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## Redox regulation of the actin cytoskeleton and its role in the vascular system

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### Abstract

The actin cytoskeleton is critical for form and function of vascular cells, serving mechanical, organizational and signaling roles. Because many cytoskeletal proteins are sensitive to reactive oxygen species, redox regulation has emerged as a pivotal modulator of the actin cytoskeleton and its associated proteins. Here, we summarize work implicating oxidants in altering actin cytoskeletal proteins and focus on how these alterations affect cell migration, proliferation and contraction of vascular cells. Finally, we discuss the role of oxidative modification of the actin cytoskeleton in vivo and highlight its importance for vascular diseases.

### Keywords

Redox regulation; Actin cytoskeleton; Vascular disease

## INTRODUCTION

The cytoskeleton serves mechanical, organizational, and signaling functions within the cell [1, 2]. Because of its multiple roles, perturbation of the cytoskeleton is important in vascular physiology and pathophysiology [3–7]. Oxidation of cytoskeletal proteins has been observed in various cellular processes and vascular diseases [8–11], but our understanding of the role of these oxidative modifications is far from complete. Determining how oxidation of these proteins alters their function may provide a new perspective on vascular disease progression. Accordingly, this review presents an overview of our current knowledge of how actin cytoskeleton proteins are regulated by oxidative species and the potential role of cytoskeletal oxidation in the vascular system.

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## ACTIN CYTOSKELETON STRUCTURE

Actin is a family of abundant and highly conserved cytoskeletal proteins that are expressed in nearly all eukaryotic cells and act as a scaffold to maintain cell shape and internal organization [1]. In humans, three classes of actin isoforms have been identified in different tissues: alpha ( $\alpha$ )-actin (muscle variants, contractile structures), beta ( $\beta$ )-actin (nonmuscle variants, meshworks) and gamma ( $\gamma$ )-actin (smooth muscle and nonmuscle variants, stress fibers) [12, 13]. Monomeric actin has a globular shape (G-actin), which strongly binds to adenosine di- or tri-phosphate and divalent cations such as  $Mg^{2+}$  or  $Ca^{2+}$  (ATP/ADP-G-actin). In vivo,  $Mg^{2+}$  is usually bound to actin; however,  $Ca^{2+}$  binding is observed frequently upon actin isolation [14]. ATP-G-actin with concomitant ATP hydrolysis promotes filamentous-actin (F-actin) assembly [15] into microfilaments in the cytoskeleton and thin filaments in the contractile apparatus [13]. Actin filaments are dynamic, with nucleation, polymerization, branching, and crosslinking highly regulated by extra- and intracellular signal-mediated modification of actin itself and actin binding proteins. These processes play a crucial role in cell shape, movement, and proliferation as well as contraction [1, 16].

## THE ACTIN CYTOSKELETON AS A TARGET OF OXIDANTS

### Source and types of oxidative species in vascular cells

Redox signaling regulates physiological and pathological responses at different levels ranging from cells to tissues to biological systems. Many reactive oxygen species (ROS), including superoxide ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $HO\bullet$ ), as well as reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ( $ONOO^-$ ), are produced in biological systems [17]. Among these,  $O_2^{\bullet-}$  and  $H_2O_2$  are important signaling molecules in all kinds of vascular cells, including endothelial cells, smooth muscle cells, fibroblasts and perivascular adipocytes [10, 18].

Intracellular oxidative species can be formed by many different enzymes, of which mitochondrial respiratory enzymes and NADPH oxidases (NOXes) are major sources [19, 20]. Almost 95% of  $O_2^{\bullet-}$  is generated from mitochondria by electron transport chain complexes [21]. This non-specific production of  $O_2^{\bullet-}$  is generally scavenged by superoxide dismutase (SOD), which converts it to  $H_2O_2$ , a relatively stable molecule that reacts with proteins [21, 22] and contributes to the constitutive intracellular redox environment. In contrast, NOX family proteins are transmembrane enzymes whose major function is to transfer electrons from NADPH to oxygen to produce  $O_2^{\bullet-}$  in localized intra- or extracellular compartments. Although  $O_2^{\bullet-}$  does not penetrate the plasma membrane or the membrane of the intracellular organelle in which it is produced, once it is converted into  $H_2O_2$  it can cross membranes and function as a signaling molecule [19]. The NOX family contains five NOXes (NOX1 to NOX5) and two dual oxidases (DUOX 1 and DUOX2). NOX1, NOX2, NOX4 and NOX5 are expressed and active in the vascular system. NOX1, NOX2 and NOX4 require a second transmembrane subunit, p22phox, and DUOXes require DUOX activator1/2, for activity [23, 24]. In addition, cytosolic regulatory partners are important in NOX maturation and expression. NOX1 interacts with NOXO1 or p47phox and NOXA1 as well as Rac; NOX2 is regulated by p47phox and p67phox; and NOX4 is constitutively active but can be regulated by poldip2 [19]. NOX5 and DUOXes are

specifically activated by calcium [23, 25]. These regulatory mechanisms ensure tight temporal and spatial control of ROS release. While all NOX enzymes directly produce  $O_2^{\bullet-}$ ,  $O_2^{\bullet-}$  is dismutated to  $H_2O_2$  before release from NOX4 or by SOD in the case of the other NOXes. Degradation of  $H_2O_2$  is controlled by peroxidases such as peroxiredoxins, catalase and glutathione peroxidase, an essential step in the regulation of signaling. Both  $O_2^{\bullet-}$  and  $H_2O_2$  have been implicated in oxidative modification of DNA, lipids and proteins [26], thus altering cellular functions in both physiological and pathological conditions [11, 17, 27].

### Mechanisms of protein oxidation

Proteins are the most common signaling targets of ROS/RNS due to their many redox-sensitive sites [28, 29]. Oxidation of proteins can be classified into two types: reversible and irreversible [30, 31]. Cysteine (Cys) and methionine (Met) residues on proteins are typical targets of reversible oxidative modification because of their low pKa thiols that react with oxidative species [32–34]. Reversible oxidation occurs in the presence of ROS such as  $H_2O_2$ ,  $O_2^{\bullet-}$ , and RNS like peroxynitrite [35, 36], initially producing sulfenic acid residues (-SOH) that can then react with glutathione (GSH) or other sulfhydryl groups to form glutathione disulfide (GSSG), intra- or extra-molecular disulfide bonds (RS-SR'), and S-glutathionylated proteins (R-SSG) [36–39] (Figure 1). S-nitrosylation (SNO) is another important reversible oxidative modification of cysteines induced by nitric oxide, nitroxy, and peroxynitrite [40] (Figure 1). S-nitrosylation in particular plays a role in integrin-dependent cell adhesion [41], cytoskeleton remodeling and cell migration [42, 43] as well as cardio protection [44–46]. These reversible modifications have been considered to play multiple beneficial roles in cellular physiological processes and may protect target proteins from further irreversible and perpetual damage [47–52].

However, excess production of ROS may convert -SOH to sulfinic acid ( $SO_2H$ ) and sulfonic acid ( $SO_3H$ ) derivatives, which are irreversible and may affect protein-protein interaction and function [48, 53–55] (Figure 1). Other irreversible oxidative modifications include carbonylation (aldehyde and ketone derivatives) and tyrosine nitration (3-nitrotyrosine) [56–59], which result in permanent configuration changes and affect functionality of target proteins. Irreversible modifications are generally associated with oxidative damage of proteins and are considered as biomarkers of oxidative stress in pathological processes [60–63].

A great number of signaling molecules, including receptor tyrosine kinases, phosphatases, and transcription factors such as NF- $\kappa$ B (nuclear factor- $\kappa$ B), AP-1 (activator protein-1) and Hic-5 (hydrogen peroxide-inducible clone-5), have been shown to contain redox-sensitive cysteine residues [64–66]. Oxidative modification of these molecules alters activity, interaction with other proteins, and/or subcellular localization, which in turn affects downstream signaling. Since many cytoskeletal-associated proteins are particularly sensitive to redox regulation [8], in the following paragraphs we will focus on those cytoskeletal proteins that have been directly shown to be redox-regulated, and discuss their impact on basic cell behaviors such as adhesion, migration, proliferation and contraction.

