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REVIEW

Lysine acetylation of cytoskeletal proteins: Emergence of an actin code

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Reversible lysine acetylation of nuclear proteins such as histones is a long-established important regulatory mechanism for chromatin remodeling and transcription. In the cytoplasm, acetylation of a number of cytoskeletal proteins, including tubulin, cortactin, and the formin mDia2, regulates both cytoskeletal assembly and stability. More recently, acetylation of actin itself was revealed to regulate cytoplasmic actin polymerization through the formin INF2, with downstream effects on ER-to-mitochondrial calcium transfer, mitochondrial fission, and vesicle transport. This finding raises the possibility that actin acetylation, along with other post-translational modifications to actin, might constitute an "actin code," similar to the "histone code" or "tubulin code," controlling functional shifts to these central cellular proteins. Given the multiple roles of actin in nuclear functions, its modifications might also have important roles in gene expression.

Introduction

Covalent posttranslational modifications (PTMs) of proteins are integral to cellular regulation. While some of these modifications are essentially irreversible, reversible PTMs can rapidly regulate cellular functions in response to stimuli. Phosphorylation is easily the most well-studied reversible PTM. However, a growing number of other reversible PTMs have been described, including acetylation, other types of acylation (malonoylation, succinylation, butyrylation, crotonylation, and palmitoylation), methylation, ubiquitination, and SUMOylation (Ringel et al., 2018; Sabari et al., 2017; Barnes et al., 2019).

Protein acetylation occurs on primary amines and can be divided into two types: N-terminal (Nt) acetylation and lysine acetylation (Drazic et al., 2016). For both types, acetyl–coenzyme A (CoA) is the acetyl donor (Fig. 1 A). Nt-acetylation occurs on over 80% of human proteins and is typically considered irreversible. Much of this acetylation is mediated by a family of N-acetyltransferases (NATs) and is largely cotranslational in the cytosol, but can occur post-translationally.

Lysine acetylation, which is the focus of this review, is widespread in cells (Narita et al., 2019). As an energetically favorable reaction, acetylation can occur nonenzymatically, and clearly does so in the mitochondrial matrix, where the high acetyl-CoA level and elevated pH are favorable (Wagner and Payne, 2013). However, a great deal of regulated lysine acetylation takes place in other compartments through enzymes known as lysine acetyltransferases (KATs), while deacetylation is mediated through lysine deacetylases (KDACs; Narita et al., 2019; Fig. 1 A). It should be kept in mind that acetylation is only one form of acylation, and there is rapidly expanding knowledge of other acylations, which are competitive with acetylation, on histones and other proteins (Narita et al., 2019; Barnes et al., 2019; Ringel et al., 2018).

Lysine acetylation has three clearly identifiable biochemical effects (Fig. 1 A). First, acetylation neutralizes the positively charged lysine residue, which can induce changes in enzymatic activity, interactions with other proteins, and/or conformation within the protein. Second, acetylation blocks other lysine modifications, such as methylation or ubiquitination. Given that acetylation and methylation produce different chemical effects on lysines (eliminating versus preserving positive charge, respectively), these two modifications can have opposing functional effects (Rice and Allis, 2001). Third, acetylation provides a binding site for bromodomain-containing proteins (Fujisawa and Filippakopoulos, 2017).

Acetylation occurs in several cellular compartments, but the most well-studied events are nuclear. Histone acetylation was discovered >50 yr ago (Allfrey et al., 1964; Verdin and Ott, 2015), and there is an extensive literature of acetylation effects on chromatin structure. Many of the nonhistone acetylation sites identified by proteomics are on transcription factors or other nuclear proteins (Narita et al., 2019). This situation is not surprising, considering that the majority of KATs and KDACs are heavily enriched in the nucleus, and that bromodomain-containing

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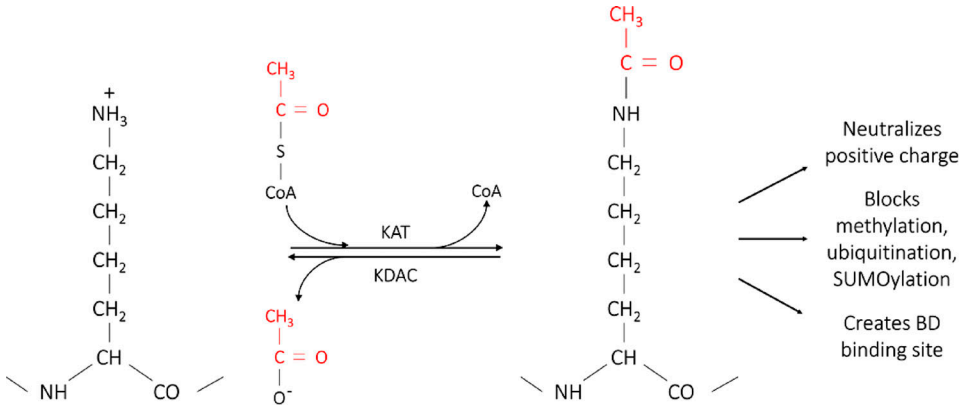

Figure 1. **Lysine acetylation.** Enzymatic lysine acetylation occurs through KATs, with the acetyl group donated by acetyl-CoA. KDACs remove the modification. Acetylation has three general effects: neutralizing lysine's positive charge, blocking other modifications to the lysine, and creating a binding site for bromodomain-containing proteins. BD, bromodomain.

proteins appear to be almost entirely nuclear (Fujisawa and Filippakopoulos, 2017).

Extensive lysine acetylation occurs in the mitochondrial matrix; however, this acetylation appears to be mainly nonenzymatic (Wagner and Payne, 2013; Wagner and Hirschey, 2014; Weinert et al., 2015; Davies et al., 2016; James et al., 2017). While enzymatic acetylation has been reported in mitochondria, there is no currently identified mitochondrial KAT (Scott et al., 2012; Fan et al., 2014). Therefore, the roles of the three mitochondrial KDACs might be to reverse detrimental nonenzymatic lysine acetylation.

This review focuses on lysine acetylation in the cytoplasm, specifically on the actin and microtubule cytoskeletons. For a recent review of noncytoskeletal lysine acetylation in the cytoplasm, please see Narita et al., 2019. While comparatively less studied than the other compartments, examples of functionally relevant cytoplasmic lysine acetylation are increasingly frequent. In addition, the function of actin in both the cytoplasm and the nucleus (Plessner and Grosse, 2019; Percipalle and Vartiainen, 2019), as well as the acetylation-based regulation of an actin polymerization factor (INF2) that is now known to polymerize actin in both compartments (A et al., 2019a; Wang et al., 2019), make actin acetylation an intriguing mechanism for nuclear/cytoplasmic communication.

