Actin Filaments—A Target for Redox Regulation

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Actin and its ability to polymerize into dynamic filaments is critical for the form and function of cells throughout the body. While multiple proteins have been characterized as affecting actin dynamics through noncovalent means, actin and its protein regulators are also susceptible to covalent modifications of their amino acid residues. In this regard, oxidation-reduction (Redox) intermediates have emerged as key modulators of the actin cytoskeleton with multiple different effects on cellular form and function. Here, we review work implicating Redox intermediates in post-translationally altering actin and discuss what is known regarding how these alterations affect the properties of actin. We also focus on two of the best characterized enzymatic sources of these Redox intermediates—the NADPH oxidase NOX and the flavoprotein monooxygenase MICAL—and detail how they have both been identified as altering actin, but share little similarity and employ different means to regulate actin dynamics. Finally, we discuss the role of these enzymes and redox signaling in regulating the actin cytoskeleton in vivo and highlight their importance for neuronal form and function in health and disease. © 2016 Wiley Periodicals, Inc.

Key Words: actin; NADPH oxidase; MICAL; redox; post-translational modification

Introduction

Actin filaments contribute to cellular (including neuronal) physiology in a ubiquitious context—including determining the shape and morphology of virtually all cells,

promoting both chemotaxis and cell motility, regulating neurite outgrowth, synaptic connectivity, as well as vesicle trafficking, and orchestrating numerous other cellular functions and organismal behaviors [Pollard and Cooper 2009; Fletcher and Mullins, 2010; Kevenaar and Hoogenraad, 2015]. The regulation of the actin filament (F-actin) cytoskeleton depends on a diverse array of molecular mechanisms that involve actin-associated proteins, including some of the best known such as the Arp 2/3 complex and ADF/cofilins, and well as signaling modulators such as Rho family small GTPases and assorted extracellular ligands and receptors [Pollard and Cooper, 2009; Hung and Terman, 2011; Gonzalez-Billault et al., 2012]. Collectively, these molecular components control F-actin dynamics within both physiological and pathological contexts.

Many of the best characterized actin regulatory proteins use noncovalent means to control actin organization and dynamics [Pollard and Cooper, 2009; Blanchoin et al., 2014], but actin and its regulatory enzymes, like other cellular proteins, are also targets for different types of post-translational modifications (PTMs) [Terman and Kashina, 2013]. One such PTM, which has emerged as a key means to affect multiple different biological processes, is through the oxidation-reduction (Redox) modification of amino acid residues [Walsh, 2006]. So too, Redox-mediated alterations have been found to regulate the dynamic properties of actin, as well as control specific actin regulatory proteins and signaling components [Terman and Kashina, 2013; Wilson and Gonzalez-Billault, 2015]. Studies also indicate that Redox signaling plays important roles in vivo, including within neurons, to control the form and function of the actin cytoskeleton. Yet, similar to the best known PTM, phosphorylation, which plays disparate roles in regulating protein function and cellular behaviors, the functional consequences of Redox-mediated PTM of actin and its regulatory proteins depends on the particular protein affected. Furthermore, not all Redox signaling results in a similar effect, in that multiple residues on actin and its regulatory

proteins have been found to be susceptible to Redox regulation with varying biochemical, cellular, and physiological consequences. Moreover, the Redox intermediates that control these effects range from diffusible oxidants that may have little ability to discriminate among different residues to enzymes that selectively target specific residues on actin. Herein, we provide an overview of the current understanding of the Redox regulation of actin. We also focus on two important Redox enzymes in this regard—NADPH oxidases and MICAL flavoprotein monooxygenases—and discuss how they both have been characterized as targeting actin, but use different mechanisms to exert their effects on the actin cytoskeleton. We also review the current understanding of the cellular role of these enzymes and pay particular attention to highlighting their effects on the form and function of the nervous system.

Actin—A Target of Redox Species

Following their identification and purification of actin over 70 years ago, Brunó Straub and his colleagues conducted biochemical studies that indicated that the presence of an oxidizing agent not only inhibited the polymerization of globular actin, but it also dismantled polymerized actin [Feuer et al., 1948]. While the physiological relevence of these observations were unclear, a vast and dizzying array of literature has since emerged into this Redox-mediated regulation of actin, revealing much about actin's susceptibility to Redox intermediates and making it clear that Redox agents don't simply effect actin in a singular manner. Notably, a large number of physiologically and nonphysiologically relevant reactive oxygen, nitrogen, and lipid reagents have been used over the years to test the Redox susceptibility of actin and this has revealed that actin is susceptible to Redox-mediated PTMs including oxidation, nitrosylation, nitration, as well as the addition of glutathione and carbonyl moieties (glutathionylation and carbonylation, respectively) [Terman and Kashina, 2013]. Collectively, these results have revealed that Redox-mediated post-translational effects on actin are widely variable and are critically dependent on the monomeric versus polymeric state of actin and the particular amino acid residues on actin that are modified.

Actin binds ADP/ATP and Mg^{2+}/Ca^{2+} (among many other small molecules and proteins) and this binding regulates the polymerization properties of actin including the ability of single actin proteins (G-actin) to polymerize and form filaments. The effects that particular Redox reagents have on actin varies considerably depending on which of these small molecules are bound and/or the presence of filamentous versus globular forms of actin. For example, while treatment with hydrogen peroxide and other oxidants greatly reduces the ability of Ca^{2+}-bound G-actin to polymerize [DalleDonne et al., 1995; Milzani et al., 1997; Milzani et al., 2000; Lassing et al., 2007; Hung et al., 2011],

Mg^{2+}-bound G-actin is largely resistant to the adverse effects of hydrogen peroxide and related oxidants [Balin and Barany 1967; Muhlrad et al., 1968; Hung et al., 2010a; Hung et al., 2011]. These differences appear to be due to changes in the conformation and surface exposed residues present when actin is polymerized in the presence of Ca^{2+} versus Mg^{2+}, and those residues that are exposed in G-actin versus F-actin [Lusty and Fasold 1969; Collins and Elzinga, 1975; Konno and Morales 1985; Lin et al., 1990; Dalle-Donne et al., 2002; Guan et al., 2003; Guan et al., 2005; Lassing et al., 2007; Takamoto et al., 2007]. These in vitro observations suggest that specific conditions might make actin more susceptibile to Redox regulation in vivo – and there are examples of these in vivo differences [Duke et al., 1976; Lamb and Posterino, 2003; Gross and Lehman, 2013]. Along these lines, it is also important to note that some of these Redox modifications like oxidation and glutathionylation are reversible, while other modifications such as carbonylation are not [see below; Castro et al., 2013; Terman and Kashina 2013]. In short, it has become clear that the effects of Redox-mediated post-translational modifications including oxidation, nitrosylation, nitration, glutathionylation and carbonylation of actin cannot be simply grouped together as causing one effect on the biochemical properties of actin – and so a full understanding of the sites on actin that are modified by Redox intermediates and how each of these modifications affects the properties of actin is an important research goal.

