Trends in Biochemical Sciences

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Review

Actin Post-translational Modifications: The Cinderella of Cytoskeletal Control

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Actin is one of the most abundant proteins in eukaryotic cells and the main component of the microfilament system. It plays essential roles in numerous cellular activities, including muscle contraction, maintenance of cell integrity, and motility, as well as transcriptional regulation. Besides interacting with various actin-binding proteins (ABPs), proper actin function is regulated by post-translational modifications (PTMs), such as acetylation, arginylation, oxidation, and others. Here, we explain how actin PTMs can contribute to filament formation and stability, and may have additional actin regulatory functions, which potentially contribute to disease development.

Highlights

Post-translational modifications of actin affect its folding and structure, as well as interaction with actin-binding proteins, and thus interfere with cytoskeleton dynamics.

The actin N-terminal acetyltransferase, NAA80, was recently identified, thus solving a 30-year-old mystery on the final step of actin's unique and conserved N-terminal maturation process.

Acetylation and arginylation compete for actin's N terminus, both affecting filament formation, interaction with actin-binding proteins, and cell motility.

Actin oxidation of Met44 and Met47 by the MICAL enzymes promotes, in synergy with cofilin, the disassembly of actin filaments and is linked to cancer development.

Toxin-mediated modifications of actin may lead to actin filament aggregation, and in some cases cell death.

The Fundamentals of Actin Functionality

Actin (see Glossary) accounts for up to ~15% of the total protein level in muscle cells and 1–3% in nonmuscle cells. It exists in both a monomeric globular state (**G-actin**) and polymerized filamentous state (**F-actin**; Figure 1A), and the switch between the two states is highly dynamic. The actin filaments play crucial roles in countless cellular functions, including muscle contraction, cell signaling, as well as cell integrity and motility [1]. The multifunctionality of actin is based on three pillars (Figure 1B): chaperonin-assisted folding [2], interactions with **actin-binding proteins (ABPs)** [1], and **post-translational modifications** (**PTMs**; Figure 1C) [3]. Numerous studies and reviews describe the influence ABPs have on the actin **cytoskeleton**. In this review, however, we describe the most recent findings on actin PTMs shedding light on a crucial, but often overlooked, aspect of actin biology.

Actins represent a family of isoforms which are highly similar in sequence (≥93% sequence identity) and each conserved throughout evolution. Based on their amino acid sequences, six isoforms were described and classified according to the tissues in which they were found in mammals and birds: four muscle forms; α-skeletal, α-cardiac, α-smooth, γ-smooth, and two nonmuscle cytoplasmic actins: β-cytoplasmic and γ-cytoplasmic [4]. α, β, and γ refer to their respective mobility during isoelectric focusing, which is exclusively due to the number (3/4) and nature (Asp/Glu) of the N-terminal acidic residues. For example, the **N terminus** of β-cytoplasmic actin is Ac-DDDIAALVV- while that of γ-cytoplasmic actin is Ac-EEEIAALVI-. The four underlined residues constitute the only differences in a total of 375 residues present in these two isoforms, emphasizing their conserved nature. Despite their sequence and structural similarities, actin isoforms display both overlapping and unique cellular roles (reviewed in [5]). This has been clearly demonstrated in mice where knockout of β-actin results in embryonic lethality [6,7], while γ-actin-deficient mice show developmental defects, but are viable [8,9]. Although these remarkably different effects are not yet fully understood, it is known that these two isoactins display distinct intracellular localization patterns [5]. Further *in vitro* experiments reveal that mixtures of isoactins in filaments could affect polymerization dynamics, stability, and interactions with ABPs [5,10]. On top of these subtle differences, PTMs could contribute by affecting actin structure, localization, and function. Most PTMs will affect the isoactins in a similar manner, given the actin sequence

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similarities. However, as described later in this review, there are clear cases of isoform-specific PTMs contributing to differentiated functions.

Post-translational Modifications: The Underrated Players of Actin Cytoskeleton Dynamics

The first actin PTM, N-terminal (Nt) acetylation, was reported for skeletal muscle actin in 1966 by Gaetjens and Bárány [11], and later identified in all other actin isoforms. Today, more than 140 PTMs have been described in eukaryotic actin sequences ([3] and http://www.phosphosite.org). Some actin PTMs are quantitative and reversible, whereas others are rare, affecting only a minority of the molecules that make up the cellular actin pool. Thus, many actin PTMs should be considered as partial modifications. Actin PTMs are found on 94 different side chains (Table 1, Key Table) which constitute about 45% of the residues that can be modified. Specifically, new phosphorylation, ubiquitination, and SUMOylation sites have been identified by global proteomics analyses in recent years [12–19]. Interestingly, we have noticed regions where the frequency of PTMs is significantly lower than average (regions: 95–145, 240–256, and 331–354). This follows the overall accessibility of the side chain residues in the actin structure, though loss of ATP/ADP or internal cleavages could also induce partial denaturation, resulting in unspecific low-level modifications. It is currently not clear to which extent the latter contribute to actin's cellular role, or whether they should be considered as structural noise. Furthermore, our knowledge about the regulation, reversibility, and the interplay between individual PTMs remains limited. Given the high number of reported actin PTMs and the absence of detailed studies for most of them, we focus here predominantly on recent reports covering **Nt-acetylation**, **Nt-arginylation,** and oxidation of actin. We discuss their molecular and physiological consequences, and their potential role in disease development.

Structural and Regulatory Implications of Actin PTMs

Although not all actin PTMs appear at the same time on the same molecule, and some PTMs have only been reported in particular organisms, their sheer number poses a serious challenge for a global understanding of their regulatory mechanisms. For instance, how can an actin molecule, whose primary role is to generate dynamic filaments composed of geometrically conserved building blocks repeated over several thousand times, give rise to these structures when decorated with potentially structure disturbing PTMs? How can both G- and F-actin interact in a dynamic and rigorously controlled manner with a plethora of ABPs when carrying this large number of modifications? PTMs can however participate in the structural architecture of actin and modify their filaments. One of the best known examples is the structure of arthrin, a 55-kDa heavy form of actin first observed in insect muscle thin filaments [20]. This insect actin, which is monoubiquitinated at Lys118 (Table 1), appears at every seventh subunit along the filament long pitch helices. It was suggested that arthrin regulates muscle contractile activity [20]. A more recent report on structural regulation of the actin filament network refers to Nt-arginylation of β-actin by arginyl-tRNA protein transferase 1 (**ATE1**). In this case, Nt-arginylated actins form normal filament structures. Non-Nt-arginylated actin isolated from ATE1 knockout (KO) cells, on the other hand, forms bundles and aggregates, resulting in shorter filaments. On a cellular level this leads to disorganization of lamellipodia and filopodia, an effect which is attributed to altered interactions with ABPs [21].