Cytosolic KATs and KDACs

The identity of KATs and KDACs in the cytoplasm remains an issue that is not fully resolved. Thus far, at least 21 human KATs have been identified, and a number of other proteins might possess KAT activity (Narita et al., 2019; Table S1). The 20 currently identified human KDACs fall into two broad groups: the NAD^+-dependent sirtuins and the zinc-dependent KDACs (Table S2). It should be noted that several KATs and KDACs, while having homology to their respective groups, have no apparent enzymatic activity or are more suited to addition/removal of other acyl groups (Narita et al., 2019).

While most KATs and KDACs are considered to be exclusively nuclear, for some there is evidence for a cytoplasmic pool, though there are conflicts between studies. Possible explanations for the conflicts could include cell type–specific localization, cell state–specific localization, and use of overexpressed constructs that may or may not mimic endogenous localization. We have summarized our interpretation of the literature, classifying KATs and KDACs as "yes," "probable," "possible," or "no" for the presence of at least a portion of the protein in the cytosol (Table S1 and Table S2). Some of the firmer conclusions are as follows. For KATs, some of the major nuclear enzymes display at least a subpopulation in the cytosol, including p300, CBP, PCAF, and Tip60. In addition, α-tubulin acetyltransferase (αTAT1) is cytoplasmic and is thought to be dedicated to tubulin (Shida et al., 2010; Akella et al., 2010), although extensive testing for other substrates has not been performed. KDACs that clearly have both a cytosolic population and bona fide KDAC activity are HDAC6 and Sirt2. In addition, there is some evidence that HDAC6 and Sirt2 can interact directly (North et al., 2003; Nahhas et al., 2007).

Cytoskeletal targets of lysine acetylation

An increasing number of cytosolic proteins have been shown to be regulated by acetylation, including chaperones such as HSP90, autophagy factors, and proteins involved in signal transduction (Narita et al., 2019). For cytoskeletal proteins, tubulin acetylation is by far the best characterized (Janke and Magiera, 2020), but actin itself as well as two actin-binding proteins have been shown to be functionally acetylated (Zhang et al., 2007; Li et al., 2017; A et al., 2019a).

Acetylation and the tubulin cytoskeleton

The fundamental unit of eukaryotic microtubules is the heterodimer of α- and β-tubulin, which polymerize end to end to form an unstable linear protofilament. Protofilaments then assemble laterally into a more stable microtubule, typically consisting of 13 protofilaments with a diameter of ∼25 nm (Brouhard and Rice, 2018; Manka and Moores, 2018). In all microtubules, protofilaments are in the same orientation, with β-tubulin at the "plus" end and α-tubulin at the "minus" end (Fig. 2 A). Both tubulins bind GTP, but only β-tubulin is capable of GTP hydrolysis and exchange. GTP hydrolysis changes the protofilament

conformation, resulting in instability and rapid disassembly at the plus end in a process called catastrophe (Brouhard and Rice, 2018; Manka and Moores, 2018). Microtubules are nucleated by the γ-TURC complex, which is enriched at centrosomes but is also found at additional locations (Petry and Vale, 2015). While many microtubules are highly dynamic, alternating between growth and catastrophe (Brouhard and Rice, 2018; Manka and Moores, 2018), some are relatively stable, for example, ciliary microtubules as well as microtubule subsets in neuronal axons and dendrites (Baas and Black, 1990; Baas et al., 2016) and many cultured mammalian cells (Webster et al., 1987; Gundersen and Bulinski, 1988).

Tubulin acetylation. The best-studied tubulin lysine acetylation event is of K40 on α-tubulin, first identified in *Chlamydomonas reinhardtii* (L'Hernault and Rosenbaum, 1985; Piperno et al., 1987), where most acetylation is in cilia but a subset of cytoplasmic microtubules is also acetylated (LeDizet and Piperno, 1986). Most cultured mammalian cells have a similar subset of cytoplasmic Ac-K40–containing microtubules (Piperno et al., 1987), and acetylated tubulins are enriched in the long microtubules of mammalian neuronal axons and dendrites (Hammond et al., 2010). Two interesting features of the K40 acetylation site are that 1) it is not universally conserved in eukaryotes, since yeasts lack the site, and 2) the residue is not on all α-tubulin genes within a species; for example, K40 is present in seven of the eight human α-tubulins. Other acetylation sites have been identified on α- and β-tubulin (Chu et al., 2011; Sadoul and Khochbin, 2016), but several lines of evidence suggest that K40 appears to be the manor site (LeDizet and Piperno, 1987; Akella et al., 2010; Shida et al., 2010).

The KAT catalyzing tubulin acetylation is αTAT1 (also called MEC-17), and displays a strong preference for microtubules over tubulin dimers (Akella et al., 2010; Shida et al., 2010). Plausible αTAT1 sequences are found in all ciliated organisms but not in yeasts (Shida et al., 2010). While *Chlamydomonas* expresses an αTAT, plants do not have a clear homologue, despite evidence for K40 acetylation (Aström, 1992; Smertenko et al., 1997). Some acetylated tubulin is still detectable in certain tissues from αTAT1 knockout (KO) mice (Kim et al., 2013) and αTAT1 KO zebrafish (Akella et al., 2010), perhaps through a different KAT, ESCO2 (Lu et al., 2018). For KDACs, two enzymes have been identified in mammals, HDAC6 and Sirt2 (Hubbert et al., 2002; North et al., 2003), but the contexts in which one or the other is dominant have not been fully elucidated. HDAC6 and Sirt2 might interact (North et al., 2003), and the SIRT2-HDAC6 complex might be the entity that binds microtubules (Nahhas et al., 2007).

K40 faces the microtubule lumen, as opposed to tubulin C-terminal tail PTMs, which are on the tubule exterior (Fig. 2 B). This location raises the issue of access for both αTAT1 and KDACs. For αTAT1, entry from either microtubule end has been shown (Szyk et al., 2014). An alternative mechanism is through "holes" along the microtubule created by mechanical stress or microtubule severing (Schaedel et al., 2015; Vemu et al., 2018), although whether KATs or KDACs could access these holes before they are rapidly repaired is unclear.