## Oxidation of actin

Actin itself is susceptible to oxidation and the effects of ROS/RNS on actin function have been extensively studied for almost 70 years [67–70]. As recently reviewed [67], the six cysteines in  $\beta/\gamma$ -actin and five cysteines in  $\alpha$ -actin can all be oxidized, with Cys374 being the most redox-sensitive. Details on the oxidation of each residue are given in Table 1; however, in general, oxidation is thought to slow polymerization/elongation of G-actin [71, 72] and make F-actin more fragile [73]. Still, it should be noted that some of these studies were designed with high concentrations of oxidants (e.g., mM  $H_2O_2$ ) instead of physiological levels (e.g., nM- $\mu$ M  $H_2O_2$  [74]). Recent work suggests that in some systems, low, physiologically relevant ROS production can promote actin polymerization and formation of stress fibers [69, 75, 76].

In addition to cysteine oxidation, actin is also directly oxidized on Met44 and Met47 [77]. This oxidation is mediated by a family of proteins called MICALs (Molecule Interacting with CasL), which include a monooxygenase domain to promote the conversion of methione to methionine sulfoxide [77]. Oxidation by MICAL causes decreased inter-actin contacts leading to F-actin disassembly. The resulting actin monomers are slower to reassemble and fragment more easily once reassembled. Additionally, MICAL oxidation of actin enhances cofilin binding to F-actin. The severing function of cofilin and modified actin interactions work synergistically to promote formation of monomeric actin upon MICAL-driven oxidation [78]. It appears that Met44 oxidation is the key residue for this actin disassembly, because Met44Leu actin mutants are resistant to MICAL-induced disassembly, while Met47Leu mutants are not [77].

Beyond the specific oxidized residues and oxidizing agents (Table 1), there are numerous other variables that determine how actin responds ROS/RNS and which residues are susceptible (Table 2). For example, although Cys17 is not very reactive, when actin is bound to myosin II it becomes more susceptible to oxidation [79]. Alternatively, G-actin contains sites of oxidation that are masked by polymerization of actin [80]. Cations (for example  $Ca^{2+}$  influx) can also determine if actin is oxidized and may modify the impact of this oxidation [67, 70, 81]. These complexities continue to be investigated, and suggest that the effect of actin oxidation is specific to the cell type, the intracellular redox environment and ongoing cellular processes.

The consequences of actin oxidation vary by the oxidative modification and the cell type, but have not been studied extensively. In cardiomyocytes, S-nitrosylation of  $\alpha$ -actin correlates with enhanced relaxation and impaired contraction [82]. In endothelial cells, oxidation of actin appears to be essential for proper cell migration, because pharmacologic reduction of ROS impairs endothelial cell migration and actin polymerization [76].  $\beta$ -actin S-glutathionylation causes the disassembly of the actin-myosin complex, which is a necessary step for successful cell adhesion and migration [69]. Additionally, oxidation of  $\beta$ -actin via the formation of a mixed disulfide bond between Cys374 and glutathione is necessary for cell spreading in response to integrin engagement [69]. To our knowledge, the effect of actin oxidation on mitosis/cytokinesis has not been directly investigated. Depletion of MICAL in developing zebrafish embryos results in small heart size and cardiomyocytes with altered morphology and decreased expression of cardiac muscle specific genes [83]. Knockdown of

MICAL can also lead to decreased cell viability [84] and increased apoptosis [85], suggesting that Met44 oxidation may regulate these functions as well. Indeed, Met44Cys actin is unable to form colonies in yeast [86] and, oxidation of actin on Cys374 (and Cys285) promotes actin aggregation, which delays yeast cell cycle reentry [9].

### Oxidation of actin binding proteins

Actin binding proteins are not only involved in regulating polymerization and depolymerization of actin, but also mediate its physiological functions. While there are many actin binding proteins, to our knowledge only two have been shown to be directly oxidized in the context of the cardiovascular system: myosin II and cofilin.

**Myosin II oxidation**—Myosin II is a motor protein that generates force by stepping along actin as it hydrolyzes ATP to ADP [87]. It can be divided into two classes: non-muscle (NM) myosin II and muscle myosin II. Non-muscle (NM) myosin II is expressed in almost all cell types [88] and is involved in diverse cellular process such as cell adhesion, migration, and division. In the context of cell division, the force generated from filaments made of NM-myosin II multimers interacting with F-actin is required for contractile ring formation during cytokinesis [89, 90]. Additionally, NM-myosin II enables cell rounding at the onset of mitosis and correct mitotic spindle positioning [91, 92]. During migration, NM-myosin II helps establish cell polarity and is involved in focal adhesion maturation and disassembly [87]. NM-MyosinII-actin rearrangement is also associated with endothelial cell permeability and barrier dysfunction [93]. In muscle cells, such as striated, cardiomyocytes and vascular smooth muscle cells (VSMCs), both muscle- and NM-myosin II are expressed [87]. Muscle myosin II plays a critical role in cell contraction. In this process, muscle myosin II reversibly binds to actin filaments and converts chemical signals into mechanical force and movement [94, 95].

Both NM- and muscle- myosin II have been proposed to be redox sensors within cells, because both are reversibly oxidized and this oxidation inhibits their interaction with actin and their ATPase activity [96]. This regulatory oxidation occurs at Met394 of protist NM-myosin II [97], which is equivalent to a cysteine that is glutathylated in mammalian cardiac and skeletal muscle myosin II [96]. However, Fiaschi and colleagues [98], who examined myosin oxidation in the context of cell spreading, suggested that only NM-MHC is under redox control. Using mass spectrometry, they showed that NM-myosin II is in a reduced form in detached rounded cells, while cells in the process of attaching and spreading contain oxidized NM-myosin II, which correlates with its interaction with actin. NM-myosin II and actin interact strongly in rounded cells or spread cells when ROS generation is inhibited, but not in control spread cells [98], suggesting that oxidation of NM-myosin II could be a physiologically relevant method of controlling the interaction of NM-myosin II and actin. Further proof of the consequence of direct NM-myosin II oxidation will require creation of oxidation-insensitive mutants of NM-myosin II.

It should be noted that myosin II is composed of 2 heavy chain molecules (MHC), each with a head that binds to actin and ATP, a neck that is wrapped in myosin light chains (MLC), and a tail where the heavy chain molecules bind to each other. The activity of myosin II is

regulated by MLC and a variety of kinases and phosphatases that are also involved in cell division and migration; for example, Rho kinase (ROCK), citron kinase, and myosin light chain kinase (MLCK) phosphorylate MLC and activate myosin II, while myosin phosphatase dephosphorylates MLC and inactivates myosin II [91]. As discussed below, MLC is indirectly regulated by oxidation-sensitive signaling cascades; however, the direct oxidation described above only occurs on MHC.

**Cofilin oxidation**—Cofilin is an actin binding protein that regulates actin dynamics by severing F-actin filaments and promoting F-actin depolymerization [99]. This promotes actin rearrangement by breaking down old F-actin filaments and generating more G-actin and barbed ends, the ingredients necessary for polymerization of new actin filaments [100] during cell migration (reviewed in [101]) and contraction [102]. The reorganization of actin by cofilin is also important during cell proliferation, as overexpression/mutation of cofilin leads to accumulation of cells in the G1-phase of the cell cycle [103], incorrect mitotic spindle positioning [104], and cytokinesis defects [105]. As described above, actin oxidation by MICAL can enhance cofilin binding. However, cofilin itself can also be directly oxidized. Cofilin oxidation increases the F-actin:G-actin ratio in the cell by preventing cofilin from severing actin [106–109], but multiple mechanisms have been described for how this occurs. Some studies suggest that cofilin oxidation prevents cofilin binding to actin [106, 108, 109], while others indicate that cofilin still binds actin but does not induce depolymerization when oxidized [107] and can even cause formation of rod-shaped actin/cofilin aggregates [110]. Phosphorylation of serine 3 is known to inhibit the interaction of cofilin with actin as well [99], but it is currently under debate if this site is phosphorylated when cofilin is oxidized [108] or if oxidation promotes dephosphorylation [106, 107].