Given the multifunctional nature of actin, one can expect that the final outcome of this high number of PTMs could be extremely complex. Some PTMs will affect steady-state filament growth by blocking one of the filament ends or reducing the concentration of polymerization competent monomers. Some PTMs may interfere with the actin-ABP equilibrium or drive actin

Glossary

Actin: family of multifunctional globular proteins that are able to polymerize into filaments and interact with a multitude of actin-binding proteins. The family consists of at least six isoforms in humans (α-skeletal, α-cardiac, α-smooth muscle, γ-smooth muscle, β-cytoplasmic, and γ-cytoplasmic).
Actin-binding proteins (ABPs): ABPs are signaling pathway-controlled actin interactors, which regulate the polymerization and depolymerization of actin filaments as well as their organization in the cytoskeleton network. Known ABPs include: the Arp2/3 complex, profilins, gelsolin, formins, and cofilin.
ATE1: arginyl-tRNA-protein transferase 1 catalyzes the attachment of arginine to the N terminus of an acceptor protein or to internal amino acid side chains.
Cytoskeleton: highly organized protein network in all domains of life (prokaryotes, archaea, and eukaryotes), consisting of hundreds of proteins which are interconnected by filaments (actins), tubules (tubulins), and the cytokeratin network.
F-actin: polymeric, filamentous form of actin.
G-actin: monomeric, globular form of actin.
MICAL: MICALs (molecule interacting with CasL) are cytosolic, multidomain enzymes that belong to a family featuring monooxygenase activity. They reversibly oxidize Met44 and Met47 of F-actin.
NAA80/NatH: Nα-acetyltransferase protein 80/N-terminal acetylation complex H belong to the N-terminal acetyltransferase (NAT) family that, together with the NatB complex, are involved in the unique N-terminal maturation process of actin by acetylating its N terminus. The NAT enzyme family provides Nt-acetylation for about 80% of the human proteome.
Nt-acetylation: addition of an acetyl group (Ac) to the N terminus of a protein. Nt-acetylation is catalyzed by N-terminal acetyltransferases using acetyl-CoA as donor.
Nt-arginylation: addition of an arginine residue (Arg/R) to the N-terminus of a protein. Nt-arginylation

molecules towards degradation pathways. And if this is not yet sufficiently complex, PTMs may enhance or switch off each other's effects by crosstalking mechanisms. The circuits that are produced could function via loops that on their turn activate novel circuits. These quantum bits of modifications are most likely not simply noise, but could push the cell following stochastic mechanisms towards a reversible or irreversible destiny. For instance, Tyr53 can be a target for phosphorylation, but also for nitration during oxidative stress. Similarly, Cys374 is highly reactive and can accept different types of modifications (Table 1). It is not clear whether these modifications will result in the same effect because they display a different chemical nature. An interesting example of the complexity involves some prominent ABPs like ADF/cofilin, gelsolin (Figure 1D), profilin, and DNase I (Figure 1E). Profilin binds to two regions in actin (Figure 1E), while cofilin interacts with actin via three sites (Figure 1D) [1,22]. Part of these sites overlap with each other. Thus, modifications in actin could tilt the balance by which these two ABPs exert their control on actin assembly. N-terminal maturation of actin is an elegant example where a particular protein modification depends on the previous one. Here, the successive actions of methionine aminopeptidases, N-terminal acetyltransferases, and ATE1 result in most actin molecules being Nt-acetylated, whereas a minority is Nt-arginylated (discussed later in this review).

is catalyzed by the arginyltransferase ATE1 using arginyl-tRNA as donor.
N terminus: start of a protein or a polypeptide, which has a free amino group ($-NH_2$). The amino group is usually positively charged at physiological pH (7.4). N-terminal modifications will mask or change this charge.
Post-translational modifications (PTMs): protein modifications that are added after the protein has been fully translated and/or folded. Modificationsolecules, such as oxidation and acetylation, to the addition of polypeptides such as SUMOylation and ubiquitination.
ROS: reactive oxygen species are highly reactive molecules and free radicals derived from oxygen that contributes to oxidative stress, which leads to various diseases.

N-Terminal Processing of Actin: A Unique Maturation Mechanism

Actins are first synthesized as precursor molecules which are further N terminally processed by successive actions of N-terminal acetyltransferases and aminopeptidases. This process was first described by Redman and Rubenstein in the early 1980s [23], and only recently more details on the players have become available. The six expressed mammalian actin isoforms are divided into two categories based primarily on the nature of their unprocessed N-terminal sequences (Figure 2A) [4]. For class I actins (nonmuscle β- and γ-actin) the initiator methionine (Met1) is directly followed by three acidic amino acids (MDDD-/MEEE-). The actin maturation process begins when the nascent N terminus is cotranslationally Nt-acetylated by NatB, which also acetylates other eukaryotic proteins beginning with MD-/ME- [26]. Normally, acetylation of acidic N termini ensures that Met1 is retained, but in an unusual twist from nature's side the Nt-acetylated Met1 is removed by a still unidentified aminopeptidase. The neo-N terminus (DDD-/EEE-) is then Nt-acetylated by the recently identified **NAA80/NatH** generating the mature actin protein [27–29]. For class II actins (striated and smooth muscle actins) an additional cysteine residue (MCD/E-) complicates the N-terminal processing. In this case, Met1 is cotranslationally removed by methionine aminopeptidase followed by Nt-acetylation of the exposed cysteine, presumably by NatA. Finally, an unknown aminopeptidase removes the acetylated cysteine and the processed acidic N terminus is then reacetylated, most likely by NAA80 [27,28], thereby completing the maturation process.

N-terminal actin maturation gained new attention when it was discovered that the processed N terminus of β-actin (DDD-) can either be acetylated by NAA80 or arginylated by ATE1 [30]. Nt-arginylation of β-actin is found to occur on Asp3 after the protein has undergone sequential removal of both the first and second amino acid (RDD-) (Figure 2A) [30]. This modification profile has not been observed on any other actin isoforms. However, it would be interesting to understand why Asp3 is not further Nt-acetylated, which should be thermodynamically a more favorable reaction over the arginylation step. A recent structural analysis indicates that DD-starting actin forms a poor substrate for NAA80 [29]. Alternatively, subcellular variations in the substrate concentrations, as well as the enzyme amounts and activities, could lead to local competitions. Indeed, a recent study suggests that Nt-arginylated β-actin in mouse embryonic fibroblast (MEF) cells is concentrated at the leading edge of lamellipodia, and is thus mainly linked to active migration [31]. Moreover, non-Nt-arginylated actin forms filamentous