The biochemical and cellular effects of K40 acetylation in the microtubule lumen are still being elucidated. Stable microtubules tend to be more acetylated in several cellular contexts, including (1) ciliary microtubules, with HDAC6 required for ciliary resorption (Pugacheva et al., 2007); (2) cultured cells (LeDizet and Piperno, 1986); and (3) neuronal axons and dendrites (Tas et al., 2017). Some biochemical results are at odds with these cellular results, since acetylated microtubules depolymerize faster and make weaker inter-protofilament interactions (Portran et al., 2017; Eshun-Wilson et al., 2019). Interestingly, these altered interactions also make acetylated microtubles more flexible, and more able to resist mechanical breakage (Portran et al., 2017). In cells, this property may make acetylated microtubules more resistant to stresses (Xu et al., 2017), which damage microtubules (Schaedel et al., 2015). However, it is unclear whether the association between microtubule acetylation and cellular microtubule stability is causative or fortuitous (the more stable microtubules having a greater opportunity to get acetylated). A second reported consequence of K40 tubulin acetylation is preferential kinesin-1 based transport in cells (Reed et al., 2006; Cai et al., 2009; Tas et al., 2017). However, biochemical assays could not recapitulate a similar kinesin-1 preference for acetylated microtubules (Walter et al., 2012; Kaul et al., 2014). It may be that cellular microtubules are also subject to other modifications that favor kinesin-1 interaction, or that the biochemical assays lack a cofactor, such as a microtubule-associated protein, that facilitates kinesin-1 recognition of acetylated microtubules. A third consequence of K40 tubulin acetylation may be to provide direct binding sites for mitochondrial inner proteins (Ichikawa and Bui, 2018), which reside in the mitochondrial lumen, as suggested by recent cryo-EM data (Ma et al., 2019). The microtubule lumen is a relatively unexplored world, but with an almost 20-nm diameter (approaching that of an ER tubule), it has the potential to act as a unique microenvironment. Indeed, actin filaments have been observed in the microtubule lumen (Paul et al., 2020).

Physiologically, tubulin acetylation is not essential for life, since KOs of αTAT1 in mice and worms, or the K40R mutant of the sole acetylatable α-tubulin in worms, result in almost complete loss of detectable acetylated tubulin yet lead to no major developmental or structural abnormalities (Akella et al., 2010; Shida et al., 2010; Kalebic et al., 2013a; Kim et al., 2013). In addition, *Tetrahymena* mutants for αTAT1 still produce cilia (Akella et al., 2010), as do mammalian cultured cells (Shida et al., 2010). However, lack of tubulin acetylation causes a number of clear specific effects, including loss of touch sensation in *Caenorhabditis elegans* and mice (Akella et al., 2010; Shida et al., 2010; Morley et al., 2016), dentate gyrus abnormalities in mice (Kim et al., 2013), and sperm structural and motility defects in mice (Kim et al., 2013; Kalebic et al., 2013b). Interestingly, αTAT1 is necessary for assembly of special microtubules containing 15 protofilaments in *C. elegans* touch receptor neurons (Cueva et al., 2012; Topalidou et al., 2012). One imagines that additional specific defects will be revealed in time.

Acetylation and the actin cytoskeleton

Actin is a 43-kD monomer that can assemble into a two-stranded right-handed helical polymer in eukaryotes (Fig. 2, D–F; Pollard et al., 2001; Blanchoin et al., 2014). Actin filaments have a

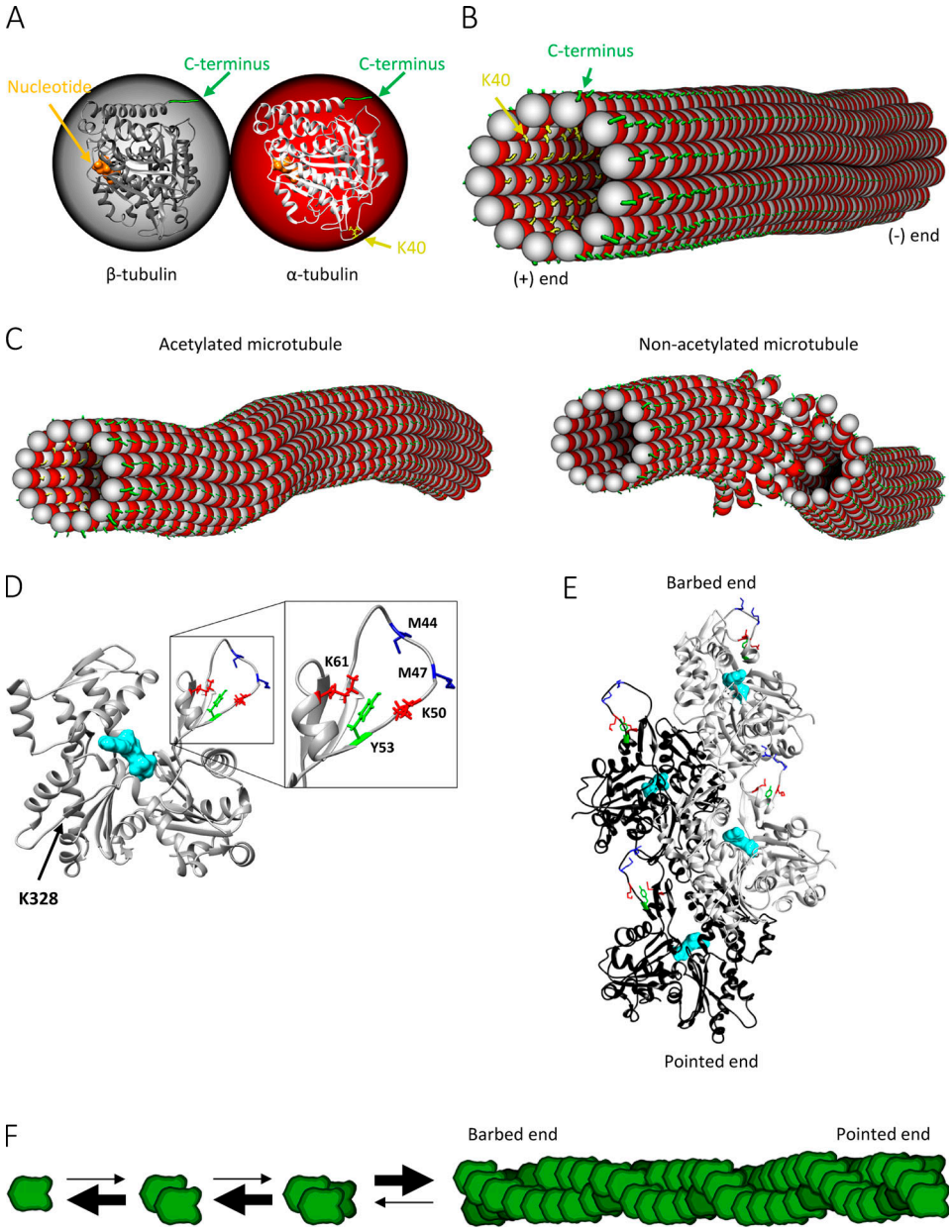

Figure 2. **Tubulin and actin acetylation. (A)** Tubulin dimer of α- and β-tubulin. K40 of α-tubulin is the predominant acetylation site. Also shown are the C termini of α- and β-tubulin, which are subject to multiple PTMs. Molecular structure model from PDB accession no. 4U3J. **(B)** The microtubule, with C termini to the exterior and K40 in the lumen. **(C)** Acetylated microtubules engage in less inter-protofilament contacts, making them more flexible than nonacetylated microtubules, resulting in more resistance to mechanical compression because they bend instead of breaking, perhaps explaining why stable cellular microtubules are highly acetylated. **(D)** Ribbon model of actin monomer, with bound ATP (cyan). Zoom is the D-loop of subdomain 2, showing residues subject to PTMs: K50 and K61 (acetylation), M44 and M47 (oxidation), and Y53 (phosphorylation). Molecular structure model from PDB accession no. 4PKG. **(E)** Model of actin filament of four subunits (ADP-bound), showing the D-loop oriented to exterior. Molecular structure model from PDB accession no. 6DJN. **(F)** Cartoon of actin polymerization, showing unfavorable dimerization and trimerization steps, with subsequent elongation more favorable. Actin monomers add to the barbed end almost exclusively in cells.