Actin—Redox Susceptible Residues and Redox Effectors

There are six different actin genes in mammals and in some lower organisms such as Drosophila, with a high-degree of conservation between them, including between muscle and nonmuscle (including neuronal) actins [Bunnell and Ervasti, 2011]. Modification of cysteine (Cys) residues is the best characterized site of Redox regulation [Go and Jones, 2013] and actin has 5-6 cysteine residues (depending on the specific actin) (Figs. 1A and 1C). Both in vitro and in vivo studies have revealed that the cysteine residues on actin are highly susceptible to Redox intermediates – including reactive oxygen, nitrogen, and lipid species. In particular, the Redox-mediated post-translational modification of actin's cysteine residues and their effects on the physicochemical properties of actin has been widely explored because of the availability and ease of use of diverse chemical oxidative reagents that target cysteine [reviewed in Kielley 1964; Muhlrad et al., 1968; Mannherz and Goody, 1976; Gergely, 1966; Dalle-Donne et al., 2001; Hung and Terman, 2011]. Cys-374 is actin's most Redox susceptible amino acid residue, undergoing oxidation, glutathionylation, carbonylation, and nitrosylation (Figs. 1A and 1C; [reviewed in Terman and Kashina,
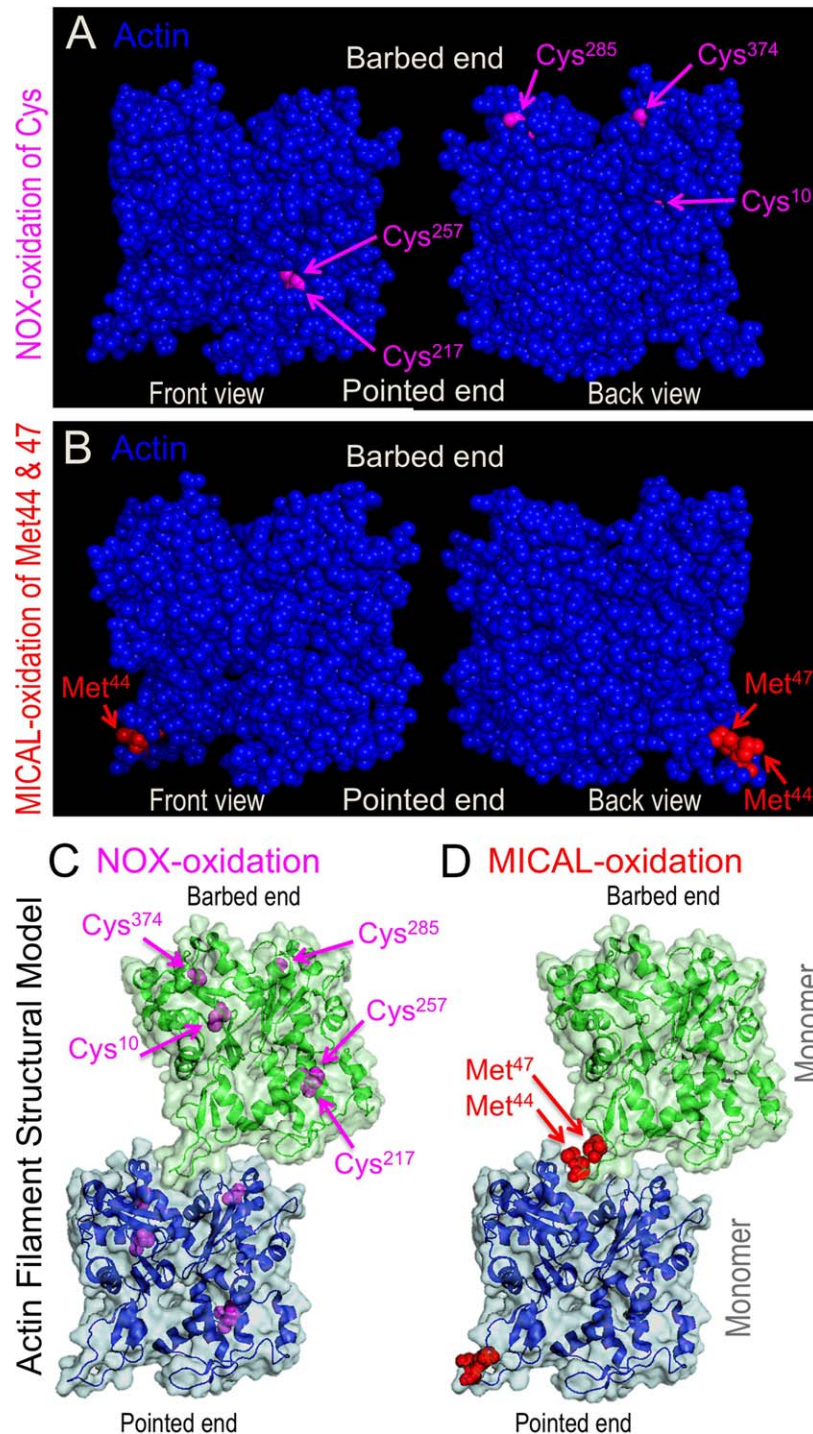
Fig. 1. Structural models of actin and F-actin denoting the individual amino acid residues modified by NOXs and MICALs. **A**: The cysteine (Cys)−374 residue is thought to be a major target of NOX-mediated oxidation of actin, while other cysteine residues in actin including Cys-10, Cys-217, Cys-257, Cys-285, and Cys-272 (not present in this actin) are also likely to be targets of NOX-mediated oxidation (see text). **B**: MICAL specifically oxidizes the Met-44 and Met-47 residues of actin [Hung et al., 2011]—with the oxidation of the Met-44 residue being the primary means by which MICAL induces the disassembly of F-actin [Hung et al., 2011]. Front and back views of the same actin subunit are shown on the left and right sides, respectively. **C, D**: Structural model of actin subunits and their fit within a structural model of a filament including general intersubunit interactions. **C**: The amino acids susceptible to NOX oxidation and their position within the filament. **D**: The amino acids oxidized by MICAL and their position within the filament. The residues oxidized by MICAL, Met-44, and Met-47, are at the critical interface between adjacent actin monomers. In particular, they are situated within the D-loop of actin at the pointed end of one actin monomer, where they associate with residues within the barbed end of another actin monomer [Hung et al., 2011; Galkin et al., 2015; von der Ecken et al., 2015]. Structures are based on PDB identifier: 2ZWH [Oda et al., 2009].

2013]. For example, oxidation of Cys-374 often occurs in purified actin samples with air-exposure, aging, and freeze-thawing [Balin and Barany, 1967; Ishiwata, 1976; Xu et al., 1998; Tang et al., 1999] and this results in disulfide bond formation between two actin monomers [Ishiwata, 1976; Tang et al., 1999]. Reducing agents such as DTT reverse these effects and are added to purified actin samples to prevent this abnormal dimerization. Likewise, subjecting purified actin to reactive oxygen, nitrogen, and lipid species results in modification of Cys-374 and induces both intermolecular disulfide bond-mediated actin aggregation [Ishiwata 1976; Gayarre et al., 2006; Farah et al., 2011] and intramolecular disulfide bond formation with Cys-285 [Farah et al., 2011]. This Cys-374 modification also has other effects on actin including decreasing its polymerization and elongation rate, increasing its critical concentration, and weakening filaments [Stournaras et al., 1990; Shartava et al., 1995; Dalle-Donne et al., 1999, 2000, 2002, 2003; Aldini et al., 2005, 2007; Gayarre et al., 2006; Pizarro and Ogut, 2009]. Yet, it should also be noted that the nature of the Redox modification on Cys-374 is also important since its modification in some cases allows for relatively normal actin properties [Tsapara et al., 1999; Dalle-Donne et al., 2007] – and because of its reactivity, Cys-374 has been used to label actin, such as creating pyrene-labeled actin, and is widely used to characterize the biochemical properties of actin [Kouyama and Mihashi, 1981; Tait and Frieden 1982; Cooper et al., 1983]. In any case, further characterization of this Redox-modified Cys-374 residue is needed since Redox-mediated alterations of Cys-374 regulate actin dynamics under different conditions in cells [Wang et al., 2001; Fiaschi et al., 2006; Gayarre et al., 2006; Aldini et al., 2007; Thom et al., 2008; Farah et al., 2011; Sobierajska et al., 2014] and occur in irreversible sickled cells [Shartava et al., 1995]. Likewise, future work should focus on the other cysteine residues within actin including Cys-10 [Duke et al., 1976; Gross and Lehman 2013; Berndt et al., 2014], Cys-217 [Hamnell-Pamment et al., 2005; Thom et al., 2008; Fedorova et al., 2010a; Su et al., 2013], Cys-257 [Thom et al., 2008; Fedorova et al., 2010a; Su et al., 2013; Berndt et al., 2014], Cys-272 [Lassing et al., 2007], and Cys-285 [Shartava et al., 1995; Chen et al., 2008; Thom et al., 2008; Farah et al., 2011; Su et al., 2013; Figueiredo-Freitas et al., 2015] that are susceptible to Redox regulation—including Cys modifications that have been associated with decreasing the ability of actin to polymerize and interact with specific regulatory proteins including profilin [Lassing et al., 2007; Farah et al., 2011] (Figs. 1A and 1C).

In contrast to the extensive body of literature focusing on the Redox susceptibility of actin's cysteine residues, far fewer studies have focused on the Redox regulation of actin's other amino acid residues. For example, methionine residues, unlike cysteine residues, do not rapidly react with hydrogen peroxide in vitro and at physiological pH [Kim