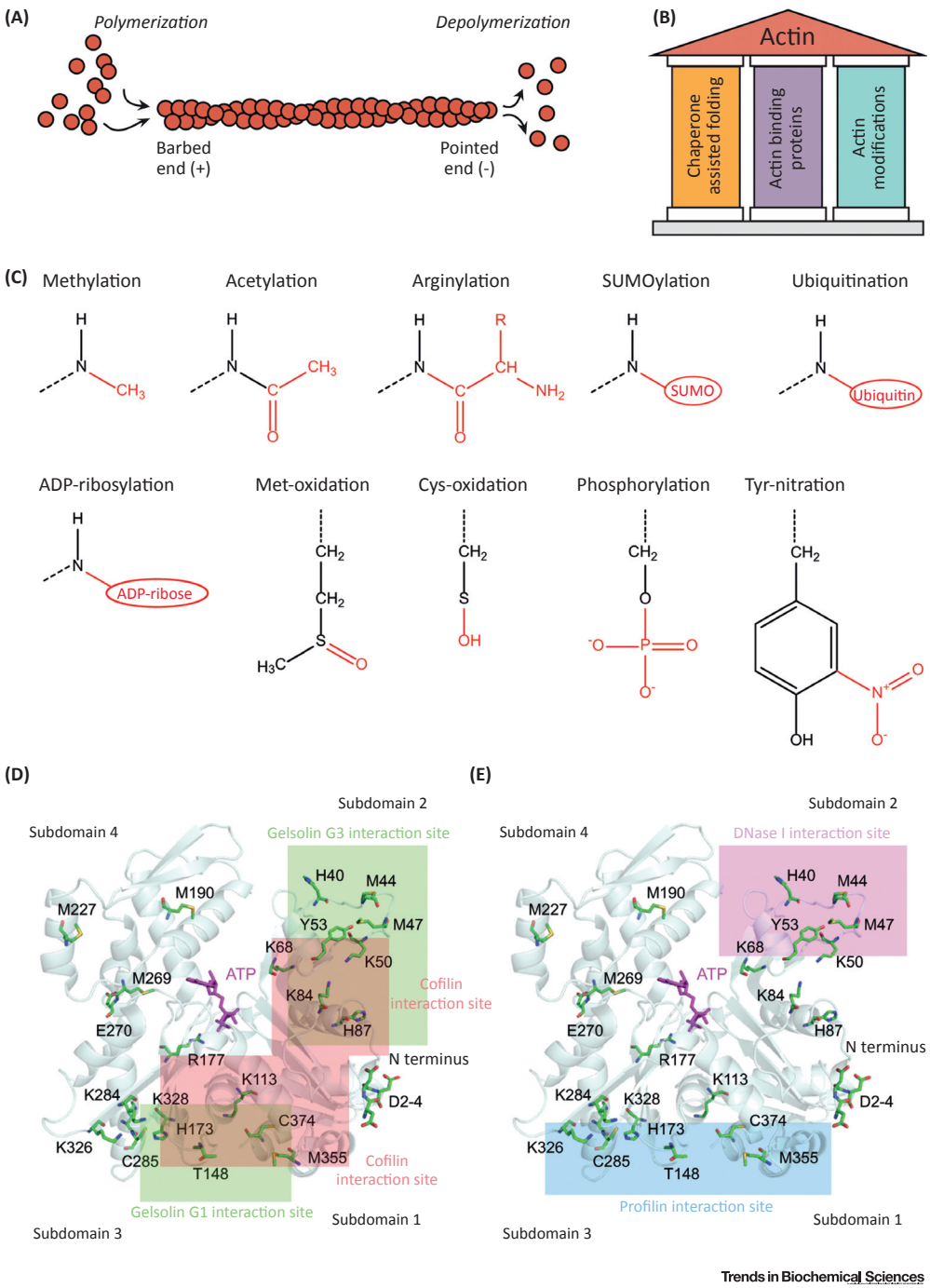


Figure 1. Post-translational Modifications of Actin. (A) Actin can be present as free monomers called G-actin (red circles), or polymerize into microfilaments known as F-actin (red chains). The switch between the two states is highly dynamic and partly regulated by post-translational modifications (PTMs). (B) The three pillars supporting the multifunctionality of actin: chaperone-assisted folding, actin binding proteins, and PTMs. (C) Structural formulae of major actin PTMs. Actin molecules can be post-translationally modified by, for example: methylation, acetylation, arginylation, SUMOylation, ubiquitination, ADP-ribosylation, methionine oxidation, cysteine oxidation, phosphorylation, and tyrosine nitration (attachment shown in red). (D) and (E) β-actin structure (PDB: 2BTF) [85] showing ATP (magenta) and selected amino acid residues (color code for atoms: carbon, green; nitrogen, blue; oxygen, red; cysteines, yellow) carrying PTMs. Interaction interfaces for (D) the gelsolin subunits G1 and G3 (green box) and cofilin (burgundy box), as well as (E) DNase I (pink box) and profilin (blue box) are highlighted, demonstrating that many amino acid residues that are part of these interfaces are subjects of modifications, and thus PTMs can interfere with ABP binding.

Key Table

Table 1. Major Post-translational Modifications of Actin Where Modified Residues Are Numbered According to Class I Actins (β/γ-Actin)

Actin PTM	Modified residues[a]
Acetylation	**Met1**, **Asp2**, **Glu2**, **Cys2**[d], **Asp3**[d], Lys50[b], **Lys52**[d], Lys61, Lys68, **Lys113**[b], Lys191[b], Lys193[d], Lys213[b], Lys315[b], **Lys326**[b], **Lys328**[b]
ADP-ribosylation	Arg28[c], Arg95[c], **Thr148**[b], **Arg177**[b], Arg206[c], Arg372[c]
Arginylation	**Asp3**, Ser52[b], **Ser54**[d], **Ile87**[b,d], Phe90, **Gly152**[d], **Leu295**[d], Asn299[b,d]
Carbonylation	**His40**, **His87**[b], **His173**, Cys374[b]
Crosslinking	**Lys50/Glu270**[b]
Disulfide bond	**Cys285**[b], **Cys374**[b]
Glutathionylation	Cys217[b], **Cys374**[b]
Methylation	Lys18[b], Lys68[b], His73[b], **Lys84**, Ile87[b,d], Asn299[b,d], Lys326[b,c]
Tyrosine nitration	Tyr53[b], Tyr69[b], Tyr91[b], Tyr198[b], Tyr218[b], Tyr240[b], **Tyr294**[b], Tyr362[b]
S-nitrosylation	Cys217[b], **Cys257**[b], Cys285[b], **Cys374**[b]
Oxidation	Cys17, **Met44**, **Met47**, Trp81[d], Met82, Trp88[d], Met178, **Met190**, Cys217[b], **Met227**, Cys257[b], **Met269**, Cys272[b], **Cys285**[b], Met235, Trp342[d], **Met355**, Trp358[d], **Cys374**[b]
Phosphorylation	Ser14, Ser33, Ser52[b], **Tyr53**[b], Ser60, Thr66, Tyr69[b], Thr77, Thr89, Tyr91[b], Tyr143, Thr148[b], S155, Thr160, Thr162, Tyr166, Tyr169, Thr186, Tyr198[b], Ser199[b], Thr201, Ser201[d], Thr202, Thr203, Tyr218[b], Thr229, Ser233, S235, Ser239, Tyr240[b], Thr249, Thr262[d], S265, S271, Tyr294[b], Thr297, S300, Tyr306, Thr318, Ser323, Thr324, Ser324[c] Tyr362[b], Ser365
SUMOylation	Lys61[b], **Lys68**[b], Lys84[b], **Lys113**[b], **Lys284**[b], Lys291[b], Lys315[b], Lys326[b], Lys328[b]
Ubiquitination	Lys18[b], Lys50[b], Lys61[b], Lys68[b], Lys84[b], **Lys113**[b], **Lys118**[c] Lys191[b], Lys213[b], Lys215, Lys238, Lys284[b], Lys291[b], Lys315[b], Lys326[b], Lys328[b], Lys359

[a]Highlighted in **bold**: amino acid modifications described in this review.
[b]Amino acid resides known to be modified by two or more PTMs.
[c]Only described in non-mammalian actins.
[d]Modified residues that are observed in class II actins (α-cardiac, α-smooth, α-skeletal, and γ-smooth) where the N terminus starts with MC-.