There are four cysteines (Cys39, Cys80, Cys139, Cys147) and a methionine (Met115), that can be oxidized in cofilin. However, there is disagreement about whether specific cysteines [107, 109, 110], all cysteines [108], or methionine [106] residues are required for cofilin inhibition and if intra- [107, 108] or intermolecular [110] disulfide bonds are formed upon oxidation. These details likely vary based on stimulus and cellular conditions. Cofilin oxidation during cell division has not been studied; however, cofilin oxidation can induce apoptosis. Oxidized and Ser3-phosphorylated cofilin localizes to mitochondria where it promotes opening of the permeability transition pore to allow cytochrome C release and apoptosis onset [108]. It has also been proposed that ROS generated at the leading edge of a migrating cell cause cofilin oxidation on Cys139 and Cys147 to inhibit cofilin:actin binding and consequently promote actin polymerization toward the front of the cell to help it migrate [109].

### **Oxidation of proteins in signaling cascades that regulate the actin cytoskeleton**

Actin cytoskeletal signaling networks are composed of a large group of proteins such as integrins, small GTPases, kinases, phosphatases, ion channels and transporters. These proteins not only directly regulate actin assembly, organization and function in response to different cellular stimuli, but also instigate signaling cascades and networks that influence cell growth, migration, contraction and survival. ROS can alter actin cytoskeletal signaling by directly oxidizing these regulatory proteins, by influencing upstream molecules that then

affect these downstream targets, or, as with integrin regulation, by modulating protein or mRNA expression.

**Integrin oxidation**—Integrins are a family of cell-cell and cell-matrix binding transmembrane adhesion molecules that mediate firm contacts of cells with the extracellular matrix (ECM) [111, 112]. They are among the most abundant cell surface receptors and are expressed in all types of cells [113]. In mammalian cells, the integrin receptor family contains 18  $\alpha$  and 8  $\beta$  subunits that link non-covalently in at least 24 known combinations of  $\alpha\beta$  subunit heterodimers [114, 115]. Integrin ligation induces promigratory intracellular signaling cascades from the “outside in”. On the other hand, activation from “inside out” also occurs by virtue of soluble signals promoting protein and inositol phosphorylation events that regulate formation of nascent focal contacts. Integrins cluster on the cell surface in focal adhesion complexes containing signaling adapter molecules and cytoskeletal structures. Through integrating the insoluble ECM with the intracellular cytoskeleton, integrins transduce mechanical signals that influence gene expression.

There is convincing evidence that integrins are redox-regulated [116–120]. Conformational changes in the integrin structure suggest a mechanism for this redox-regulation of integrin activity. Cysteine residues within the genu domain and calf-2 domain of the integrin  $\alpha$ -subunit have been considered to be the redox-regulated sites within the integrin ectodomain [121]. For example, integrin  $\alpha 7\beta 1$  is oxidized by  $H_2O_2$  derived from NOX4 at two specific cysteine residues [121]. The cysteine residues in each EGF-domain of the  $\beta$  integrin subunit, which stabilize the stalk domains [122–125], do not appear to be oxidized by  $H_2O_2$  [121]. Overall, redox-dependent cleavage of the  $\alpha$ -subunit influences the transition between a bent/inactive and extended/active conformation of the integrin ectodomain [121]. These conformational transitions induce the physical separation of the two stalks and lead to changes within the transmembrane and cytoplasmic domains [126, 127], which transduces a signal to integrin-associated molecules [112, 128, 129].

In addition to direct oxidative modifications, integrins are also indirectly affected by ROS-mediated regulation of their expression. In liver sinusoidal endothelial cells exposed to prolonged high glucose, integrin  $\alpha v\beta 3$  and laminin expression are upregulated, and this response is significantly inhibited by N-acetyl-cysteine (NAC), suggesting that the expression of  $\alpha v\beta 3$  is dependent on ROS levels in these cells [130]. In addition, ROS increase  $\alpha v$  and decrease  $\alpha 5$  expression in endothelial cells, affecting adhesion to vitronectin or fibronectin [131]. In VSMCs, downregulation of the Nox4 activator Poldip2 increases  $\beta 1$  integrin expression, leading to increased collagen I expression [132].

**Rho family GTPase oxidation**—Rho family GTPases act as molecular switches, “on” while GTP-bound and “off” while GDP-bound, whose primary function within the cell is to regulate the actin cytoskeleton [133, 134]. Of these GTPases, RhoA, Rac1, and Cdc42 are the best studied, and all three are required for cell migration and proliferation (Figures 2 and 4). During cell division, RhoA enhancement of linear actin polymerization and myosin II activation is essential for cell rounding and contractile ring ingression, and it is equally important that the opposing functions of Rac1 (actin branching and myosin II inactivation) are turned off during these processes (Figure 4) [92, 135]. Rho GTPases are also central

regulators of cell migration. Their complex role in this process has been recently reviewed [136], but in brief, Rac1 and Cdc42 generate cell protrusions, while RhoA generates the force necessary to move. This activity is tightly regulated by a number of proteins. Guanine nucleotide exchange factors (GEFs) activate GTPases by promoting the release of nucleotide from GTPases. Since GTP is ten times more abundant than GDP within the cell, nucleotide release promotes GTP binding and enhances downstream effector binding. GTPase activating proteins (GAPs) catalyze GTP hydrolysis to GDP to turn the GTPase “off.” Post-translational modifications also regulate Rho GTPase activity. Prenylation of the C-terminus of the GTPase is required for membrane localization and Guanine Nucleotide Dissociation Inhibitors (GDIs) bind to the prenyl group to stabilize the GTPase and prevent membrane association. Phosphorylation can activate and inactivate specific Rho GTPases (reviewed in [137]). Similarly, oxidation can both enhance and inhibit Rho GTPase activity, as detailed below.

The Rho family of GTPases consists of 20 proteins; about half of which contain a conserved redox sensitive motif GXXXXGK(S/T)C, including a cysteine that is solvent-exposed and in contact with bound nucleotide [138]. In vitro,  $O_2^{\bullet-}$ , nitrogen dioxide ( $\bullet NO_2$ ), or  $HO\bullet$  treatment enhances GDP dissociation from RhoA, Rac1, and Cdc42 400- to 600-fold, while NO or  $H_2O_2$  treatment enhances exchange approximately 5- and 10-fold, respectively [138]. Addition of radical scavengers allows re-binding of the nucleotide [138]. This nucleotide release is likely due to disruption of hydrogen bonds between the GTPase and nucleotide. Therefore, in cellulo, where the reducing potential is high and GTP is abundant, ROS/RNS should promote release of bound nucleotide, similar to a GEF, to activate Rho GTPases [139].

Such a scenario is the case for Rac1, which has been shown to be activated by ROS/RNS both in vitro [138, 140] and in cellulo [139–142]. Early work by Heo and colleagues [138] showed that the dramatic increase in Rac1 nucleotide dissociation induced by  $O_2^{\bullet-}$  in vitro has no effect on a Rac1 C18S mutant, suggesting that activation of Rac1 is due to direct oxidation at Cys18. Upon oxidation of cysteine thiols to sulfenic acid, cysteines become highly reactive with glutathione and S-glutathiolation is commonly observed [143]. Therefore, it is not surprising that in cellulo Rac1 is glutathiolated, and that mass spectrometry analysis of purified Rac1 in the presence of oxidized glutathione suggests that this occurs at Cys18 [140]. Purified glutathiolated Rac1 has a 200-fold enhanced rate of nucleotide exchange compared to non-oxidized Rac1 [140].