diameter of ∼7 nm, with all monomers facing the same direction, creating a polar filament in which a "barbed" end is more dynamic than the "pointed" end. Actin is an ATPase, and monomers hydrolyze bound ATP after addition to the filament, with subsequent phosphate release favoring filament disassembly. Many mammalian nonmuscle cells contain >10 million actin molecules, or 100–200 µM cytoplasmic actin (Hatch et al., 2016; A et al., 2019b).

Mammals contain six actin genes (Perrin and Ervasti, 2010), four of which are found predominantly/exclusively in muscle. The two nonmuscle actins, β- and γ-actin, differ in only four amino acids at the N terminus. Mouse models show that β-actin is more important for overall viability, but γ-actin–deficient mice have multiple developmental abnormalities (Perrin and Ervasti, 2010). Non-muscle actin has an essential role in a large number of diverse cellular processes. Major actin-based

structures include lamellipodia, filopodia, stress fibers, and the cytokinetic ring. In these structures, individual actin filaments turn over in minutes (stress fibers; Amato and Taylor, 1986) or seconds (lamellipodia; Theriot and Mitchison, 1991). There are also multiple additional populations of short, highly dynamic filaments that assemble and disassemble transiently for specific functions, including during endocytosis (Kaksonen and Roux, 2018), endocytic recycling (Simonetti and Cullen, 2019), Golgi dynamics (Fucini et al., 2000; Colón-Franco et al., 2011; Ramabhadran et al., 2011; Kage et al., 2017), mitochondrial damage (De Vos et al., 2005; Li et al., 2015; Kruppa et al., 2018; Fung et al., 2019), autophagy (Coutts and La Thangue, 2015; Kast et al., 2015; Mi et al., 2015; Hu and Mullins, 2019), response to increased cytoplasmic calcium (Shao et al., 2015; Ji et al., 2015; Wales et al., 2016), and phagocytosis (May and Machesky, 2001). In almost all of these processes, the role of actin is to move membranes, which it can do either through the force of its own polymerization or by being a track for myosin motors (Pollard et al., 2001; Blanchoin et al., 2014).

Assembly of actin is controlled by several families of actin assembly factors, including two important to this review: the Arp2/3 complex and formin proteins (Chesarone and Goode, 2009; Dominguez, 2016). Assembly factor regulation is crucial to specify when and where individual actin filament pools occur. As discussed below, an Arp2/3 complex activator, cortactin, is regulated by acetylation. Two of the 15 mammalian formins, INF2 and mDia2, are also regulated by acetylation, with mDia2 getting directly acetylated, whereas INF2 regulation occurs through acetylation of actin itself. We will address acetylation of actin first, followed by cortactin and then mDia2.

Acetylation of actin. Mammalian actins contain 19 lysines, which are generally conserved throughout eukaryotic actins. High-throughput proteomic studies reveal acetylation of at least seven lysines, with K50, K61, and K328 being the most frequently cited (Table S3). One difficulty with these analyses is that they cannot distinguish between the six mammalian actins as all lysines are in completely conserved regions.

Until 2019, there was only one report of functional importance for lysine acetylation: K326/K328 acetylation affecting *Drosophila* flight muscle (Viswanathan et al., 2015). In this study, acetyl-mimetic mutants of K326 and K328 of the cardiac actin protein were overexpressed in *Drosophila* flight muscle. While WT cardiac actin had no effect, the K326Q/K328Q mutant resulted in muscle degeneration and decreased flight ability. The conclusion was that K326/K328 acetylation might alter both tropomyosin and myosin II binding, resulting in sarcomere contraction defects.

Intriguingly, acetylation of a low percentage of actin can inhibit specific actin polymerization pathways, those involving INF2 (A et al., 2019a). In view of INF2's important roles in cells and its role in two diseases, its regulation mechanism is of high significance. INF2 stimulates rapid and transient assembly of actin filaments throughout the cytosol, stimulated by increased cytoplasmic calcium (Shao et al., 2015; Ji et al., 2015; Wales et al., 2016; Chakrabarti et al., 2018). Downstream effects of this cytoplasmic actin polymerization include mitochondrial fission (Korobova et al., 2013; Chakrabarti et al., 2018) and altered transport vesicle dynamics (Andrés-Delgado et al., 2010; Madrid et al., 2010), mediated by two splice variants: endoplasmic reticulum–bound INF2-CAAX and cytosolic INF2-nonCAAX, respectively. An important general role for INF2 might be immobilization of cytoplasmic components, which might facilitate specific interactions (Korobova et al., 2013; Bayraktar et al., 2020). Curiously, INF2 also affects microtubule acetylation in an indirect manner, through transcriptional control of αTAT1 via the myocardin-related transcription factor (MRTF)–serum response factor (SRF) pathway (Fernández-Barrera et al., 2018). Exciting recent work shows a role for INF2-CAAX in the nucleus, polymerizing actin filaments that elongate into the nucleoplasm, and that appear to be involved in chromatin remodeling (Wang et al., 2019). Physiologically, INF2 has been shown to have wide-ranging roles (Labat-de-Hoz and Alonso, 2020). INF2 mutations link to two human diseases, focal segmental glomerulosclerosis (Brown et al., 2010) and Charcot-Marie-Tooth disease (Boyer et al., 2011), and INF2 plays roles in placental development (Lamm et al., 2018) and ischemia-reperfusion pathogenesis (Zhang and Yu, 2018).

