of active Cdc42, a Rho family small GTPase, and a key regulator of the actin cytoskeleton and polarity (Wedlich-Soldner et al. 2003). This process involves amplification of stochastic Cdc42 activity to cross a certain threshold and establish polarity. In this system Cdc42 mediates the polymerization of actin cables on which the unconventional myosin V transports more Cdc42 towards the membrane and amplifies random signals into a stable site of active Cdc42 that interacts with actin cables (Wedlich-Soldner et al. 2003; Li and Gundersen 2008).

1.4 Thoughts About the Roles of Actin Associated Adhesion Structures in Acquisition of Polarity

How does the assembly of the actin cytoskeleton contribute to embryonic morphogenesis and acquisition of polarity? In multicellular organisms, cytoskeleton-anchored adhesions of cells to the ECM or to their neighbors play a key morphogenetic role. Consequently, the characterization of cell adhesions, and their structural and functional diversity was extensively pursued in recent years (Wolfenson et al. 2013; Winograd-Katz et al. 2014; Lecuit and Yap 2015; Priya and Yap 2015).

Adhesive interactions, as part of the cells' communication and interactions network, are highly complex processes that enable the sensing of the chemical and physical properties of external surfaces. These environmental cues can be further integrated by the cells, and activate signaling processes that regulate cell proliferation, survival, differentiation, and migration. Furthermore, specific classes of cell adhesions, mainly integrin mediated cell–ECM adhesions (Meldolesi 2016), and cadherin-mediated cell–cell adherens junctions (AJ) (Harris and Tepass 2010) have an inherent association with the actin cytoskeleton, and consequently, play a key role in cellular mechanics, including cell polarization processes.

It is noteworthy that in addition to integrin- and cadherin-mediated adhesions, discussed here, cells contain a wide variety of additional adhesive mechanisms, which affect cell behavior and fate. To mention just a few – cells can bind to the connective tissue and basement membrane via a variety of lectins and proteoglycan receptors, as well as intermediate filament-anchored hemidesmosomes (McDonald and Mecham 1991). Similarly, the canonical inter-cellular junctional complex of epithelial cells consists of apical tight junctions, which seal epithelial sheets, and sets the apical-basolateral polarity. Along their lateral membranes, epithelial cells communicate via gap junctions and form robust cytokeratin adhesions, namely desmosomes (Alberts 2015). In this article we will focus only on the actin-associated, integrin- and cadherin-mediated junctions.

Cellular characterization of integrin-mediated ECM adhesion was (and still is) strongly based on the investigation of focal adhesions (FA) and related structures. FAs are specialized and defined regions (commonly measuring 0.25–2 by 2–10 μm) along the ventral plasma membrane, which are directly interacting with the substrate. Moreover, FAs are associated via their cytoplasmic faces with the termini of bundles of actin filaments, known as stress fibers. The molecular composition of FAs was addressed by a wide variety of experimental approaches, ranging from immunofluorescence microscopy to biochemistry to advanced genomics and proteomics, yielding a long list of proteins that belong to the so-called integrin adhesome (Table 1; Zaidel-Bar et al. 2007; Zaidel-Bar and Geiger 2010; Horton et al. 2016). Among them are "scaffolding proteins" (e.g., talin, vinculin, paxillin, and zyxin) that physically bridge, directly or indirectly, between the cytoplasmic domains of integrins and the actin cytoskeleton, and "signaling proteins" (e.g., a variety of kinases, phosphatases, and regulators of GTPases), which can affect cell behavior, as well as the fate of the adhesion site itself (Zaidel-Bar and Geiger 2010). In recent years much information has accumulated not only on the physiological roles of the integrin adhesome, but also on its involvement in human diseases (Winograd-Katz et al. 2014).

FAs, and additional forms of integrin-mediated contacts with the ECM such as focal complexes, fibrillar adhesions, podosomes, and invadopodia (Geiger and Yamada 2011), play key roles in the induction of cell spreading in a wide variety of tissue cells (e.g., mesenchyme-derived fibroblasts, epithelial cells, and endothelial cells), followed by anterior–posterior polarization. The mechanism underlying this process involves a combination of integrin-mediated signaling and scaffolding. Interaction with the ECM was shown to activate the small GTPases Rac1 and

Table 1 The molecular architecture of integrin- and cadherin-mediated adhesions

Major protein	Cadherin	Integrin	Both	All
Actin and regulators	19	10	7	36
Adaptors	29	47	18	94
Adhesion receptor	22	40	2	64
Cytoskeletal	–	–	3	3
GAPs	6	10	3	19
GEFs	2	14	3	19
GTPases	4	4	2	10
Lipid kinases	–	–	2	2
Lipid phosphatases	–	4	1	5
S/T kinases	4	1	4	9
S/T phosphatases	–	3	1	4
Y kinases	3	4	6	13
Y phosphatases	7	4	5	16
Motor proteins	4	–	1	5
Proteases	4	2	1	7
Channels	2	4	–	6
Ub ligases	1	3	–	4
Chaperones	–	3	–	3
All	107	153	59	319

The major families of molecular components, associated with the cadherin and integrin adhesomes, are presented. This table shows that both types of adhesions are highly enriched with actin, actin regulators, and adaptor proteins that link the adhesion receptors to the actin cytoskeleton. Moreover, many of the actin regulators and adaptors are associated with both integrin and cadherin adhesions. The two adhesion types contain, in addition, multiple signaling molecules, most prominently small GTPases and their regulators, as well as kinases and phosphatases (mainly tyrosine specific). These signaling molecules are believed to play an important role in regulating cell behavior and fate, as well as the assembly and stability of the adhesion site

CDC42, which trigger cell spreading by activating lamellipodial and filopodial protrusion (Price et al. 1998; Lawson and Burridge 2014). Nascent adhesions, which form under the expanding lamellae, are consequently exposed to mechanical stress, which promotes their growth and maturation. This is followed by the nucleation and assembly of stress fibers that run between FAs, and apply contractile forces to the two FAs associated with its ends. This Rho A-activated actomyosin contractility applies a stable tension, via the stress fibers to FAs (~5.5 nN/μm^2; see Balaban et al. 2001), which was shown to be crucial for maintaining the integrity of the FA-stress fiber complex.

How are integrin-mediated FAs involved in regulating cell polarization? Examination of cells, plated on a rigid surface, indicated that cell spreading on the substrate is, initially, a radial process, leading to the development of circular ("fried-egg") morphology, with peripheral, mostly radially oriented FAs. The rate

and extent of spreading and FA formation vary between cells, and are usually affected by ligand density and other surface features (e.g., Reinhart-King et al. 2005). Upon longer incubation, the radial symmetry is usually broken (most prominently in fibroblasts), and the cells tend to polarize, and assume an elongated morphology. In cells with migratory properties, the two ends of the major axis of the cells are distinct; one end developing a protrusive lamellipodium ("leading edge"), while the opposite end is dominated by focal adhesions ("trailing edge"). The acquisition of such anterior–posterior polarity plays a crucial role in physiological processes such as embryonic development and wound healing, as well as pathological states like invasive and metastatic migration of cancer cells. This transition from radial to axial symmetry is regulated by both the rigidity of the underlying substrate and the cell's adhesion-signaling system. Specifically, it was shown that cell polarization/ elongation depends on substrate rigidity, and is usually preceded by polarization of the peripheral FAs (Fig. 4). Furthermore, it was shown, by siRNA screening, that the polarization process is tightly regulated by tyrosine kinases within the adhering cells (for details, see: Prager-Khoutorsky et al. 2011).

Actin associated cell–cell AJs also play key roles in tissue coherence, primarily in epithelial, endothelial, and cardiac muscle cells (Geiger et al. 1983; Volberg et al. 1986). In simple epithelial cells (e.g., intestinal or renal epithelia) they are usually organized as sub-apical rings, located just below the apical tight junctions (Farquhar and Palade 1963), while in other cell types they form patchy adhesions of variable sizes and distributions (Fig. 3). The adhesion receptors of AJs are different cadherins, which commonly mediate calcium-dependent homotypic interactions (between similar cells) and act in a homophilic manner (interacting with the same cadherin type on the neighboring cell). Just like integrins, in ECM adhesions, cadherins interact with actin via a network of scaffolding and signaling molecules, referred to, collectively, as the "cadherin adhesome" or "cadhesome" (Table 1; Guo et al. 2014; Zaidel-Bar et al. 2015). These components, collectively, contribute to the mechanical stability of the inter-cellular junction, and to its capacity to generate and transmit adhesion signals. Moreover, some of these molecules (e.g., vinculin, α-actinin, and VASP) are present in both types of adhesions, while others are associated only with focal adhesions (e.g., paxillin, ILK) or only with AJs (e.g., α and β catenin).

The regulation of AJ formation and stability is based on the same general principles which operate in integrin adhesions, including mechanical stimulation and integration (Lecuit and Yap 2015), coordinated activation of stimulatory GEFs and inhibitory GAPs (Braga and Yap 2005), and the recruitment of different kinases and phosphatases (Bertocchi et al. 2012). While the regulatory mechanisms underlying cell–cell and cell–ECM adhesion appear to be quite similar (Table 1), the two types of adhesions are far from being up- and down-regulated simultaneously. On the contrary; commonly, the state of organization of the two types of adhesions is reciprocally related. Whether the mechanism whereby these adhesion systems are coordinated and/or differentially regulated is not clear. Different possibilities are considered, including the activation of specific signaling pathways, stimulation of a mesenchymal-epithelial transition-like process, and direct mechanical cross-talk

between the two structures. Whichever the underlying mechanism is, the transition from epithelial to mesenchymal characteristics leads to the loss of apical-basal polarity and (in some cases), to acquisition of a "migratory" phenotype, based on anterior–posterior polarity.

1.5 The Roles of Mechanical Forces in Acquisition of Polarity

Mechanical forces are major regulators of biological processes. These forces can be divided into two types according to their source: intrinsic forces, developed inside the cell, and extrinsic, "environmental" forces, generated by whole body movements, muscle activity, blood flow, and the like. Mechanical forces affect multiple cellular processes, ranging from cell architecture to signal transduction to cell fate determination (Iskratsch et al. 2014; Zaidel-Bar et al. 2015).

The actin cytoskeleton is not only force generator, but is also sensitive to mechanical perturbations, which affect its polymerization (Goeckeler and Wysolmerski 1995; Hirata et al. 2008; Jegou et al. 2013), turnover (Wilson et al. 2010), and the assembly of actin-rich structures such as stress fibers and lamellipodia (Giuliano et al. 1992; Svitkina et al. 1997; Hotulainen and Lappalainen 2006; Goeckeler et al. 2008; Naumanen et al. 2008; Senju and Miyata 2009). As mentioned above, actin associated adhesion structures, both cell–cell and cell–ECM, are also sensitive to mechanical cues that alter their adhesive properties, their molecular composition, and signal transduction (reviewed in Geiger et al. 2001; Schiller and Fassler 2013; Wolfenson et al. 2013; Ladoux et al. 2010; Sumida et al. 2011; Priya and Yap 2015).

With such profound impact of cellular mechanics on the cytoskeleton and associated adhesion structures, it is not surprising that mechanical forces are major regulators of polarity, including front-back polarity (Vicente-Manzanares et al. 2009), apical-basal polarity (Yu et al. 2008), PCP (Lee et al. 2012; Walters et al. 2006), and left-right polarity (Naganathan et al. 2014; Tee et al. 2015).

To illustrate the mechanisms, we would like to describe here the "nodal model." Ciliated cells at the node of the mouse embryo (the node is a transient structure, a cavity at the posterior part of the notochord) rotate clockwise and generate a directional flow that can be detected by sensory structures and translated into expression of specific genes and establishment of left-right polarity. Defects in the assembly (Nonaka et al. 1998; Takeda et al. 1999) or motility (Supp et al. 1997) of cilia give rise to left-right polarity defects. Moreover, an artificial flow system was able to rescue motile cilia mutants and flip left-right polarity, providing direct evidence for the role of mechanical forces in the establishment of left-right symmetry (Nonaka et al. 2002).

2 Novel Mechanisms in the Establishment of Planar Cell Polarity

2.1 Some Basic Facts

PCP is a tissue-level phenomenon, which plays a fundamental role in tissue morphogenesis. For instance, PCP regulates collective cell migration and convergent extension (a process whereby tissue extends on one axis and narrows down in the orthogonal direction) (Wallingford et al. 2000; Darken et al. 2002; Goto and Keller 2002), as well as mitotic spindle orientation (Lake and Sokol 2009; Segalen and Bellaiche 2009) and cilia positioning and function (Park et al. 2006; Park et al. 2008; Tarchini et al. 2013). Studies in vertebrate model organisms showed that mutations in PCP genes may give rise to many developmental defects and diseases including neural tube closure defects, inner ear and heart development defects, wound healing defects, kidney disease, and cancer (Munoz-Soriano et al. 2012). In humans PCP defects are associated with neural tube closure defects such as spina bifida and craniorachischisis (Lei et al. 2010; Seo et al. 2011; Robinson et al. 2012), and were also reported in several models of human cancers (Hatakeyama et al. 2014).

At the molecular level, two groups of genes control PCP: the "core-PCP" and the "Fat-Dachsaus" groups, yet the mode of molecular interaction between the two pathways is not well understood. *Frizzled* and disheveled are core-PCP proteins; however, they are also important regulators of the *Wnt* signaling pathway. Therefore the core-PCP pathway is often referred to as non-canonical *Wnt* pathway. An important hallmark of these proteins is their asymmetric localization within the cells. Only *Celsr* (also known as *flamingo*), a transmembranal atypical cadherin core-PCP protein, is localized on both the anterior and posterior sides of the cell where it creates homodimers that connect neighboring cells (Fig. 2b), all the other PCP proteins in the two groups exhibit asymmetric distribution (reviewed in McNeill 2010; Devenport 2014; Sebbagh and Borg 2014; Sokol 2015).

2.2 Involvement of External Mechanical Forces in Planar Cell Polarity

One of the major challenges in the PCP studies is to identify the upstream cue(s) that provide directionality and determine the global axis of PCP. Recently it was shown that *wingless (Wg)* and *Wnt4* function redundantly to orient PCP in the *Drosophila* wing disc and affect the axis of polarity by regulating the interaction between two core-PCP proteins [*frizzeled* and *Vangl (Van Gogh)*] (Wu et al. 2013). An important work on the same tissue suggested that mechanical forces also affect the orientation of the PCP axis and couple PCP establishment with wing morphogenesis (Aigouy et al. 2010).

During the development of the drosophila wing, the tissue PCP changes its orientation. In early stage PCP is oriented towards the edge of the wing, later on it reorients along the proximal-distal axis (Classen et al. 2005). Live imaging

experiments conducted by Aigout and coworkers (Aigouy et al. 2010) demonstrated that the PCP reorientation is temporally correlated with cell and tissue shape changes. At the tissue level the shape dynamics involve the contraction of the wing hinge that loses health of its size while the blade becomes more elongated and narrows down. At the cellular level, oriented cell divisions, changes in cell shape, and establishment of new contacts can be detected. By severing the contracting hinge the authors demonstrated that PCP reorientation and tissue/cell dynamics are coupled. Laser ablation experiments showed that hinge contraction alters force distribution in the tissue from isotropic to anisotropic (Aigouy et al. 2010). Together, these data suggest a key role for mechanical forces in PCP orientation in the fly.

During gastrulation mechanical forces strain the developing skin in *Xenopus*. At the same time skin planar axis is established. These PCP cues will then guide the development and coherent function of multiciliated epithelial cells at the surface of the tadpole. Chien et al. (2015) noted that ventralized Xenopus embryos that exhibit many patterning defects manage to establish PCP. Since these abnormal embryos do undergo gastrulation that strains the developing skin, the authors asked whether PCP establishment is regulated by mechanical cues. To check this hypothesis, the authors exposed isolated ectoderm that normally does not establish PCP to external forces. By doing so Chien et al. nicely demonstrated that mechanical forces are very effective in establishing the axis of PCP in the developing Xenopus skin (Chien et al. 2015).

2.3 Involvement of Internal Mechanical Forces in Planar Cell Polarity

While external forces (hinge contraction) play a key role in wing development, wing cells are not indifferent bystanders in the process. Wing cells actively generate internal forces that respond to the external forces and together affect wing morphogenesis (Etournay et al. 2015). Moreover, recently, a tissue-specific knockdown of *Wdr1* (restricted to the mouse epidermis) demonstrated a key role for internal, actomyosin-generated forces in PCP establishment in a mammalian system (Luxenburg et al. 2015).

During the development of the mouse skin, epidermal cells of the basal layer establish PCP that orients the growth of hair follicles towards the anterior of the embryo (Fig. 2). This process results in a highly ordered pattern of hairs throughout the mouse fur. Defects in the core PCP gene *Frizzled6* give rise to an abnormal hair pattern (Guo et al. 2004; Wang et al. 2006). Early in epidermal development [embryonic day (E)12.5] core PCP proteins can be detected throughout the cortex of the cell, a day later early signs of anterior–posterior enrichment can be detected and 2 days later (E14.5) a polarized pattern is readily evident (Devenport and Fuchs 2008). E14.5 is also the first time point in development in which hair follicles begin their development.

The differentiation of keratinocytes involves dramatic changes in cell shape as cells of the basal layer alter their cuboidal architecture and turn flat. Recently,

Luxenburg et al. (2015) demonstrated that cytoskeleton-driven cell shape changes take place within the basal layer at the same time that the tissue establishes PCP. During this process the basal layer of epidermal cells becomes more compact and changes their orientation in the tissue. The knockdown of *Wdr1*, a scaffolding protein that enhances cofilin mediated actin severing activity, negatively affects both cell shape dynamics and the establishment of PCP. In line with these PCP defects, hair follicle orientation and molecular asymmetry are abnormal without *Wdr1*. Laser ablation experiments showed that boundary tension is significantly reduced without *Wdr1*, highlighting the involvement of this gene in the ability to generate and maintain cytoskeleton-derived forces and cortical tension in the cell.