Unlike Rac1 and Cdc42, RhoA has an additional cysteine (Cys16) in its redox sensitive motif, GXXXXCGK(S/T)C, that complicates GTPase activation [138, 144]. Upon  $\bullet NO_2$  treatment (3–5  $\mu M$ ), oxidation of the GTPase can be observed by mass spectrometry as a disulfide bond formed between Cys16 and Cys20 [144]. This disulfide bond can only form when the GTPase is nucleotide-free; however, once the disulfide bond has formed, RhoA is unable to bind nucleotides or interact with the GEF, Vav2 [144]. Therefore, it has been proposed that at low levels of ROS, Cys20 is oxidized which promotes nucleotide exchange to activate RhoA; however, at higher levels of ROS disulfide bonds form between Cys16 and Cys20 to inactivate RhoA [144]. Consistent with the joint interaction of Cys16 and Cys20 reducing RhoA activity, treatment of RhoA with phenylarsine oxide (PAO), which crosslinks



neighboring thiol groups, inactivates RhoA [145]. Further complicating the matter, NO treatment of recombinant RhoA has been described to cause S-nitrosylation and inactivation of RhoA, yet it is unclear on which cysteines this occurs [146].

Under normal cellular conditions, with a high reducing potential and abundant GTP, physiological levels of peroxide activate RhoA seemingly via direct oxidation [139]. Aghajanian and colleagues [139] knocked down RhoA and then expressed wild type or C16/20A mutant RhoA in fibroblasts. Upon stimulation of these cells with various concentrations of H<sub>2</sub>O<sub>2</sub> (0.1–10 μM), only WT RhoA was activated, suggesting that redox-sensitive Cys16 and/or Cys20 is required for H<sub>2</sub>O<sub>2</sub>-induced activation of RhoA. Importantly, the C16/20A mutant was still able to be activated by GEF-mediated stimulation and inhibited by C3 toxin, indicating that the ROS-induced activation of RhoA was driven by direct oxidation of these residues [139]. Despite this observation, controversy remains within in cellulo literature, some of which shows that RhoA is activated by ROS/RNS [139, 147–153] and others of which describe inhibition or reduced expression of RhoA by ROS/RNS [145, 146, 154, 155]. The controversy may be partially due to the fact that in addition to direct oxidation, ROS/RNS affect many regulators of Rho family GTPases. For example, p190RhoGAP is activated when low molecular weight PTP is oxidized [155], but p115RhoGEF is also activated indirectly by OONO- and H<sub>2</sub>O<sub>2</sub>-induced kinase activation [148], and ROS likely also cause release of RhoGDI from RhoA [156]; therefore, the outcome of redox signaling is hard to predict with respect to RhoA activity, and is likely specific to cellular conditions.

Although the effect of Rho family GTPase oxidation on migration and cell division has not been directly examined, it is clear that Rho family oxidation controls the cytoskeleton, because only cells expressing WT RhoA and not oxidation-resistant C16/20A RhoA showed increased stress fibers in response to H<sub>2</sub>O<sub>2</sub> [139]. Similarly, expression of the oxidation-mimetic C18D Rac1 induces significantly more lamellipodia compared to wild type Rac1 [140]. Since ROS promote excessively high levels of Rac1 activity, one would predict that this should inhibit cell proliferation. RhoA oxidation is more complex, depending on the level of ROS and if both cysteines or just one is oxidized. Therefore, disentangling the redox regulation of RhoGTPases during migration and proliferation is an area ripe for investigation. Since RhoA activity [157], ROS production [158], and VSMC proliferation and migration [159] are all elevated in atherosclerotic plaques, this would be a particularly interesting system to use to determine the relevance of Rho GTPase oxidation.

**IQGAP oxidation**—IQGAP is a Rac1 and Cdc42 effector with numerous roles in actin and microtubule organization that is highly expressed in endothelial cells [160]. In brief, it can directly bind to actin to facilitate actin cross-linking and inhibit polymerization, and can also regulate many other cytoskeletal proteins. For example, it activates N-WASP to promote actin branching and binds mDia to promote actin elongation [160]. IQGAP1 localizes to the leading edge of migrating cells, where it enhances filopodia and lamellipodia formation and migration [161]. Of interest, IQGAP1/3 also appears to be required for proper localization of RhoA to the cleavage furrow during telophase, and its suppression leads to impaired cytokinesis [162].

Several years ago, Kaplan and colleagues [163] reported IQGAP oxidation following vascular endothelial growth factor (VEGF) treatment and hindlimb ischemia. Additionally, using DCP-Bio1 (a compound that binds to cysteine sulfenic acid) for immunofluorescence, they found that cysteine sulfenic acid co-localized with IQGAP, p47phox (a regulatory component of NOX2), and F-actin at the leading edge of migrating cells, suggesting that these proteins are likely oxidized at this location [163]. Previous studies showed that in migrating cells, IQGAP recruits NOX2 to the leading edge to stimulate ROS production there [160, 164]. Therefore, it seems that IQGAP both enhances ROS production and is a target of ROS production during cell migration. However, it remains unclear exactly how IQGAP oxidation regulates its many functions.

**Protein tyrosine phosphatase oxidation**—One important class of cytoskeleton-related enzymes with extremely low pKa active-site catalytic cysteine residues is protein tyrosine phosphatases (PTPs), which have high sensitivity to redox inactivation. PTPs regulate cytoskeletal signal transduction pathways and participate in cell cycle control by dephosphorylating proteins [155, 165, 166]. Recently, oxidation has emerged as an important regulatory mechanism of PTPs. The oxidation of PTPs has been demonstrated for PTP1B [167], Src homology phosphatase (SHP)-2 [168], PTEN [169], receptor-PTP $\alpha$  [170], and low-molecular-weight protein tyrosine phosphatase (LMW-PTP) [171, 172]. LMW-PTP is involved in the control of mitogenic and adhesive signals [173, 174] and its redox regulation shows a peculiarity above all other PTPs. Two of the eight cysteine residues present in LMW-PTP are modified by H<sub>2</sub>O<sub>2</sub> treatment, namely Cys12 and Cys17 [175]. Normally, activation of its receptor by the pro-migratory platelet-derived growth factor (PDGF) is limited by the LMW-PTP, but in the presence of ROS, LMW-PTP is modified by direct oxidation on Cys12 and Cys17 to form an inactivating disulfide bond, thus amplifying PDGF signaling [171, 176]. Another study showed that S-glutathionylation of LMW-PTP regulates VEGF-mediated focal adhesion kinase activation [177], an important step in focal adhesion formation. These examples suggest that oxidative regulation of LMW-PTP is necessary for efficient adhesion and migration.

**Src oxidation**—Src family kinases are critically involved in the control of cytoskeletal organization and in the generation of integrin-dependent signaling responses in fibroblasts, inducing tyrosine phosphorylation of many signaling and cytoskeletal proteins [178]. Redox regulation of Src is a central feature of cell adhesion, as the elimination of ROS by antioxidant treatment almost completely abolishes Src activation in this situation [179]. This observation is further supported by the ability of antioxidants to inhibit p125FAK/Src association and p125FAK phosphorylation and is in agreement with data showing that ROS are necessary for integrin signaling during fibroblast adhesion and spreading [180, 181]. Src itself is redox sensitive, since each Src domain contains cysteine residues with pKa's low enough to be oxidized by physiological levels of ROS [182, 183]. Direct oxidative modifications on Src have been reported on, for instance, Cys277 [184] in the linker region, Cys245 [179], Cys122 and Cys164 [185] in the SH2 domain, and Cys487 [179] in the kinase domain. Oxidation of cysteine residues in the SH2 domain causes inter-molecular disulfide bridge formation and disruption of internal Tyr527-SH2 domain interaction; this conformational change promotes auto phosphorylation at Tyr416 and leads to activation of

Src [186]. Oxidation of Cys245 and Cys487 further activates an already active Src [179]. On the other hand, oxidative inhibition of the Src-specific PTP, which de-phosphorylates Tyr418 in the activation loop [187], and oxidative regulation of C-terminal Src-Kinase (CSK), which phosphorylates the auto-inhibitory C-terminal Tyr527 [185], also influences Src activity. In cell-based studies it is often difficult to differentiate the direct effect of redox regulation on Src activity from the indirect effects through Src regulators. Because both activation [179] and inactivation [188, 189] of Src in response to oxidative stress have been reported, it is not clear whether direct oxidation of Src or simply inactivation of the PTPs that dephosphorylate Src predominates.