Acetylated actin (Ac-actin) inhibits INF2 through an intriguing mechanism (A et al., 2019a, 2019b). A complex between Ac-actin and cyclase-associated protein (CAP) inhibits INF2, by binding two regulatory regions in INF2, the diaphanous inhibitory domain (DID) and the diaphanous autoregulatory domain (DAD; Fig. 3 A). In other formins, the DID and DAD bind tightly in an auto-inhibitory interaction (Lammers et al., 2005; Wallar et al., 2006), but INF2's DID/DAD interaction is weak and insufficient for inhibition of the purified protein (Ramabhadran et al., 2013). However, both DID and DAD are required for cellular INF2 regulation, suggesting the existence of an additional inhibitory molecule, which turns out to be CAP/Ac-actin. The KAT responsible for actin acetylation in this context is unclear, but HDAC6 appears to be the relevant deacetylase. HDAC6 inhibition reduces INF2-mediated actin polymerization in cells (A et al., 2019a). Two lysines in subdomain 2 of actin, K50 and K61 (Fig. 2 D), appear relevant for INF2 regulation (A et al., 2019b). In biochemical assays, K-to-Q "acetyl-mimetic" mutations of these positions are potent INF2 inhibitors, only when complexed with CAP. In cellular assays, expression of either K50Q- or K61Q-actin blocks calcium-stimulated actin assembly. Interestingly, CAP/Ac-actin appears to bridge the DID and DAD to affect inhibition, instead of bolstering a direct auto-inhibitory interaction between DID and DAD (A et al., 2019b).

These studies suggest that INF2 activation in cells is through HDAC6-mediated actin deacetylation, releasing INF2 from the inhibitory CAP/Ac-actin complex (Fig. 3 A). The role of Ac-actin in regulation of INF2 has a number of exciting cellular implications. First, since INF2-CAAX plays roles in mitochondrial calcium uptake and mitochondrial fission (Korobova et al., 2013; Ji et al., 2015; Chakrabarti et al., 2018), this system may act as a metabolic sensor, through the level of the KAT substrate acetyl-CoA (Su et al., 2016; Sivanand et al., 2018), to modulate mitochondrial function. Second, since INF2 influences nuclear actin and downstream nuclear functions (Wang et al., 2019), regulation through actin acetylation might extend to the nucleus. Third, disease-linked INF2 mutations (Brown et al., 2010; Boyer

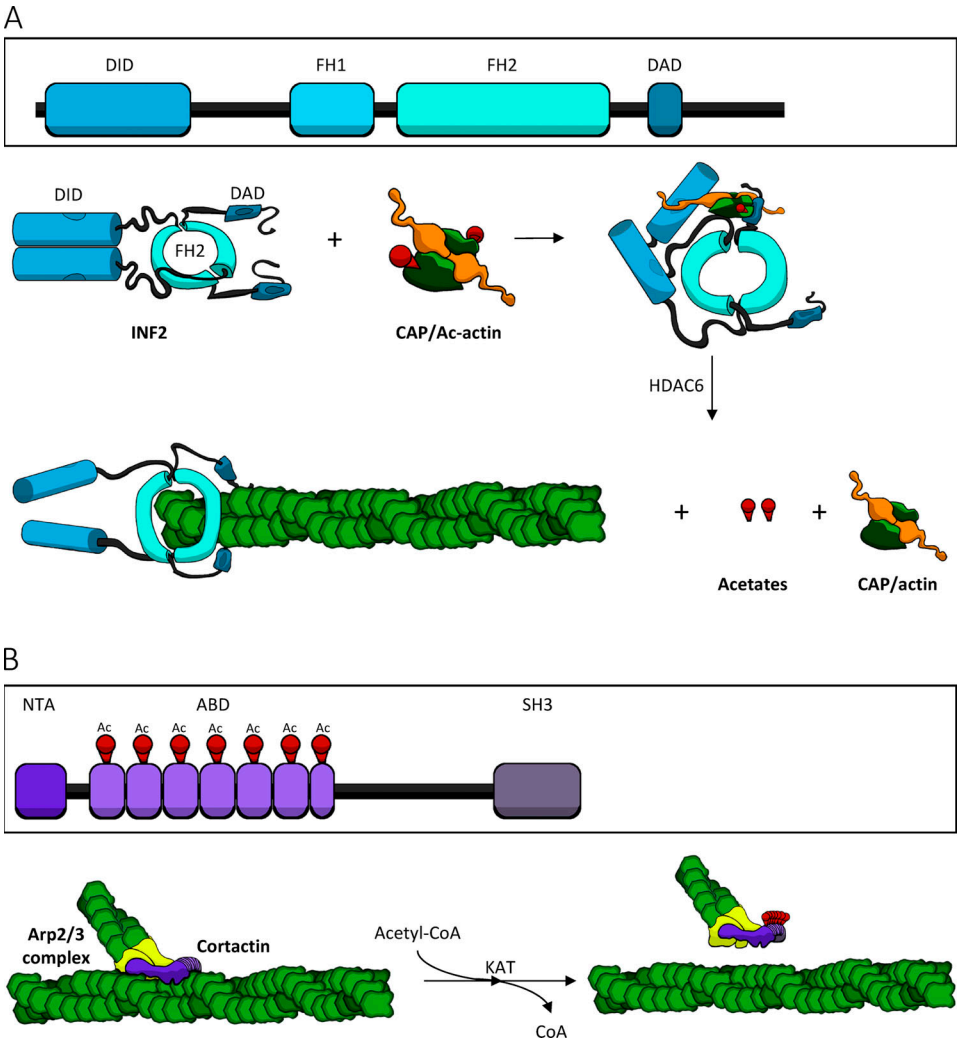

Figure 3. **Acetylation-based regulation of actin dynamics through INF2 and cortactin. (A)** INF2 regulation. Top: Bar diagram of INF2, with the FH1 and FH2 domains being involved in actin polymerization, and the DID and DAD domains being regulatory. Bottom: INF2 dimer inhibition by complex between CAP and Ac-actin, with CAP/Ac-actin serving as a bridge between DID and DAD domains. Deacetylation of actin by HDAC6 releases this bridge, allowing the FH2 domain to interact with actin. For simplicity, CAP/actin is shown as a dimer, although evidence suggests that CAP is hexameric. Also, shown here is interaction between CAP/Ac-actin and only one of the two INF2 subunits in the INF2 dimer, again for simplicity. **(B)** Cortactin regulation. Top: Bar diagram of cortactin, with the NTA interacting with Arp2/3 complex, the ABD consisting of 6.5 repeat regions and interacting with actin filaments, and a C-terminal SH3 domain that interacts with multiple proteins. Each repeat of the ABD is subject to acetylation. Bottom: Speculative model for acetylation-based cortactin regulation. Cortactin bound to both Arp2/3 complex and the mother filament at an Arp2/3 complex–mediated branch. Cortactin acetylation lowers the affinity of the ABD for actin filaments, which could enhance branch disassembly. NTA, N-terminal acidic region.

et al., 2011) are poorly regulated by CAP/Ac-actin (A et al., 2019a; Bayraktar et al., 2020), suggesting an effect on this interaction. Fourth, the concept that a low percentage of Ac-actin can affect overall actin polymerization through binding to regulatory proteins might be more general; low amounts of actin acetylated on specific lysines could even serve to regulate processes not related to actin polymerization.