et al., 2014], and so other oxidative reagents such as hypochlorous acid have been used to test the susceptibility of actin's methionine residues to Redox modification. These studies have revealed that several of the 16 methionine (Met) residues within actin including Met-44, Met-47, Met-176, Met-190, Met-227, Met-269, and Met-355 are susceptible to oxidation in vitro and their oxidation has been linked to functional impairment of actin [Dalle-Donne et al., 2002; Milzani et al., 2000]. Moreover, recent work performed during the characterization of the Mical flavoprotein monooxygenase (see below; [Hung et al., 2011]) has revealed that the specific oxidation of the Met-44 residue of actin causes striking disassembly and decreased polymerization of actin filaments (Figs. 1B and 1D; [Hung et al., 2011]). This Met-44 residue is within the D-loop of the subdomain 2 portion of actin that is crucial for actin subunit contacts (Figs. 1B and 1D; [Galkin et al., 2015; Hung et al., 2011; von der Ecken et al., 2015]). When this specific residue is oxidized, it brings a negative charge (i.e., a significant dipolar character with a negative charge centered on the oxygen) into the monomer-monomer contact region inducing severing of actin filaments (Fig. 1D; [Hung et al., 2011]). Notably, similar to other Redox-affected residues on actin, the Redox susceptibility of these methionine residues including Met-44 varies depending on actin's conformation, ionic state, and polymerization condition [Dalle-Donne et al., 2002; Guan et al., 2003; Guan et al., 2005; Hung et al., 2011; Takamoto et al., 2007]. Therefore, it is possible that particular in vivo conditions and disease states may make some of these methionine residues more susceptible to Redox-regulation. It is also noteworthy that other actin amino acids, including specific tyrosine, histidine, and tryptophan residues, are also susceptible to Redox-induced modifications, although the functional significance of these modifications are unknown. For example, specific tyrosine residues have also been found to be nitrated in vivo (Tyr-294; [Zhan and Desiderio, 2004]), while other tyrosine residues are nitrated following peroxynitrite anion treatment (Tyr-53, Tyr-198, and Tyr-362; [Aslan et al., 2003]), during aging (Tyr-218; [Kanski et al., 2005]), within amyotrophic lateral sclerosis (ALS) mouse models (Tyr53; [Casoni et al., 2005]) and within patients with sickle cell disease (Tyr-91, Tyr-198, and Tyr-362; [Aslan et al., 2003]). Likewise, treatment of purified actin with reactive lipid species induces the carbonylation of specific histidine residues (His-40, His-87, His-173; [Dalle-Donne et al., 2007]) and X-ray irradiation results in oxidation of Trp-79, Trp-86, Trp-340, and Trp-356 amino acid residues (Fedorova et al., 2010a,b).

Identifying the cellular sources of Redox intermediates is also a crucial direction in characterizing the role and physiological importance of the Redox modifications of actin. In particular, while the effects that different chemicals and nonspecific Redox intermediates have on actin have been

well characterized, it has been unclear until more recently whether Redox processes that specifically target actin also exist. For instance, enzymes which produce reactive oxygen, nitrogen, and lipid species including NADPH oxidase [Kang et al., 1999; Moldovan et al., 2000; Wojciak-Stothard et al., 2005; Barth et al., 2009; Dailianis et al., 2009; Munnamalai and Suter, 2009; Ullevig et al., 2012; Bhopale et al., 2015], lipoxygenases [Lepley and Fitzpatrick, 1994; Kang and Vanderhoek, 1998; Rice et al., 1998; Miller et al., 2001; Provost et al., 2001; Fiaschi et al., 2006; Munnamalai and Suter, 2009], xanthine oxidase [Rosado et al., 2002], myeloperoxidase [Thom et al., 2008], and nitric oxide synthase (NOS) [Jaffrey et al., 2001; Webb et al., 2001; Banan et al., 2003; Su et al., 2007; Harris et al., 2008; Dailianis et al., 2009; Bhopale et al., 2015] have been linked to the modification of actin. However, actin constitutes only one set of proteins that are modified by these enzymes since they diffusibly release reactive species, and so questions have arisen regarding the specificity of these effects. Likewise, actin is one of the most abundant cellular proteins, raising concerns that actin might be modified nonspecifically by diffusible reactive oxygen, nitrogen, and lipid species produced by these enzymes. Moreover, it has been suggested that actin might even serve to "soak-up" these species and/or to protect the cell against redox intermediates [Dalle-Donne et al., 2003, 2007; Farah et al., 2011]. Therefore, many important directions remain to be explored related to the identity and specific mechanisms of action of the Redox effectors of actin dynamics and their physiological and pathological roles. However, recent work has provided a new understanding of these important concepts and below we focus on these emerging properties by highlighting two of actin's Redox effectors: 1) NADPH oxidases, which are enzymes well-known to release reactive oxygen species to exert their effects, and 2) the recently characterized F-actin disassembly factor Mical, which is a Redox enzyme that specifically and directly oxidizes actin.

NADPH Oxidases: F-Actin Effects and Cellular Functions (with a Focus on the Nervous System)

Pro-oxidation species such as superoxide, hydrogen peroxide and nitric oxide play critical roles as signaling molecules owing to the fact that their concentrations can be enzymatically regulated [Bigarella et al., 2014]. In this context, NADPH oxidase (NOX) enzymes have important functions. The NOX enzymatic family comprises 7 members called NOX 1–5 and Duox 1–2 [Bedard and Krause, 2007]. NOXs catalyze the conversion of molecular oxygen (O_2) into the superoxide radical (O_2^-), a short-lived molecule that is rapidly converted both spontaneously or enzymatically into hydrogen peroxide (H_2O_2) [Nunez et al., 2012] (Fig. 2). The superoxide and hydrogen peroxide are released extracellularly and can reach the intracellular compartment through either diffusion, as in the case of H_2O_2, or via membrane-enclosed vesicles, in the case of superoxide [Lassegue et al., 2012; Valdivia et al., 2015] (Fig. 2). NOX proteins may also mediate signaling via ROS production in endosomal compartments, or "redoxosomes" [Oakley et al., 2009; Valdivia et al., 2015]. NOX-derived ROS have been implicated in targeting intracellular signaling proteins in a paracrine, autocrine, and intracrine manner [Valdivia et al., 2015].

The main NOXs expressed in the central nervous system of mammals are NOX 1, 2, and 4 [Sorce et al., 2012] (Fig. 2). NOX2 is the best-characterized family member and appears to be the main NOX expressed in neurons [Lambeth, 2004; Bedard and Krause, 2007; Nayernia et al., 2014]. NOX2 (also called gp91[phox]) is a plasma membrane protein of approximately 50 kDa spanning 6 transmembrane domains, with both NADPH and FAD binding sites towards the intracellular space [Harper et al., 1985; Rotrosen et al., 1990; Paclet et al., 2004; Groemping and Rittinger, 2005]. These distinguishing catalytic subunits (i.e., NOX 1-5, Duox 1-2) are also typically joined by up to five variable regulatory phox subunits to form the NADPH oxidase complex. For example, NOX2 forms a complex with p22[phox], a 22 kDa plasma membrane protein that confers further stabilization to NOX2 [Dinauer et al., 1987; Parkos et al., 1988; Ambasta et al., 2004]. The complex NOX2/p22[phox] interacts with 4 additional molecular partners: p40[phox], p47[phox], p67[phox] and the Rho GTPase Rac [Lambeth 2004] (Fig. 2). The assembly of these interactors depends on signaling mechanisms involved in ROS production. To enhance ROS synthesis, protein kinase PKC phosphorylates p47[phox], which allows for its translocation to the plasma membrane to meet the NOX2/p22[phox] complex [Groemping and Rittinger, 2005]. After phosphorylation, p47[phox] undergoes a conformational change that releases its own autoinhibitory domain, promoting the interaction with the NOX activator p67[phox] [Groemping and Rittinger 2005; Nauseef 2004; Sumimoto et al., 2005]. p47[phox] is usually referred as the organizer of NOX complex assembly, which allows increasing ROS synthesis over basal levels in response to signaling mechanisms. Finally, Rac and p40[phox] bind the complex through a p67[phox] interaction [Glogauer et al., 2003; Bokoch and Zhao, 2006]. Rac, which is a well known regulator of F-actin dynamics, has 3 family members (Rac1, Rac2 and Rac3) [Heasman and Ridley, 2008]. In neutrophils, the knock down of Rac2 but not Rac1 decreases ROS production via NOX, suggesting a family member dependent modulatory effect [Roberts et al., 1999; Ambruso et al., 2000; Kim and Dinauer, 2001]. Rac1 is expressed ubiquitously, Rac2 is mainly expressed in hematopoietic cells [Didsbury et al., 1989], and Rac3 is present in developing neurons [de Curtis, 2014]. In neurons, both Rac1 and Rac3 are positively associated with F-actin polymerization, axonal growth and neuronal polarity [Gonzalez-Billault et al., 2012]. Additional studies will be required to dissect which Rac family member is required for