**Focal adhesion kinase activation by oxidants**—Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase important in integrin signaling pathways controlling VSMC migration and proliferation [190]. ROS induce activation of FAK and promote cell motility in endothelial cells in a dose- and time-dependent manner [191]. FAK consists of a central catalytic domain, which is flanked by large amino- and carboxy-terminal regions. The amino-terminal sequence binds the  $\beta$ 1-integrin intracellular domain and the carboxy-terminus contains two proline-rich sequences that bind to SH2 domain-containing proteins, such as Src, paxillin or Graf [192, 193]. Activation of FAK is associated with its phosphorylation on several specific tyrosine residues, Tyr397, Tyr925, and Tyr577 [194]. FAK is autophosphorylated on Tyr397 when bound to activated  $\beta$ 1-integrins, which is crucial in cell migration and cell cycle progression [192, 195, 196]. It has been shown that ROS induce tyrosine phosphorylation of FAK on Tyr397, Tyr925, and Tyr577, leading to increased kinase activity. The phosphorylation on Tyr397 was observed in both actin-rich and membrane-cytosol fractions, indicating that ROS-induced FAK phosphorylation is likely involved in adhesion [197]. This phosphorylation by ROS was suggested to occur through tyrosine phosphatase inhibition rather than activation of tyrosine kinases [191, 197]; however, ROS-induced phosphorylation of FAK on certain tyrosine residues is partly sensitive to tyrosine kinase inhibitors, suggesting that alternative mechanisms may contribute [191], such as ROS-induced Src activation [198]. The complexity of this pathway, with multiple redox-sensitive kinases and phosphatases but no known direct oxidation, makes it imperative to study redox activation of FAK in different cells and cellular context.

**Protein kinase C oxidation**—The protein kinase C (PKC) family is composed of serine/threonine protein kinases that are involved in a variety of pathways to regulate cell growth, adhesion, migration, differentiation and gene transcription [199–201]. Traditionally, PKC isoforms are activated by  $\text{Ca}^{2+}$ , which itself is regulated by ROS, and diacylglycerol (DAG), and require phosphorylation on serine/threonine for full activation [202]. In recent years, ROS, especially  $\text{H}_2\text{O}_2$ , have been proposed to be an upstream activator of PKC [203, 204]. For example, PKC can be activated by  $\text{Ca}^{2+}$  induced by ROS and mediates Rho GTPase activation during focal complex formation in the early stage of cell spreading [153, 201, 205]. In bovine pulmonary artery endothelial cells, cyclic strain stimulates mitochondrial ROS release, which induces PKC-dependent phosphorylation of FAK [206]. Conversely, many studies have found that PKCs regulate NOX1-3 by phosphorylating the indispensable subunit p47phox [207–209]. Taken together, these studies suggest that PKCs are both upstream and downstream of ROS.

Several reports indicate that PKC contains redox-sensitive cysteines [210, 211]. PKCs have two pairs of zinc fingers within their regulatory domains, the sites of DAG binding. Each zinc finger is composed of six cysteine residues and two zinc atoms, which render the regulatory domain susceptible to redox regulation [202, 210]. The C-terminal catalytic domains of PKCs also contain cysteine residues that are sensitive to oxidative modifications and affect kinase activity [212, 213]. For instance, thiol oxidation and release of zinc from zinc finger motifs are necessary for  $O_2^{\bullet-}$ -mediated activation of PKC [213]. However, the redox regulation of PKC is complex. While ROS such as  $O_2^{\bullet-}$  [214, 215] and  $H_2O_2$  [216] activate PKC, hydroxyl radical decreases PKC activity [217]. Furthermore, even when mild ROS activate PKC, prolonged exposure leads to further oxidation and inactivation [203]. Because activation of PKC is regulated at multiple levels, including phosphorylation, membrane insertion [218, 219], and proteolytic cleavage [220], the full range of effects of oxidation is unclear and remains to be further explored.

**Oxidation of calcium channels and transporters**—Cytosolic homeostasis of  $Ca^{2+}$  is essential for cytoskeletal-mediated cell behaviors, most notably cell contraction, migration and growth, and several ion channels and transporters that regulate  $Ca^{2+}$  flux have been shown to be responsive to oxidation. Cytosolic  $Ca^{2+}$  influx is regulated by L-type voltage-gated  $Ca^{2+}$  channels (LTCC), which contain an  $\alpha_1C$ -subunit that is sensitive to inactivation by oxidation [221–223]. Inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) is another major  $Ca^{2+}$  channel that releases  $Ca^{2+}$  from the sarcoplasmic reticulum (SR) into the cytosol and is conformationally regulated by oxidative species [224]. Oxidation of Cys34–Cys42 of the IP<sub>3</sub>R reduces its activity in line with the conformational changes, indicating that Cys34 and Cys42 may serve as reduction sensors. In addition, it has been suggested that Cys65 may act as an oxidation sensor [224, 225].

Calcium reuptake, and therefore reduction of cytosolic calcium, is also redox sensitive. Sarco-/endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA), the major  $Ca^{2+}$  reuptake effector on SR, contains almost 25 cysteine residues that are easily oxidized, especially Cys674, which is the most redox-sensitive. Reversible S-glutathiolation of Cys674 enhances the SERCA activity [226–228]. However, irreversible oxidative modifications including sulfonylation of Cys674 and nitration of Tyr294/295 decrease SERCA activity [229]. The sodium-calcium exchanger (NCX) is another significant mechanism for cytosolic  $Ca^{2+}$  removal. NCX is mainly located on plasma membrane. It mediates efflux of one single  $Ca^{2+}$  ion in exchange for the import of three  $Na^+$  ions [230, 231]. NCX can be activated in the presence of ROS, such as  $H_2O_2$  or that produced by xanthine oxidase with hypoxanthine in vitro [232–234]. However, strong ROS like hypochlorite inhibit NCX activity [235]. The potential mechanisms of these effects may involve disulfide bond formation by direct redox modification and phosphorylation by PKC [236, 237]. Moreover, the plasma membrane  $Ca^{2+}$ -ATPase, which is responsible for a more modest cytosolic  $Ca^{2+}$  extrusion, is also suppressed by oxidative species [238, 239].

Although direct oxidative modification has effects on these  $Ca^{2+}$  channels and transporters, oxidative modification of upstream kinases plays a role in  $Ca^{2+}$  regulation as well. In particular, LTCC [240–242] and NCX [236, 237, 243] are substrates of protein kinases such as PKC (see above) and  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CAMKII, see below),

which both can be regulated by ROS. The complexity of the intracellular  $\text{Ca}^{2+}$  system, together with multiple levels of redox sensitivity, makes it challenging to separate the effects of direct oxidation by ROS and redox-sensitive kinase activity on  $\text{Ca}^{2+}$  channels and transporters.