Acetylation of cortactin. Cortactin is a 62-kD protein containing an N-terminal Arp2/3 complex binding sequence, an actin filament–binding domain (ABD), a proline-rich region, and a C-terminal SH3 domain (Fig. 3 B). Most metazoans have a cortactin gene, but not yeasts or plants (Scherer et al., 2018). Cortactin enhances the activity of Arp2/3 complex, which assembles new actin filaments by binding to the side of an existing filament (the "mother filament") to generate branched filament networks (Fig. 3 B; Schnoor et al., 2018). Arp2/3 complex activation generally requires mother filament binding and binding to a nucleation-promoting factor (Siton-Mendelson and Bernheim-Groswasser, 2017). Cortactin synergizes with other nucleation-promoting factors to enhance Arp2/3 complex nucleation activity (Helgeson and Nolen, 2013), and can stabilize branches by binding both Arp2/3 complex and the mother filament (Fig. 3 B; Weaver et al., 2001).

Cortactin plays roles in many Arp2/3 complex-mediated processes, including lamellipodial assembly (Zhao et al., 2013), invadopodia assembly (Bowden et al., 1999; Oser et al., 2009), dendritic spine dynamics (Hering and Sheng, 2003; Catarino et al., 2013; Lin et al., 2013), and secretory processes (Sung

et al., 2011; Sinha et al., 2016). There may also be Arp2/3 complex–independent cortactin functions (Schnoor et al., 2018).

Cortactin is acetylated on multiple lysines in the ABD (Zhang et al., 2007). Analysis of Cos-7 cells suggests that single lysines in each of the seven ABD repeats are targets, with ABD-7 (K309) being the most avidly targeted (Zhang et al., 2007). The enzymes that acetylate and deacetylate cortactin are still not entirely clear. Possible KATs include PCAF in HeLa cells (Zhang et al., 2007), CBP in COS-7 cells (Ito et al., 2015), and p300 in HEK293T cells (Zhang et al., 2009). KDACs include HDAC6 and Sirt2 in HeLa cells (Zhang et al., 2007), Sirt1 in HEK293T and H549 cells (Zhang et al., 2009; Ito et al., 2015; Motonishi et al., 2015), and HDAC8 in smooth muscle cells (Li et al., 2014).

Biochemically, cortactin acetylation appears to decrease actin filament binding, with acetylation of at least four sites in the ABD needed for significant effects (Zhang et al., 2007). The effect of cortactin acetylation on Arp2/3 complex activity has not been studied, but one speculation would be that it causes weaker mother filament binding, in turn causing weaker nucleation and/or branch stability (Fig. 3 B). In cells, cortactin acetylation inhibits cell migration (Zhang et al., 2007, 2009; Ito et al., 2015) and has effects on post-synaptic density organization in neurons (Catarino et al., 2013). Acetylated cortactin is highly enriched in the nucleus, and cortactin deacetylation appears to promote translocation to the cytosol (Ito et al., 2015; Motonishi et al., 2015). It is unclear whether cortactin has a specific nuclear function, or whether nuclear translocation of acetylated cortactin serves to down-regulate its cytoplasmic functions. Finally, decreased cortactin acetylation has recently been associated with actin-dependent mitochondrial fragmentation (Lovy et al., 2020).

Acetylation of mDia2. The three mammalian Dia formin family members (mDia1–3) are related to the *Drosophila* protein Diaphanous, which assembles a number of actin-based structures, including the cytokinetic ring (Castrillon and Wasserman, 1994; Homem and Peifer, 2008; Nowotarski et al., 2014). mDia2 plays several cellular roles and is the likely formin necessary for mammalian cytokinetic ring assembly (Watanabe et al., 2008). All mDias mediate both actin nucleation and elongation, with mDia2 also having actin filament bundling activity (Harris et al., 2006).

Acetylation of K970 on mDia2 has been reported (Li et al., 2017). This residue is in the actin-interacting FH2 domain of mDia2, and the crystal structure of another formin FH2 domain complexed with actin suggests that the analogous residue points toward the actin but does not make direct contact (Thompson et al., 2013). While it is unclear whether K970 acetylation directly influences mDia2 interactions with actin, the acetyl-mimetic mutant blocks cytokinetic ring assembly and enucleation of erythrocyte precursors (Li et al., 2017). HDAC6 appears to be the deacetylase for mDia2, and its inhibition also blocks these cellular processes (Li et al., 2017). This lysine is conserved in mDia1 as well as in *Drosophila* Diaphanous and in the *C. elegans* cytokinetic formin, but not in mDia3. It will be interesting to see whether acetylation of FH2 residues constitutes a common regulatory mechanism for other formins.

Acetylation in the context of other actin PTMs: "The actin code"

Both histones and tubulin are subject to a variety of PTMs that regulate their functions, collectively known as the "histone code" and "tubulin code" (Gardner et al., 2011; Verhey and Gaertig, 2007; Park and Roll-Mecak, 2018; Janke and Magiera, 2020). Actin is also subject to a number of PTMs other than acetylation (Table S3). Many of these modifications have not been studied functionally and, because actin is abundant, some modifications could represent off-target enzymatic reactions or low levels of nonenzymatic activity. Excellent reviews have covered the breadth of actin PTMs in general (Terman and Kashina, 2013; Varland et al., 2019). Here, we focus on several select modifications that have been studied biochemically and in cells (Fig. 2 D).

One well-studied actin modification is methionine oxidation of M44 and M47 by molecules interacting with CasL protein 1 (MICALs), a family of NADPH-dependent oxido-reductases in vertebrates (Alto and Terman, 2018). MICAL preferentially oxidizes polymerized actin over actin monomers, and actin oxidation leads to rapid depolymerization, both by inherent instability of the oxidized filament and through increased severing by cofilin (Hung et al., 2011; Grintsevich et al., 2016, 2017). Actin is the only known MICAL substrate. Methionine sulfoxide reductases catalyze the reverse reaction (Alto and Terman, 2018). MICAL-mediated actin oxidation is important in actin disassembly during processes such as axonal guidance and cytokinesis (Hung et al., 2010; Frémont et al., 2017).

A second actin modification found routinely in high-throughput proteomics is tyrosine phosphorylation, with 13 of the 15 actin tyrosines identified as phosphorylation sites in multiple studies (Table S3; Hornbeck et al., 2015). Only one site, tyrosine 53 (Y53), has been studied functionally. Y53-phosphorylated actin from *Dictyostelium* has deficiencies in both nucleation and elongation (Liu et al., 2006). In mammalian neurons, the level of Y53-phosphorylated actin is altered by cell stimulation, and might be relevant to neuronal maturation and synaptic plasticity (Bertling et al., 2016).