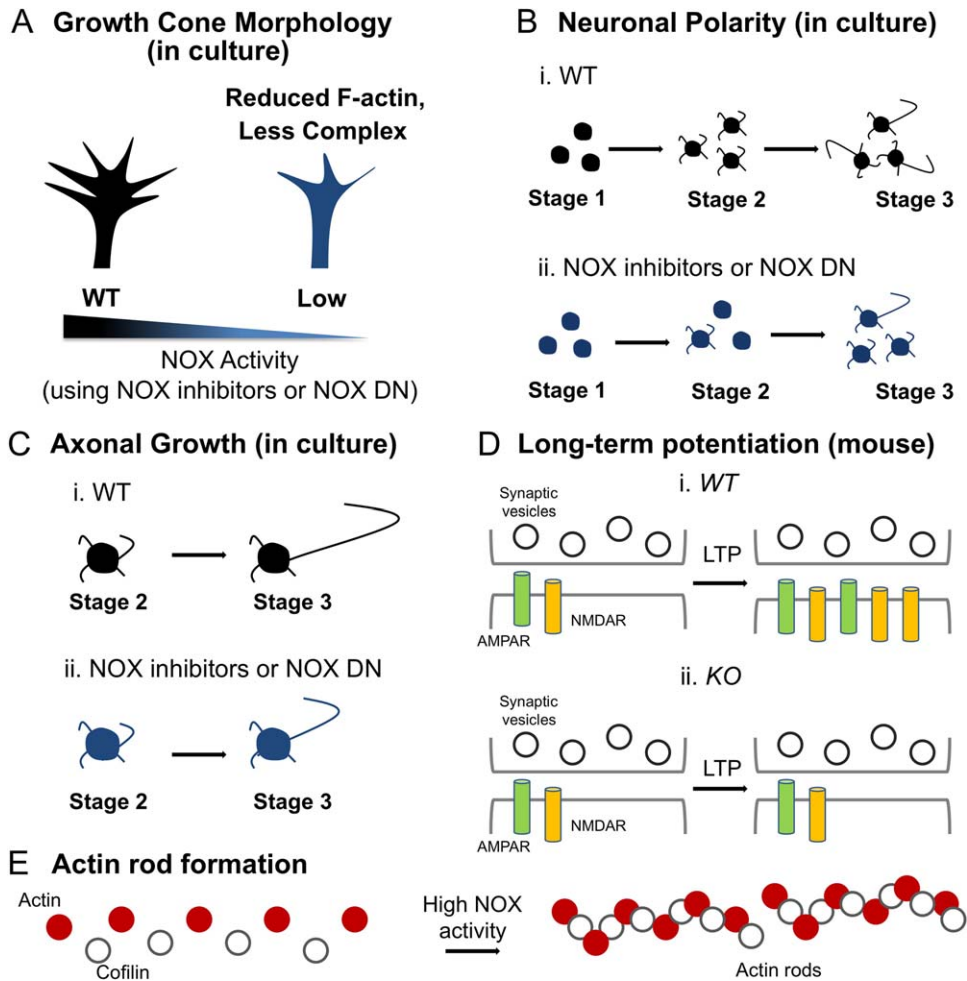## Conformation and subunits of neuronal NOX complexes
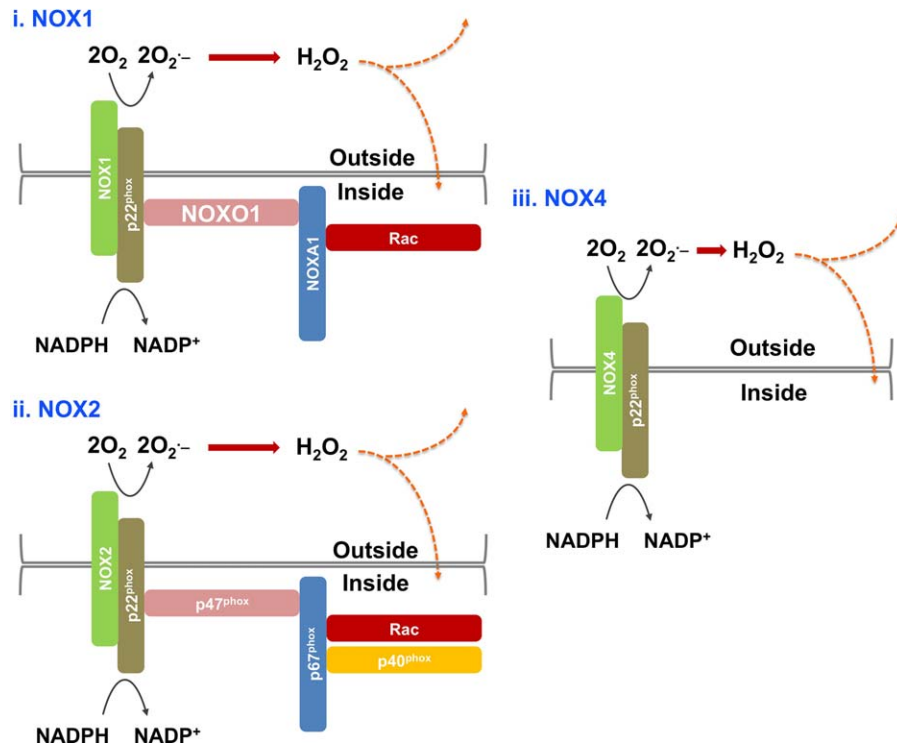
Fig. 2. Main NOX complexes described in neurons. NADPH oxidases are a family of membrane-associated multisubunit enzymes that generate reactive oxygen species (ROS) to regulate diverse systems. Members of the Nox family include NOX1-5 and Duox1-2; with NOX1, NOX2 (also called gp91^{phox}) and NOX4 being the main Nox family members described to date in neurons. Members of the Nox family catalyze the conversion of O_2 into $O_2^{.-}$ (superoxide), which can be converted into H_2O_2 (hydrogen peroxide) [Lassegue et al., 2012; Valdivia et al., 2015]. These ROS are released extracellularly and can reach the intracellular compartment through either diffusion, as in the case of H_2O_2, or via membrane-enclosed vesicles, in the case of superoxide [Lassegue et al., 2012; Valdivia et al., 2015]. Nox proteins may also mediate signaling via ROS production in endosomal compartments, or "redoxosomes" (not shown; reviewed in [Oakley et al., 2009]). NOX-derived ROS have been implicated in modifying intracellular signaling proteins in a paracrine, autocrine, and intracrine manner [Valdivia et al., 2015]. It should also be noted that the distinguishing catalytic subunits (i.e., NOX 1–5, Duox 1–2) are also typically joined by five variable regulatory phox subunits to form the NADPH oxidase complex. (**i-ii**) The NOX1 and NOX2 complexes share common features, such that NOXO1 and p47^{phox} play similar roles including assembling and organizing the plasma membrane subunits (i.e., NOXs and the stabilizing factor p22^{phox}) with the cytosolic subunits (i.e., p67^{phox}, NOXO1, Rac, and p40^{phox}) of the complex. Note also that NOXA1, which is present in the NOX1 complex, is homologous to p67^{phox}, the activator subunit of the NOX2 complex. (**iii**) The NOX4 complex lacks the cytosolic subunits and only displays the plasma membrane-associated proteins NOX4 and p22^{phox}. Nox proteins with the exception of Nox4, which is constitutively active, are silent in resting cells [Lassegue et al., 2012].

ROS production in neurons, which could clarify whether the pool of Rac required for ROS production is the same as that which regulates F-actin dynamics and neuronal polarization. Considering this, both Rac and NOX might be involved in a positive feedback loop that regulates the synthesis of ROS required for neuronal polarization through the regulation of the actin cytoskeleton.

NADPH oxidases have been found to have many targets in cells and regulate multiple different cellular events [reviewed in Katsuyama 2010; Brandes et al., 2014; Sirokmany et al., 2016]. Among these, NOX family enzymes have been linked to regulating the organization of the actin cytoskeleton through different proteins in a number of contexts and cell types [reviewed in Terman and Kashina 2013; Stanley et al., 2014; Valdivia et al., 2015]. In particular,

since NADPH oxidases release ROS/H_2O_2 to exert their effects, they appear to target specific cysteine residues in proteins – and Cys374 is recognized as a predominant target for NOXs on actin, with effects that lead to oxidation, glutathionylation, and carbonylation of this residue [Figs. 1A and 1C; reviewed in Stanley et al., 2014; Valdivia et al., 2015]. Likewise, components of the NOX complex have been found to associate/co-localize with actin in different cells and evidence also indicates that the actin cytoskeleton plays an important role in facilitating the ability of NOX enzymes to be activated at distinct locations to result in focused ROS production [reviewed in Terman and Kashina, 2013; Stanley et al., 2014; Valdivia et al., 2015]. Work in neurons also supports the link between the NADPH oxidase complex and actin. In particular, although still poorly

A Growth Cone Morphology (in culture)

Reduced F-actin, Less Complex

WT — Low

NOX Activity
(using NOX inhibitors or NOX DN)