**Calmodulin oxidation and calcium/calmodulin dependent protein kinase II activation by oxidants**—Calmodulin (CaM) is a  $\text{Ca}^{2+}$  binding protein that mediates many effects of  $\text{Ca}^{2+}$ . For example, the formation of the  $\text{Ca}^{2+}$ /CaM complex activates MLCK, which in turn regulates actin-myosin interaction and VSM contraction [244]. CaM contains four EF-hand  $\text{Ca}^{2+}$  binding sites and at least six redox-sensitive methionines. While oxidation of Met36, Met51, Met71, Met72 and Met145 lead to CaM degradation [245, 246], oxidation of Met144 and Met145 affects the tertiary structure of the C-lobe, resulting in destabilization and decreasing substrate affinity [247–249].

CaMKII is a CaMK isoform with many functions, as it phosphorylates various proteins involved in migration [250], proliferation [251] and ion channel activity [252] in many vascular cells. CaMKII is activated by CaM binding to its autoinhibitory regulatory domain [253–255]. The methionines on CaMKII are also sensitive to oxidative species. Erickson et al. [256] reported that oxidative modification on Cys280/Met281 in the  $\alpha$  isoform or Met281/282 in the  $\beta$ ,  $\gamma$ , and  $\delta$  isoforms leads to increased CaMKII activation. Methionine sulfoxide reductase A (MsrA) inactivates CaMKII by reducing oxidized methionine residues [256]. Moreover, it has been suggested that  $\text{Ca}^{2+}$  and CaM are required for oxidative modification on CaMKII [256].

Based on current knowledge,  $\text{Ca}^{2+}$  and CaM are more potent regulators of CaMKII activation than direct oxidative modification [257–260]. How CaM is oxidized *in vivo* and how oxidation might affect its interaction with CaMKII remains unclear. Since both CaM and CaMKII play important roles in calcium/CaM signaling and affect a wide range of  $\text{Ca}^{2+}$ -mediated cellular functions [261, 262], further investigation of the interplay of oxidation with activation/inactivation of this complex is warranted.

## REDOX REGULATION OF THE ACTIN CYTOSKELETON IN CELL PHYSIOLOGY

### Cell adhesion and migration

Mounting evidence suggests that ROS, particularly  $\text{H}_2\text{O}_2$  derived from NADPH oxidases, are necessary for cell movement [17, 263]. When cell surface receptors like receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) bind to extracellular stimuli such as growth factors and chemoattractants, ROS are generated at the cell surface as well as within intracellular compartments and react with specific proteins to regulate their activity and function [17, 20]. Early studies showed simply that ROS promote cell migration, because treatment with ROS-degrading enzymes suppressed growth factor- and chemoattractant-induced migration in endothelial cells, neutrophils, fibroblasts and VSMCs [264–268]. Moreover, it has been shown that ROS are produced during migration in these cells [264, 265, 267, 268]. In particular, PDGF one of the most potent migratory factors for

VSMCs, activates NOX1 to produce H<sub>2</sub>O<sub>2</sub>, which is required for PDGF-induced migration [269–272]. Migration in response to this peptide is attenuated by pre-treatment with antioxidants such as NAC, the glutathione peroxidase (GPx) mimetic, ebselen, and catalase [269]. VSMCs from NOX1 deficient mice display reduced PDGF- and basic fibroblast growth factor (bFGF)-induced migration [273, 274], while VSMCs from NOX1 transgenic mice exhibit enhanced migration [273]. NOX4 has also been shown to be critical for VSMC migration in response to PDGF [147], and both NOX1 and NOX4 play a role in VSMC migration in response to other agonists such as insulin-like growth factor-I [134], VEGF [275] and angiotensin II [266].

Migration begins when a cell is exposed to an environmental signal such as a chemoattractant or growth factor [276, 277]. Initially, cell membrane extensions protrude from the plasma membrane either as spike-like filopodia or broad lamellipodia [278]. Protrusive forces, generated by actin polymerization and increased actin branching, are needed for the formation of these structures at the leading edge of the cell [16]. Integrins on the tips of these lamellipodia mediate the first contacts with the substrate and thus stabilize the membrane extensions. If appropriate integrin ligands are sensed, signals are conveyed into the cells, resulting in recruitment of further integrins to these sites and the assembly of cytoskeletal and signaling molecules into focal contacts, which consequently mature into focal adhesions [279, 280]. Next, cell contraction via engagement of actin-myosin interactions occurs, facilitated by the Rho signaling pathway [156]. Focal adhesions mature and strengthen at the leading edge, while their dissolution in the rear allows the cell to contract and move forward [281]. Redox regulation of the actin cytoskeleton and its associated proteins is highly integrated into the entire cycle of migration, beginning with lamellipodium formation [282]. The growth factor or chemoattractant binds receptor tyrosine kinases or G protein-coupled receptors and activates a GEF and consequently Rac and CDC42 [276, 283, 284]. Rac and CDC42 separately activate WASP homologue (WH) domain-containing proteins neural WASP (NWASP) and WAVE, activating the ARP2/3 complex and resulting in extension and branching of F-actin [285–287] (Figure 2). Because both Rac and CDC42 are redox-sensitive, it is very likely that branched F-actin formation is controlled by ROS. Another potential point of redox regulation is the involvement of cofilin in F-actin dynamics. Extension of F-actin requires free-barbed ends [288, 289], which cofilin provides by its severing function [290, 291]. Thus, free-barbed ends formed by cofilin activation, along with the released G-actin, enable formation of new actin filaments [292]. As noted above, cofilin itself can be directly oxidized. It was reported recently that elevation of H<sub>2</sub>O<sub>2</sub> in migrating cells results in cofilin oxidation on Cys139 and Cys147. Oxidation-resistant cofilin impedes cell spreading, adhesion and directional migration [109]. This evidence is in line with recent studies that showed physiological oxidative modifications on cytoskeletal proteins are required for cell motility [69, 75, 76]. Perhaps more importantly, Slingshot1L (SSH1L) phosphatase, which activates cofilin by dephosphorylating its inactive form, is also mediated by ROS. In PDGF-treated VSMCs, H<sub>2</sub>O<sub>2</sub> derived from NOX1 oxidizes the scaffold protein 14-3-3, leading to disruption of the SSH1L/14-3-3 complex and activation of SSH1L, activating cofilin [293, 294]. Cofilin is returned to its inactive form by LIMK-mediated phosphorylation [295]. LIMKs, in turn, are phosphorylated and activated by Rho and its downstream kinase ROCK as well as Rac and

its downstream kinase p21-activated kinase (PAK) [295, 296] (Figure 2). Because Rho and Rac are redox-sensitive, LIMK is also likely to be dependent upon ROS, although this possibility has not yet been studied in detail.

The next step in migration—leading edge attachment to the substrate—is mediated by integrin binding, a phenomenon that has been closely linked to ROS [115]. Integrin engagement occurs upon binding with specific sequence motifs of extracellular matrix proteins [115, 281]. Subsequently, the activated integrins recruit actin binding proteins such as talin [297], paxillin [298], vinculin [299], and  $\alpha$ -actinin [300], as well as signaling proteins such as Src, FAK and integrin-linked kinase [301], creating focal adhesions (Figure 2). In fibroblasts, ROS are dramatically increased due to NOX and 5-lipoxygenase activation during the adhesion process [181]. Because integrin signaling pathways contain a range of redox-sensitive kinases, phosphatases, GTPases and transcription factors, altered ROS levels likely affect focal adhesions at multiple levels. For example, inhibition of LMW-PTP by direct oxidation enhances FAK and focal adhesion maturation [181]. Two other important redox-sensitive protein kinases involved in integrin-mediated signaling are Src and FAK [302, 303] (Figure 2). PDGF activates Src during VSMC migration, and inhibition of Src attenuates PDGF-induced VSMC migration [302]. Integrin-mediated activation of FAK leads to phosphorylation of paxillin, thus regulating its translocation to focal adhesions and promoting focal adhesion formation [303]. At the same time, autophosphorylation of FAK on tyrosine 397 promotes its association with Src [304, 305]. This phosphorylation is essential for FAK-induced focal adhesion disassembly, which is important as the cell begins to move [306]. Thus, it has been suggested that FAK–Src signaling interrupts focal adhesion maturation by promoting disassembly and in turn promoting adhesion turnover as the cell extends [307]. Since  $H_2O_2$  induces FAK autophosphorylation and activates Src, ROS are almost certainly physiological mediators of focal adhesion turnover. Furthermore, FAK–Src signaling [308] leads to the activation of several downstream signaling cascades, including the mitogen-activated protein kinase (MAP kinase)/ERK pathway [309], which in turn phosphorylates and activates MLCK [310] and alters the rate constant of FA disassembly [311]. Although MAPK/ERK are not known to be directly modified by ROS in this context, they experience redox regulation by ROS-activated upstream molecules such as Src, PKC and PAK [312–314] (Figure 2).