In the mouse epidermis core PCP proteins undergo endocytosis on mitosis and will be recycled to the cell surface of the daughter cells (Devenport et al. 2011). When core-PCP mitotic internalization is blocked, cell shape changes that normally accompany mitosis (e.g., mitotic rounding (Luxenburg et al. 2011) abolish the localization of core PCP proteins and planar polarity (Shrestha et al. 2015). This observation further supports a key role for cell shape dynamics in the PCP.

3 Cytoskeletal Involvement in the Acquisition of Left-Right Polarity

3.1 Some Basic Facts

The establishment of left-right symmetry takes place early in development. In *C. elegans* and *Xenopus* left-right cues are generated by the actin cytoskeleton and can be detected at the single cell embryo stage (Danilchik et al. 2006; Naganathan et al. 2014). In most vertebrates left-right polarity cues affect the development of many organs, including the heart, lungs, stomach, liver, gallbladder, pancreas, spleen, and the brain. Moreover, many of the large arteries and veins also establish left-right asymmetry during development (Casey and Hackett 2000; Levin 2005). In cultured cells left-right polarity is evident by consistent handedness of cytoskeletal structures (Tee et al. 2015) and consistent directional migration under both confined and isotropic conditions (Nonaka et al. 2002; Xu et al. 2007; Tamada et al. 2010; Yamanaka and Kondo 2015).

A range of left-right polarity defects was described in humans, from complete reversal of all organs known as "*situs inversus*" to a range of partial defects known as "partial *situs inversus*," "*situs ambiguus*," "*heterotaxy*," or "*heterotaxia*." Some of these conditions may be fetal or negatively affect the health of the affected individual (Casey and Hackett 2000).

While defects in left-right polarity were described in snails more than a century ago, the first molecular components that mediate it were described only 20 years ago (Levin et al. 1995). Since then studies in several model organisms and in cultured cells identified a variety of molecular mechanisms that establish, maintain, and amplify left-right polarity. These mechanisms involve both the actin and the microtubule cytoskeleton and their motor proteins, as well as external forces and

ion fluxes (for recent reviews, see Coutelis et al. 2014; Hamada and Tam 2014; Yoshiba and Hamada 2014).

3.2 Key Roles for the Actin Cytoskeleton in Left-Right Polarity: Insights from Cultured Cells

The notion that the actin cytoskeleton plays a role in left-right polarity is well established. Wan et al. (2011) demonstrated this phenomenon in many different cell types. In this experimental system cells were cultured in a confined environment and cell alignment and migration were analyzed to determine left-right polarity. Strikingly, all types of cells exhibit handedness. Interestingly, normal and transformed skin fibroblasts exhibit opposite handedness, suggesting that chirality might be altered in specific pathological states, such as cancer. Treatment with drugs that modulate actin dynamics alters handedness, emphasizing the key role of the actin cytoskeleton in this type of polarity (Wan et al. 2011).

Recently Tee and coworkers (2015) provided intriguing insights into the roles of the actin cytoskeleton in left-right polarity. These authors cultured fibroblasts on circular adhesive islands and tracked the actin cytoskeleton by video microscopy. A sequence of five distinct patterns was detected that included a shift from isotropic to chiral pattern with defined handedness. The isotropic pattern was made of radial actin fibers growing from peripheral focal adhesions towards the cell center and transverse fibers that move centripetally. The shift to a chiral pattern takes place at the level of the radial fibers, which uniformly tilt to the same direction, inducing the swirling of the transverse fibers.

To gain mechanistic insights into the establishment of left-right polarity in this system the authors studied the structural, molecular, and dynamic properties of the chiral actin pattern. They demonstrated that while the radial and traverse actin fibers interact physically, their molecular composition is different: the actin bundling protein α-actenin is enriched in the radial fibers while the traverse fibers are enriched in myosin II. Moreover, myosin II motor activity is essential for the assembly of both radial and traverse fibers and its activity within the traverse fibers propel their movement along the radial fibers that rely on formin-mediated actin polymerization for their assembly. Overexpressing of α-actinin flips the handedness of the actin network, suggesting a key role for actin bundling in the development of cell chirality.

3.3 Key Roles for the Actin Cytoskeleton in Left-Right Polarity: Physiological insights

The aforementioned studies demonstrate that the actin cytoskeleton has an intrinsic ability to establish handedness under experimental conditions. A recent study by Naganathan et al. (2014) demonstrated that the actin cytoskeleton plays a key role in establishing left-right polarity also in a physiologically relevant system, the

C. elegans embryo. More than a decade ago Munro et al. (2004) showed that sperm entry into *C. elegans* egg induces flow of actin and myosin that polarizes PAR proteins and establishes anterior–posterior polarity. More recently Naganathan et al. (2014) revisited these early events in *C. elegans* development, and found that while cortical flow is readily detectable along the anterior–posterior axis, an orthogonal flow can also be detected, leading the anterior and posterior parts of the embryo to counter-rotate, relative to each other. Apparently a significant portion of myosin II activity is dedicated to the generation of torque that propels both the establishment of chirality and anterior–posterior flow. This process is regulated by Rho signaling, and subtle changes in this pathway can alter torque without affecting tension, affecting left-right but not anterior–posterior polarity. This mechanism is relevant also at the 4-cell stage at which *C. elegans* handedness is established. Interestingly, manipulating Wnt signaling genes that are known to play a role in left-right symmetry altered chiral actin flow. This observation further emphasizes the link between left-right polarity and actomyosin activity.

4 Conclusion

Cells rely on internal and/or external cues to establish asymmetry. In recent years it became evident that mechanical forces applied by the pericellular environment or generated inside cells, by the cytoskeleton, function as important upstream signals that trigger the establishment of cell and tissue polarity.

In the last decade advances in high-resolution microscopy, ECM nano- and micro-fabrication, as well as computational analysis shed new light on the molecular mechanisms underlying the mechanobiology of cell polarity. That said, many key questions are still poorly understood. For instance, it will be important to understand the cross-talk between chemical and mechanical cues in these processes and the identity of the diverse proteins that mediate the acquisition of polarity. These studies will, hopefully, advance our knowledge of cell polarity, with relevance to diverse physiological and pathological processes, including embryonic development, stem cell differentiation, and cancer biology.

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Molecular Control of Actin Dynamics In Vivo: Insights from *Drosophila*

Lena Brüser and Sven Bogdan

Contents

Abstract

The actin cytoskeleton provides mechanical support for cells and generates forces to drive cell shape changes and cell migration in morphogenesis. Molecular understanding of actin dynamics requires a genetically traceable model system that allows interdisciplinary experimental approaches to elucidate the regulatory network of cytoskeletal proteins in vivo. Here, we will discuss some examples of how advances in *Drosophila* genetics and high-resolution imaging techniques contribute to the discovery of new actin functions, signaling pathways, and mechanisms of actin regulation in vivo.

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1 Introduction

The actin cytoskeleton is essential for many biological functions in eukaryotic cells. The building block is the 42 kDa monomeric globular actin (G-actin) protein that can undergo cycles of self-assembly into filamentous actin (F-actin). Actin filaments contribute not only to mechanical stability of cells but also provide the mechanical forces to drive cell shape changes and cell migration during morphogenesis. Cells have evolved numerous actin binding proteins that maintain the pool of actin monomers, promote actin nucleation, restrict the length of actin filaments, and cross-link filaments into networks or bundles (Campellone and Welch 2010; Firat-Karalar and Welch 2011; Pollard and Borisy 2003; Pollard and Cooper 2009; Rottner and Stradal 2011). Thus, these accessory proteins determine the mechanical and dynamic properties of actin filaments and differentially control the organization of actin filaments into higher-order structures adapted to fulfill distinct cellular functions. Many aspects of actin assembly and disassembly can be recapitulated in vitro and in cell culture models. Reconstitution experiments greatly advance our understanding of how these molecules act on actin turnover and how they generate distinct cellular actin structures (Bugyi and Carlier 2010; Caceres et al. 2015; Cooper and Schafer 2000; Loisel et al. 1999; Vignaud et al. 2012). One of the key challenges for cell and developmental biologists, however, is to understand how these cytoskeletal proteins act in concert to shape cells and build tissues and organs in vivo.

The most powerful strategy to determine the function of a gene of interest is to remove it from a biological system and study the resulting phenotype. The fruit fly *Drosophila* has emerged as such a powerful genetic model organism to understand the mechanisms of cytoskeletal organization and dynamics as well as how the actin cytoskeleton is coupled to development and physiology. Genome-scale RNAi high-throughput screens in *Drosophila* cell culture lines have led to the identification of conserved components regulating cell shape and cell morphology (D'Ambrosio and Vale 2010; Kiger et al. 2003; Rohn et al. 2011). *Drosophila* cell lines such as the macrophage-like *Drosophila* S2R+ cells are well suited for high-resolution microscopy imaging (Fig. 1), for example, by using the structured illumination microscopy (SIM), a wide-field technique that doubles both lateral (100 nm) and axial (250 nm) resolution (Gustafsson et al. 2008). Importantly, S2R+ cells rapidly take up long double-stranded RNAs (dsRNA) added to the medium, causing efficient target knockdown. Comprehensive dsRNA libraries allow large-scale screens to systematically identify the cytoskeletal regulators. The whole toolbox of central cytoskeletal regulators is conserved in flies with reduced gene redundancy as

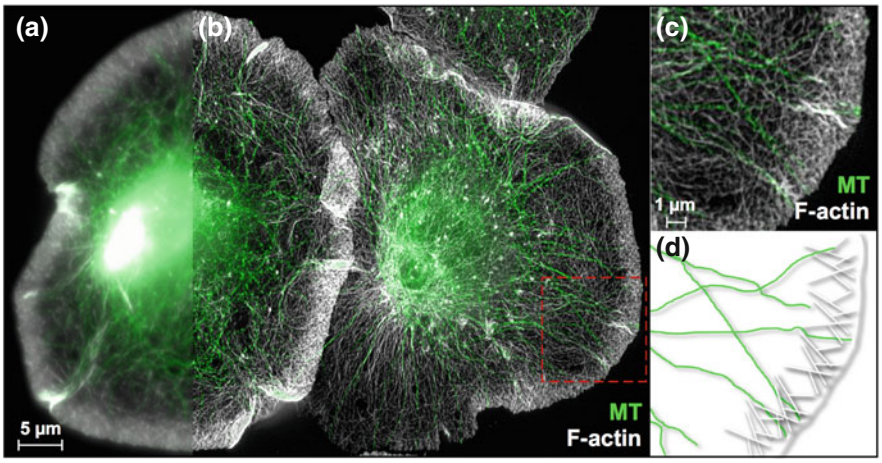

Fig. 1 The actin and the microtubule cytoskeleton of *Drosophila* S2R+ cells. Maximum intensity projection image of S2R+ cells stained with phalloidin-Alexa488 (F-actin) and Tubstain-TexasRed (microtubules). (**a**) Part of the entire image visualized by conventional wide-field microscopy and (**b**) imaged by structured illumination microscopy (SIM). The dense zone of actin network at the periphery corresponds to the lamellipodium. (**c**) A subset of the lamellipodium is enlarged from image (**b**). Scale bars are shown. (**d**) Schematic drawing of a branched actin network (*white-grey*) at the lamellipodium tip and single microtubules (*green*). Image was taken from Zobel and Bogdan (2013)

compared to vertebrates. Numerous techniques further allow combinatorial genetics by which gene functions, gene dosage, or a number of cytoskeletal genes can be manipulated simultaneously in vivo. Advances in live cell imaging and high-resolution microscopy further complement these genetic analyses (Hadjieconomou et al. 2011; Kanca et al. 2014; McMahon et al. 2008; Supatto et al. 2009; Zobel and Bogdan 2013). Comparable studies of the migratory cell behavior in a multicellular context with different genetic backgrounds are still not feasible in a vertebrate model system such as mice and zebrafish.

Remarkably, 75% of human disease-related genes have *Drosophila* homologs suggesting that flies can serve as a valuable model for human diseases (Reiter et al. 2001). Consistently, recent studies indeed highlighted the translational impact of *Drosophila* studies for genetic and age-related diseases or pathological dysfunctions (Bier 2005; Rieder and Larschan 2014; Wangler et al. 2015). Defects in membrane protrusions and cell migration are a major cause of several human diseases resulting in developmental malformations, immune diseases, and cancer metastasis. Members of the Wiskott–Aldrich syndrome protein (WASP) family are a prominent group of conserved cytoskeletal regulators, and their dysfunction results in aberrant cell protrusions, cell motility, differentiation, and increased invasion.

2 The Wiskott–Aldrich Syndrome Protein Family Members: Conserved Key Drivers of Actin Dynamics

The founding member of the WASP protein family was first identified in humans, whose loss-of-function results in the Wiskott–Aldrich Syndrome (WAS), an X-linked immunodeficiency, characterized by reduced numbers of small platelets (microthrombocytopenia), eczema, and an increased incidence of autoimmunity and malignancies (Derry et al. 1994; Imai et al. 2003; Maillard et al. 2007; Villa et al. 1995). The severity of WAS correlates with distinct mutations, ranging from complete loss-of-function to reduced gene function or expression (Jin et al. 2004; Stewart et al. 1999). Null mutations have a broad impact on different actin-mediated cellular processes in both adaptive and innate immune cells including abnormal differentiation of platelets and lymphocytes, defects in immune synapse formation, impaired phagocytosis and podosome formation (Bouma et al. 2007, 2011; Devriendt et al. 2001; Poulter et al. 2015; Recher et al. 2012; Thrasher and Burns 2010; Tsuboi 2007). While the expression of human WASP is restricted to the hematopoietic system, the related members such as N-WASP or the WASP family verprolin homologous proteins (WAVE) are more broadly expressed in mammals. Consistently, loss of the ubiquitously expressed N-WASP or WAVE2 in mice results in early lethality caused by severe neuronal and cardiovascular defects (Bear et al. 1998; Cotta-de-Almeida et al. 2007; Miki et al. 1996; Snapper et al. 1998; Xia et al. 2013; Yan et al. 2003). Similarly, a deficiency of the *Wiskott–Aldrich syndrome protein and SCAR Homolog* causes early embryonic lethality (Linardopoulou et al. 2007; Xia et al. 2013). However, whether excessive autophagy observed in *wash* deficient mice causes the lethality remains unclear (Xia et al. 2013). The developmental roles of the *WASP homolog associated with actin, membranes, and microtubules* WHAMM and the Junction mediating and regulatory protein JMY, two recently identified new members of the WASP proteins in mammals, have not yet been addressed (Campellone et al. 2008; Zuchero et al. 2009).

Compared to mammals, the *Drosophila* genome contains only single gene copies of *wave, wasp,* and *wash*, therefore analyses are not complicated by redundancy (Ben-Yaacov et al. 2001; Liu et al. 2009a; Zallen et al. 2002). Insects like *Drosophila* have subsequently lost a common ancestor of WHAMM and JMY (Kollmar et al. 2012; Veltman and Insall 2010). Instead, *Drosophila* has several uncharacterized genes encoding predicted Wiskott–Aldrich homology region 2 (WH2) domain proteins (Weiss and Schultz 2015). One of these proteins is WHAMY, which originated from a *wasp* gene duplication and underwent a sub-neofunctionalization. However, WHAMY lacks nucleation promoting factor (NPF) activity, but promotes exceptionally fast actin filament elongation (Brinkmann et al. 2016). By contrast, *Drosophila* WASP, WAVE, and WASH are potent NPFs in vitro (Fricke et al. 2009; Liu et al. 2009a; Tal et al. 2002; Zallen et al. 2002). Like their human counterparts, they possess a common C-terminal WCA domain, which is required and sufficient to activate the actin-related protein (Arp) 2/3 complex, a central nucleator of branched actin filaments in eukaryotic

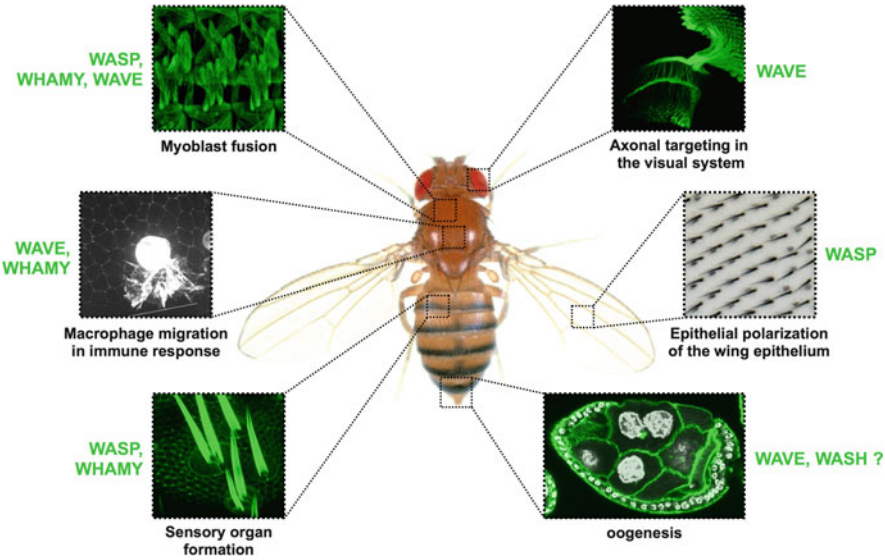

Fig. 2 Actin driven developmental processes controlled by Wiskott–Aldrich syndrome protein (WASP) family members in *Drosophila*. Despite their similar biochemical properties WASP proteins fulfill distinct cellular functions in vivo during *Drosophila* development. For references see text

cells (Machesky et al. 1994; Machesky and Gould 1999; Machesky and Insall 1998; Rohatgi et al. 1999).

In *Drosophila*, WASP proteins differentially regulate distinct aspects of Arp2/3 function in development (Fig. 2). Similar to human WASP loss-of-function mutations, flies lacking zygotic WASP function are viable and complete all stages of development, but die as young adults (Ben-Yaacov et al. 2001; Bogdan et al. 2005). Recent data further imply synergistic functions between WASP and WHAMY during morphogenesis. Double mutants are late-embryonic lethal and show severe defects in morphogenesis (Brinkmann et al. 2016).