B Neuronal Polarity (in culture)

i. WT

Stage 1 — Stage 2 — Stage 3

ii. NOX inhibitors or NOX DN

Stage 1 — Stage 2 — Stage 3

C Axonal Growth (in culture)

i. WT

Stage 2 — Stage 3

ii. NOX inhibitors or NOX DN

Stage 2 — Stage 3

D Long-term potentiation (mouse)

i. *WT*

Synaptic vesicles — LTP
AMPAR — NMDAR

ii. *KO*

Synaptic vesicles — LTP
AMPAR — NMDAR

E Actin rod formation

Actin — High NOX activity — Actin rods
Cofilin

Fig. 3. Contribution of NOX to actin-dependent neuronal processes in physiological and pathological contexts. In A-D, WT designates *wild-type* (or *no treatment*), inhibitors designate inhibitors to NOX, DN designates dominant negative proteins that decrease NOX funtion, and *KO* designates *NOX* knockout. **A**: Inhibition of NOX2 activity using NOX inhibitors (e.g., apocynin, VAS2870, gp91 ds-tat) and other tools (e.g., ectopic expression of the mutant P156Q p22^{phox} dominant negative [DN]) reduce the content of F-actin in the growth cone of both aplysia bag cells and hippocampal neurons in culture [Munnamalai and Suter, 2009; Wilson et al., 2015]. **B, C**: Loss of function of NOX2 (e.g., using VAS2870, gp91 ds-tat or the mutant P156Q p22^{phox} dominant negative [DN]) blocks proper polarization of hippocampal neurons in culture, as well as the break of the symmetry of stage 2 neurons (B) and axonal growth (C) [Olguin-Albuerne and Moran, 2015; Wilson et al., 2015]. **D**: Analyses of *NOX2 (gp91^{phox})* and *p47^{phox}* knockout mice, which serve as models for chronic granulomatous disease (CGD) that affect humans and carry point mutations in NOX proteins (NOX2, p47^{phox}, p67^{phox} and p22^{phox}) [Pao et al., 2004; Cole et al., 2013], develop an impaired long-term potentiation (LTP) response, a method to evaluate synaptic plasticity characterized by an increase in the expression and translocation of AMPA and NMDA receptors (AMPAR and NMDAR, respectively) to the post-synaptic plasma membrane [Kishida et al., 2006]. **E**: High ROS levels due to NOX over-activation in Alzheimer's disease induce the aggregation of actin and cofilin in a 1:1 ratio in hippocampal neurons, leading to the formation of actin rods and neuronal dysfunction [Walsh et al., 2014; Minamide et al., 2010].

characterized in mammalian neurons, NOX proteins including NOX1, NOX5 and DUOX have a dynamic expression pattern during early development of the nervous system of zebrafish (as assayed in zebrafish [0-2 days post fertilization]), while cybb2/NOX2 is stably expressed during this period of development [Weaver et al., 2016]. More indepth examination has revealed that components of the NOX complex such as p40^{phox} colocalize with F-actin in growth cones [as assayed using the mollusc Aplysia; [Munnamalai et al., 2014] (Fig. 3A). NOX2, p47^{phox} and p22^{phox} have also been detected in growing axons of both

mature and immature hippocampal cultured neurons [Serrano et al., 2003; Tejada-Simon et al., 2005; Wilson et al., 2015]. These 3 proteins have also been detected within the axonal tips/growth cones of young polarizing neurons (2 days in vitro) [Wilson et al., 2015].

Functional studies have also provided a link between NADPH oxidase, physiological ROS, and actin dynamics in neurons. In particular, the acquisition of neuronal morphology occurs after the last post-mitotic division of the neural precursor, in a process called the establishment of neuronal polarity [Caceres et al., 2012]. Here, axonal

specification occurs approximately 1 day after neuronal differentiation and the break of the cellular symmetry occurs through an actin dependent mechanism [Dotti et al., 1988; Bradke and Dotti, 1999]. Inhibition of the NOX complex activity through both dominant negative proteins and pharmacological agents inhibits neuronal polarization and axonal growth [Wilson et al., 2015] (Figs. 3B and 3C). Likewise, neuronal expression of a mutant isoform of p22^{phox} that inhibits ROS production via NOX1, NOX2 and NOX3 [p22^{phox} P156Q; Kawahara et al., 2005], decreases both the lamellar organization of actin in stage 1 cultured hippocampal neurons (the initial neuronal morphology before axonal specification) as well as growth cone filopodial dynamics within their elongating axons [Fig. 3A; Wilson et al., 2015]. Neurite outgrowth is also decreased after NOX inhibition in both cerebellar granule neurons of mice and Aplysia neurons, and work in Aplysia has linked this to changes in actin cytoskeletal structure and dynamics [Munnamalai and Suter, 2009; Munnamalai et al., 2014; Olguin-Albuerne and Moran, 2015]. Interestingly, cultured cerebellar granule neurons isolated from NOX2 null mice exhibited normal neurite outgrowth compared to those from wild type mice. These animals also exhibited normal ROS levels, which may suggest compensatory mechanisms and/or that mice also expresses other NOX forms, like NOX1 and NOX4, according to previous reports [Sorce et al., 2012; Olguin-Albuerne and Moran, 2015]. In short, the influence of NADPH oxidase/ROS production on the activity of actin and actin-related proteins has just begun to be explored in neurons. For instance, both Rac1 and Cdc-42 activities are decreased after NOX2 inhibition in cultured hippocampal neurons [Wilson et al., 2015], which suggests that molecular regulators of F-actin dynamics are also dependent on redox balance/signaling in neurons. These results further support the notion that the NOX complex is spatially correlated with the actin-rich sites of a neuron, like the periphery of axonal growth cones and emerging axons and neurites, supporting a functional relationship between NOX proteins and actin in neurons.

In addition to links between NOX during early neuronal development and axon extension, there are also emerging links between NOX and dendritic spine remodeling, synaptic plasticity, and synaptic function, which are also dependent on actin [reviewed in Hotulainen and Hoogenraad, 2010; Kevenaar and Hoogenraad, 2015]. In this regard, it is interesting that glutamate signaling activated by an NMDA receptor agonist induces superoxide synthesis via NOX2 [Brennan et al., 2009; Reyes et al., 2012]. This raises the possibility of links between glutamatergic activity, synaptic plasticity, dendritic spine remodeling and actin cytoskeletal dynamics in a NOX/redox dependent manner. Previous findings also suggest that humans with deleterious point mutations in gp91^{phox}, p47^{phox}, p67^{phox} and p22^{phox} proteins develop cognitive impairments and lower intellectual coefficients compared to controls [Pao et al., 2004;

Cole et al., 2013]. These mutations trigger an inherited disease called Chronic Granulomatous Disease (CGD), in which 70% of the affected individuals have point mutations in the X-linked $gp91^{phox}$ (NOX2) gene, 25% have mutations in $p47^{phox}$, and 5% have mutations in either $p67^{phox}$ or $p22^{phox}$ [Pao et al., 2004]. Electrophysiological analyses of $gp91^{phox}$ (NOX2) and $p47^{phox}$ knockout mice also show that mutations in either of these genes results in decreased LTP responses compared to wild type animals and proposes that the lack of NOX proteins affects memory and learning consolidation processes in a murine model [Kishida et al., 2006] (Fig. 3D).

Specific neurological disease processes have also been linked to NOX and its production of ROS. Alzheimer's disease, for example, has been characterized by the development of actin rods within neurites, which consist of cofilin and actin aggregation in a stoichiometric ratio (1: 1 = actin: cofilin) [Minamide et al., 2010]. These rods have been linked to inducing a loss of synaptic function by blocking vesicle trafficking across dendrites and by sequestering cofilin within the somato-dendritic compartment, affecting LTP responses [Cichon et al., 2012]. The amyloid-β (Aβ) peptide, as well as the proinflammatory cytokines TNF-α and IL-6, induce actin rod formation through a NOX dependent mechanism in both cortical and hippocampal cultured neurons [Walsh et al., 2014]. Superoxide produced by NOX (and further interconversion into other ROS) oxidizes cofilin's cysteines, triggering disulfide bridge formation and cofilin aggregation [Klamt et al., 2009; Bernstein and Bamburg, 2010; Bernstein et al., 2012; Walsh et al., 2014; Bamburg and Bernstein, 2016] (Fig. 3E). In addition, sustained excitotoxic glutamatergic activity, as well as energy deprivation (i.e.: mithocondrial dysfunction), also induce rod formation but through a NOX-independent (but perhaps not a ROS-independent; see for example, [Nunez et al., 2012; Villegas et al., 2014; Rojas et al., 2015]) mechanism [Walsh et al., 2014]. NOX-derived ROS are also involved in neuronal degeneration of spinal cord neurons [Kuhn, 2014]. In particular, proinflammatory cytokines TNF-α and IL-1β induce the collapse of spinal cord neuronal growth cones through a NOX2 dependent mechanism [Kuhn, 2014]. Likewise, elevated ROS synthesis triggers aberrant actin reorganization in affected growth cones after spinal cord injury [Kuhn, 2014] and the observed neurodegeneration after spinal cord injury has been hypothesized to be linked to NOX and alterations to the actin cytoskeleton [Walsh et al., 2014]. Together, these findings support the notion that NADPH oxidase driven ROS production controls the physiology and pathology of neurons. Future work should focus on further analysis of these and other nervous system-related effects linked to NOX proteins [Nayernia et al., 2014], as well as more-indepth analysis of NOX expression patterns in the nervous system [Nayernia et al., 2014] and the

examination of *NOX* mutants for neuronal-related phenotypes [Sirokmany et al., 2016].