aggregates *in vitro*, while ATE1 KO cells show impaired lamella formation and cell migration (Figure 2B) [30]. Acetylation enhances the negative nature of the N terminus, by neutralizing the free α-amino group, while arginylation on the other hand decreases the negative charge density. It is therefore not surprising that both modifications play a role in cytoskeleton morphology and affect actin's polymerization kinetics [27,30]. NAA80 specifically Nt-acetylates β- and γ-actin, and presumably also acetylates the N terminus of class II actins [27–29]. Furthermore, NAA80's activity regulates actin cytoskeleton dynamics and cell morphology by reducing actin filament assembly as well as filopodia and lamellipodia formation, which ultimately decelerates cell migration (Figure 2B) [27,32]. Most of the data on actin's structure reveal large fluctuations in the N terminus (amino acid residues 1–6), indicating disorder. The Nt-acetylation effect observed on actin filament elongation is therefore difficult to explain. The N terminus is also not positioned in close proximity to the monomer–monomer interface, making a direct effect less likely. However, the introduction of conformational changes cannot be excluded. Most likely the effect is induced by contacts with ABPs, since the N terminus of both monomeric and filamentous actin is exposed on the surface where it can interact with a number of regulatory proteins, such as myosin [33], and potentially formins [27,34]. Indeed, early studies on genetically engineered yeast actin demonstrated that the negative nature of actin's N terminus enhances the activation of myosin's ATPase activity [35].