Unlike human WASP, a role of *Drosophila* WASP in the hematopoietic system has not yet been found. *wasp* mutant flies display locomotion defects and a partial loss of sensory bristles (Ben-Yaacov et al. 2001; Bogdan et al. 2005). The phenotypic analysis of mutant flies revealed important roles in sensory organ development, muscle development, and spermatogenesis (Berger et al. 2008; Mukherjee et al. 2011; Ben-Yaacov et al. 2001; Bogdan et al. 2005; Rotkopf et al. 2011). The loss of sensory bristles in *wasp* and *arp2/3* mutants phenocopies *Notch* loss-of-function and is caused by a cell fate transformation (Ben-Yaacov et al. 2001). Additional work further suggests that the WASP-Arp2/3 pathway is not involved in Notch receptor endocytosis or its processing in the signal-receiving cell, but rather plays an important role in the trafficking of Delta-positive vesicles from the basal area to the apical cortex of the signal-sending pIIb cell (Rajan et al. 2009). Since Notch plays a critical role in cell fate determination in the hematopoietic

system, the clinical features of WAS patients such as thrombocytopenia and defects in the maturation of progenitor T and B lymphoid cells might be similarly caused by a defective Notch signaling. However, consistent data are still not available that further support this conclusion. Recent studies on WAS-linked thrombocytopenia and T-cell differentiation defects rather uncover an Arp2/3-independent function of nuclear WASP on Notch signaling by chromatin remodeling or transcriptional reprogramming in human T-cells (Sadhukhan et al. 2014; Sarkar et al. 2014; Taylor et al. 2010). A role of nuclear WASP in flies has not yet been addressed, but given the available genetic toolkit in flies these studies would certainly provide further insights into the nuclear function of WASP-related proteins.

By contrast, the conserved functions of *Drosophila* WASP and mammalian N-WASP in muscle cell fusion, spermatid maturation, and their release clearly depend on Arp2/3 functions and require an intact C-terminal WCA domain (Massarwa et al. 2007; Mukherjee et al. 2011; Rotkopf et al. 2011; Gruenbaum-Cohen et al. 2012; Xiao et al. 2014). Analyses of the underlying cellular and molecular processes perturbed in mutant flies and mice revealed a remarkably conserved requirement of WASP/N-WASP in the formation of invasive podosome-like protrusions mediating myoblast fusion in flies and mammals (Chen 2011; Sens et al. 2010; Shilagardi et al. 2013). These data also suggest a general role of WASP/N-WASP in the formation of invasive cell adhesion structures not only in the physiology of muscle cell fusion and blood–testis barrier integrity, but also in the pathophysiology as previously observed in malignant cells (Frugtniet et al. 2015; Tang et al. 2013; Yu et al. 2012).

Compared to WASP, *Drosophila* WAVE rather plays a crucial role in most morphogenetic cell movements during development, including blastoderm development, axonal growth of the central nervous system, oogenesis, and photoreceptor axon targeting in the larval visual system (Stephan et al. 2011; Zallen et al. 2002). Comparable studies in different model systems confirmed a pivotal function of WAVE proteins in promoting lamellipodial protrusions and cell migration in all eukaryotic cells (see more details in the next paragraph). The developmental functions of the third member of the *Drosophila* WASP protein family, WASH, are less understood. The initial phenotypic analysis of a *wash* deletion mutant suggests an essential function of WASH during early oocyte development (Liu et al. 2009a). However, the underlying cellular requirement of WASH in oogenesis remains unclear. A more recent study indicates a conserved cellular function of *Drosophila* WASH in retromer-dependent endocytic recycling (Dong et al. 2013).

3 The WASP Family Verprolin Homologous Protein (WAVE): A Key Regulator in Shaping Cells and Driving Cell Migration in all Eukaryotic Cells

Among all WASP proteins, the cellular functions and the molecular regulation of WAVE proteins are best understood. WAVE is a conserved central Arp2/3-regulator driving lamellipodial protrusions and cell migration in all eukaryotic

cells (Ibarra et al. 2005; Takenawa 2005). WAVE is found in a conserved hetero-pentameric complex, the WAVE regulatory complex (WRC) (Chen et al. 2010; Eden et al. 2002). This complex always comprises five subunits: Sra1/Cyfip1, Kette/Nap1, Abi, HSPC300/Brick1, and WAVE. Within the WRC, the nucleation promoting activity of WAVE towards the Arp2/3 complex is inhibited by intracomplex sequestration of its Arp2/3 activating WCA domain. All WRC components localize at protruding lamellipodial tips of motile and non-motile cells such as *Drosophila* S2R+ cells (Zobel and Bogdan 2013). WAVE-depleted S2R+ cells exhibit a dramatic reorganization of the actin cytoskeleton (Zobel and Bogdan 2013). The whole actin meshwork in the lamellipodium is disrupted and loosely packed actin bundles remain in knockdown cells (Zobel and Bogdan 2013). Similar RNAi experiments and loss-of-function studies demonstrated that all WRC components are crucial for the integrity of the complex. Removal of one of the subunits results in the degradation of the whole WRC causing similar phenotypes (Bogdan and Klambt 2003; Kunda et al. 2003; Rogers et al. 2003). Re-expression of each subunit in a mutant background fully restores the WRC integrity in vivo (Stephan et al. 2011). The molecular mechanism of how the complex protects the individual subunits from proteosomal degradation has not been addresed yet. Tyrosine phosphorylation of N-WASP is known to initiate its degradation through ubiquitination (Suetsugu et al. 2002). Both, WAVE and Abi become highly tyrosine-phosphorylated mediated by the non-receptor tyrosine Abelson (Abl), but whether phosphorylated WAVE and Abi proteins are more suceptible to ubiquitin-dependent proteasomal degradation has not been analyzed in more detail (Cestra et al. 2005; Leng et al. 2005; Stephan et al. 2008). The WRC not only protects from protein degradation, but also regulates the activity and localization of WAVE. In response to upstream signals, the WRC is both recruited to the membrane and triggered to release its inhibition on WAVE (Chen et al. 2010; Stephan et al. 2011). Many WRC ligands have been described so far, and it has been suggested that cooperative events are required to achieve optimal activity of the WRC in vivo (Krause and Gautreau 2014). One of the central WRC ligands is the small Rho-GTPase Rac1. Rac1 directly binds to Sra-1 and activates the WRC by allosterically releasing the bound Arp2/3-activating VCA domain of WAVE (Chen et al. 2010). However, Rac1 only binds the WRC with low affinity, thus, a very high concentration of activated (GTP bound) Rac1 is required to activate the WRC in vitro (Chen et al. 2010). Recent studies demonstrate that simultaneous interactions between activated Rac1, Arf6, Ena/VASP proteins, and acidic phospholipids further enhance WRC activity (Chen et al. 2014b; Koronakis et al. 2011; Oikawa et al. 2004).

4 *Drosophila* Macrophages: An Efficient In Vivo Model System to Study Cell Shape Control and Directed Cell Migration in Immune Response

RNAi high-throughput screens in *Drosophila* cell lines have successfully been performed to identify regulators of cell morphology in cell culture (Kiger et al. 2003; Liu et al. 2009b; Rogers et al. 2003; Rohn et al. 2011). More recently, in vivo RNAi methodology has become an important tool for reverse genetics in the physiological context of the fly. Using the GAL4/UAS system (Brand and Perrimon, 1993), tissue- or cell-specific expression of transgenic double-stranded RNAs (dsRNAs) can be achieved to efficiently suppress gene functions. Genome-scale RNAi libraries (Dietzl et al. 2007; Ni et al. 2009) are available to screen for any *Drosophila* gene in vivo and makes the dissection of signaling pathways on a genome-wide level feasible. *Drosophila* immune cells have recently gained popularity for in vivo RNAi studies of cell shape and cell migration. Flies have several types of circulating immune cells, collectively named hemocytes. The majority of *Drosophila* hemocytes are macrophages that begin to phagocytose microbial invaders immediately upon infection. Similar to mammals, these macrophages also play an important role in tissue remodeling during development and in wound response. *Drosophila* hemocytes originate from distinct hematopoietic waves from the embryo to the adult (Krzemien et al. 2007; Markus et al. 2009; Ramond et al. 2015). Pupal macrophages have recently been established as an in vivo cell culture model system that combines the advantages of cultured cells with a genetically tractable in vivo model system (Chen et al. 2014b; Moreira et al. 2013; Sander et al. 2013). Different from embryonic macrophages, targeted expression of RNAi transgenes in the *Drosophila* pupa is highly efficient and phenotypes are not masked by maternally loaded proteins (Mohr et al. 2010; Sander et al. 2013). Immune cells can easily be isolated from white prepupae by rupturing the cuticle and the actin cytoskeleton as well as the localization of endogenous proteins can be visualized at high spatial resolution using SIM (Fig. 3a; Sander et al. 2013). Unlike hemocyte-like S2R+ cells, pupal macrophages are polarized, spread on cover glass forming a broad lamellipodial actin filament network at the cell front and actin stress fiber-like structures at the cell rear (Fig. 3a). Macrophage-specific RNAi mediated gene knockdown can specifically be induced by the *hml*Δ-Gal4 driver, which is expressed in almost all larval blood cells and pupal macrophages. Suppression of Arp2/3-mediated actin polymerization in *arp2* or *wave* depleted cells results in a complete loss of lamellipodial protrusions as previously shown for S2R+ cells (Rogers et al. 2003; Zobel and Bogdan 2013). Knockdown cells display an overall reduction of the actin meshwork and exhibit prominent filopodia consisting of densely packed bundles of actin filaments (Fig. 3b). Recent loss- and gain-of function studies further revealed an important function of WHAMY, but not WASP in membrane protrusions and cell migration in macrophages (Brinkmann et al. 2016). Overexpression of WHAMY leads to a strong induction of filopodia presumably due to the filament elongation activity of WHAMY. WHAMY binds activated Rac1 and localizes at lamellipodial

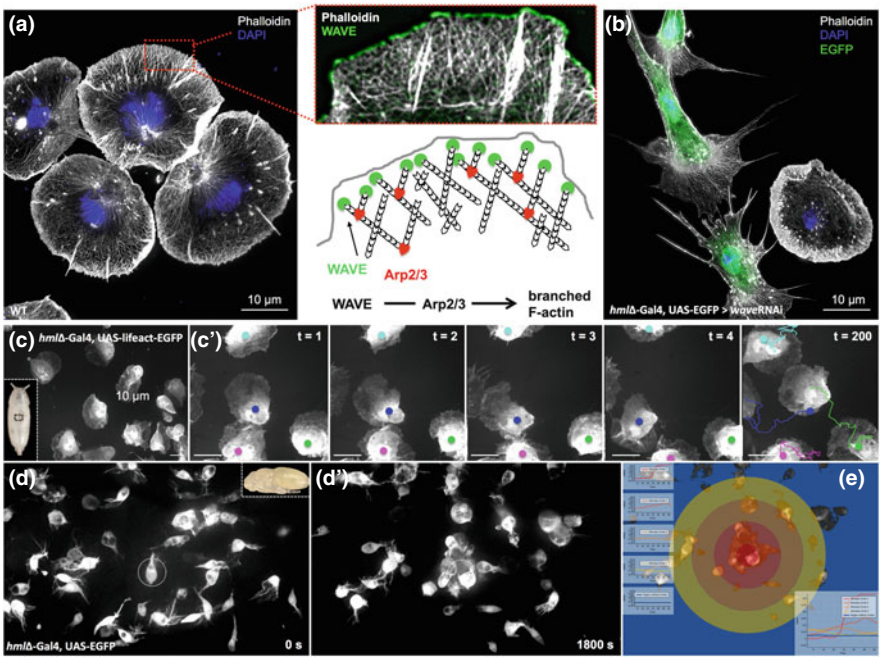

Fig. 3 *Drosophila* pupal macrophages – a versatile tool for combined ex vivo and in vivo imaging of actin dynamics at high resolution. Structured illumination microscopy (SIM) and spinning disc microscopy time-lapse images of isolated *Drosophila* pupal macrophages. (**a**) Representative SIM image of wild type pupal macrophages stained with phalloidin (*white*, F-actin) and DAPI (*blue*, nuclei). *Left*: Higher magnification of a lamellipodium stained for endogenous WAVE (*green*) and F-actin (*white*) at the leading edge. Image was taken from Sander et al. 2013. (**b**) Representative SIM image of a macrophage-specific RNAi-mediated knockdown of *wave* marked by EGFP expression resulting in distinct cell morphological defects. Image was taken from Sander et al. 2013. (**C**, *C′*) Frames of a time-lapse spinning disc microscopy video of prepupal wild type macrophages expressing specifically Lifeact-EGFP to visualize cell migration and F-actin dynamics. The *rectangle* in the white prepupa indicates the recording position. Tracks of migrating macrophages are analyzed using ImageJ and the plug-in for manual tracking. (**d**) Migratory behavior of a wild type pupal macrophage (20 h APF) upon cell wounding (specific cell ablation). Migration is depicted before (**d**) and after wounding (*D′*). (**e**) A *H*istogram-based *M*acrophage *M*igration *S*core (HMMS) allows a quantification of the migration behavior. The score utilizes local histograms around the laser-induced wound to measure the macrophage concentration over time as recently described (Lammel et al. 2014)

tips of S2R+ cells and primary macrophages depending on both CRIB domains. In contrast to WAVE, loss of WHAMY function does not impair lamellipodia formation, but rather regulates cell spreading and contributes to cell motility (Brinkmann et al. 2016). Thus, competition between WHAMY and the WRC for Rac1 might disturb the balance between nucleation and elongation activity and therefore might contribute to the observed overexpression phenotype.

Spinning disc confocal microscopy of macrophages in living pupae further allows to dissect the in vivo requirement of gene functions in cell motility and

cell migration (Lammel et al. 2014; Sander et al. 2013). At the onset of pupa formation, most cells are still attached to the internal body wall like sessile larval macrophages. Shortly after pupariation, however, the cells form polarized lamellipodial protrusions and start to migrate randomly to redistribute from the dorsal patches of the body wall (Fig. 3c). Pupal macrophages use integrin-mediated cell adhesion when moving over the two-dimensional surface of the body wall. Mutations in the β-integrin subunit, encoded by the myospheroid (mys) gene result in an aberrant migratory cell behavior of prepupal macrophages at this stage without any cell polarization defects (Moreira et al. 2013). Recent work revealed that the activation of macrophage polarization and motility at prepupal stages requires ecdysone, the central molting steroid hormone in insects (Sampson et al. 2013). Ecdysone treatment of non-polarized isolated larval macrophages promotes cell polarization and cell motility in vitro, thus recapitulates the cell-autonomous effect of ecdysone on actin dynamics at the onset of pupal development (Sampson et al. 2013). Consistently, microarray analysis of hemocytes identified a large set of migratory genes up-regulated by ecdysone signaling at the onset of metamorphosis, including *wave*, *arpc*5, and *rac*2 with known functions in cell motility and cell shape. Remarkably, macrophages also require ecdysone signaling for bacterial phagocytosis and to become wound-responsive during metamorphosis (Regan et al. 2013). Interestingly, steroid hormones such as estrogen and progesterone can also act on macrophage activation during the mammalian wound healing response although it remains unclear whether the underlying molecular mechanisms are conserved between flies and humans (Goulding 2004; Routley and Ashcroft 2009).

Dispersed mid-pupal stage macrophages remain highly motile, patrolling for infection or dying larval cells. Their capacity to respond to wounds has further been increased with pupal age. Upon laser-induced single cell ablation or wounding of the epithelium wild type cells immediately switch from a random into a directed cell migration mode (Fig. 3d, e). Cells move over long distances and become clustered at the wound sites, where they immediately start to engulf dead cells (Fig. 3d, e). The major driving force for macrophage migration is the formation of Rac-WAVE-Arp2/3-dependent protruding lamellipodia (Evans et al. 2013; Sander et al. 2013; Stramer et al. 2005). Most cells depleted for Rac, Arp2/3 complex, or WAVE fail to migrate towards the wound (Sander et al. 2013). Some *wave* deficient cells located close to the wound still show some response sending long filopodial protrusions towards the wound (Sander et al. 2013). Recent work revealed that directed macrophage migration not only depends on Arp2/3-branched actin nucleation at the cell front but also requires the actin nucleating activity of formins such as the homologue of the FHOD formin at the cell rear (Lammel et al. 2014). Different from WAVE, FHOD localizes at the cell rear of migrating macrophages. The phenotypic analysis of *fhod* mutant cells further suggests that FHOD acts as an effector of the Rho-dependent kinase mediating actomyosin-based retraction at the trailing edge of migrating macrophages (Lammel et al. 2014). An important function in cell motility has recently been proposed for human FHOD1. Human FHOD1 expression is up-regulated during epithelial–mesenchymal transition

(EMT) and its overexpression in melanoma and breast cancer cells promotes cell migration and cell invasion (Jurmeister et al. 2012) (Gardberg et al. 2013). However, the physiological function of mammalian FHOD1 in cell migration has not yet been addressed in a knockout model (Bechtold et al. 2014).

5 Coupling the Membrane to Actin Cytoskeleton in Directed Cell Migration During Wound Response

The key drivers for single cell migration in mammals such as Rac, WAVE, the Arp2/3 complex, and formins seem to be equally essential for directed cell migration of *Drosophila* macrophages. However, the regulatory network of how additional actin binding proteins coordinate cell migration in vivo is less understood. Also still unknown are the molecular mechanisms that drive the directionality of WAVE-dependent lamellipodial protrusions in response to wound signals. Moreover, how membrane signals are coupled to the cell motility machinery remains a key question in the field (Fig. 4).