A third characterized actin modification is methylation of histidine 73 (Johnson et al., 1967; Elzinga, 1971; Wilkinson et al., 2019). This modification occurs on the majority of actin molecules from both muscle and nonmuscle cells, and actin appears to be the sole substrate of the methyltransferase SETD3 (Wilkinson et al., 2019). Methylation somewhat destabilizes the folding of actin monomers but also modestly increases the polymerization rate (Yao et al., 1999; Nyman et al., 2002; Wilkinson et al., 2019). The methyl-H73 residue also occupies a key position in the nucleotide "sensor" loop of actin, whose orientation changes significantly between ADP- and ATP-actin (Graceffa and Dominguez, 2003). SETD3 KO mice display a number of phenotypes associated with dysfunctional smooth muscle contraction (Wilkinson et al., 2019).

An interesting feature of most of these modifications, including the acetylation of K50 and K61 described earlier, is that they are in a particular region of actin, at or near the "D-loop" of subdomain 2. The D-loop is the least structured region of actin, and is oriented to the outer surface of the filament (Fig. 2 E).

Because it is at the pointed end of the monomer, the D-loop does not interact with barbed end–binding proteins like profilin, WH2 motifs, or formins, but does interact with tropomodulin and DNase 1 (Dominguez and Holmes, 2011; Rao et al., 2014) as well as myosin (von der Ecken et al., 2016). The D-loop and surrounding subdomain 2 residues also undergo significant changes between ADP- and ATP-bound actin (Graceffa and Dominguez, 2003). Given the importance of nucleotide turnover in cellular actin dynamics (Blanchoin et al., 2014; Pollard et al., 2001), alterations in this region could have profound effects.

One question is whether these D-loop modifications have any of the following effects: (1) direct effects on actin polymerization; (2) indirect effects on actin polymerization, by altering interactions with one or more actin-binding proteins; or (3) regulatory effects on other proteins. Despite the fact that the D-loop makes important intersubunit contacts in filaments (Chou and Pollard, 2019), modifications of residues in this region have variable effects on actin polymerization or filament stability. Methionine oxidation clearly destabilizes actin filaments and enhances cofilin action, resulting in rapid depolymerization (Hung et al., 2011; Grintsevich et al., 2016, 2017). Conversely, mutations of K50 or K61 do not change the polymerization properties of actin itself, but affect INF2 regulation (A et al., 2019b). Y53 phosphorylation inhibits but does not prevent polymerization (Liu et al., 2006). Interestingly, an R62D mutant is used experimentally to act as a nonpolymerizable variant, and this mutant cannot incorporate into cellular filaments (Posern et al., 2002). Therefore, despite the growing knowledge of actin filament structure (Rao et al., 2014; Chou and Pollard, 2019), actin modifications should be tested individually for polymerization effects.

Considering the high concentration of actin (100–200 µM) in many mammalian cell types (Hatch et al., 2016; A et al., 2019b), modification of sufficient actin to have a significant effect on overall cellular polymerization may be difficult. However, the modified actin might rather serve to "poison" specific actin polymerization pathways, as was elegantly shown for host cell actin modified by a *Vibrio cholerae* toxin, actin crosslinking domain (Heisler et al., 2015). In the case of actin oxidation, the effect on actin disassembly may be local, due to subcellular localization of MICAL, its acute activation by stimuli such as nerve growth factor, and the fact that cofilin-mediated severing unleashes a number of additional mechanisms for actin turnover (Alto and Terman, 2018).

Rather than affecting actin polymerization itself, modified actin could be used as a regulatory mechanism, an example being the regulatory role of K50 and K61 acetylation on INF2 activity (A et al., 2019a, 2019b). This regulatory function could also explain the low stoichiometry of some modifications. Given the high cytoplasmic actin concentration, modification of a small percentage may be sufficient for effective regulation of lower-abundance proteins.

Finally, the close proximity of the modifications described here, in and around the D-loop of actin, could suggest their combinatorial effects. In other words, one modification might favor a second modification, or doubly modified actin might have enhanced or specific functional effects. No information on this possibility is currently available.

Roles for actin in the nucleus

Nowhere has acetylation been more studied than in the nucleus. Interestingly, actin is known to play important roles in the nucleus as well. Actin is at significantly lower concentrations in the nucleus compared with cytosol, but flux between the two compartments is high, with 33% of nuclear actin turned over per minute (Dopie et al., 2012). Actin is imported in a cofilin-bound form through the importin Ipo9, and exported in a profilin-bound form through exportin 6 (Stüven et al., 2003; Dopie et al., 2012). Some of actin's nuclear roles may be played right on the chromatin. Chromatin immunoprecipitation suggests that actin is present near transcription start sites of numerous genes when weakly transcribed, but on highly transcribed genes can also be found on gene bodies (Sokolova et al., 2018).

Actin is involved in a broad spectrum of nuclear functions and appears to act in three forms in the nucleus: (1) as a stable subunit of large, multiprotein complexes; (2) as actin monomers; and (3) as actin filaments (Fig. 4).

At one end of the spectrum is the use of actin and/or actin-related proteins (Arps) as components of chromatin-remodeling complexes (Clapier et al., 2017; Klages-Mundt et al., 2018). These mega-dalton ATP-using molecular machines are extremely intricate in their catalytic mechanisms (Clapier et al., 2017; Willhoft and Wigley, 2020). Actin itself is a fundamental component of INO80 chromatin remodeling complexes from yeast to humans, in which it forms a subcomplex with two Arps, Arp4 and Arp8 (Fig. 4; Willhoft and Wigley, 2020), using binding interfaces that do not resemble those in actin filaments (Knoll et al., 2018). In addition, actin and Arp4 also form part of a module in the NuA4/Tip60 complex, which is predominately a nuclear KAT (Ikura et al., 2000; Wang et al., 2018). In both INO80 and NuA4/Tip60, it is an actin monomer, rather than a filament, which is part of the complex. Actin appears to be ATP-bound in these complexes (Knoll et al., 2018), but it is not clear whether it serves as an ATPase as part of the catalytic mechanism. In addition, actin monomers themselves probably do not dynamically exchange into/out of the complexes.