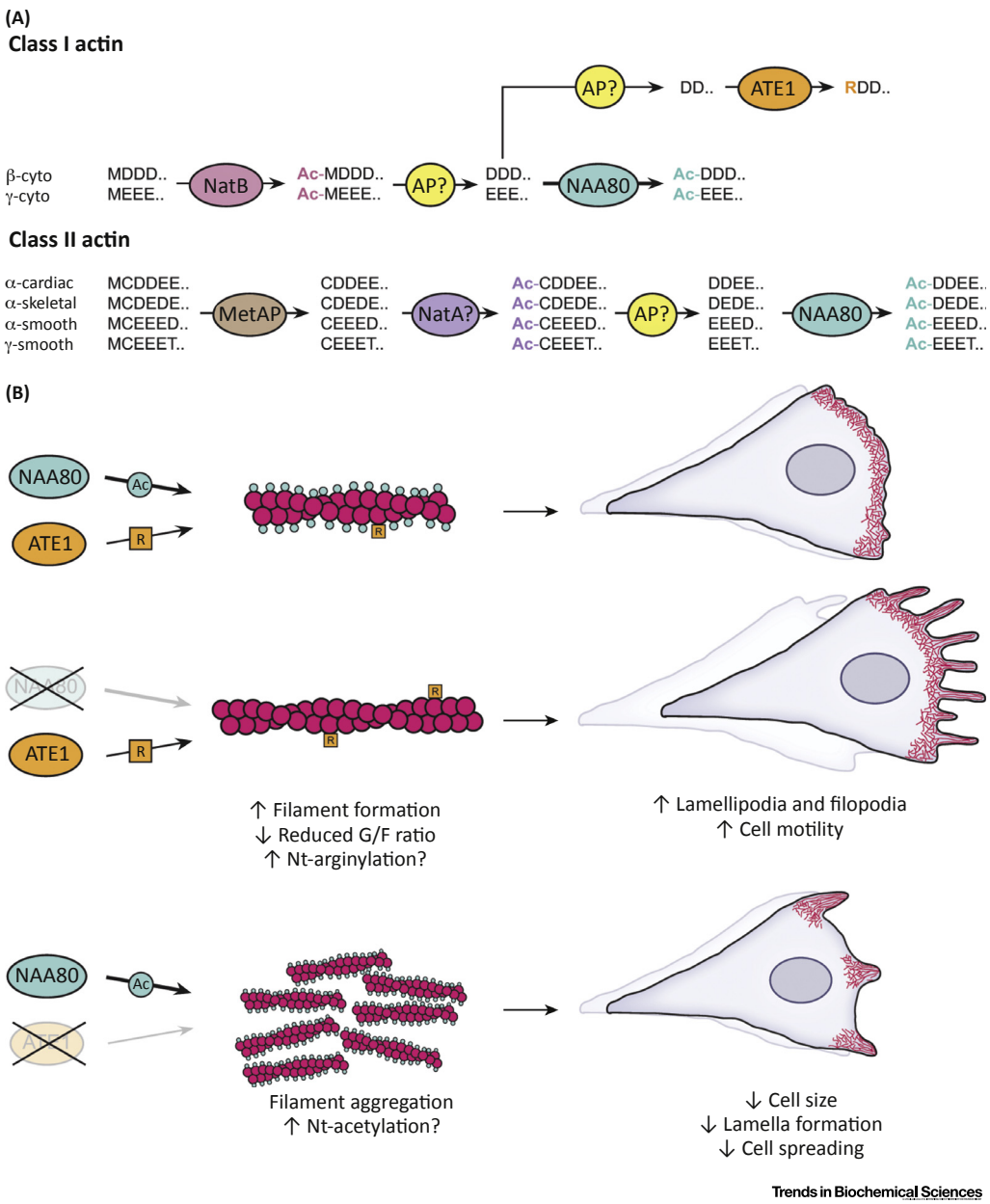


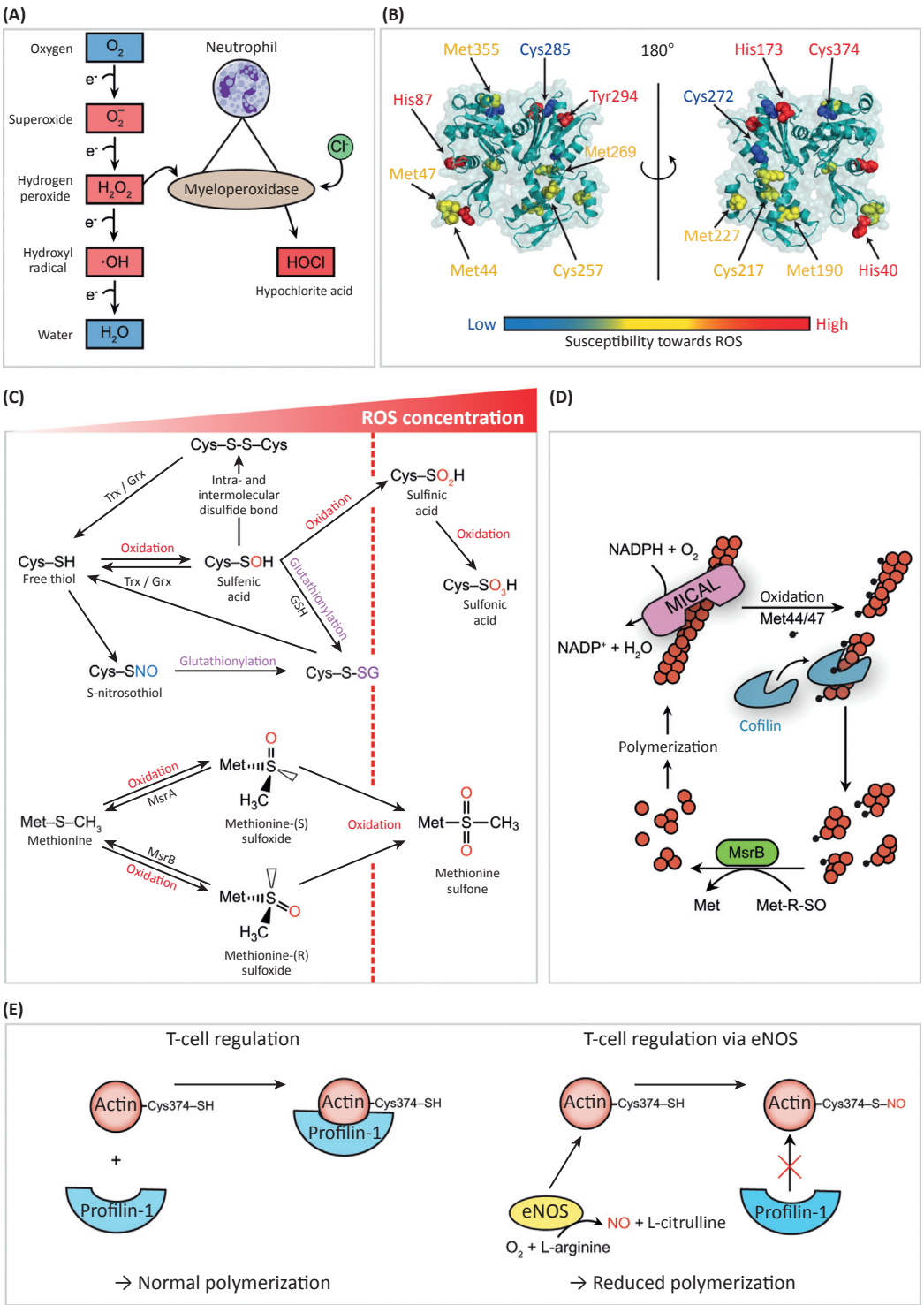
Figure 2. Actin's Unique N-Terminal Maturation Process and Functional Consequences of Nt-Modifications on the Actin Cytoskeleton. (A) Actins are first synthesized as precursor molecules which are rarely detected in their native state owing to a unique N-terminal maturation process. For class I actins (β/γ-actin) the nascent N termini are cotranslationally acetylated by NatB, followed by removal of the acetylated Met1 by a still unidentified aminopeptidase (AP). Finally, the newly exposed acidic N termini (DDD-/EEE-) are acetylated by NAA80/NatH (Ac-DDD-/Ac-EEE-). A few β-actin N termini will not be Nt-acetylated, instead they undergo further proteolytic processing, and the new N termini (DD-) are then Nt-arginylated by arginyl-tRNA protein transferase 1 (ATE1) (RDD-). In the case of class II actins (α-actins and γ-smooth muscle actin), methionine aminopeptidase (MetAP) removes Met1 at the ribosome followed by acetylation of Cys2 presumably by NatA (Ac-CD/E-). Subsequently, the acetylated Cys residue is removed by an unknown aminopeptidase and the resulting acidic N terminus is finally acetylated by NAA80 (Ac-D/E-). (B) Acetylation and arginylation (top) changes the N-terminal charge density and affects actin structure and function. In the absence of NAA80-mediated acetylation of actin's N terminus (middle), actin filament elongation and depolymerization are accelerated. Moreover, NAA80 HAP1 knockout cells show increased lamellipodia and filopodia formation, and compared to control cells have increased cell motility, as shown by scratch wound assay and chemotaxis migration. Consequently, NAA80 acts as a natural brake for cell movement. Nt-arginylation prevents actin from aggregating *in vitro* (bottom). ATE1 knockout mouse embryonic fibroblasts (MEFs) appear smaller than control cells, and fail to form normal lamella, causing impaired cell movement. ATE1 is thought to regulate active migration at the leading edge. Ac, acetyl; R, arginine.

Stressed Actin: A Regulatory Pathway

For a long time, actin modifications caused by oxidative stress were considered to be exclusively destructive. Oxidative stress is caused by reactive oxygen species (**ROS**; Figure 3A), including: hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and reactive nitrogen species (RNS), such as nitric oxide (NO). ROS are byproducts of physiological redox regulation, but are also actively produced by neutrophils of the innate immune system [36]. At low ROS concentrations the thiol groups (SH) of cysteines can be oxidized to sulfenic acid or be glutathionylated (Figure 3B,C). These modifications can be reversed by redox proteins, such as thioredoxin and glutaredoxin. Moreover, the accessible methionine residues can be reversibly oxidized to two methionine sulfoxide diastereomers (Met-S-SO and Met-R-SO; Figure 3C). Met-(S/R)-SO can be reduced in humans by four stereoselective methionine sulfoxide reductases: one MsrA, and three MsrBs (B1, B2, B3) [37]. It should be noted that the oxidation of Met to Met–SO converts its hydrophobic side chain into a hydrophilic moiety. Consequently, the oxidation/reduction process could have a profound effect on actin's structures and ABP-interactions. Oxidative stress further leads to the formation of disulfide bridges or mixed disulfide bonds with glutathione (Figure 3C). The most vulnerable target in actin is Cys374, which can form an intramolecular disulfide bond with Cys285 (Figure 3B), the latter causing delayed dissociation between actin and spectrin [38,39], and reduced actin filament dynamics [40]. However, S-glutathionylation of Cys374 appears crucial for the disassembly of the actomyosin complex, thus promoting contraction of the cytoskeleton during cell spreading and the formation of stress fibers [41]. H_2O_2/HOCl mainly target accessible cysteine and methionine residues in G-actin, (Cys272, Cys285, Cys374, Met44, Met47, Met190, Met227, Met269, and Met355), which are more solvent-exposed than others (Figure 3B), especially when not buried inside actin filaments [42]. When applying high ROS concentrations, cysteine and methionine residues can become irreversibly modified (sulfinic and sulfonic acid, and methionine sulfone). In addition, new modifications occur, such as tyrosine nitration (Tyr294) [43] and histidine carbonylation (His40, His87, and His173) [38,44,45]. These modifications usually impair actin polymerization and destabilize F-actin bundles [40,46]. Especially in the case of severe oxidative stress, actin carbonylation accumulates and leads to aggregation of actin [47]. Consequently, these irreversible modifications inhibit cell proliferation, motility, and reduce cell viability. It is noteworthy that the many studies that identified actin modifications upon ROS treatment were performed *in vitro*, and thus the physiological relevance is not always obvious.

Despite their destructive nature, ROS have in recent years been shown to act as signaling molecules under physiological conditions, and their induced modifications are key regulators in certain cellular pathways. **MICAL**-mediated methionine oxidation was discovered to initiate F-actin depolymerization [48]. The MICAL enzymes belong to the class of flavoprotein monooxygenases, using NADPH and H_2O_2 to stereoselectively oxidize Met44 and Met47 (Met44/47) of actin to Met-R-SO [48–50]. MICALs bind directly to F-actin, enhancing its catalytic activity [48,51]. The oxidation of Met44/47, which depends on the ADP/ATP nucleotide-binding state of F-actin, destabilizes actin filaments and initiates their disassembly. It further causes conformational changes of F-actin, which increases the susceptibility for cofilin (an F-actin depolymerizing factor), and thus accelerates filament disassembly (Figure 3D) [52].