Different from immune cell chemotaxis in mammals, a functional requirement of G-protein-coupled receptors (GPRCs) as chemokine receptors has not yet been described in *Drosophila*. Previous studies clearly demonstrate that the molecular machineries used by *Drosophila* macrophages to chemotax during development and inflammation are different. Developmental dispersal of macrophages is controlled by the platelet-derived growth factor/vascular endothelial factor (PDGF/VEGF) related receptor Pvr and its ligands Pvf2 and Pvf3. The ligands provide the trophic signal for macrophage survival and serve as guidance cues (Bruckner

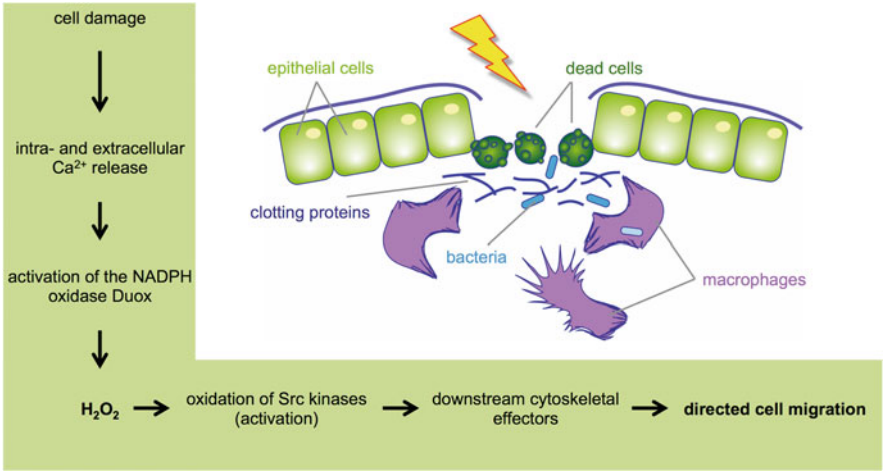

Fig. 4 Hydrogen peroxide might be an universal immediate wound signal released by damaged cells. Proposed model how hydrogen peroxide might act as an immediate wound signal released upon wounding (Moreira et al. 2010; Yoo et al. 2012). For details see text

et al. 2004; Cho et al. 2002). After developmental dispersal, embryonic macrophages also become responsive to wound stimuli due to downregulation of developmental cues such as Pvf2 and Pvf3 (Moreira et al. 2010). Interestingly, chemotaxis of macrophages towards wounds, but not Pvf-directed developmental dispersal strongly depends on phosphatidylinositol 3-kinase (PI3K) (Wood et al. 2006), a canonical signaling module downstream of GPRCs. The receptors that act upstream of PI3K-mediated wound response have not been identified yet. However, there is growing evidence that at the wound site hydrogen peroxide acts as an universal immediate wound signal released by damaged cells in the fly as well as in zebrafish embryo (Moreira et al. 2010; Niethammer et al. 2009). Hydrogen peroxide is generated by the NADPH oxidase Duox, which is in turn activated by an intracellular calcium flash induced immediately upon wounding of *Drosophila*, zebrafish, and *C. elegans* epithelia (Razzell et al. 2013; Wang et al. 2013; Moreira et al. 2010; Yoo et al. 2012). How cells decode this damage signal is not known. A possible sensor of hydrogen peroxide might be Src family tyrosine kinases such as Lyn (Yoo et al. 2012). Lyn contains a conserved cysteine residue. The oxidation of this Cys regulates Lyn kinase activity and is required for zebrafish neutrophil chemotaxis to hydrogen peroxide and wounds (Yoo et al. 2012). This cysteine is not only conserved in related *Drosophila* non-receptor tyrosine kinases such as Src42A and Abelson (Abl), but likewise in the Eph receptor tyrosine kinase (Eph). The oxidation of these kinases might be an important regulatory mechanism of how hydrogen peroxide directly controls directed migration of *Drosophila* macrophages. Hydrogen peroxide can also act directly on cytoskeletal effector proteins as recently demonstrated for the actin binding protein cofilin (Cameron et al. 2015). Interestingly, Src kinases, Abl and Eph are putative ligands of the WRC containing one or more WRC interacting sequences (WIRS). This conserved six-peptide motif directly binds a conserved surface on the WRC formed by the Sra-1 and Abi subunit (Chen et al. 2014a). Given the low complexity of this motif a large variety of putative WIRS ligands have been found including cell adhesion receptors, guidance receptors, ion channels, and G-protein-coupled receptors (Chen et al. 2014a; Fig. 5). Many of these membrane proteins contain one or more conserved WIRS in their cytoplasmic domain, an important criterion for their functional requirement. Consistently, the WIRS binding surface of the WRC is evolutionarily well conserved from sponges to humans. Two point mutations in the highly conserved contact residues on Abi (AbiΔWIRS) abolish binding to all WIRS ligands analyzed so far (Chen et al. 2014a). A functional requirement of WIRS interactions for directed macrophage migration in *Drosophila* has not been addressed yet. However, recent studies demonstrate that WIRS interactions play a pivotal role in different WRC-dependent morphogenetic cell movements (Chen et al. 2014a). Introducing the same mutations in Abi results in defects of retinal axon targeting and oogenesis in *Drosophila*. Female flies lacking the WIRS interactions are partially sterile and show a prominent egg elongation defect. The cellular mechanism that drives egg elongation is based on collective migration of follicle cells. Recent advances in live imaging changed our view of how cells migrate as an epithelial sheet to shape tissue and organs.

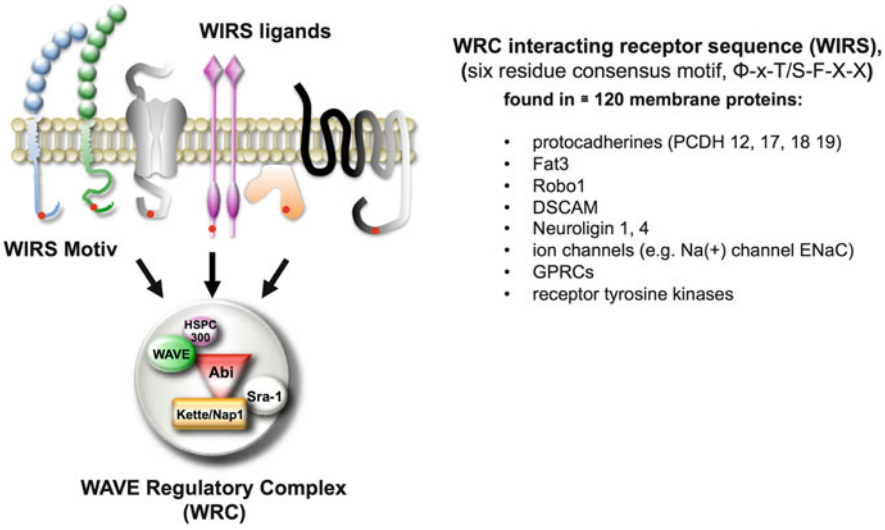

Fig. 5 The WAVE regulatory complex (WRC) links diverse membrane receptors to the actin cytoskeleton. Schematic drawing of how the WRC can directly be recruited to membranes through a conserved peptide motif (WIRS) found in a variety of transmembrane and membrane-associated proteins. *Left*: Some of the WIRS ligands are listed that bind the WRC in pull-down assays (Chen et al. 2014a)

6 *Drosophila* Egg Chambers: Insights into Conserved Gene Functions Required for Collective Cell Migration and Cell Invasion

The *Drosophila* ovary has served as a powerful model for study a wide range of cellular processes and researchers have uncovered new mechanistic insights in cell–cell communications during collective cell migration and invasion (Bastock and St Johnston 2008; Horne-Badovinac and Bilder 2005; Robinson and Cooley 1997). The developing eggs, so-called egg chambers, consist of a cyst of 16 interconnected germline cells, one oocyte, and 15 nurse cells, surrounded by a simple follicle epithelium (Fig. 6). Early stage egg chambers are spherical, but progressively elongate along the anterior–posterior (A-P) axis as they grow. Recent advances in live imaging and ex vivo culture conditions of egg chambers have led to the discovery of a new type of morphogenetic movement that drives axis elongation during fly oogenesis (Bastock and St Johnston 2011; Bilder and Haigo 2012; Haigo and Bilder 2011; He et al. 2011). Elongating egg chambers rotate around their circumferential axis. As it turned out, the collective migration of follicle cells drives this rotational movement (Cetera et al. 2014). Follicle cells migrate as an epithelial sheet forming filopodial protrusions at the cell front rather than sheet-like lamellipodial protrusions found in single migrating cells (Fig. 7). While follicle cells migrate, they create a planar polarized basement membrane mainly consisting

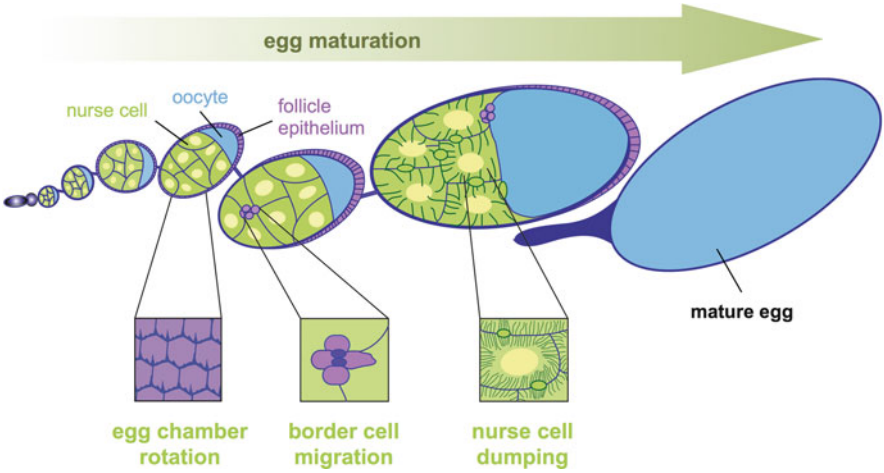

Fig. 6 An overview of *Drosophila* oogenesis. Schematic drawing of egg chambers of increasing age up to the mature egg. *Bottom*: Magnified view of three important actin-driven processes, egg chamber rotation, border cell migration, and nurse cell dumping. For details see text

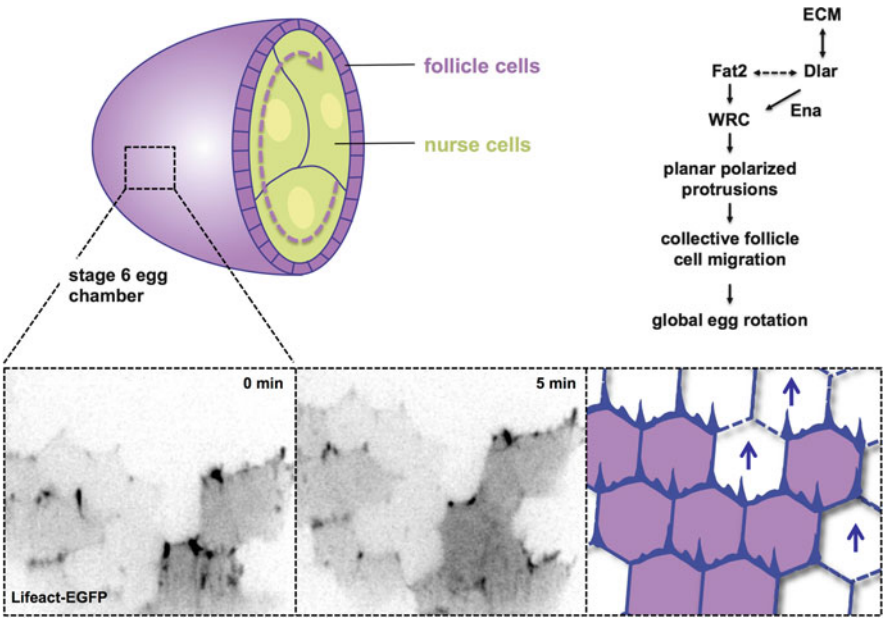

Fig. 7 Rotational movement of elongating egg chambers is driven by collective migration of follicle cells. *Left side*: Two frames of a spinning disc microscopy time-lapse movie of an ex vivo cultured egg chamber expressing lifeact-EGFP in a mosaic pattern. Wild type actin-rich cell protrusions are formed at the cell front in the direction of the movement (*arrows*, *right*). Cells migrate perpendicular to the anterior–posterior axis of the developing egg, with an average maximum speed of about 0.5 μm/min (Haigo and Bilder 2011). *Right side*: Proposed model of how Fat2 acts through the WRC to drive collective cell migration during global egg rotation

of collagen fibrils. These fibrils align along the direction of movement and form a kind of molecular corset restricting radial expansion of the egg (Bilder and Haigo 2012; Haigo and Bilder 2011). The basal actin cytoskeleton in turn aligns along the polarized extracellular matrix (ECM) and forms parallel actomyosin bundles orientated perpendicular to the A-P axis. Once rotational movement stops at mid-stage egg chambers, the basal actomyosin network in the follicle cells starts to contract in an oscillating manner which further forces egg elongation.

Which molecules drive the global egg chamber rotation? Genetic studies have already revealed a close functional relationship between the ECM, cell adhesion receptors, and the actin cytoskeleton during egg chamber elongation. Mutations in genes encoding components of basement membrane such as laminin, collagen IV, their receptors such as βPS integrin, but also loss of small RhoGTPases such as Rac1, Rac2, and Rho1 result in a common round-egg phenotype indicative of axis elongation defects (reviewed in Gates 2012). Two recent studies unraveled an important role of the WRC and its interaction partner Ena/Vasodilator-stimulated phosphoprotein (Ena/VASP) in egg chamber elongation (Cetera et al. 2014; Chen et al. 2014b). The loss of either the WRC or its interaction to Ena and WIRS ligands results in abnormally shaped, round eggs (Cetera et al. 2014; Chen et al. 2014b). In vivo membrane labeling using a Neuroglian-GFP trap revealed that migrating follicle cells form membrane protrusions extending in the direction of rotational movement (Cetera et al. 2014). WAVE and Ena are enriched at the tips of these membrane protrusions and a model has been proposed, in which WAVE defines the protrusive edge of each follicle cell and WAVE-dependent lamellipodia drives collective follicle cell migration (Cetera et al. 2014). A key open question is by which mechanism the symmetry-breaking occurs that recruits the WRC to the cell front (Cetera and Horne-Badovinac 2015). Prime candidates are WIRS receptors that might control cell chirality. A recent study identified the atypical cadherin Fat2 as a novel WIRS ligand and explored a molecular pathway, in which Fat2 and the receptor tyrosine phosphatase Dlar act through the WRC to control collective cell migration during *Drosophila* oogenesis (Squarr et al. 2016; Sturner and Tavosanis 2016). Live imaging analyses of ex vivo cultured egg chambers revealed large actin-rich protrusions formed at tricellular junctions where Fat2 and the WRC already accumulate at early stages of developing egg chambers. These whip-like protrusions are thought to interact with the ECM to synchronize directed cell migration and to drive the morphogenetic movement (Squarr et al. 2016; Sturner and Tavosanis 2016).

Rotational movements that drive morphogenesis might be conserved in mammals. Supporting this notion, similar rotational motions have recently been identified in cultured cysts of mammalian epithelial cells under more physiological three-dimensional (3D) culture conditions. Like the follicle epithelium, the spherical monolayer of polarized cells form a planar polarized actin cytoskeleton at their basal surface which is aligned in the same direction of their migration (Tanner et al. 2012; Wang et al. 2013). The similar morphology of the protrusions in *Drosophila* and human epithelial cells further suggests that the molecular mechanisms driving tissue rotation might be also conserved (Squarr et al. 2016;

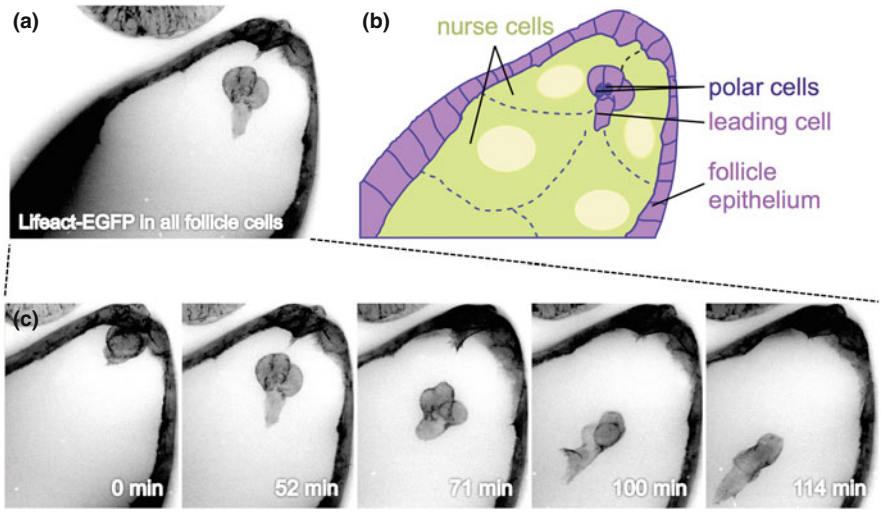

Fig. 8 Border cell migration – a powerful system to identify genes driving collective cell migration and cell invasion. (**a**) Image of the anterior region of an ex vivo cultured stage 9 egg chamber expressing lifeact-EGFP in all follicle cells (see below). (**b**) Schematic drawing of the egg chamber shown in **a**. (**c**) Frames of a spinning disc microscopy time-lapse movie Wild type actin-rich cell protrusions are formed at the cell front in the direction of the movement (*arrows, right*). Cells migrate perpendicular to anterior–posterior axis of the developing egg, with an average maximum speed of about 0.5 μm/min (Haigo and Bilder 2011)

Sturner and Tavosanis 2016). Consistently, the human homologue of Fat2 localizes at the tips of membrane protrusions and at intercellular epithelial junctions (Moeller et al. 2004). Thus, it will be of great interest to address whether conserved molecular machineries including Fat cadherins, Dlar, and the WRC coordinate collective cell migration that might also drive elongation in other developing organs.

As rotational movement of the entire follicle epithelium ceases, another fascinating collective cell behavior of follicle cells, referred to as border cell migration, can be observed. Border cell migration has been analyzed extensively as an excellent genetic model of collective cell migration and cell invasion (Montell et al. 2012; Pocha and Montell 2014; Rorth 2012; Rosales-Nieves and Gonzalez-Reyes 2014; Starz-Gaiano and Montell 2004). Different from the rotating follicle epithelium, only a small group of up to eight cells from the anterior follicle epithelium is specified to become motile. The border cells surround two non-motile so-called polar cells (Fig. 8). They undergo an EMT, and form prominent membrane protrusions, delaminate from the follicle epithelium, and migrate as a cohort in between the nurse cells (Fig. 8). Once they arrive at the anterior border of the oocyte, the border cells migrate dorsally towards the oocyte nucleus where the polar cells that have been carried along the migrating border cells form a pore in the micropyle, a specialized egg appendage through which the sperm can enter.