MICAL Flavoprotein Monooxygenases: F-Actin Effects and Cellular Functions (with a Focus on the Nervous System)
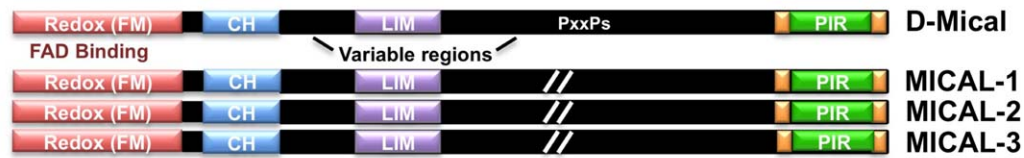
The multi-domain flavoprotein monooxygenase Mical has recently provided an important new understanding of the Redox regulation of actin dynamics and its physiological importance. The MICAL family of enzymes (referred to as MICAL in vertebrates, Mical in invertebrates, and collectively called MICAL) and the presence of their Redox enzymatic moiety was first noted in conjunction with the identification of Mical as a binding partner of the transmembrane guidance receptor Plexin (Fig. 4A; [Terman et al., 2002]). Mical was found to bind with Plexin, which serves as a receptor for members of the large Semaphorin family of axon/cellular guidance cues [Kolodkin and Tessier-Lavigne, 2011], and regulate Semaphorin/Plexin effects on axon guidance and connectivity [Terman et al., 2002]. These results became even more intriguing when it was determined that the integrity of Mical's Redox (Flavoprotein Monooxygenase) enzymatic moiety was critical for its effects on these neuronal behaviors [Terman et al., 2002]. However, the cellular role of Mical and its biochemical function was only recently characterized when it was determined that Mical uses it's Redox enzymatic domain to bind to F-actin and that Mical modifies the organization of the actin cytoskeleton in vitro and in vivo in a Redox-dependent manner [Hung et al., 2010a]. Provocatively, and in contrast to work with NADPH oxidases (see above), these studies also indicated that Mical was not simply releasing a diffusible oxidant such as H_2O_2 to cause effects on F-actin – since diffusible oxidants including cell-lethal levels of H_2O_2 had no effect on F-actin disassembly and H_2O_2 did not mimic the effects of Mical [Hung et al., 2010a, 2011]. Moreover, the H_2O_2 scavenger catalase (and other types of reductants such as DTT and thioredoxin/thioredoxin reductase) did not affect Mical-mediated F-actin disassembly [Hung et al., 2011, 2013]. Further analysis uncovered that Mical needed to be in close approximation with F-actin [Hung et al., 2011], and that binding to F-actin (but not G-actin) activates the enzymatic activity of Mical [Hung et al., 2011; Zucchini et al., 2011; McDonald et al., 2013], which then induces Mical to oxidize actin filament subunits [Hung et al., 2011]. Turning to mass spectrometry analysis revealed that Mical selectively oxidizes two methionine residues on actin, Met-44 and Met-47 (Figs. 1B and 1D; [Hung et al., 2011]) and mutagenesis analysis demonstrated that mutating the Met-44 residue on actin abolishes Mical's effects on F-actin in vitro and in vivo (Figs. 4B–4D; [Hung et al., 2011]).
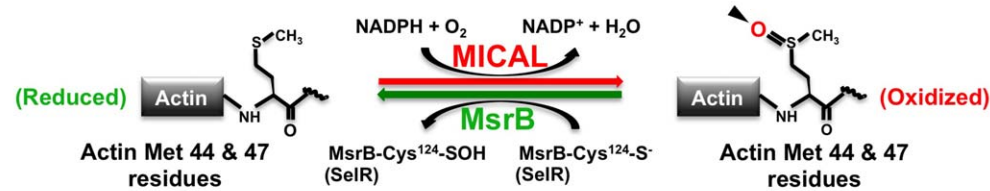
Mical, therefore, is a new type of actin effector – a direct actin regulatory enzyme that uses F-actin as a specific substrate and employs a catalytic oxidation-dependent post-translational mechanism to selectively control actin dynamics. Moreover, Mical's specific effects on actin lead to an instability of the F-actin cytoskeleton – such that Mical-mediated oxidation of the Met-44 residue of actin triggers both F-actin depolymerization and aberrant F-actin reassembly (Figs. 4B and 4D; [Hung et al., 2010a, 2011]). In this way, Mical's ability to destabilize the F-actin cytoskeleton is also meaningful in light of its important role downstream of repulsive guidance cues like Semaphorins, which have long been characterized for their disruptive effects on the F-actin cytoskeleton (Fig. 4D; [Luo and Raper, 1994; Hung and Terman, 2011]). While much remains to be learned about the enzymatic and biochemical properties of MICALs and their effects on actin, recent work also reveals that these Mical-mediated effects on actin dynamics are reversible by members of the SelR/MsrB family of methionine sulfoxide reductase enzymes [Hung et al., 2013; Lee et al., 2013]. In particular, biochemical and mass spectrometric studies identified that SelR/MsrB proteins directly and specifically reverse the effects of MICALs on actin – by de-oxidizing actin – and restoring its normal polymerization properties (Fig. 4B; [Hung et al., 2013; Lee et al., 2013]). Likewise, in vivo studies revealed that this MICAL-SelR system regulates actin dynamics in multiple cells including during axon guidance, synaptogenesis, mechanosensory organ formation, muscle development, and Semaphorin/Plexin repulsive signaling (Fig. 4D; [Hung et al., 2013]). These results, therefore, reveal that the MICALS and SelR/MsrBs comprise a reversible Redox cellular signaling system that controls actin cytoskeletal organization (Fig. 4D). Moreover, methionine has a unique oxidation pattern in that two stereoisomers (an *R*-isomer and an *S*-isomer) can be produced by oxidation. Actin was found to be stereo-specifically oxidized in the *R*-isomer conformation by the MICALs (and reduced by SelR/MsrB) [Hung et al., 2013; Lee et al., 2013] – providing a first demonstration that the stereospecific oxidation/de-oxidation of specific methionine residues (similar to the reversible phosphorylation of serine, threonine, and tyrosine residues) is a means to precisely modulate protein function.

Thus, unlike diffusible oxidants that induce random protein modifications [Shacter, 2000; Stadtman et al., 2003; Kim and Gladyshev, 2007], MICAL-mediated oxidation of actin is substrate, residue, and stereo-specific. It is also preferentially activated by one form of its actin substrate, the polymerized form, thereby further revealing unusual features of this new actin regulatory Redox enzyme. There is one Mical family member in Drosophila and three mammalian MICAL family members with a conserved domain organization, including their N-terminally located Redox enzymatic moiety (Fig. 4A; [Terman et al., 2002]). An
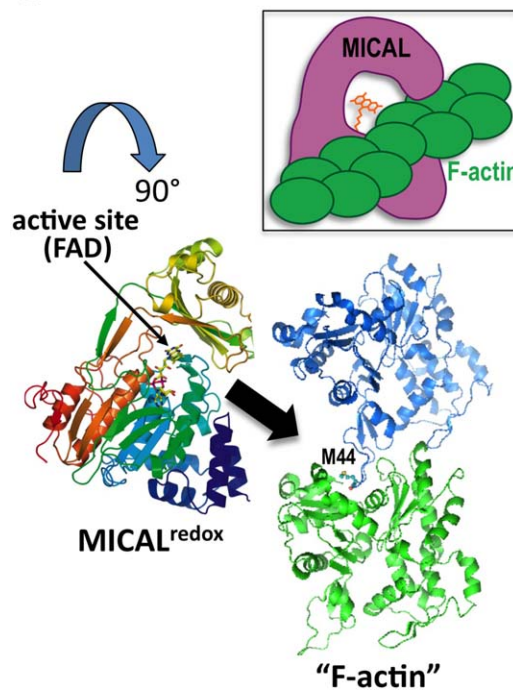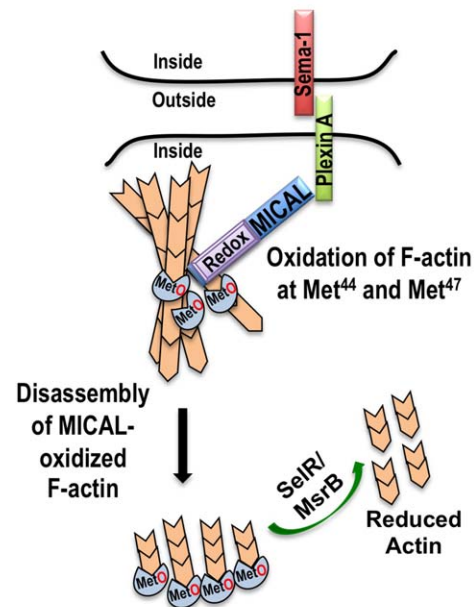
Fig. 4. The MICAL and SelR/MsrB Reversible Redox Actin Regulatory System. **A**: The MICAL family of proteins includes one Drosophila family member (D-Mical) and three vertebrate family members (MICALs 1-3) that share a conserved domain organization including the critical flavoprotein monooxygenase (Redox [FM]) domain. Each family member also contains a calponin homology (CH) domain, a LIM domain, a proline-containing (PxxPs) region, and the C-terminal plexin-interacting region (PIR). The length of the variable regions of MICAL family proteins varies and it has been designated as (//) in the diagram [Terman et al., 2002]. **B**: In the presence of its coenzyme NADPH, Mical adds an oxygen to both the Met-44 and Met-47 residues on F-actin. Mical oxidizes these two residues stereospecifically in the R conformation and generates actin Met-44-R-sulfoxide and Met-47-R-sulfoxide (actin$^{Met(R)O-44}$ $^{\&}$ ^{47}) [Hung et al., 2011, 2013]. SelR/MsrB family proteins specifically reduce MICAL-oxidized actin [Hung et al., 2013; Lee et al., 2013]. **C**: Model of the interaction between MICAL and F-actin. The model suggests a possible mechanism by which the active site of Mical gains access to the poorly accessible Met-44 residue, which is buried within F-actin. In the model (based on published structures; PDB IDs are 2BRY, 2ZWH; [Nadella et al., 2005; Siebold et al., 2005], rotating the Mical structure by 90° (from the top of the page down) provides one mechanim to envision how the active site (small arrow) of Mical accesses (large arrow) the Met-44 (M44) residue – and as illustrated in the inset. **D**: Model of Mical Redox-mediated F-actin disassembly and cellular remodeling. Based on published data [Hung et al., 2010a, 2011, 2013], Semaphorin/Plexin association induces a conformational change in Plexin to allow Mical to bind to Plexin. Binding to Plexin activates Mical to associate with F-actin and oxidize the Met-44 and Met-47 residues of F-actin. Mical-mediated oxidation of F-actin induces actin filament disassembly. SelR/MsrB family proteins counteract Mical's effects on F-actin disasembly and cellular remodeling.