Another signaling molecule, NO, which is enzymatically generated by the endothelial nitric oxide synthase (eNOS), has important functions in T-cell regulation and activation [53]. eNOS colocalizes with F-actin near the Golgi, and modifies Cys374 by S-nitrosylation. This impairs binding to profilin-1, resulting in reduced actin polymerization and relocalization inside the cell (Figure 3E) [54]. These examples demonstrate the importance of actin oxidation as a regulatory factor and not only as a 'killer' modification.



Trends in Biochemical Sciences

Figure 3. ROS-Mediated Actin Modifications and Regulation by MICAL Enzymes. (A) Reactive oxygen species (ROS) are products of redox reactions and are generated when molecular oxygen (O_2) is not completely reduced to water (H_2O), resulting in superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH).

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Physiological Consequences of Actin PTMs

Actin PTMs play a central role in many biological processes, including neurodevelopment. Neurons have an elaborate network of actin filaments, especially in dendritic spines and growth cones. Dynamic phosphorylation of Tyr53 regulates F-actin turnover rates by destabilizing long actin filaments. Moreover, it promotes the stability of shorter actin filaments, which facilitates a faster reorganization of the cytoskeleton in dendritic spine maturation [55], a process crucial for learning and memory formation. Neuronal development also depends on the activity of NADPH oxidase 2 (Nox2), which regulates the distribution of H_2O_2 in neurons [56]. Nox2 colocalizes with F-actin bundles in the periphery of neuronal growth cones where its H_2O_2-producing activity regulates F-actin dynamics and neurite outgrowth [57]. Furthermore, ATE1 was recently shown to be crucial for normal neuronal outgrowth and migration in mice [58]. It was suggested that ATE1's role in brain development arises from cotargeting of ATE1 and β-actin mRNAs to the growth cones, resulting in a local synthesis of arginylated β-actin that regulates neurite outgrowth. Moreover, *ATE1*$^{-/-}$ mice die during embryogenesis, most likely due to defective heart and vascular development [59]. The exact underlying molecular mechanism(s) for the role of arginylation in cell motility [30], embryogenesis [59], and tissue development [58,60] is not completely understood, given that ATE1 has more than one protein target.

Actin PTMs are also essential for effective cytokinesis and proper cell division. The dioxygenase ALKBH4 localizes to the contractile ring where it demethylates K84me1 of actin, thus creating a binding site for nonmuscle myosin II. ALKBH4-deficient cells display defective cleavage furrow organization, resulting in cytokinesis failure and formation of multinucleated cells [61]. After cleavage furrow ingression and midbody formation, actin must be cleared from the abscission site to enable membrane scission by the ESCRT machinery. This is achieved by GTPase Rab35 activation of MICAL1, which is then recruited to the abscission site where it promotes rapid depolymerization of F-actin from both ends, leading to an efficient clearing of F-actin [62]. Cytoskeletal reorganization can be also initiated by the Abl kinase, which phosphorylates the Tyr500 of MICAL1, enhancing its activity [63]. Since the Abl kinase responds to a number of stimuli, such as the semaphorin/plexin complex, or the growth factors EFG and PDGF, MICAL activation and subsequent actin oxidation has a broad spectrum of physiological consequences [64,65].

Actin shuttles between the cytoplasm and nucleus in an ABP-dependent manner. In the nucleus, actin is thought to facilitate chromatin remodeling and gene transcription. MICAL2 induces F-actin depolymerization in the nucleus, enabling newly restored G-actin to act as a transcriptional regulator in serum response factor signaling [66]. Moreover, nuclear actin can be SUMOylated in a process that requires Lys68 and Lys284 [67,68]. It has been speculated that SUMOylation regulates nuclear trafficking and actin structure [67].

Due to actin's abundance and role in muscle cells, it is not surprising that actin PTMs participate in the contractile machinery by modulating the electrostatic interactions between F-actin, tropomyosin, and myosin. For example, acetylation of Lys326 and Lys328 masks positive charges that are crucial for proper thin filament regulation [69,70]. Expression of pseudo-acetylated cardiac actin

Hypochlorite acid is actively produced by the enzyme myeloperoxidase in neutrophils of the innate immune system as defense mechanisms towards invading pathogens. (B) β-actin structure (PDB: 2BTF) [85] highlighting surface exposed Cys, Met, His, and Tyr residues that are susceptible towards ROS-mediated modifications. (C) ROS target mainly Cys and Met residues of actin in a concentration-dependent manner, resulting in a variety of PTMs (disulfide bond formation, nitrosylation, glutathionylation, multilevel cysteine oxidation, and stereoselective methionine oxidation). Most of these PTMs are reversed by redox enzymes (Trx, thioredoxin; Grx, glutaredoxin; MsrA, methionine sulfoxide reductase A; MsrB, methionine sulfoxide reductase B). High ROS concentrations lead to irreversible modifications (sulfinic and sulfonic Cys oxidation, Met sulfone). (D) MICAL enzymes bind to F-actin and catalyze in an NADPH-dependent reaction the oxidation of Met44 and Met47 to Met-R-SO, initiating depolymerization. In addition, this attracts the F-actin severing protein cofilin, thereby accelerating the depolymerization effect. Met-R-SO can be reduced to Met by MsrB, which allows actin to enter a new polymerization cycle. (E) Nitric oxide (NO) is involved in T-cell regulation and activation. eNOS S-nitrosylates Cys374 of actin, impairing profilin-1 binding, and thus reducing actin polymerization rates.

(K326Q, K328Q, and K326Q/K328Q) in indirect flight muscles of *Drosophila melanogaster* leads to perturbed muscle structure and function as well as disrupting flight performance [71]. Masking of Lys326 and Lys328 is thought to alter the electrostatic interactions with tropomyosin (Glu181) and/or myosin (Glu286), destabilize the inhibitory positioning of tropomyosin, and thereby enhance actomyosin formation causing muscle hypercontractility [71]. Actin acetylation might therefore be crucial for proper muscle function. Indeed, the K328Q actin mutation causes nemaline myopathy with muscle stiffness and hypertonia [72].

The Role of Actin PTMs in Diseases

The actin cytoskeleton has an indisputable role in human development and failure to orchestrate the dynamic interplay between actin and ABPs could lead to actin-related diseases, a concept which was further elaborated by Rubenstein and Wen (Box 1) [73]. They described a regulatory allosteric system in human actins that appear prone to disease-causing mutations. Some of the most effective mutations colocalize with a PTM hotspot in an otherwise poorly modified region. We therefore suggest similar molecular pathophysiology upon dysfunctional actin modifications, especially with regard to ABP interactions (Box 1).