Consequently, disruption of border cell migration leads to female sterility (Montell et al. 1992; Montell 2003).

Forward and reverse genetic screens have led to the identification of numerous genes that orchestrate the specification, delamination, and the guidance of border cell migration. An obvious initial question was why only border cells become migratory and invasive, whereas the other follicle cells stay in their epithelial layer? One of the key signals is the cytokine Unpaired, which activates the JAK-STAT (Janus Kinase/Signal Transducer and Activator of Transcription) pathway in the border cells (Beccari et al. 2002; Silver et al. 2005). Unpaired is secreted by the polar cells, so only those follicle cells closest to the polar cells receive the highest JAK/STAT signaling and adopt the invasive cell fate (Silver and Montell 2001). While JAK/STAT signaling specifies the spatial selection of the migratory cells, ecdysone levels provide the temporal signal controlling the start of border cell migration (Bai et al. 2000). Both, the ecdysone and JAK/STAT pathways synergize during border cell migration and the integration of their spatial and temporal signals requires by the BTB protein Abrupt (Jang et al. 2009). Abrupt acts as a repressor of ecdysone signaling, which is normally lost from border cells in response to JAK-STAT activity. This contributes to the spatial pattern of increased ecdysone response to the anterior follicle cells, including the border cells (Godt and Tepass 2009). Remarkably, ecdysone and JAK-STAT signaling are not only important for the initiation, but rather continuously required throughout border cell migration as demonstrated by temperature shift experiments with temperature-sensitive mutants (Silver et al. 2005). This suggests a functional requirement and continuous transcriptional regulation of JAK-STAT target genes throughout border cell migration.

A systematic analysis of the transcriptional switch inducing migration of border cells upon JAK/STAT activation revealed a significant up-regulation of about 300 genes (Borghese et al. 2006; Wang et al. 2006). Among these, the homophilic cell–cell adhesion molecule E-cadherin, microtubule- and actin cytoskeleton-regulators were found. Surprisingly, a large cluster of "muscle-specific" genes that are known to be required for myoblast fusion were also identified, namely *rolling pebbles* (rols) and *myoblast city* (mbc) (Borghese et al. 2006; Wang et al. 2006). During myoblast fusion, Mbc mediates the cell-type specific activation of Rac1 and in turn activation of WAVE/SCAR to promote an invasive actin-associated structure termed FuRMAS (fusion-restricted myogenic-adhesive structure), that promotes cell fusion (Haralalka et al. 2011). Thus, fusion proteins such as Mbc might also contribute to the formation of similar finger-like protrusions in border cells promoting the invasion in between the nurse cells. Border cell clusters indeed exhibit a clear front-back polarity with one or two leading cells that form invadopodia-like actin-rich protrusions (Fulga and Rorth 2002). Consistently, the highest Rac activity can be measured in the leading cell and light-mediated activation revealed that Rac sets the direction of membrane protrusions and the direction of migrating border cells (Wang et al. 2010). These data also imply a regulatory feedback loop of Rac activity that stabilizes forward-directed protrusions, thus maintains the front-back polarity between leading and follower cells. More recent

data clearly demonstrate that E-cadherin mediates this mechanical feedback loop promoting direction sensing through Rac during border cell migration (Cai et al. 2014). Rac activation is thought to be mediated in response to chemotactic cues. Two important receptor tyrosine kinase (RTK) pathways have been identified, the EGF receptor (EGFR) and PDGF/VEGF receptor (PVR) signaling cascades function redundantly to guide border cells (Duchek and Rorth 2001; Duchek et al. 2001; McDonald and Montell 2005; McDonald et al. 2003, 2006). Photoactivated Rac can substantially rescue defects in the directional movement in border cells expressing dominant-negative EGFR/PVR suggesting that Rac normally acts downstream of these receptors (Wang et al. 2010). However, whether asymmetric Rac activation at the cell's leading edge is mediated by a direct interaction with these RTKs or whether there is an amplification mechanism that increases Rac activity have not yet been explored. Nor is it known how Rac acts on the actin machinery. Suppression of the central Rac effector, the WRC clearly affects border cell migration as hemocyte migration and rotational movement (K. Brinkmann and S. Bogdan, unpublished observations).

Thus, the principles of single and collective cell migration might be similar, but there seems to be a multitude of variations on a common theme. Exploring the nature of these variations in cell adhesion and communication will be the driving force for researchers to better understand the underlying mechanisms of cell migration in the future. New genetic approaches and high-resolution microscopy techniques allow important new insights into molecular principles of conserved regulatory networks that act on cellular structures and cell dynamics. Advanced optogenetic tools for the manipulation of endogenous proteins in single cells combined with quantitative single-molecule imaging approaches will further be important to dissect evolutionarily conserved modules in actin nucleation, in flies, and mammals.

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From Cytoskeleton to Gene Expression: Actin in the Nucleus

Tiina Viita and Maria K. Vartiainen

Contents

Abstract

Although most people still associate actin mainly with the cytoskeleton, several lines of evidence, with the earliest studies dating back to decades ago, have emphasized the importance of actin also inside the cell nucleus. Actin has been linked to many gene expression processes from gene activation to chromatin remodeling, but also to maintenance of genomic integrity and intranuclear movement of chromosomes and chromosomal loci. Recent advances in visualizing different forms and dynamic properties of nuclear actin have clearly advanced our understanding of the basic concepts by which actin operates in the nucleus. In this chapter we address the different breakthroughs in nuclear actin studies, as well as discuss the regulation nuclear actin and the importance of nuclear actin dynamics in relation to its different nuclear functions. Our aim is to highlight the fact that actin should be considered as an essential component of

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the cell nucleus, and its nuclear actions should be taken into account also in experiments on cytoplasmic actin networks.

Keywords
Actin • Gene expression • Nuclear actin • Nuclear organization

1 In and Out of the Nucleus: Regulation of Nuclear Actin Levels

Already over 40 years ago, a protein with similar properties as actin was found in nuclear isolation experiments and subsequent studies suggested a role for actin in transcription (Clark and Merriam 1977; Clark and Rosenbaum 1979; Jockusch et al. 1971, 1974; Lane 1969; Lestourgeon et al. 1975). Although these early studies faced harsh criticism, developments mainly during the last decade have firmly both placed actin in the nucleus and linked it to many essential nuclear processes, many of which are related to gene expression.

Like all proteins, actin is synthesized in the cytoplasm, and thus it has to be imported into the nucleus to contribute to nuclear processes. Nuclear import and export of proteins takes place through the nuclear pore complexes (NPCs), which form channels through the nuclear envelope. The transport can be either passive diffusion of small molecules and proteins (approximately less than ~30–40 kDa) or active, energy-dependent transport, assisted by nuclear transport receptors, of larger molecules [reviewed in (Wente and Rout 2010)]. The size of actin, at 42 kDa, is at the limit of passive diffusion. Nevertheless, dedicated nuclear transport receptors for actin have been described for both nuclear import (Dopie et al. 2012) and export (Stuven et al. 2003), and hence actin seems to use the active transport route to travel between the nucleus and cytoplasm (see Fig. 1 for a schematic view on nuclear actin dynamics). Photobleaching assays revealed that actin actually shuttles very rapidly in and out of the nucleus, and therefore the cytoplasmic and nuclear pools of actin are in constant communication with each other (Dopie et al. 2012). The nuclear export mechanism of actin was first discovered while the Görlich-lab was charactering a novel nuclear transport factor, exportin-6. This protein seems to be rather restrictive in exporting actin, and perhaps some actin-associated proteins. Profilin is needed for efficient interaction between Exportin-6 and actin, and thus for the formation of the actin export complex (Stuven et al. 2003), but the biochemical details are not known. The nuclear import mechanism of actin was examined by comparing the nuclear import rate of differently sized actin constructs in a fluorescence recovery after photobleaching assay (FRAP). The nuclear import rate of actin did not decrease even when tagged with two GFP moieties, thus indicating that like export, also nuclear import of actin is an active process. We further identified Importin-9 as the nuclear import receptor for actin and also here, a small actin monomer-binding protein, cofilin, seems to be required for the assembly of the actin transport complex. The need for actin monomer-binding proteins in nuclear

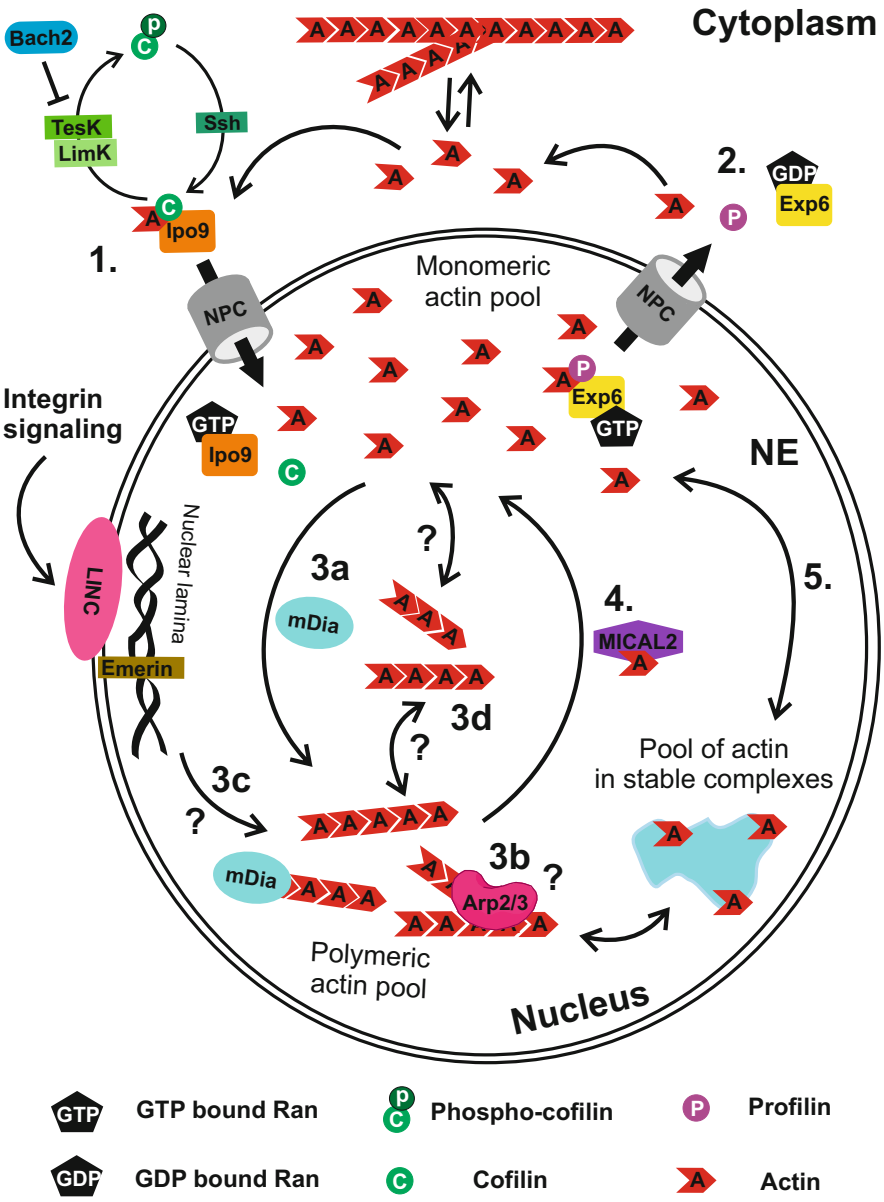

Fig. 1 Schematic of actin dynamics inside the nucleus. (*1*) Actin is imported as monomer to the nucleus and Importin-9 (Ipo9) mediates nuclear import together with cofilin (C). Cofilin can be phosphorylated and thus inactivated by Tes and Lim kinases (TesK and LimK) and dephosphorylated to its active form by Slingshot (Ssh). Bach2 regulates TesK levels to maintain active cofilin. (*2*) Exportin-6 (Exp6), together with profilin (P), mediates nuclear export of actin. Actin is also exported as a monomer. (*3*) Polymerization of nuclear actin can be promoted by several mechanisms. (**a**) Serum stimulation and cell adhesion activate nuclear actin polymerization through the formins mDia1/2. (**b**) Arp2/3 complex can branch actin filaments, but although found in the nucleus, it is unclear if Arp2/3 complex nucleates actin filaments there, and is thus indicated with a *question mark*. (**c**) Also emerin has been linked to nuclear actin polymerization,

transport of actin can also stem from the fact that actin is transported across the NPC as a monomer, and the availability of actin monomers limits both nuclear import and export rate of actin (Dopie et al. 2012). Therefore binding events in either cytoplasm or nucleus affect nucleo-cytoplasmic shuttling rate of actin, and this may be used to regulate nuclear actin levels. Indeed, these levels vary from one cell to another in cultured mammalian cells. Systematic analysis of cellular parameters affecting the amount of nuclear actin revealed that levels of export competent actin monomers dictate nuclear transport of actin at steady-state (Skarp et al. 2013), confirming that binding events, including polymerization, especially in the nucleus influence the quantity of nuclear actin. Related to this notion, MICAL-2 is a nuclear, atypical actin-regulatory protein, which can oxidase methionine 44 in actin, and thus induce redox-dependent depolymerization of nuclear actin, which leads to decreased nuclear actin (Lundquist et al. 2014).

The fact that actin uses an active transport mechanism for both nuclear import and export indicates that the balance between cytoplasmic and nuclear actin levels needs to be tightly regulated to maintain cellular homeostasis. In fact, depletion of either importin-9 or exportin-6, thus either decreasing or increasing nuclear actin levels, respectively, diminishes transcription (Dopie et al. 2012), highlighting the need for accurate control of nuclear actin levels. It is therefore not too surprising that altered nuclear actin levels have been documented both during pathological conditions and during various cellular stresses. Already early on, nuclear accumulation of actin was reported in various cellular stress events, such as upon heat shock (Welch and Suhan 1985), DMSO treatment (Fukui and Katsumaru 1979), ATP depletion (Pendleton et al. 2003), and recently also during replication stress, which also induces nuclear localization of actin polymerization regulators IQGAP1 and the small GTPase Rac1 (Johnson et al. 2013). However, how the increased nuclear actin may contribute to the cellular stress response is still unclear. Increased nuclear actin levels have also been reported upon cellular senescence (Kwak et al. 2004) and in certain diseases, such as intranuclear rod myopathy (IRM) (Domazetovska et al. 2007).

In some cell types, actin is accumulated to the nucleus to serve a specific purpose. In *Xenopus laevis* oocyte, nuclear export of actin is down-regulated by post-transcription control of exportin-6 to acquire high nuclear actin levels. These oocytes have huge nuclei, which require additional mechanical support from a filamentous actin meshwork to stabilize the nuclear structure (Bohnsack et al. 2006). This same actin meshwork also protects ribonucleoprotein droplets, such as nucleoli and histone locus bodies, against gravity in these large nuclei (Feric

Fig. 1 (continued) but the mechanism is not clear, and is hence indicated with a *question mark*. (**d**) Actin can also form small punctate polymers inside the nucleus, but how they are assembled and their relationship with larger actin filaments still remain unclear, and are thus marked with a question mark. (*4*) Mical2 can promote depolymerization of nuclear actin filaments through oxidation. (*5*) Besides the monomeric and polymeric actin pools, a large fraction of intranuclear actin is found in stable complexes. These could represent actin bound to, for example, in chromatin remodeling complexes and other transcription related complexes

and Brangwynne 2013). Whether nuclear export of actin is regulated by other mechanisms in different biological contexts remains to be explored. Nevertheless, a comparative RNAi screen performed in both *Drosophila* SR2+ and mammalian cells revealed proteins, whose depletion leads to nuclear accumulation of actin. This study showed that nuclear actin regulators are among the core set of evolutionary conserved actin regulators, underscoring the importance of nuclear actin control in various organisms. The function of two of the hits, CDC73 and CDC2l5, was conserved both in mammals and in flies, and these two factors are linked to transcription and alternative splicing, respectively. In fact, many of the hits from the *Drosophila* screen are components of the spliceosome (Rohn et al. 2011), which suggests that regulation of nuclear actin might be linked to different RNA related processes. Nuclear translocation of actin is also linked with differentiation of HL-60 cells to macrophages upon phorbol 12-myristate 13-acetate (PMA) treatment. Translocation of actin into the nucleus could be blocked with specific inhibitors against different kinases, which indicates that these kinases could regulate either nuclear import or export of actin. Interestingly, chromatin immunoprecipitation-on-chip assays revealed actin-binding to a broad range of different gene promoters upon PMA treatment and knockdown of β-actin decreased expression of selected genes (Xu et al. 2010). In this case it therefore seems that nuclear actin levels are increased to regulate the expression of specific sets of genes required to execute a differentiation program.

Decreased nuclear actin has been linked to quiescence. Spencer et al. showed that treatment of epithelial cells with the extracellular matrix protein laminin 111 (LN1), which has been shown to induce quiescence, resulted in lower nuclear β-actin levels compared to proliferating cells. Reduced nuclear actin levels were linked to reduced transcription (Spencer et al. 2011), similarly as decreased nuclear actin upon depletion of its nuclear import receptor, Importin-9 (Dopie et al. 2012). At present it is not known if decreased nuclear actin upon quiescence is due to decreased import or enhanced export. Phosphorylation state of cofilin plays an important role in nuclear import of actin (Dopie et al. 2012, 2015). A recent genome-wide RNAi screen in cultured *Drosophila* cells identified several novel regulators of nuclear import of actin, and a subset of these were conserved in mammalian cells. Four of these factors (Chinmo, Rack1, Shi and Cpb) regulate the phosphorylation status of Twinstar (Dopie et al. 2015), which is the cofilin ortholog in flies. Chinmo, which is a transcriptional repressor (Zhu et al. 2006), was shown to regulate nuclear actin levels in both mammalian and *Drosophila* cells, as well as in fly tissues, by controlling the expression of the cofilin kinase TesK (Cdi in *Drosophila*). Few of the identified hits affected RanBP9 (fly ortholog of importin-9) levels by an unknown mechanism. Curiously, the remaining hits were functionally very diverse, being implicated in processes such as membrane trafficking and cellular energy metabolism, potentially linking nuclear actin control to many cell biological processes (Dopie et al. 2015).