additional family of conserved proteins, the MICAL-Likes, have also been indentified that are similar to MICALs but lack the N-terminal Redox enzymatic region [Terman et al., 2002]. The Met-44 and Met-47 residues that the MICALs oxidize are also conserved in all actins from invertebrates to humans, indicating that MICALs are likely to play prominent roles in modulating actin dynamics in both neuronal and non-neuronal tissues. Indeed, expression analysis reveals that MICALs exhibit widespread expression in both neuronal and non-neuronal tissues. For example, collectively, the *MICALs* appear to be ubiquitously expressed including being found in (among other tissues) brain, skeletal muscle, heart, fibroblasts, lung, kidney, bone marrow, thymus, spleen, liver, and testis – as well as in various cell lines and cancer cell lines [reviewed in Hung and Terman, 2011; Zhou et al., 2011b; Giridharan and Caplan, 2014]. More specifically, for instance, they are also collectively expressed in most, if not all areas of the central and peripheral (CNS and PNS) nervous systems [reviewed in Hung and Terman, 2011; Zhou et al., 2011b; Giridharan and Caplan, 2014 and see also Lein et al., 2007; Hawrylycz et al., 2012; Miller et al., 2014]. Functional studies have also gone on to reveal prominent localization of MICALs with F-actin structures and important roles for MICALs in regulating multiple neuronal and non-neuronal actin dependent events. For instance, multiple different cell culture assays using primary cells and cell lines including neurons/neuronal-like cells (dorsal root ganglion neurons, hippocampal neurons, PC12 cells), fibroblasts (mouse embryonic fibroblasts [MEFs], NIH 3T3 cells), endothelial cells (rat brain capillary endothelial [RBEC] cells), kidney cells (podocytes, HEK 293 cells, COS-7 cells), and cancer cell lines (HeLa cells, 786-O kidney cancer cells, MERO-14 pleural cancer cells) have been used to characterize the effects of MICALs on F-actin dynamics and morphology [Schmidt et al., 2008; Grigoriev et al., 2011; Hung et al., 2011; Morinaka et al., 2011; Giridharan et al., 2012; Hung et al., 2013; Lee et al., 2013; Lundquist et al., 2014; Van Battum et al., 2014; Aggarwal et al., 2015; Hou et al., 2015; Mariotti et al., 2016]. Likewise, both genetic and knockdown experiments in invertebrates and mammals indicate important roles for MICALs in non-neuronal functions including cell viability [Ashida et al., 2006; Zhou et al., 2011a; Loria et al., 2015; Mariotti et al., 2016], skeletal muscle morphology and function [Beuchle et al., 2007; Hung et al., 2013], immunity [Lee et al., 2013], podocyte (kidney) cell shape and function [Aggarwal et al., 2015], and cardiovascular integrity [Lundquist et al., 2014; Hou et al., 2015]. Multiple different cellular, knockdown, and genetic experiments from invertebrates to mammals have also characterized roles for MICALs in a range of neuronal functions including axon growth, extension, and guidance [Terman et al., 2002; Schmidt et al., 2008; Hung et al., 2010a, 2013; Morinaka et al., 2011; Lundquist et al., 2014; Van Battum et al., 2014], synapse development [Beuchle et al.,

2007; Hung et al., 2013; Van Battum et al., 2014], dendrite morphology [Kirilly et al., 2009; Rumpf et al., 2014], mechanosensory organ formation [Hung et al., 2010a, 2011, 2013], neuronal cell migration and positioning [Bron et al., 2007], and vesicle trafficking/fusion at the plasma membrane [Grigoriev et al., 2011; Van Battum et al., 2014; Bachmann-Gagescu et al., 2015] (Fig. 5).