Abnormal cell invasion and metastasis is a hallmark of cancer, two processes in which the actin cytoskeleton plays a dominant role. Therefore, it is not surprising that actin PTMs have been linked to cancer development and tumorigenesis. Both NAA80 and ATE1 KO cells display defective cell motility [27,30], which is a common feature among cancer cells and contributes to invasion and metastasis. Reduced ATE1 expression has been reported in various human cancers [74]. Moreover, ATE1 KO MEFs exhibit defective contact inhibition which is thought to support the uncontrolled growth in dense cultures and invasive behavior in Matrigels [74]. The direct tumorigenic potential of NAA80-deficient cells has yet to be investigated. Nevertheless, several somatic mutations in *NAA80* and *ATE1* are reported in the COSMIC cancer database (v86, released 14 Aug 2018) [75]. For example, the mutation profile of *NAA80* in human cancers includes 45 missense mutations and two frameshift mutations that, as far as we know, have not been characterized. The frameshift deletion mutation p.E92fs*5 (resulting in 92 out of 308 amino acid residues) should, in theory, give rise to a catalytic-inactive form of NAA80. Furthermore, several of the residues that are affected by missense mutations (W105R, R107H, R112H, F123S, G190D, L194Q, P258A, P266L, P267S, P283L, G298W, and I308M) are evolutionarily conserved, implying that these residues may be important for NAA80's structure and function [29]. It is currently not known whether any of these *NAA80* mutations have disease-causing effect(s). But one could speculate that some of them affect NAA80's activity and actin Nt-acetylation, thus altering cytoskeleton dynamics and promoting tumor progression.

The emerging role of the MICAL enzyme family in F-actin disassembly, a key element of cell motility and migration, has placed the MICALs at the new horizon of cancer research. MICAL1 expression was directly linked to increased cell migration and invasiveness in various melanoma and breast cancer models [76–78]. ROS production by MICAL1, which promotes epithelial–mesenchymal transition (EMT), and thus metastasis formation, was linked to typical EMT-dependent signaling cascades, such as semaphorin/plexin [76] and Rab35 signaling, as well as the PI3K/AKT pathway [77] and the EGF-induced MAPK/ERK pathway [78]. Regulation of EMT was also linked to MICAL2 expression. Gastric and renal epithelial cancer cells show an increase in EMT upon MICAL2 expression, and a reduced viability, motility, and invasiveness when MICAL2 is depleted from these cells [79].

Actin PTMs also play exceptional roles in the development of infectious diseases. Several bacterial pathogens release toxins that induce ADP-ribosylation and crosslinking, of actin,

Box 1. Pathogenic Actin Allosteric Regulatory System: A New Concept

Bartlett, Rubenstein, and their colleagues, hypothesized the existence of a pathogenic actin allosteric regulatory system where the binding of ABPs initiates conformational changes in structural networks, affecting actin filament formation and stability [73,86,87]. Central to the hypothesis is actin's pathogenic helix (Lys113–Thr126), which extends from the filament surface to the strand–strand interphase, and the C-terminal helix (Val370–Phe375; Figure I). The two helixes are interconnected via interactions between Glu117 and His371. Moreover, Lys113 in the pathogenic helix extends towards the actin–actin interface where it forms an ionic bridge with Glu195 of an actin subunit in the opposing strand. This filament-stabilizing interaction is modulated by Arg256 on the cross-strand monomer, giving rise to a triangular unit. Together, these interacting structural elements are thought to constitute an allosteric system where surface binding of ABPs may induce conformational changes that propagate throughout the actin molecule and affect filament dynamics. The pathogenic helix is a mutational hotspot and implicated in several actinopathies, including nemaline myopathy, Baraitser–Winter syndrome, and deafness (reviewed in [73]). For example, two missense mutations in γ-actin (K118M and K118N) can give rise to nonsyndromic deafness. A study using yeast actin revealed that both mutations affect the structure and function of the DNase I binding loop, and in the case of K118N resulted in faster filament formation [88]. The K113E mutation in α-actin is associated with nemaline myopathy [89] and was recently reported to suppress actin catch-slip bonds [90]. In yeast, expression of K113E actin leads to growth defects and defective actin polymerization [91]. Within the broad repertoire of actin PTMs we notice that both Lys113 and Lys118, which are members of the pathogenic helix, are located within a region that is highly PTM silent. Residues 96–142 do not carry PTMs, except for Lys113 which can undergo acetylation, SUMOylation, or ubiquitination. We assume that unintended Lys113 modifications could induce effects similar to the different phenotypes described for the mutations at this site. Another example is Cys257, which is highly susceptible to ROS-induced modifications [3]. Missense mutations in the neighboring residue Arg256 are implicated in several diseases [73]. Interestingly, different mutations of Arg256 cause various symptoms where R256C and R256H are associated with TAAD aneurysms, while only the R256C mutation causes cerebral aneurysms. Finally, we note that several residues within the C-terminal helix are modified, including Cys374 which is highly reactive. Together, these effects emphasize the functional importance of this region for proper actin function.

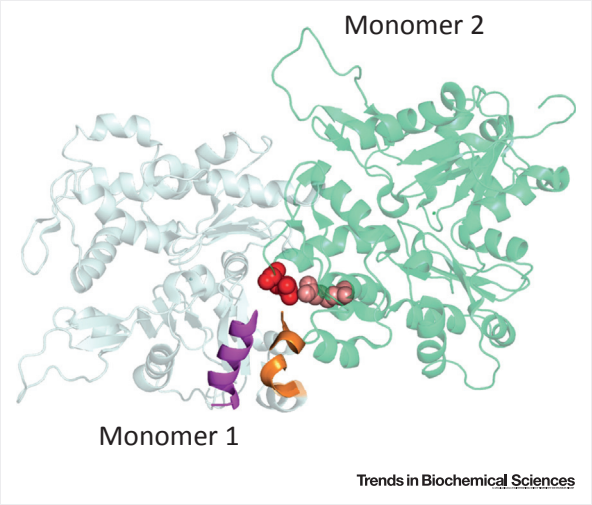

Figure I. Actin's Pathogenic Helix. Shown is a model structure of two α-skeletal actin monomers (PDB: 2ZWH) [92]. The interaction between two protomers in an actin filament is dependent on the pathogenic helix (magenta, Lys113–Thr126) and the C-terminal helix (orange, Ala365–Phe375) of monomer 1 (cyan), and the residues Glu195 (red spheres) and Arg256 (pink spheres) of monomer 2 (green).

which interfere with the host cells' ability to polymerize actin (Box 2). Although other toxic effectors are secreted by pathogens into the host cell, the actin-modifying toxins take a key role in altering the host cell cytoskeleton to the advantage of the pathogen (Box 2).

Competition for Actin's N Terminus: Nt-Acetylation versus Nt-Arginylation

Nt-arginylation was only reported for β-, but not γ-actin, in which the DDD-actin N-terminal sequence was converted into an RDD-actin sequence, turning a −3 charged N terminus