2 Regulation of Nuclear Actin Dynamics

Assembly of actin monomers to filaments is necessary for many different cellular events, such as maintaining the cell shape, cell movement, and division [reviewed in (Pollard and Cooper 2009)]. Hence, actin polymerization is a key feature for actin's operations in the cytoplasm, but the polymerization status of nuclear actin has been subject to a great debate. Conventional, phalloidin-stainable actin filaments cannot be seen in most nuclei. Certain antibodies raised against special forms of monomeric actin show strong nuclear staining in cells, suggesting that nuclear actin could be mainly monomeric or takes specific conformations inside the nucleus (Schoenenberger et al. 2005). Moreover, biochemical experiments have indicated the presence of monomeric actin in all nuclear complexes studied in detail so far. These include heterogeneous nuclear ribonucleoproteins (hnRNPs) (Obrdlik et al. 2008; Percipalle et al. 2002, 2003), positive elongation factor b (pTEFb) (Qi et al. 2011), and INO80 chromatin remodeling complex (Fenn et al. 2011; Kapoor et al. 2013) (see also below).

However, recent results have firmly established that nuclear actin can polymerize, and even form canonical, phalloidin-stainable filaments in certain situations (see Fig. 1 for a schematic of nuclear actin dynamics). First evidence for this came from the studies by McDonald et al., which revealed with a FRAP assay on GFP-actin that there are different actin pools with distinct kinetic properties in the nucleus and these pools resembled monomeric and filamentous actin pools found in the cytoplasm (McDonald et al. 2006). Further FRAP studies revealed that, in addition to monomeric and polymeric pools, a substantial fraction of actin is present in a third, stable pool, from where actin is exchanged rather slowly (Dopie et al. 2012). This likely represents actin bound to numerous nuclear complexes. Visualization of actin filaments in the nucleus has been a challenge, but recent publications presenting new, nuclear-targeted actin probes have tackled this problem. First Belin and colleagues introduced new probes based on known actin-binding domains, which could identify both monomeric and polymeric forms, and which were targeted to the nucleus with a nuclear localization signal (NLS) (Belin et al. 2013). R1EN contains an RPEL motif from the actin-regulated transcription coactivator megakaryocytic acute leukemia (MKL1, also known as MAL or MRTF-A, see also below 3.1), which binds actin monomers (Guettler et al. 2008). Co-localization studies suggest that actin monomers could be components of nuclear speckles (Belin et al. 2013). Another probe, Utr230-EN, contains a truncated version of the utrophin actin-binding domain and binds to actin filaments, which appear as punctate manner in the nucleus. These structures are excluded from the chromatin-rich regions and did not co-localize with RNA polymerases. These polymers could therefore contribute to the viscoelastic structure of the nucleoplasm (Belin et al. 2013). Almost at the same time Baarlink and colleagues showed for the first time signal-induced actin filament assembly inside the nucleus. They visualized nuclear actin filaments upon serum stimulation with another nuclear actin probe, NLS-Lifeact (Baarlink et al. 2013). Lifeact is small peptide probe derived from budding yeast protein Abp140, which binds both

monomeric and filamentous actin (Riedl et al. 2008). These rapidly and transiently formed actin filaments seen upon the serum stimulation are nucleated by diaphanous-related formins (mDia1/2), and can also be stained with phalloidin (Baarlink et al. 2013). On the other hand, also formin-2 (FMN2), together with Spire-1/Spire-2, can induce nuclear actin assembly. This pathway has been linked to DNA damage-induced actin assembly, and required for efficient clearance of DNA double strand breaks and nuclear oxidation response upon genotoxic stress (Belin et al. 2015). Further evidence for signal-induced filamentous actin formation in the nucleus has been witnessed during cell adhesion. For this, the Grosse-lab used another actin probe based on nuclear targeted actin chromobody, which is a small antigen-binding domain derived from heavy chain antibodies of camelids. This probe revealed nuclear actin polymerization upon cell spreading and fibronectin stimulation, therefore linking actin polymerization inside the nucleus to integrin signaling. However, the actin filaments in spreading cells were thicker and shorter than upon serum stimulation. Nevertheless, they seemed to be also nucleated by mDia1/2, and additionally required the Linker of Cytoskeleton and Nucleoskeleton complex (LINC) (Plessner et al. 2015). LINC complex is formed from outer nuclear membrane KASH-domain proteins, which span the perinuclear space between the nuclear envelope and bind to inner nuclear membrane SUN-domain proteins. In the nucleus, LINC is connected to the nuclear lamina and in the cytoplasm to all three cytoskeletal filament systems [reviewed in (Chang et al. 2015)].

Curiously, nuclear lamina may have an important role in coordinating nuclear actin dynamics, as many of its components have been reported to bind actin. These include both A- and B-type lamins, where the tail binds actin at least in vitro (Simon et al. 2010). On the other hand, Holaska et al. found actin when searching for emerin-binding proteins in nuclear extracts of HeLa cells. Emerin is an integral inner nuclear membrane protein containing a LEM domain, with numerous roles reported in gene expression, cell signaling, and nuclear organization [reviewed in more detail (Berk et al. 2013)]. Further biochemical experiments revealed that emerin binds to pointed ends of actin filaments, and could thereby promote actin polymerization (Holaska et al. 2004), which has also been corroborated by functional studies. Ho and colleagues showed that emerin is mislocalized in lamin A/C deficient cells. This results in defective nuclear actin polymerization: a phenotype that can be rescued by ectopic expression of emerin, and the actin-binding ability of emerin was needed for the rescue (Ho et al. 2013). Moreover, a recent RNAi screening study identified *Drosophila* lamin and Nup98 as putative regulators of nuclear actin polymerization, and their depletion altered GFP-actin mobility in FRAP assays (Dopie et al. 2015). Nevertheless, further experiments are needed to elucidate how these new regulators and other ABPs control nuclear actin dynamics.

Also numerous other ABPs have been found in the nucleus, including, for instance, actin-filament nucleating factor junction-mediating and regulatory protein (JMY) (Coutts et al. 2007; Zuchero et al. 2009), monomeric actin-binding proteins thymosin β4 (Huff et al. 2004), and cyclase-associated protein-2 (CAP2) (Peche et al. 2007), as well as actin capping proteins gelsolin-like capping protein (CapG) (De Corte et al. 2004) and tropomodulin (Kong and Kedes 2004). Furthermore, also

Rho GTPases and their effectors, which are master regulators of cytoplasmic actin dynamics, have been localized to the nucleus [reviewed in (Rajakyla and Vartiainen 2014)], but their relevance in regulating actin in this compartment warrants further studies. These novel developments therefore clearly demonstrate that nuclear actin can polymerize. It is also apparent that the nuclear polymerization process is under as tight control as cytoplasmic actin dynamics. Due to nuclear presence of numerous ABPs, it is intriguing to speculate that traditional actin treadmilling could take place also in the nuclear environment, but more studies are needed to reveal the exact function of polymerized nuclear actin and to characterize the regulatory pathways in detail.

3 Actin in Gene Expression

3.1 Actin in Gene Activation

Regulation of gene expression is clearly one of the key functions of the nucleus. Intriguingly, actin has been linked to nuclear complexes throughout the gene expression process (Fig. 2). The best known actin-regulated transcription factor is serum response factor (SRF), which controls the expression of many cytoskeletal genes in response to changes in actin dynamics. The signal from the actin cytoskeleton to SRF is mediated by the transcription coactivator MKL1 (also known as MAL or MRTF-A) (Miralles et al. 2003). MKL1 binds actin monomers through its RPEL-repeat and this way acts as an actin monomer sensor. Actin inhibits nuclear import of MKL1, because actin-binding masks the NLS embedded in the RPEL-repeat of MKL1 (Mouilleron et al. 2011; Pawlowski et al. 2010; Vartiainen et al. 2007). Actin-binding is required for nuclear export of MKL1 by an unknown mechanism, and also prevents MKL1 from activating SRF in the nucleus. Upon stimulations that promote actin polymerization, the actin monomer levels decrease, and actin-free MKL1 accumulates in the nucleus and activates SRF-mediated transcription (Vartiainen et al. 2007). Actin therefore regulates MKL1 activity at three levels: nuclear import, nuclear export, and activation of target gene transcription. Hence nuclear actin itself is a key player in the regulation of MKL1 localization and activity (Vartiainen et al. 2007), and this notion is also supported by the study showing that formin-dependent actin polymerization inside the nucleus activates MKL1 (Baarlink et al. 2013). Moreover, impaired nuclear actin polymerization upon emerin mislocalization in the lamin A/C deficient cells as discussed above results in reduced nuclear accumulation of MKL1 and in decreased expression of SRF target genes. Interestingly, also lamin A/C mutations linked to cardiomyopathy lead to a similar phenotype, which may contribute to the disease etiology (Ho et al. 2013). Also modulation of MICAL-2 expression, which regulates nuclear actin polymerization by oxidation, results in altered MKL1/SRF-mediated gene expression in vitro and in vivo (Lundquist et al. 2014).

There is also evidence that polymerized actin could regulate the expression of specific genes. For example, actin oligomers can activate toll-like receptor (TLR)

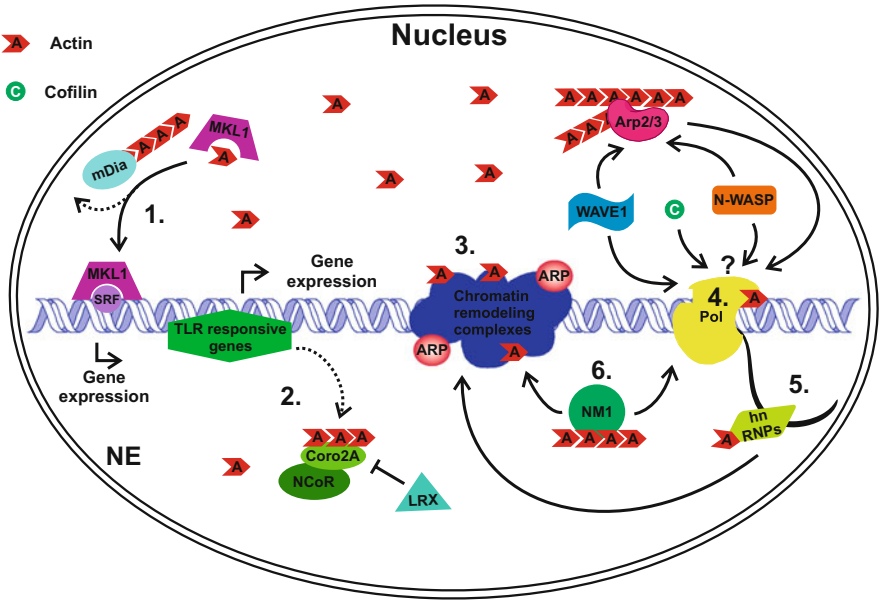

Fig. 2 Different functions of nuclear actin. Actin has been linked with nuclear functions ranging from the gene activation to pre-mRNA processing. (*1*) Actin polymerization by mDia1/2 in the nucleus releases MKL1 from the actin monomer to activate SRF and enables expression of SRF targeted genes. (*2*). Expression of Toll-like receptor (TLR) responsive genes requires clearance of NCoR repressor complex from the promoter. Actin oligomers bind to the NCoR subunit Coronin2A (Coro2A) and prevent liver X receptor (LRX) from blocking NCoR turnover. (*3*) Actin is a part of different chromatin remodeling complexes, such as SWI/SNF and Ino80. Actin seems to work together with actin related proteins (ARP) in these complexes. (*4*) Actin and different ABPs, such as Arp2/3 complex, N-WASP, cofilin (C) and WAVE1, have been linked to RNA polymerase function. The nuclear function of these ABPs may be actin-independent, and this is indicated with a *question mark*. (*5*) Actin interacts with several hnRNPs and this interaction may be required for linking pre-mRNA processing to chromatin remodeling, and thus to maintenance of an active chromatin state. (*6*) Actin may function together with motor protein myosin, most notable nuclear myosin 1c (NM1), in both RNA polymerase and chromatin remodeling-related functions

responsive genes and stimulate promoter clearance. When coronin 2A, a subunit of nuclear receptor co-repressor complex (NCoR), interacts with actin polymers, NCoR turnover can be induced by TLRs (Huang et al. 2011). Actin polymerization also activates retinoic acid (RA) induced *HoxB* expression (Ferrai et al. 2009) and is required for transcriptional reactivation of the pluripotency gene *Oct4*. Overexpression studies with different actin polymerization regulators identified transducter of Cdc42-dependent actin assembly 1 (TOCA1), as the actin polymerization inducer upon *Oct4* activation (Miyamoto et al. 2011). Also cytoplasmic actin dynamics can regulate the activity of transcription factors, and thus indirectly influence nuclear events. Indeed, Presequence Protease 2 (PREP2) (Haller et al. 2004) and Yin-Yang1 (YY1) (Favot et al. 2005) are transcription factors,

which are bound to actin filaments in the cytoplasm and depolymerization of actin filaments releases them to the nucleus to activate transcription. In addition, also the growth regulating Hippo-pathway is modulated by actin dynamics at several levels [reviewed in (Piccolo et al. 2014; Rajakyla and Vartiainen 2014)]. This indicates that actin can control gene expression by regulation of transcription factor localization and activity, in both the cytoplasm and the nucleus.

3.2 Actin in Chromatin Remodeling Complexes

Dynamic changes in chromatin architecture are critical to permit access of the transcription machinery to the transcribed genes. Actin has been linked to both ATP-dependent chromatin remodeling complexes, which remodel the nucleosomes, and to histone acetyltransferases (HATs), which modify histones to create a chromatin environment favoring transcription [reviewed in (Lee and Workman 2007)]. In different species, actin has been identified in at least SWI/SNF, Ino80, SWR1, NuA4, Tip60, and SRCAP complexes (Cai et al. 2005; Fuchs et al. 2001; Galarneau et al. 2000; Ikura et al. 2000; Mizuguchi et al. 2004; Shen et al. 2000; Zhao et al. 1998). Notably, actin is often present in these complexes together with actin related proteins (Arps), which share the same basic actin fold. Curiously, out of the 10 known Arps, six (Arp4-Arp9) are localized mainly to the nucleus, and are essential subunits of many chromatin remodeling complexes [reviewed in (Oma and Harata 2011)]. Helicase-SANT (HSA) domain has been described as the binding platform for Arp-actin and Arp-Arp modules in many of these complexes (Szerlong et al. 2008). The exact function of actin has not been described for every complex, but in the case of Brg1 chromatin remodeling complex (SWI/SNF) it has been suggested that actin and Arp4 would form heterodimers and with this mechanism work together to maintain the integrity of the complex (Nishimoto et al. 2012). Actin has also been suggested to regulate Brg1 ATPase activity (Zhao et al. 1998). Within the Ino80 chromatin remodeler, actin and two Arps, Arp4 and Arp8, form a stable subcomplex by binding to the HSA domain of Ino80 ATPase (Szerlong et al. 2008). Detailed investigation of this subcomplex with structural biochemical assays showed that Arp4 likely binds monomeric actin via its barbed end. Arp4, assisted by Arp8, can also depolymerize actin filaments (Fenn et al. 2011). Also Shen-lab showed by using biochemical and genetic assays that actin operates as a monomer in the Ino80 complex, because its barbed end is not accessible for polymerization. They also showed that the subdomain 2 of actin contributes to chromatin binding of this complex (Kapoor et al. 2013). Together these studies reinforce the idea that actin is monomeric in the Ino80 complex, because Arp8 and Arp4 prevent actin polymerization by capping the barbed end of actin, while the pointed end interacts with chromatin (Fenn et al. 2011; Kapoor et al. 2013).

Also ABPs can influence the activity of chromatin remodeling complexes. The Wiskott-Aldrich syndrome protein and SCAR homologue (WASH) is an activator of the Arp2/3 complex and linked to endosomal sorting (Duleh and Welch 2010).

However, a recent study demonstrated that in the nucleus WASH associates with the nucleosome remodeling factor (NURF) complex, and assists its interaction with the c-Myc gene promoter. Importantly, the VCA-domain of WASH, which is required for promoting actin nucleation, is required for this process, which induces hematopoetic cell differentiation (Xia et al. 2014).

3.3 Nuclear Actin in RNA Polymerase Transcription

RNA polymerases are large protein complexes that transcribe the DNA to RNA. Biochemical studies have shown that actin co-purifies with all three RNA polymerases: Pol I (Fomproix and Percipalle 2004), Pol II (Egly et al. 1984; Smith et al. 1979), and Pol III (Hu et al. 2004). Although the precise mechanism by which actin interacts with different polymerases is unknown, actin seems to be required for their function. For example, actin antibodies inhibit Pol I transcription. Actin also seems to partner with nuclear myosin I (NM1), when activating Pol I (Philimonenko et al. 2004). Many myosins, which are motor proteins interacting with actin filaments, have been found inside the nucleus [reviewed in (de Lanerolle and Serebryannyy 2011)]. Most studies so far have focused on NM1, an isoform of myosin IC. Together with polymerized actin, motor activity of NM1 is required for the recruitment of Pol I to genes (Ye et al. 2008). The interplay between NM1 and actin has also been linked to chromatin modifications that activate ribosomal RNA (rRNA) transcription. NM1 is part of a chromatin remodeling complex B-WICH. Actin and SNF2h, a component of B-WICH, compete for binding to NM1, and it has thus been speculated that NM1 would be moving between B-WICH and actin bound Pol I complexes, and in this way regulate the different functions of Pol I (Percipalle et al. 2006; Sarshad et al. 2013). Regulation of NM1 activity by GSK3β phosphorylation seems to be especially important for activating rRNA transcription after mitosis (Sarshad et al. 2014).