An emerging body of work, therefore, is beginning to characterize the attributes of MICAL family Redox enzymes and these studies have revealed critical functions for these Redox enzymes in multiple different neuronal and non-neuronal physiological events. Yet, an extensive number of questions remain to be answered regarding the MICALs. For example, the different MICALs are heavily alternatively spliced [Hung and Terman 2011; Terman et al., 2002], so it is possible that different splice forms may dictate their localization and subcellular functions. In this regard, MICALs have been best characterized for their role in the cytoplasm, but MICAL splice variants have also been found to have functions in regulating the organization of actin in the nucleus [Lundquist et al., 2014]. Likewise, MICALs were first functionally characterized as operating downstream of Semaphorin/Plexin repulsive signaling [Aggarwal et al., 2015; Hou et al., 2015; Hung et al., 2010a; Schmidt et al., 2008; Terman et al., 2002], but a growing body of data have also provided links between MICAL and growth factors such as nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) [Ashida et al., 2006; Hou et al., 2015; Lundquist et al., 2014], as well as cell adhesion molecules [Van Battum et al., 2014]. In a related way, emerging evidence has begun to link MICAL with multiple neuronal and non-neuronal diseases and abnormalities including cancer [Ashida et al., 2006; Ho et al., 2012; Loria et al., 2015; Lundquist et al., 2014; Mariotti et al., 2016], diabetic nephropathy [Aggarwal et al., 2015], blood brain barrier dysfunction [Hou et al., 2015], muscular dystrophy [Marotta et al., 2009], liver disease [Chambers et al., 2011], prion susceptibility [Marbiah et al., 2014], epilepsy [Luo et al., 2011], Alzheimer's disease [Muller et al., 2007], aging [Aenlle et al., 2009], skeletal anomalies [Bredrup et al., 2011], and obesity [Li et al., 2013]. Likewise, dominant de novo mutations of MICALs substrate residues on actin, the Met-44 and Met-47 residues, have been characterized in both skeletal muscle and smooth muscle actins in human patients and they lead to actin accumulation defects that resemble the effects of mutating MICALs, as well as muscular dystrophies and vasculature disorders [Hoffjan et al., 2011; Hung et al., 2010b; Laing et al., 2009; Regalado et al., 2014]. Furthermore, disruptions to the SelR/MsrB enzymes that reverse MICAL-mediated effects on actin dynamics have also been linked to neurodegenerative disorders, aging processes, and deafness (e.g., [Ahmed et al., 2011; Kim and Gladyshev 2007; Kim et al., 2016]). It is also possible that MICALs may have other direct substrates
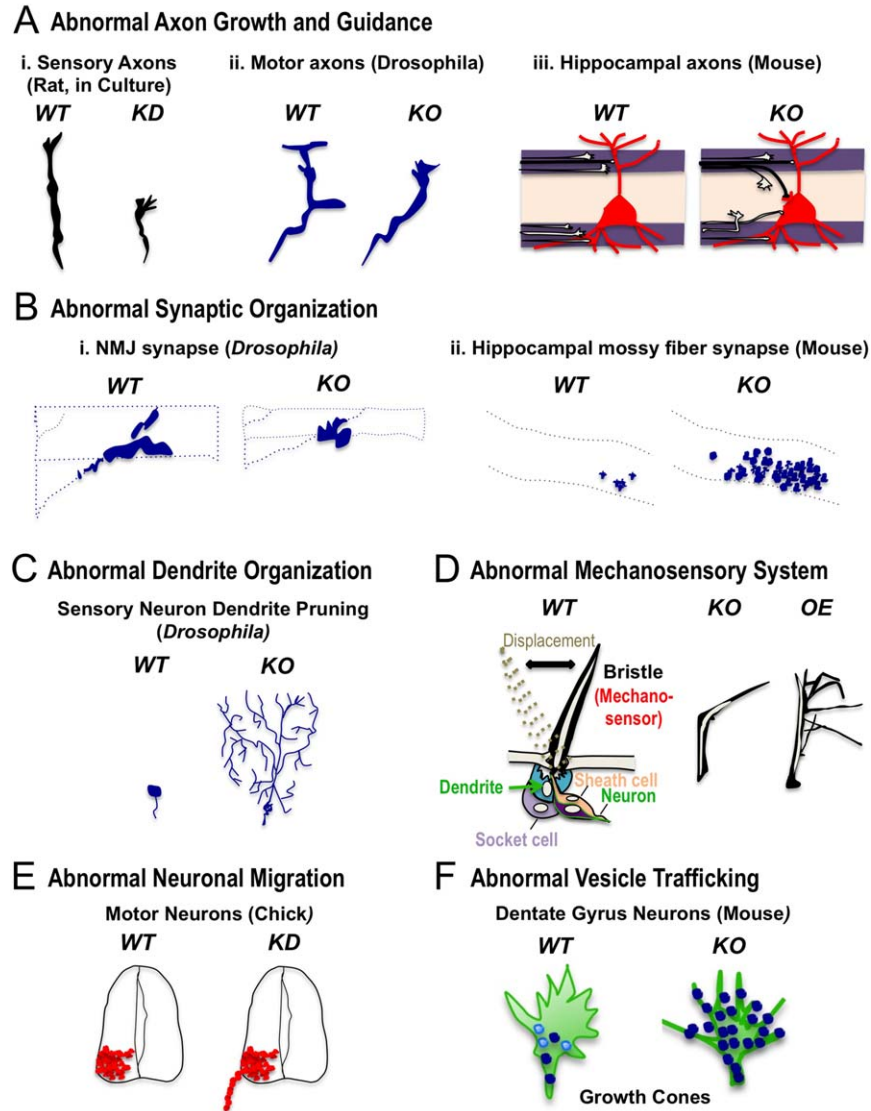
Fig. 5. MICAL family Redox Enzymes are Essential for the Proper Form and Function of the Nervous System. In A-F, WT designates *wild-type*, KD designates *MICAL* knockdown, KO designates *Mical* mutant or *MICAL* knockout, and *OE* designates increased Mical expression. These defects and many in non-neuronal cells and tissues (see main text) are consistent with MICALs role in disassembling F-actin – including resulting in the presence of more stable F-actin in response to decreasing *MICAL* function, and less stable/disassembled F-actin in response to increasing *MICAL* function. **A**: MICALs are required for proper axon growth and guidance. **i.** Loss of *MICAL* (*KD*, *MICAL-2* shRNA-mediated knockdown) in dorsal root ganglia (DRG) neurons decreases their rate of axon growth [Lundquist et al., 2014]. **ii.** Loss of *Mical* (*KO*, Drosophila *Mical$^{-/-}$* mutants) results in defective motor and CNS (not shown; [Hung et al., 2010a]) axon guidance in vivo [Terman et al., 2002]. **iii.** Loss of *MICAL* (*KO*, Mouse *MICAL-1$^{-/-}$* knockout mutants) results in defective hippocampal mossy fiber axon projections in vivo [Van Battum et al., 2014]. **B**: MICALs are essential for proper synaptic organization. **i.** Loss of *Mical* (*KO*, Drosophila *Mical$^{-/-}$* mutants) results in shorter and condensed synapses at nerve-muscle synapses (neuromuscular junction [NMJ]) in vivo [Beuchle et al., 2007]. **ii.** Loss of *MICAL* (*KO*, Mouse *MICAL-1$^{-/-}$* knockout mutants) generates abnormal (ectopic) synapses in the hippocampus in vivo [Van Battum et al., 2014]. **C**: MICALs are required for proper dendrite organization, as depicted here where a loss of *Mical* (*KO*, Drosophila *Mical$^{-/-}$* mutants) prevents the pruning and disassembly of dendrites that normally occurs in vivo [Kirilly et al., 2009]. **D**: MICALs are required for proper formation of mechanosensory organs, as depicted here where a loss of *Mical* (*KO*, Drosophila *Mical$^{-/-}$* mutants) generates mechanosensors (bristles) that do not form normally in vivo and contain more F-actin [Hung et al., 2010a]. Increasing the levels of *Mical* in bristles (*OE*) also alters their morphology in a different manner and results in loss of F-actin [Hung et al., 2010a]. **E**: MICALs are required for proper migration of neurons, as depicted here where a knockdown of *MICAL* (*KD*, *MICAL-3* shRNA-mediated knockdown) generates abnormally positioned motor neuron cell bodies in vivo [Bron et al., 2007]. **F**: MICALs are required for proper trafficking of vesicles in neurons, as depicted here where a loss of *MICAL* (*KO*, Mouse *MICAL-1$^{-/-}$* knockout mutants) generates an abberant accumulation and distribution of vesicles to abnormal F-actin rich regions in the periphery of axonal growth cones [Van Battum et al., 2014].

besides F-actin (i.e., proteins that activate the NADPH consumption activity of MICAL and are directly targeted by MICAL) — but in any case, it is interesing to consider that MICALs may also have indirect substrates based on their ability to release oxidants such as H_2O_2 during the course of their enzyme reaction [reviewed in Hung and Terman 2011]. For example, while MICAL does not release diffusible oxidants such as H_2O_2 to disassemble F-actin (as discussed above; [Hung et al., 2011; Hung et al., 2013; Hung et al., 2010a]), it is possible that in the course of directly oxidizing actin, MICAL might also influence the function of other nearby proteins through the release of difussible oxidants, such as has recently been reported for MICAL and the protein CRMP [Morinaka et al., 2011]. Finally, as mentioned above, the Redox susceptibility of Met-44 and Met-47 varies depending on actin's conformation, ionic state, and polymerization condition [Dalle-Donne et al., 2002; Guan et al., 2003; Guan et al., 2005; Takamoto et al., 2007; Hung et al., 2011], so it is possible that particular in vivo conditions and disease states may make these residues susceptible to abnormal Redox regulation. In particular, the identification of this new MICAL/SelR oxidant-based physiological signaling system raises the possibility that certain pathologies and traumas allow oxidants abnormal access to actin where they nonspecifically work like MICALs and aberrantly oxidize Met-44 and/or Met-47 to pathologically disrupt F-actin-based cellular functions and behaviors. In this regard, it is interesting that Met-44, as well as Met-47, show increased oxidation in vivo in response to oxidative stress [Fedorova et al., 2010a; Ghesquiere et al., 2011]. Collectively, therefore, the identification of MICAL and its reversible Redox actin regulatory system provides an unusual new system that directs cellular form and function with multiple implications for biomedicine.

Conclusions

The actin cytoskeleton is critical for many, if not all aspects of cellular behaviors and so a critical goal is to understand the factors that specify its organization and dynamic properties. It is now becoming clear that Redox-mediated effects are an important means to regulate actin dynamics and multiple different actin-dependent cellular functions. Yet, it is also clear that the Redox regulation of actin involves a diverse set of modifications – and these alter different residues on actin with distinct behavioral effects. Likewise, it has also become clear that there are different types of actin Redox effectors, and that they use different Redox species – and we describe herein the different mechanisms used by NADPH oxidases and MICAL flavoprotein monooxygenases, and the different residues on actin that they target. Thus, while exciting discoveries have been made, much remains to be investigated regarding the Redox regulation of actin and the action of the different Redox effectors. For

example, it is still incompletely understood where and when the different Redox effectors of actin are acting. Likewise, it is poorly understood how the Redox-regulation of actin fits in with more classically studied actin regulatory proteins. Similarly, whether interactions exist among the different actin Redox effectors such as NADPH oxidase and MICAL is unknown—as are potential interactions with other Redox effectors that have been linked to actin regulation such as NOS and lipoxygenases. The range of specific celluar signaling pathways that impinge on these Redox effectors is also poorly understood, but is a critical step in our understanding of the Redox-mediated regulation of the actin cytoskeleton. Indeed, given the extensive links between oxidative conditions, Redox-related post-translational alterations to actin, and multiple diseases and pathological disorders within and without the nervous system that are associated with Redox-related modifications to actin including, among others, aging, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), cardiovascular disease, Chagas' disease, chronic obstructive pulmonary disease (COPD), chronic pain, diabetes, experimental autoimmune encephalomyelitis, Friedreich's ataxia, inflammatory bowel disease, ischemia, macular degeneration, pulmonary tuberculosis, and sickle cell anemia/disease [reviewed in Terman and Kashina, 2013], it is a critical biomedical goal to understand the Redox regulation of actin.

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