Box 2. Bacterial Pathogens Cause 'Toxic' Actin Modifications

Many human pathogens (bacteria, parasites, etc.) deploy their pathogenic properties by secreting toxins into host cells via various delivery systems. These bacterial effectors are usually multiprotein complexes and exert their toxicity by targeting vital host complexes including signaling pathways, ribosomes, membrane trafficking, and the actin cytoskeleton (reviewed in [93]). A typical way how toxins affect actin is by modifying certain residues to change host cell behavior in favor of the pathogen, or even causing cell death. In particular, ADP-ribosylation (Figure I) is a toxin-linked actin modification, which is catalyzed by enzymes called ADP-ribosyltransferases (ART). ADP-ribosylation of G-actin at Arg177 by the *Clostridium botulinum* C2 toxin inhibits actin polymerization, ATP binding, and ATPase activity [94,95]. This toxin seems to prefer cytosolic β/γ-actin, however α-actin is also a potential target [93,96]. In contrast, ADP-ribosylation at Thr148 by the *Photorhabdus luminescens* toxin (TccC3) leads to polymerization of long, curled filaments, and consequently to actin aggregation. It further impairs the binding of actin severing proteins, such as gelsolin and ADF/cofilin, and reduces ATPase activity [97]. Thus, actin ADP-ribosylation inhibits various cellular functions, such as phagocytosis by the innate immune system, the first defense line against evading pathogens [98]. Another mechanism of pathogenic actin effectors is intermolecular crosslinking of actin molecules and thus impairment of their function. One of these toxins is the actin crosslinking domain (ACD) produced among others by the *Vibrio* species (known representative *Vibrio cholerae*), which crosslinks actin molecules at Lys50 and Glu270 generating nonfunctional actin oligomers. The toxicity of these actin oligomers is partially due to their ability to hijack formins [99] and various actin assembly factors [100], resulting in decreased nucleation and elongation abilities of these ABPs, and thus decreased actin polymerization in cells. The *Legionella pneumophila* effector protein RavK deploys a different strategy to disrupt the actin cytoskeleton. It does not add a new chemical group to one of the actin residues; instead it cleaves actin between the amino residues Thr351 and Phe352 thereby inhibiting actin polymerization and modulating the host's actin cytoskeleton [101].

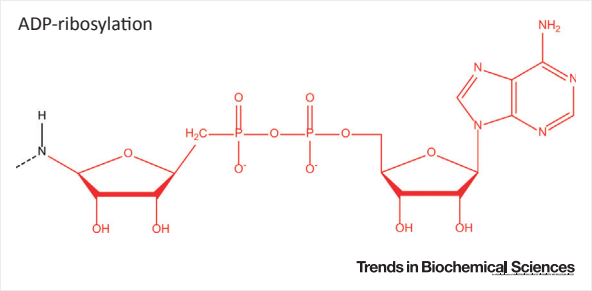

Figure I. Chemical Identity of ADP-Ribose. ADP-ribose (highlighted in red) is mainly known to be attached to actin by bacterial toxins in a reaction called ADP-ribosylation. The toxins target arginine and threonine residues, and therby impair actin functionality.

into an uncharged entity [30]. The absence of a similar modification of γ-actin (starting with EEE-) was later attributed to fast degradation of the modified isoform via the ubiquitination pathway, owing to slower translation rates thereby exposing a crucial lysine residue [80]. This finding caused high interest in the field because it was stated that approximately 40% of intracellular β-actin could be Nt-arginylated in MEFs [30], while no such modification was ever reported in β-actin purified by DNase I affinity chromatography from lysates of non-muscle cells or from various tissues. Later reports of the same group confirmed the existence of Nt-arginylation of β-actin, though at much lower levels [31]. Additional arginylation sites were later detected following high resolution mass analysis on total cellular lysates [81]. Interestingly these were not only limited to N-terminal residues, but also happened at midchain side chains [82]. The assumed enzymatic mechanisms, in which each time the participation of the same players: arginyl-tRNA and the corresponding transferase ATE1, was noticed, should be completely different. While the reaction at the N terminus follows the same mechanism of the ribosomal peptidyl transferase reaction forming a peptide bond, the proposed mechanism for midchain arginylation is thought to pass via the formation of an intermediate carboxylic anhydride which is then attacked by the α-amino group of arginine. The latter mechanism has at least two difficult points: firstly, the

same enzyme ATE1 should catalyze two completely different mechanisms, and secondly, the supposed formation of the intermediate anhydride needs much more energy than can be delivered by the cleavage of the aminoacyl bond in the arginyl-tRNA [82]. Additional elegant MS techniques revealed prominent arginylation of α-cardiac actin during heart development [82,83]. Four sites were identified: Ser54, Ile87, Gly152, and Leu295. Although these modifications might be substoichiometric, where only a few actin molecules are affected, in the particular case of polymerizing actin they could have a profound effect on the cellular organization of the myofibrillar/microfilament system, with functional implications. Indeed, in the current models of actin polymerization and branching, one modified actin monomer among a thousand intact actins could affect the final picture, by functioning either as F-actin capping entity or as initiation site for filament branching [30,84].

Concluding Remarks

Actin is a remarkable protein, both in terms of its evolutionary conservation and multifunctionality. A combination of factors is responsible for its extraordinary biological capacity. While ABPs have received considerable attention for regulating the actin cytoskeleton, numerous PTMs have lately entered the spotlight as important influencers for proper actin function, including acetylation, arginylation, and oxidation. Although new details about the impact of PTMs on actin became available in recent years, many questions remain. By uncovering NAA80 as actin's N-terminal acetyltransferase the field has taken a crucial step towards deciphering the complete maturation pathway of mammalian actins. Still, the identity of the N-terminal aminopeptidase that specifically acts on actin remains unknown. Another intriguing concept is the functionality of the different actin isoforms. To what extent do their roles overlap, and when, where, and how do they undertake their unique tasks? Related to this, how exclusive is the N terminus of actin? Are the actions of the actin modifiers NAA80 and ATE1 mutually exclusive or do they have tissue specific roles (see Outstanding Questions)? A potential synergy between a plethora of actin PTMs could possibly revolutionize our understanding of actin cytoskeleton dynamics. One of the major challenges within actin research is to differentiate between the different actin isoforms. This hurdle has become increasingly apparent as PTMs might affect actin isoforms differently. Given actin's pathophysiological role, mapping actin's modification repertoire and defining the actin processing machinery and ABP interactome will be of great basic and translational importance.

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Disclaimer Statement

The authors declare no competing financial interests.

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Outstanding Questions

Which enzyme catalyzes the removal of the Nt-acetylated initiator methionine in class I actins and/or the Nt-acetylated cysteine in class II actins? This unknown N-acetyl aminopeptidase is the last missing link to the N-terminal processing of actin. Why does actin have such a complex, and from an evolutionary point of view late developed maturation process? Is it a quality control mechanism, as most mature actins molecules exist in the Nt-acetylated form? But how, when, and where does the aminopeptidase, NAA80 and ATE1 interact and perform their activity in the cell?

Is Nt-arginylation of actin a general mechanism of cytoskeletal regulation? Does it occur in all cells and tissues, or is it specific to distinct subtypes of cells or cellular conditions? How does the cell decide if β-actin should be Nt-arginylated or not?

The importance of MICAL-catalyzed actin oxidation is thought to be clear: Met44/47 of actin are conserved from invertebrates to humans; MICALs are expressed in all organs and tissues; and several MICAL knockdown studies have demonstrated its crucial role in immune cells, skeletal muscle function, and metastasizing cancer cells. But why are there different MICAL isoforms (three main isoforms in humans)? Is it specific regulation or localization? Does MICAL activity play a role in muscle cell contraction?

Which of the different actin PTMs occur simultaneously? How do these PTMs interact with each other? Can they occur in parallel, resulting in a fine regulation of actin function, including differential ABP binding? Or are they mutually exclusive, as is the case for Nt-acetylation and Nt-arginylation? Middle-down proteomics, using endoproteinases, such as GluC (instead of trypsin) which generate larger polypeptides (5–7 kDa) for MS analysis, have emerged as a new avenue for identifying coexisting PTMs, and can be used to study actin at different cellular stages and conditions.

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