The first RNA polymerase to which actin was linked with is Pol II (Egly et al. 1984; Smith et al. 1979), which mediates transcription of protein encoding genes. The transcription by Pol II can be roughly divided into four different steps: pre-initiation, initiation, elongation, and termination [reviewed in (Shandilya and Roberts 2012)]. Hofmann et al. showed that actin is recruited to certain promoters of class II genes and has a function during the assembly of the pre-initiation complex (PIC) (Hofmann et al. 2004). Phosphorylation of the C-terminal domain (CTD) of Pol II plays a crucial role in the transitions between different steps of the transcription process. For example, the Positive elongation factor (pTEFb) phosphorylates serine 2 of the Pol II CTD and releases the polymerase from a paused state. Cyclin-dependent kinase 9 (CDK9), a subunit of pTEFb, interacts with actin monomers and the interaction seems to enhance the P-TEFb activity on Pol II (Qi et al. 2011). Interestingly, also some ABPs have been linked to Pol II function. For example, fractionation experiments have hinted that actin, phosphorylated Pol II, and the actin monomer-binding protein cofilin are present in the same complex in the nucleus, and depletion of cofilin-1 decreases

transcription as measured by fluorouridine incorporation (Obrdlik and Percipalle 2011). Also actin-filament nucleating Arp2/3 complex (Yoo et al. 2007) and its activators N-WASP (Wu et al. 2006) and WAVE1 have been linked to Pol II activity. However, the binding of WAVE1 to the transcription apparatus does not require actin polymerization, but is rather dependent on the WHD domain of WAVE1. Importantly, nuclear WAVE1 is required for normal *Xenopus* development (Miyamoto et al. 2013), but the role of actin here warrants further studies.

During Pol II elongation the freshly transcribed mRNA will immediately be in contact with different proteins, which regulate its processing and transport. Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a set of proteins, which bind to this forming mRNA [reviewed in more detail (Han et al. 2010)]. Curiously, actin has been reported to bind to several hnRNPs from different organisms. The first clue to this came from a study done in *Chironomus tentans*, where actin was found to bind Hrp36 (Percipalle et al. 2001). Further studies in human cells identified hnRNP U as a protein associated with actin and having an important role in Pol II elongation (Kukalev et al. 2005; Percipalle et al. 2002, 2003). Assays with truncated hnRNP U protein and peptides concluded that hnRNP U possesses a specific C-terminal motif, which associates with actin. This motif is conserved in both insects and mammals (Kukalev et al. 2005). In mammals the interaction between hnRNP U and actin recruits a p300/CBP associated factor (PCAF), a HAT, to the actively transcribing gene (Obrdlik et al. 2008), thus linking mRNA processing to gene activation. Of note, also in this context the interaction between NM1 and B-WICH complex likely plays a role (Almuzzaini et al. 2015). In addition, the small actin monomer-binding protein profilin, has been found in the nuclear speckles. This could suggest that profilin might be involved in pre-mRNA processing alongside with actin (Skare et al. 2003).

Finally actin has also been associated with Pol III and depletion of actin from Pol III preparations inhibited transcription of the U6 gene. Actin was found to interact with polymerase subunits RPABC2 and RPABC3, which are found in all three polymerases (Hu et al. 2004), potentially suggesting that actin might bind to all polymerases through these proteins. Even though the involvement of actin in Pol III is still quite poorly understood, also components of B-WICH (see above) have been linked to Pol III transcription (Cavellan et al. 2006). Perhaps B-WICH could be involved in all actin–NM1–polymerase interactions.

4 Actin in Intranuclear Transport

In the cytoplasm one key role for actin is to provide force for motility, either through polymerization or together with motor protein myosins. Indeed, actin is essential for the majority of cellular movement events, from muscle contraction to cell migration. Also in the nucleus, actin, quite often together with the nuclear myosin NM1, has been linked to movement events. For example, actin and myosin cooperatively reposition inducible chromosomal loci from the nuclear periphery to the nuclear interior upon transcription activation (Chuang et al. 2006). A recent

study from the Belmont-lab demonstrates that actin-dependent motion of the HSP70 transgene to nuclear speckles has functional consequences on transcriptional activation, and thus likely plays a role in optimizing the cellular response to heat shock (Khanna et al. 2014). Actin has also been linked with Cajal bodies (Gedge et al. 2005) and it promotes movement of the active U2 gene loci toward these bodies (Dundr et al. 2007). Earlier studies have suggested energy and myosin-dependent movement of PML bodies (Muratani et al. 2002), but a recent publication suggests that actin polymerization is needed for the movement of HIV-1 proviruses away from the PML bodies for their efficient activation (Lusic et al. 2013). Altogether actin seems to have a role in intranuclear movement of genes, and perhaps also nuclear bodies, but more mechanistic studies on nuclear actin are still needed. For example, it is not clear how actin could be attached to the genes to promote their movement.

In addition to movement of individual gene loci, actin has also been linked to larger nuclear rearrangements. For example, upon serum withdrawal chromosome positioning is altered considerably. This movement is fast, and can be prevented by inhibiting NM1 (Mehta et al. 2010). Again, the mechanistic details are still missing, but it is rather intriguing that also certain ABPs have been linked to regulation of nuclear organization. Analysis of mutants of the *Drosophila* WASH, the Arp2/3 complex activator mentioned also above (see Sect. 3.2), revealed abnormal nuclear morphology and disruption of both chromatin and nuclear organelle substructures. Further studies revealed that WASH associates with constitutive heterochromatin (Verboon et al. 2015). However, the role of actin in this context is not yet known.

5 Summary

Our understanding of nuclear actin has rapidly evolved during the last decade, and the development of imaging tools has played a significant role in this. For example, the ability to finally visualize polymerized nuclear actin (Baarlink et al. 2013; Belin et al. 2013, 2015) has at least partially settled the most controversial and enigmatic aspect of nuclear actin: its form. On the other hand, the description of the nucleocytoplasmic shuttling mechanism of actin (Dopie et al. 2012; Stuven et al. 2003) has provided the basis for how nuclear actin levels are regulated, and RNAi screens have identified novel regulators of nuclear actin, demonstrating that this process is an evolutionary conserved phenomenon (Dopie et al. 2015; Rohn et al. 2011). These studies not only indicate the need to tightly control nuclear actin, but may also provide tools for functional studies.

Actin has been linked to numerous essential nuclear processes, but the precise molecular mechanisms remain unclear. One main reason is that only very few direct binding partners have been identified for nuclear actin, and hence there is very little knowledge of nuclear actin biochemistry. Most of the described nuclear functions of actin relate to gene expression, but actin could also have broader role in contributing to nuclear organization (Mehta et al. 2010) and in the maintenance of genomic integrity (Belin et al. 2015), as suggested by recent data. Also many

ABPs are found in the nucleus, but little is still known about their actual nuclear functions or involvement in nuclear actin dynamics. This can be explained by the difficulty of showing nuclear specific contributions, because most of these proteins have essential functions in the cytoplasm. To overcome these issues, we need to improve tools to study nuclear actin, find direct binding partners for nuclear actin, and really pinpoint the mechanisms how actin or actin dynamics affect nuclear functions. Nevertheless, the recent progress clearly demonstrates that actin is no longer only an artifact inside the nucleus and it must be regarded as an important regulator of many different nuclear processes.

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What We Know and Do Not Know About Actin

Thomas D. Pollard

Contents

Abstract

Seven decades of research have revealed much about actin structure, assembly, regulatory proteins, and cellular functions. However, some key information is still missing, so we do not understand the mechanisms of most processes that depend on actin. This chapter summarizes our current knowledge and explains some examples of work that will be required to fill these gaps and arrive at a mechanistic understanding of actin biology.

Keywords

Actin • Arp2/3 complex • Capping protein • Cofilin • Profilin

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1 Overview of Actin in Biology

Actin is one of the most abundant proteins on earth, and through its ability to assemble into filaments actin plays important roles in many biological systems including muscle contraction, cleavage of cells during cytokinesis, protrusion of the leading edge of motile cells, separation of plasmids in prokaryotes, and maintaining the physical integrity of the cell (Pollard and Cooper 2009). If one were only interested in what happens, then in some sense we already have a basic understanding of how actin functions in these and other biological systems. On the other hand, if one is interested in how all these systems work at the molecular level, the glass is only half full. Seven decades of research on actin revealed its atomic structure, a description of its assembly, some of the most important regulatory proteins, and its organization of various cellular structures. However, a closer look reveals obvious gaps in our understanding of most of these reactions.

This chapter will review what we know and focus on the areas where more work will be required to characterize the system at a fundamental level. Many mechanistic details such as kinetic and thermodynamic constants are missing, but the most challenging frontier is only now being addressed, namely how combinations of regulatory proteins influence actin in unexpected ways.

2 Structure of the Actin Molecule and Actin Filament

Numerous high-resolution crystal structures are now available for actins from eukaryotes (Fig. 1) and prokaryotes as well as for actin-related proteins Arp2, Arp3, Arp7, and Arp9 (Dominguez and Holmes 2011). The structure of Arp1 has been determined by cryo-electron microscopy of the dynactin complex (Urnavicius et al. 2015). All actins and Arps have the same fold and highly conserved nucleotide binding sites, but surface features of Arps and prokaryotic actins differ from eukaryotic actins.

Actin polymerizes into double helical polymers with all of the subunits oriented in the same direction (Fig. 2). Based on decoration with myosin heads, one end of the filament is called the barbed end and the other the pointed end. Our understanding of actin filament structures has advanced rapidly with improvements in techniques for X-ray fiber diffraction (Oda et al. 2009) and cryo-electron microscopy (Fujii et al. 2010; Galkin et al. 2010; von der Ecken et al. 2014). Structures are also available for actin filaments complexed with capping protein, cofilin, myosin, tropomyosin, tropomodulin, and other proteins, as well as with the natural small molecule toxin, phalloidin. Polymerization changes the conformation of the actin subunit, especially flattening the twist between the two halves of the protein (Oda et al. 2009; Fujii et al. 2010; Dominguez and Holmes 2011). Actins from prokaryotes also polymerize into filaments but in many cases adopt geometries different from eukaryotic actins (Bharat et al. 2015).

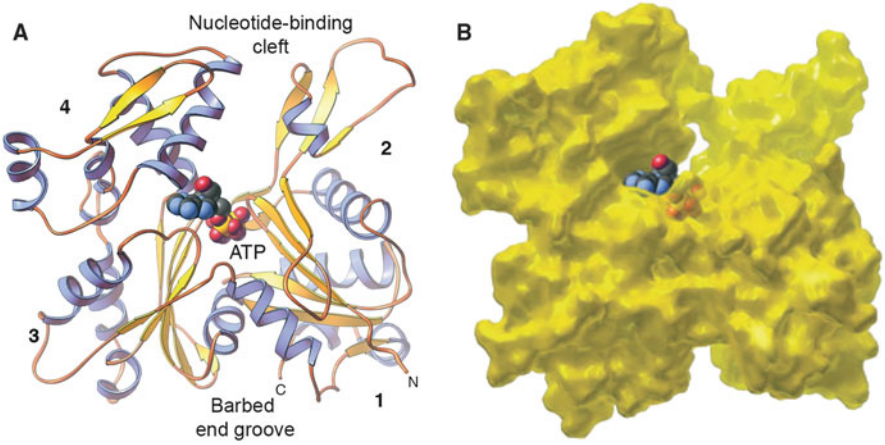

Fig. 1 Ribbon diagram (**a**) and space-filling model (**b**) of the actin molecule (PDB file 1ATN; modified from T.D. Pollard and W.C. Earnshaw, Cell Biology, second edition, Saunders)

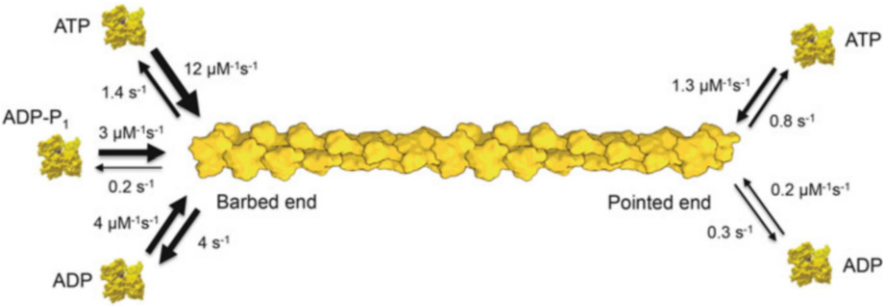

Fig. 2 Actin filament elongation rate constants from Pollard (1986) and Fujiwara et al. (2007)

3 Assembly of Actin Filaments

Spontaneous nucleation of actin filaments: Nucleation of actin filaments from purified monomers is extremely unfavorable, owing to instability of actin dimers and trimers, which dissociate on time scales of microseconds and milliseconds respectively (Cooper et al. 1983; Frieden 1983). Brownian dynamics simulations showed that the favored dimer is one with interactions along the long-pitch helix of the filament (Sept and McCammon 2001), but our current understanding of the nucleation reactions is limited to orders-of-magnitude estimates of the rates and equilibrium constants for the assembly of dimers, trimers, and longer oligomers.

Actin filament elongation: We understand many details about the kinetics and thermodynamics of the association and dissociation of actin subunits at both ends of actin filaments (Pollard 1986). The barbed end is much more dynamic than the

pointed end (Fig. 2). Association of ATP-actin is diffusion-limited at the barbed end (Drenckhahn and Pollard 1986) and theoretical calculations indicate that about 2% of collisions result in binding, a very favorable ratio which suggests some electrostatic guidance (Sept et al. 1999). Association and dissociation of subunits of ADP-P_i-actin are similar to ATP-actin at both ends of the filament (Fujiwara et al. 2007). At barbed ends ADP-actin associates slower and dissociates about 5 times faster than ATP-actin, but little is known about the mechanism. Similarly, we do not understand why subunit addition at pointed ends is much slower than the diffusion limit (Pollard 1986). The electrostatics may be less favorable (Sept et al. 1999), but the conformations of the ends are probably different.

Experiments with profilin suggest that the conformations of terminal subunits at the ends of filaments differ from monomers and change upon nucleotide hydrolysis and phosphate release (Courtemanche and Pollard 2013). For example, in spite of lower affinity for ADP-actin monomers, profilin binds ADP-bound barbed ends 200 times more strongly than AMP-PNP-barbed ends.

Differences in the conformations of the terminal subunits must contribute to the physical basis for these differences. However, remarkably little is known about the structures of actin filaments ends in spite of their obvious importance in actin biology, as they are the only sites for filaments to grow and shorten. Models of the subunits in the middle of filaments are available at near atomic resolution (Oda et al. 2009; Fujii et al. 2010; Galkin et al. 2010), but the only structural information about an actin filament end is a reconstruction of the pointed end from cryo-electron micrographs at 23 Å resolution (Narita et al. 2011). This structure showed the terminal subunit tilted by 12° over the penultimate subunit in a way that partially obstructs the binding site for an incoming subunit and might slow the dissociation of the terminal subunit. This conformation may also reduce the affinity of pointed ends for phosphate.

Hydrolysis of bound ATP: Polymerization of Mg-ATP-actin stimulates the rate of ATP hydrolysis by a factor of 5,000 from ~10^{-5}/s (Rould et al. 2006) to 0.3/s (Blanchoin and Pollard 2002). Various models for the hydrolysis reaction have been debated, but the most recent work shows that the hydrolysis reaction is stochastic, occurring randomly on polymerized actin subunits (Jégou et al. 2011). Quantum mechanics/molecular mechanics simulations show how the conformation of the subunit in the filament promotes hydrolysis by positioning the nucleophilic water favorably in the active site (McCullagh et al. 2014).

Dissociation of phosphate in the middle and at both ends of the filament: Classic experiments showed that phosphate binds to and dissociates from ADP-actin subunits buried in the middle of a filament (Carlier and Pantaloni 1986). The affinity is low, in the millimolar range, and depends on the pH, since $H_2PO_4^-$ in the ligand (Carlier and Pantaloni 1988). Both phosphate binding and dissociation are very slow, so the γ-phosphate cleaved from bound ATP dissociates with a half time of 6 min. Phosphate has a much lower affinity for ADP-actin subunits on both ends of filaments than within the polymer (Fujiwara et al. 2007). Rapid dissociation of the γ-phosphate from the terminal subunits controls the stability of filament ends,

and the lower affinity of phosphate for pointed ends than barbed ends explains the differences in their critical concentrations. At steady state in the presence of ATP the combination of slow association of ATP-actin and rapid dissociation of P_i at the pointed end makes terminal subunits much more likely to have bound ADP than the barbed end. Thus the critical concentration at the pointed end is intermediate between that of ATP-actin and ADP-actin, whereas the critical concentration at the barbed end is close to that of ATP-actin.

Unfinished business: In spite of this satisfying progress, we still seek answers to a number of fundamental questions about actin filament assembly. Why does ADP-actin bind slower and dissociate faster than ATP-actin at barbed ends? Does the conformation of the pointed end explain the slow association and dissociation of actin subunits? Why is the affinity of both filament ends for phosphate much lower than the middle of the filament? How does the bound nucleotide on the terminal subunits affect their conformations? Why do the rates of ATP hydrolysis and phosphate dissociation seem to differ greatly among actins?

4 Regulation of Actin Assembly by Other Proteins

Cells use dozens of proteins to regulate every aspect of actin assembly. These proteins maintain a pool of unpolymerized actin monomers, nucleate actin filaments, promote elongation, terminate the elongation of actin filaments by capping, promote dissociation of P_i from ADP-P_i-subunits, sever actin filaments, and crosslink filaments into higher order structures. None of the proteins act alone. Their synergisms are a major emerging trend in the field. I will explain the best understood example of synergism between actin-binding proteins and note other examples.

5 Pool of Unpolymerized Actin

Synergism between profilin and capping proteins maintains a pool of unpolymerized actin and is the classic example of cooperation in this system. The pool of unpolymerized actin is absolutely essential for the dynamics of the system where actin filaments assemble and turn over on a time scale of tens of seconds. Without a pool of unpolymerized actin subunits, new filaments could not arise or grow.