In addition to influencing focal adhesion disassembly, actin-myosin interactions regulate contraction of the cell body to propel forward movement.  $Ca^{2+}$ -dependent activation of MLCK is the major mechanism regulating VSMC contraction, but Rho GTPases also fine tune actin-myosin function [281] (see *Contraction* below for further details). ROS have been extensively linked to activation of RhoA/ROCK signaling in rat aorta [153] and in pulmonary VSMCs and endothelial cells [153, 315, 316]. ROCK phosphorylates the myosin binding subunit, myosin light chain phosphatase (MLCP), reducing its activity [317]. ROCK also acts in cooperation with MLCK to phosphorylate myosin II during migration [318]. PAKs, downstream of Rac, negatively regulate myosin II phosphorylation by phosphorylating MLCK, but also phosphorylates myosin II directly, leading to actomyosin interaction [319] (Figure 2). This mechanism may be ROS-regulated, because PAK activation in VSMCs is dependent on NOX1-generated ROS [273]. However, how ROS-

specific modification of these proteins interacts with phosphorylation signals remains to be determined.

In summary, based on the known redox-sensitivity of many cytoskeleton-related signaling molecules, as well as whole cell studies using antioxidants to inhibit migration, a clear role for targeted, specific redox regulation of migration exists (Figure 2). It is likely that cell migration occurring during both normal and pathological processes is regulated by ROS via effects on actin dynamics [320–322]. Thus, further investigations of the specific targets of ROS and how they are modified during migration of all vascular cells types is in order.

### Cell contraction

Contraction of VSMCs is integral to control of vessel tone and blood pressure, and there is increasing evidence that ROS are involved in cell contraction pathways. Since Heinle [323] showed that exogenous H<sub>2</sub>O<sub>2</sub> application induces vasoconstriction of carotid artery, it has been demonstrated that both exposure to ROS and selective depletion of endogenous ROS alter cell contractility [324]. The specific roles of ROS in VSMC contraction remain unclear, although there are several likely molecular targets. Under oxidative conditions, ROS act both upstream and downstream of intracellular Ca<sup>2+</sup> release and cytosolic Ca<sup>2+</sup> influx. ROS increase the open probability of membrane Ca<sup>2+</sup> channels and increase Ca<sup>2+</sup> release [325–327] to promote contractile bundle formation. It should be noted that most studies report that higher concentrations of ROS suppress force [328, 329]; however, mounting evidence shows that low levels of ROS increase force [324, 325, 329].

Although contractile mechanisms differ among cells and tissues, the most well-established model of cell contraction relies on actin-myosin cross-bridge cycling driven by ATP hydrolysis (Figure 3). This pathway is present in striated muscle as well as in nonmuscle cells. The repeated cycles begin with myosin activation, which occurs via phosphorylation of the myosin light chain by MLCK, a Ca<sup>2+</sup>/calmodulin-dependent process [330]. As the myosin head crawls along actin filaments, ATP is hydrolyzed. The energy produced during this process induces a conformational change in myosin, leading to continued cycles of actin-myosin complex formation, ATP hydrolysis and muscle contraction [95]. Actin-myosin complex formation is regulated by two accessory proteins bound to actin filaments, tropomyosin and troponin. In non-muscle and smooth muscle cells, the actin contractile bundles are associated with tropomyosin [317]. Here, we mainly focus on redox regulation mechanism of contraction in VSMCs (Figure 3).

Cell contraction is induced by multiple stimuli (Figure 3). When agonists such as norepinephrine and angiotensin II bind to G-protein coupled receptors, or growth factors bind to RTKs, phospholipase C (PLC) is activated. Phospholipase C $\gamma$  in particular is a redox-sensitive protein activated by recruitment of its Src homology domains to phosphotyrosine residues on activated RTKs [331]. In contrast, PLC $\beta$  isoforms, which are activated by GPCRs, do not have SH2 domains, are not regulated through tyrosine phosphorylation, and are not redox-sensitive enzymes [331]. Activation of PLCs catalyzes the formation of IP<sub>3</sub> and DAG. IP<sub>3</sub> binds to receptors in the SR to release Ca<sup>2+</sup> into the cytosol. Of note, the IP<sub>3</sub>R is targeted to proteasome degradation by H<sub>2</sub>O<sub>2</sub> [332], resulting in a decrease in Ca<sup>2+</sup> efflux from the SR. DAG, along with Ca<sup>2+</sup>, activates the redox-sensitive



PKC to promote downstream contractile signaling [95] (Figure 3). How oxidants interact with PKC during contraction is unknown. Increases in cytosolic  $\text{Ca}^{2+}$  promote the binding of CaM to MLCK, resulting in phosphorylation of MLCK and actin-myosin assembly. The small G protein RhoA and its downstream effector ROCK can also regulate the activity of MLC by promoting MLCK and inhibiting MLCP, as noted above [317] (Figure 3). ROS-induced vascular smooth muscle cell contraction mediated via activation of Rho/ROCK has been demonstrated by several experimental approaches [139, 153, 315, 333, 334].

Redox regulation of ion channels also has a well-established role in contraction. LTCCs open,  $\text{Ca}^{2+}$  influx occurs, which, together with  $\text{IP}_3\text{R}$  activation, induces  $\text{Ca}^{2+}$  release from the SR [330] (Figure 3). It has been shown that oxidative modifications suppress the activity of these channels [335], while ROS-regulated activation of CaMKII [336], PKA [337], and PKC [240] lead to an increase in  $\text{Ca}^{2+}$  current by phosphorylation of these channels.

Cell relaxation occurs as a result of decreased intracellular  $\text{Ca}^{2+}$  due to inactivation of LTCC and activation of SERCA and NCX as well as PMCA to re-uptake  $\text{Ca}^{2+}$  into the SR, and increased MLCP activity [330] (Figure 3). This complicated process is mediated by a variety of redox-sensitive protein kinases and phosphatases such as CaMKII, PKA, PKC, among others [255, 338, 339]. As with cell migration, establishing the relationship between regulation of these molecules by direct oxidative modification and phosphorylation is challenging.

Finally, it should be noted that redox regulation of NO-mediated vasodilation is well established. Aside from  $\text{O}_2^{\bullet-}$ -mediated inactivation of NO itself, which inhibits vasodilation, activation of protein kinase G (PKG) to enhance relaxation can be achieved by S-nitrosylation of Cys42 [340] (Figure 3). This cGMP-independent mechanism of activation facilitates  $\text{Ca}^{2+}$  extrusion, MLCP activation and consequently relaxation [341].

### Cell division and proliferation

Depending on the concentration and duration of ROS exposure, ROS are widely acknowledged to both promote and inhibit cell proliferation [342–344]. It appears that low levels of transient ROS promote proliferation, while higher levels and/or sustained ROS result in cell cycle arrest [344], and very high levels induce apoptosis [345, 346]. Indeed, depletion of the ROS generator NOX1 reduces cell proliferation, while NOX1 overexpression enhances proliferation [273] in an  $\text{H}_2\text{O}_2$ -dependent manner [347]. Similarly, treatment with ROS scavengers or knockdown of NOX2 or NOX4 to decrease endogenous ROS decreases endothelial cell proliferation [348], yet NOX4 can also induce VSMC differentiation via  $\text{H}_2\text{O}_2$  to have the opposite effect [349, 350].