In the middle of the spectrum is regulation of nuclear processes by more loosely bound actin monomers. One process is through MRTF (also called MAL). MRTF is a coactivator of SRF, a transcription factor that is activated by many cellular stimuli. A role for cytoplasmic actin in MRTF/SRF activity had long been known, with actin monomers sequestering MRTF in the cytoplasm, and cytoplasmic actin polymerization causing MRTF translocation to the nucleus (Posern and Treisman, 2006). In addition, it was found that nuclear actin monomers could bind MRTF, promoting its export (Vartiainen et al., 2007). Similar to the cytoplasmic situation, polymerization of nuclear actin decreases nuclear actin monomers, allowing MAL to bind SRF and activate transcription (Fig. 4; Baarlink et al., 2013; Plessner et al., 2015). Another nuclear complex that appears to contain monomeric actin as an exchangeable subunit is the histone-modifying complex ATAC (Viita et al., 2019). Interestingly, ATAC contains two histone acetyltransferases with different substrate

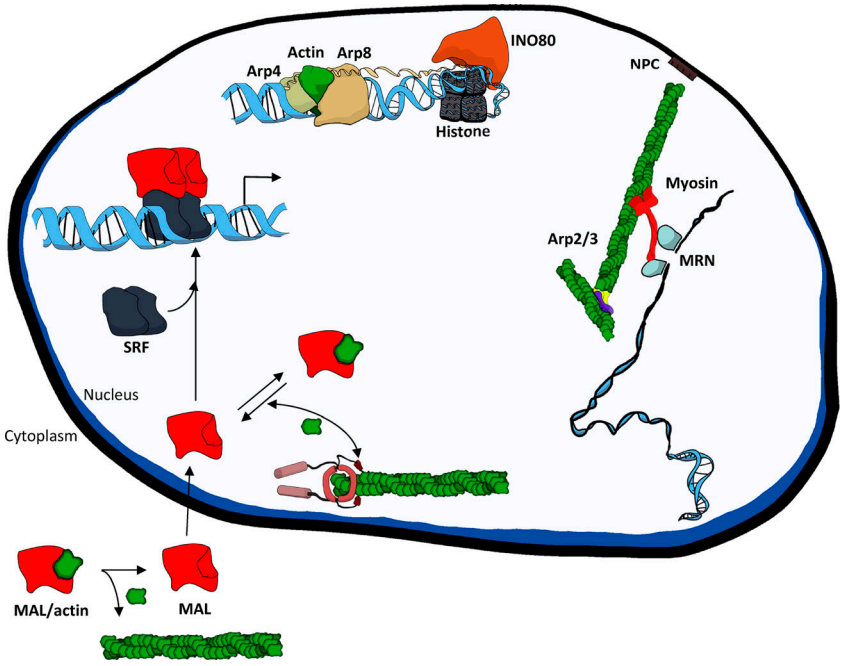

Figure 4. **Nuclear actin.** Examples of the three general types of nuclear actin function. Top: Actin monomer as a tightly bound component of multiprotein complexes. Shown here is the INO80 chromatin remodeling complex. A complex of actin and two Arps (Arp4 and Arp8) forms a module that is connected to the main complex by the helical HSA domain (yellow), and serves to specify the position of INO80 on the nucleosome. Left: Actin monomer as a reversible regulator of nuclear proteins. Shown here is actin bound to MRTF, both in the cytoplasm and in the nucleus. In both compartments, actin monomer inhibits MRTF's ability to activate transcription through SRF. Actin polymerization in both the cytosol and the nucleus releases MRTF for SRF activation. Here, the nuclear actin polymerization is formin-mediated. Right: Actin filaments as force-producing structures for nuclear dynamics. Shown here is myosin-mediated movement of a heterochromatin double-strand break (bound to MRN) along an Arp2/3 complex–polymerized actin filament to the nuclear periphery for repair. MRTF, myocardin-related transcription factor; NPC, nuclear pore complex; MRN, MRE11-Rad50-NBS1 complex.

specificities, KAT14 and GCN5 (Suganuma et al., 2008). Actin binding reduces acetyltransferase activity of KAT14 (Viita et al., 2019).

At the far end of the spectrum, there is an expanding list of roles of actin filaments in the nucleus (Caridi et al., 2019; Percipalle and Vartiainen, 2019; Plessner and Grosse, 2019; Kyheröinen and Vartiainen, 2020; Baarlink et al., 2017; Wei et al., 2020) verified by well-characterized nuclear-targeted actin probes (Belin et al., 2013; Baarlink et al., 2013; Plessner et al., 2015). There is evidence for multiple distinct processes involving nuclear actin polymerization, through Arp2/3 complex (Fig. 4; Caridi et al., 2018; Schrank et al., 2018; Tsopoulidis et al., 2019; Wei et al., 2020) and three formins: mDia1, Fmn2, and INF2 (Baarlink et al., 2013; Belin et al., 2015; Parisis et al., 2017; Liu et al., 2018; Wang et al., 2019). Deconvolving these processes mechanistically could be challenging. In addition, the bifunctional actin polymerization factor JMY can translocate to the nucleus upon DNA damage (Zuchero et al., 2012; Adighibe and Pezzella, 2018), but it is unclear whether nuclear functions of JMY involve actin (Hu and Mullins, 2019).

Regarding actin PTMs and actin's nuclear function, there are several points of interest. (1) As mentioned above, actin appears to interact with a number of nuclear KATs and KDACs. In the cases of KAT14 and HDAC1/2, actin appears to inhibit enzymatic activity directly (Viita et al., 2019; Serebryannyy et al., 2016). These associations raise the possibility that acetylation of actin itself might serve modulatory roles on acetylation dynamics. (2) Another interesting possibility is that acetylation or other PTMs of the stably bound actin in complexes like Ino80 might serve regulatory roles for these complexes. (3) Since the formin INF2 has recently been shown to mediate nuclear actin polymerization (Wang et al., 2019), actin acetylation might actually play a role in its own nuclear polymerization. (4) An additional possibility is that actin acetylation or other PTMs could alter the cytoplasmic/nuclear flux of actin monomers, especially if any of these acetylations affect cofilin or profilin binding.

Conclusions and future directions

Our knowledge of actin acetylation is nowhere near that of histones or tubulin. Nonetheless, the emerging data suggest that actin acetylation might be an important cytoskeletal regulatory mechanism and imply that its roles go beyond just regulating actin dynamics. Regulation of the acetylation process itself will be important in this respect, and may depend partly on the cell's metabolic state, through changes in acetyl-CoA levels needed for KAT activity (Su et al., 2016; Sivanand et al., 2018). Knowledge of these pathways could link the cytoskeleton with other cellular processes in novel ways.

Online supplemental material

Table S1 shows evidence for a cytoplasmic KAT population. Table S2 shows evidence for a cytoplasmic KDAC population. Table S3 shows actin PTMs from proteomic studies.

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Supplemental material

Tables S1–S3 are provided online. Table S1 shows evidence for a cytoplasmic KAT population. Table S2 shows evidence for a cytoplasmic KDAC population. Table S3 shows actin PTMs from proteomic studies.