Mechanism of action of profilin: Profilins are small proteins that bind to the barbed end of actin monomers with a slightly higher affinity for ATP-actin monomers ($K_d = 0.1$ μM) than ADP-actin monomers ($K_d = 0.4$ μM) (Vinson et al. 1998). The location of the binding site on actin (Schutt et al. 1993) explains how

steric interference prevents the profilin-actin complex from participating in spontaneous nucleation of actin filaments and elongation of pointed ends.

On the other hand, profilin-actin elongates barbed ends at the same rate as free actin monomers (Pollard and Cooper 1984). The complex binds barbed ends without interference and profilin dissociates rapidly from the barbed end. Nevertheless, high concentrations of free profilin slow barbed end elongation, presumably by occupying the barbed end and blocking the addition of subunits. For 30 years the field struggled to understand how profilin interacts with the barbed end of actin filaments (Yarmola and Bubb 2006). Recent kinetics experiments revealed how the nucleotide bound to actin influences the affinities of profilin and profilin-actin for barbed ends. Profilin has a much higher affinity for ADP-actin filament barbed ends ($K_d = 1$ μM) than AMP-PNP-actin filament barbed ends ($K_d = 226$ μM; AMP-PNP is a nonhydrolysable analog of ATP) (Courtemanche and Pollard 2013). Thus nucleotides have opposite effects on the affinity of profilin for monomers and barbed ends. This difference suggests that the conformations of barbed end subunits differ from those of monomers and change upon nucleotide hydrolysis and phosphate release. Profilin has a low affinity for the conformation of ATP-actin at the barbed end, so it dissociates rapidly, while the higher affinity of profilin for ADP-actin at the barbed end slows elongation and can even promote depolymerization (Jégou et al. 2011; Courtemanche and Pollard 2013). Minor steric clashes between profilin and actin subunits at the barbed end could explain these differences, but have not yet been documented by direct structure determination.

Synergism between profilin and capping protein: Profilin is present in high (50–100 μM) concentrations in the cytoplasm and most of the unpolymerized actin is bound to profilin (Kaiser et al. 1999), so profilin is thought to suppress spontaneous nucleation. However, profilin alone cannot explain the high concentration of unpolymerized actin, on the order of 50–100 μM in the cytoplasm, because profilin-actin would elongate actin filament barbed ends and deplete the pool of unpolymerized actin. Furthermore, the affinity of profilin for ATP-actin is relatively high but under cellular conditions the concentration of free actin monomers would be well above the critical concentration for polymerization.

The combination of profilin and capping protein explains the large pool of unpolymerized actin in the cytoplasm (Vinson et al. 1998). Capping protein blocks actin subunit addition and dissociation at the barbed ends of actin filaments (Isenberg et al. 1980), the only actin assembly reaction not inhibited by profilin. Together profilin and capping protein maintain a metastable pool of actin monomers bound to profilin orders of magnitude above the critical concentration but ready to elongate any actin filament barbed ends created by nucleation proteins. Several proteins modulate the activity of capping protein, adding a new dimension to the issue of maintaining the actin monomer pool (Edwards et al. 2014).

Some vertebrate cells also have high concentrations of thymosin-β4, which binds both the barbed and pointed ends of actin monomers and blocks all known assembly reactions (Yarmola and Bubb 2004). Thymosin-β4 acts as an actin monomer buffer, but actin exchanges rapidly on and off thymosin-β4, so it can

also bind profilin and participate in elongation of barbed ends (Pantaloni and Carlier 1993).

Unfinished business: The main questions about the actin monomer pool are how the nucleotide state of the actin filament modulates the conformation of the barbed end and its affinity for profilin and how other proteins regulate the activity of capping protein. Better quantitative data on fractions of capped and uncapped barbed ends in live cells are also vital for understanding the overall actin economy in the cytoplasm.

6 Nucleation of Actin Filament Branches by Arp2/3 Complex

Given that spontaneous nucleation of filaments by actin monomers is unfavorable and profilin suppresses nucleation, cells use other proteins to initiate new actin filaments. The two best characterized nucleation proteins are Arp2/3 complex and formins.

Arp2/3 complex consists of two actin-related proteins, Arp2 and Arp3, supported by five other protein subunits (Machesky et al. 1994). The complex assembles networks of branched actin filaments (Mullins et al. 1998) for cellular motility (Pollard and Borisy 2003) and endocytosis (Weinberg and Drubin 2012). The Arps become the first two subunits of the new filament, which forms as a branch on the sides of an existing actin filament called the mother filament (Rouiller et al. 2008) (Fig. 3).

Arp2/3 complex is intrinsically inactive, because the Arps are separated from each other (Robinson et al. 2001) and must undergo a large conformational change to initiate a new filament (Rouiller et al. 2008). This massive rearrangement involves a 30° rotation of ARPC1, ARPC4, and Arp2 relative to the rest of the complex to bring Arp2 adjacent to Arp3 (Dalhaimer and Pollard 2010). Molecular dynamics simulations showed that steric interference makes this rotation energetically unfavorable.

Interactions between Arp2/3 complex and proteins called nucleation-promoting factors and the side of an actin filament favor the conformational change and the formation of a daughter filament (Machesky et al. 1999). Nucleation-promoting factors (Campellone and Welch 2010) include the Wiskott-Aldrich syndrome protein (WASP) and related proteins (N-WASP, Scar/WAVE, WASH, WHAMM, and JMY) with V motifs that bind actin monomers and CA motifs that bind Arp2/3 complex (Marchand et al. 2001).

Arp2/3 complex has multiple binding sites for CA motifs of nucleation-promoting factors including sites on Arp2 and Arp3 (Padrick et al. 2011; Ti et al. 2011; Boczkowska et al. 2014). It is generally agreed that VCA delivers actin bound to the V motif of VCA to Arp3 and Arp2 to initiate the branch, but we currently know more about how CA binds Arp3 than Arp2. VCA binding to Arp2/3

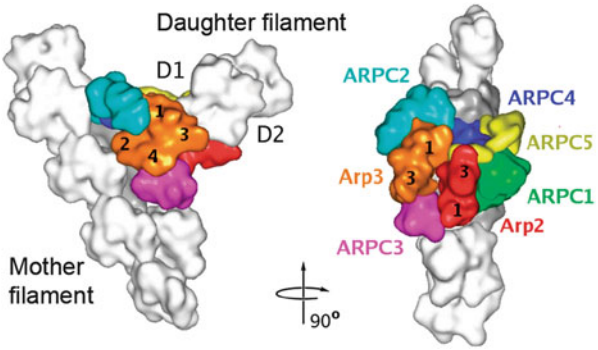

Fig. 3 Reconstruction from electron micrographs of an actin filament branch junction formed by Arp2/3 complex. Subunits in the mother and daughter filaments are *white*. Subunits of Arp2/3 complex are *colored* and *labeled*; all seven make physical contacts with the mother filament. Arp3 and Arp2 are the first two subunits of the daughter filament (Relabeled from Rouiller et al. 2008)

complex also favors binding of the complex to the sides of actin filaments (Ti et al. 2011).

Binding of actin-VCA also favors binding of Arp2/3 complex to the side of a mother filament (Ti et al. 2011) but the mechanism is not known. Single molecule observations of Smith et al. showed that most associations of Arp2/3 complex with the side of a filament are unproductive and result in rapid dissociation (Smith et al. 2013), explaining the slow rate of binding measured with pyrene-labeled Arp2/3 complex (Beltzner and Pollard 2008; Ti et al. 2011). However, the order of events along the pathway is uncertain. One scenario is that CA binding causes a partial rotation of Arp2, while subsequent binding to the mother filament provides the energy to complete the conformational change that stabilizes the interaction with the mother filament and position the Arps to initiate the daughter filament.

One nucleation-promoting factor, cortactin, stabilizes actin filament branches (Weaver et al. 2001), while tropomyosin bound to the mother filament inhibits branch formation (Blanchoin et al. 2001). Physical forces also influence branching by favoring assembly of daughter filaments on convex surfaces of mother filaments (Risca et al. 2012).

Unfinished business: How does CA bind Arp2/3 complex and promote activation? How does CA bound to Arp2/3 complex promote binding to the side of a mother filament? What is the physical pathway of activation of Arp2/3 complex? How are branches oriented productively toward the leading edge? How do branches turn over in vitro and in cells?

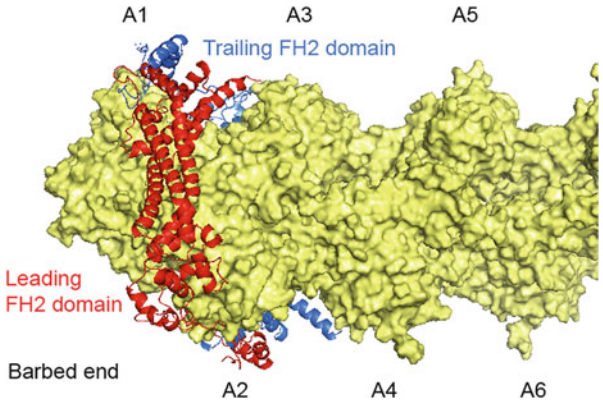

Fig. 4 Model of the barbed end of an actin filament with an associated Bni1 FH2 domain dimer. The space-filling models of the *yellow* actin subunits are numbered from the terminal subunit (*A1*). The FH2 domains are shown as *ribbon diagrams* with the leading FH2 *domain red* and the trailing FH2 *domain blue*. The model is based on a molecular dynamics refinement (Baker et al. 2015) of a crystal structure (Otomo et al. 2005)

7 Nucleation and Elongation of Actin Filaments by Formins

A family of proteins (15 human genes, 3 fission yeast genes) called formins produces unbranched filaments for cytokinesis, fungal cables and animal filopodia and stress fibers (Skau and Waterman 2015). The characteristic feature of formins is a formin homology 2 (FH2) domain; other domains with regulatory functions vary between isoforms and species (Higgs 2005). Dimers of FH2 domains nucleate the polymerization of actin filaments (Pruyne et al. 2002) and remain attached to the barbed end of actin filaments as they elongate (Kovar and Pollard 2004) (Fig. 4). The nucleation reaction is still poorly understood, but strongly favors free actin monomers rather than profilin-actin complexes (Paul and Pollard 2008).

All formin FH2 domains tested slow the elongation of actin filament barbed ends (Kovar et al. 2006). Biochemical analysis (Kovar et al. 2006; Vavylonis et al. 2006; Paul and Pollard 2009a, b), X-ray crystallography (Otomo et al. 2005) and molecular dynamics simulations (Baker et al. 2015) suggest that the FH2 domain alters the conformation of actin subunits at the barbed end in a way that prevents addition of a new subunit. One proposal is that the FH2 domain flattens the helix at the barbed end of the actin filament making it an unfavorable binding site for an incoming actin subunit. Since ends associated with a formin grow steadily, the conformation of an end is likely to fluctuate rapidly between two states, open and closed. Actin alone is open virtually all of the time, but association with FH2 domains biases the equilibrium toward the closed state. Formins slow elongation by $<5\%$ to $>95\%$, so they differ in their abilities to influence the equilibrium between the open and closed states. Small tensile forces on an actin filament slow elongation by formin Bni1p,

suggesting that subpiconewton forces influence the equilibrium between the open and closed states (Courtemanche et al. 2013).

Little is known about how formin FH2 domains remain associated with barbed ends through more than 2,000 cycles of actin subunit addition before dissociating from the end. Early speculation suggested the trailing FH2 domain steps toward the end before the new subunit arrives (Zigmond et al. 2003), but our evidence suggests that the trailing FH2 domain steps onto the newly added actin subunit (Paul and Pollard 2009a, b).

Profilin inhibits nucleation by formin FH2 domains but increases the rate of elongation, providing that an FH1 domain is associated with the FH2 domain. Depending on the concentration of profilin, elongation rates can exceed the diffusion-limited rate of free actin monomers on a free actin filament end (Kovar et al. 2006; Vavylonis et al. 2006). The explanation is that the rate-limiting step for elongation becomes the association of profilin-actin with polyproline tracts in the flexible FH1 domain rather than actin binding to the end of the filament. Formin FH1 domains bind multiple profilin-actin complexes, which transfer rapidly (>1,000/s) onto the barbed end (Paul and Pollard 2008). Although this involves more steps than simple binding of an actin subunit to the end of the filament, it is faster owing to the multiple profilin-binding sites on FH1 domains. Transfer of actin from the FH1 domain to the end of the filament is diffusion-limited and position-specific variations in the sequences of the polyproline motifs optimize the efficiency of FH1-stimulated polymerization by binding profilin weakly to sites near the FH2 domain and more strongly to distal sites (Courtemanche and Pollard 2012). Each FH1 domain of budding yeast formin Bni1p transfers actin to the barbed end of a filament independently of the other FH1 domain and structural evidence suggests a preference for actin delivery from each FH1 domain to the closest long-pitch helix of the filament.

Formins synergize with other proteins to regulate actin assembly. The first example was regulation of formin activity by Rho-family GTPases, which activate formins by overcoming intramolecular auto-inhibitory interactions (Alberts 2001; Li and Higgs 2003). Formins also compete with capping protein at barbed ends (Kovar et al. 2003; Zigmond et al. 2003; Bombardier et al. 2015; Shekhar et al. 2015). Other proteins either promote (Park et al. 2015) or inhibit (Gould et al. 2014) actin polymerization by formins.

Unfinished business: At the molecular level, more work is required to determine how formins nucleate new filaments, gate the elongation of barbed ends, and step so reliably onto new subunits added to barbed ends. At the cellular level we must determine how each of the 15 mammalian formins contributes to actin assembly and how diverse regulatory proteins contribute to the localization and activities of formins.

8 Recycling Polymerized Actin and Associated Proteins

Hydrolysis of the ATP bound to polymerized actin and dissociation of the γ-phosphate prepare the filament for disassembly. Phosphate dissociation is more important than ATP hydrolysis, because polymerized ADP-P_i actin has properties very similar to ATP-actin (Fujiwara et al. 2007). ADP-actin dissociates fivefold faster from barbed ends that ATP-actin, while ADP-actin dissociates very slowly from pointed ends (Fig. 2). However, in cells ADP-actin is unlikely to be exposed on free barbed ends owing to the very favorable addition of ATP-actin from the cytoplasmic pool. Therefore, other mechanisms are required to explain how many filaments in cells turn over in a minute or less (Theriot and Mitchison 1991).

The leading candidate to foster actin filament turnover is severing by cofilin, a small protein that binds ADP-actin monomers and filaments with higher affinity than ATP-actin (Blanchoin and Pollard 1999; Cao et al. 2006). Cofilin also promotes dissociation of the γ-phosphate from ADP-actin polymers (Blanchoin and Pollard 1999). Actin filaments saturated with cofilin have a tighter helical twist (McGough et al. 1997) and are more flexible than normal (McCullough et al. 2011). Nevertheless, these saturated filaments are very stable (Andrianantoandro and Pollard 2006). On the other hand, filaments sparsely occupied with cofilin are severed rapidly because of instability between parts of the filament with different flexibilities (Elam et al. 2013a, b; Ngo et al. 2015). Sparse occupancy occurs naturally during the aging of growing filaments, given that older parts stochastically are converted to ADP-actin and then bind cofilin. In a reconstituted system, this aging and severing mechanism keeps growing filaments at constant length but repeatedly cutting off the oldest part near the pointed end (Michelot et al. 2007). Severing promotes turnover by creating ends for the dissociation of ADP-actin. Severed filaments can regrow (Bravo-Cordero et al. 2013), but many are likely to be capped at their barbed ends, so they can depolymerize slowly by dissociating ADP-actin from their pointed ends.

Other proteins modulate actin filament severing by cofilin. For example, proteins that compete with cofilin for binding the side of actin filaments such as tropomyosin, myosin, actin interacting protein-1 (Aip1), and the tyrosine kinase Arg (Maciver et al. 1991; Elam et al. 2013a, b; Chen et al. 2015; Courtemanche et al. 2015) can either inhibit or promote severing depending on how they influence the saturation of the filament with cofilin. The protein coronin has a more specific effect on severing by recruiting cofilin to actin filaments (Mikati et al. 2015).

Cofilin (Chan et al. 2009) and a related protein GMF (Luan and Nolen 2013; Ydenberg et al. 2013) both promote dissociation of branches formed by Arp2/3 complex from their mother filaments. Cofilin destabilizes the branch by changing the conformation of the mother filament, resulting in dissociation of the branch, presumably capped on its pointed end by Arp2/3 complex. GMF interacts directly with Arp2/3 complex and dissociates the branch from Arp2/3 complex on the side of the mother filament. Both of these actions help explain why branched actin filaments are confined to a narrow zone at the leading edge of a motile cell (Svitkina et al. 1997).

Unfinished business: Although the molecular mechanism of cofilin severing has become clearer, many questions remain about how cofilin contributes to recycling actin in cells. Much more information is required about the timing of severing following actin filament assembly and the fates of fragments released into the cytoplasm.

9 Production of Force by Polymerizing Actin Filaments for Cellular Movements

Experiments with purified proteins established that elongation of free actin filaments (Footer et al. 2007) and filaments associated with a formin (Kovar and Pollard 2004) produce piconewton forces. The binding energy from subunit addition is converted into physical force with high thermodynamic efficiency. The density of actin filaments at the leading edge (Abraham et al. 1999; Mogilner and Oster 2003) is sufficiently high to produce the force required for protrusion of the membrane (Mogilner and Oster 2003). An attractive feature of the branched networks at the leading edge of motile cells is that the filaments impinge upon the inside of the plasma membrane at an angle that is favorable for filament growth to push on the membrane (Mogilner and Oster 1996). Experiments on reconstituted systems show that applied force increases the density of the filaments in branched networks and increases the force produced by the network (Bieling et al. 2016). This force-feedback is likely to enhance the effectiveness of pushing by expanding actin filament networks.

Unfinished business: The feasibility of actin polymerization driving cellular movements is established experimentally and theoretically, but remarkably little has been observed about the lifetimes of filaments engaged in pushing in living cells. The challenges include the very high density of filaments and their small sizes. Hopefully, improvements in imaging may someday make it possible to visualize the birth, elongation, capping, severing, and turnover of individual actin filaments in live cells.

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