Integrin activation (necessary for anchorage-dependent growth) and growth factor binding both stimulate ROS production that is thought to act as a signaling molecule in a variety of pathways to regulate cell division [342–344]. Indeed, ROS levels are highest during mitosis and lowest during the G1 phase of the cell cycle [351–353]. ROS levels are especially high and diffuse in prophase, and while levels of ROS drop during cytokinesis, there appear to be discrete areas of high ROS. For example, a methodology paper by Hsiegh and colleagues

[352] shows high ROS levels in what appears to be the intercellular bridge during late stages of cytokinesis.

While we have not been able to identify any reports of the actin cytoskeleton or its regulatory proteins being oxidized during proliferation, the actin cytoskeleton is intimately involved in cell division, as are many of the proteins discussed above that are known to be oxidized. With this in mind, we summarize here the indirect evidence for cytoskeletal oxidation in proliferation (Figure 4).

The role of the actin cytoskeleton in cell division has been recently reviewed in detail [92]. The dynamic ratio of G- and F-actin within a cell regulates four main aspects of cell division: 1) transcription of pro-proliferative genes; 2) cell rounding at mitosis onset; 3) mitotic spindle orientation and function; and 4) contractile ring formation/cytokinesis completion (Figure 4). The first of these functions is related to the transcriptional co-factor myocardin-related transcription factor (MRTF-A). When actin is in its monomeric (globular) form it prevents MRTF-A nuclear localization via multiple mechanisms. Nuclear G-actin binds MRTF-A and enhances its nuclear export [354]. Cytoplasmic G-actin binds MRTF-A and sequesters it in the cytoplasm. However, polymerization of actin allows the release of MRTF-A, which then translocates to the nucleus to enhance transcription of serum response factor (SRF)-target genes [355]. Depending on a variety of factors (SRF phosphorylation, cell type, etc. [356, 357]), SRF can either enhance proliferation or promote VSMC differentiation. In general, MRTF-A/SRF signaling promotes the latter. This effectively inhibits pro-growth signaling of SRF, because MRTF-A competes for a common binding surface on SRF with co-activators that promote proliferation [358]. While there are exceptions, in general oxidation of actin on cysteine or methionines (by MICALs) causes the balance between G- and F- actin to be shifted towards G-actin [70, 71, 77, 78]. However, cofilin oxidation has the opposite effect, impairing cofilin's severing functions and promoting F-actin [106–109]. Therefore, one would predict that actin oxidation will enhance cell proliferation, but cofilin oxidation will inhibit proliferation by promoting MRTF-A/SRF complex formation. However, a recent report suggests that it is more complicated than this. As expected, MICAL-2 oxidation of nuclear actin on Met44 promoted F-actin disassembly, yet surprisingly this promoted MRTF-A/SRF-driven gene expression, including cardiac muscle specific genes in developing zebrafish embryos [83]. Since differentiation and proliferation are antagonistic in muscle cells, this suggests that oxidation of nuclear actin by MICAL-2 may impair proliferation in these conditions. To complicate matters further, F-actin promotes nuclear entry of pro-proliferative transcriptional co-activators (e.g. YAP and TAZ), as well [359].

Separate from this probability, myosin II recruitment to the cell cortex generates intracellular tension that is required for cell rounding during mitosis [360]. This rounding is important for proper spindle assembly and efficient, error-free chromosome separation [361]. When myosin II is directly oxidized, its activity is inhibited [96]. Therefore, for proper cell division to occur, myosin II must be protected from the elevated levels of ROS during mitosis to enable cell rounding. Additionally, the interaction of F-actin with myosin II helps generate the forces necessary to promote contractile ring ingression and centrosome separation [92, 362]. Opposing this, branching of actin seems to inhibit contractile ring formation and thus

cytokinesis [92]. Cdc42 and IQGAP, which promote actin branching, are both directly oxidized [138, 163], yet it is unclear how this affects their roles in cell division.

All of these functions of actin are tightly regulated spatiotemporally by numerous proteins (Figure 4). Cofilin binds to F-actin and severs it to shift the balance towards G-actin [359], while formins (such as mDia) speed the assembly of F-actin [134]. Cofilin and myosin II activities are regulated by kinases, which in turn are regulated by Rho family GTPases, which are activated by specific GEFs and turned “off” by GAPs at discrete locations during mitosis and cytokinesis. For example, during anaphase, the RhoGEF Ect2 is recruited to the mitotic spindle (and held in an active conformation) through binding MgcRacGAP [363]. This causes RhoA activation directly above the central spindle, which leads to formin-induced actin polymerization to generate the contractile ring. RhoA also activates ROCK to stimulate myosin II to pull on the actin filaments and cause furrow ingression [134]. Too wide an area of RhoA activation and/or Rac1 activation at the cleavage furrow leads to cytokinesis defects, demonstrating how important it is that these GTPases are tightly regulated spatiotemporally during this process [364]. As described above, both RhoA and Rac1 can be directly oxidized to activate these GTPases [92, 139] and RhoA can also be inhibited by high levels of ROS [144].

Unfortunately, the role of ROS in cell division is difficult to predict, since different concentrations and durations of ROS signaling can have very different effects [342–344], and ROS are transient signaling molecules that must be generated at the correct time and place to regulate cellular processes. The known role of ROS in growth, coupled with the established regulation of the cytoskeleton by ROS, clearly indicate the need for further investigation into how ROS regulate the cytoskeleton in the context of cell division.

## **REDOX REGULATION OF THE ACTIN CYTOSKELETON IN VASCULAR DISEASE**

Vascular disease results in part from impaired vascular function caused by several vessel wall pathologies such as hypertensive vascular remodeling, atherosclerosis, restenosis and thrombosis [365–367]. A considerable amount of *in vivo* evidence suggests that ROS are involved in both hypertension and proatherogenic mechanisms and contribute to vascular dysfunction [368, 369]. Activation of NOXes has been demonstrated in a variety of animal models of vascular disease such as spontaneous hypertension [370], angiotensin-II-induced hypertension [371, 372], hypercholesterolemia [373] and atherosclerosis [374–376]. Many vasoactive agonists, such as angiotensin II, VEGF, PDGF, and thrombin [377–381] generate ROS. As discussed above, ROS regulate the actin cytoskeleton at multiple levels, impacting cell migration, proliferation and contraction [382]. All these process have distinct repercussions on the structure of vascular wall, suggesting that redox regulation of actin cytoskeletal signaling may play important roles in vascular remodeling and dysfunction.

In hypertension, vascular remodeling is characterized by rearrangement of vascular wall components like the actin cytoskeleton [383]. A decline in G-actin content, conforming to increased actin polymerization and F-actin formation was described [384, 385]. Actin polymerization is also believed to contribute to vascular constriction and resistance artery

remodeling in hypertension [386, 387]. Disruption of actin filaments inhibits  $\text{Ca}^{2+}$  channels in VSMC from spontaneously hypertensive rats, resulting in dysfunction of vasodilation [388]. Several redox-sensitive signaling pathways have also been suggested to link the cytoskeleton and vascular disease. For example, PKC-mediated vasoconstriction in cerebral arteries is highly dependent on cytoskeletal actin polymerization for force generation [389]. Although only a few studies have directly explored the relationship of ROS-induced cytoskeleton modification to vascular remodeling, most of the regulatory mechanisms have yet to be elucidated. The role of redox modification of cytoskeleton signaling must be considered in this context.

Studies in atherosclerosis showed that ROS modulate cytoskeletal changes in VSMCs [390, 391]. A reduction of actin binding proteins such as gelsolin and vinculin associated with actin disorganization was reported in the medial layer of human atherosclerotic coronary arteries [390, 391]. In animal models of vascular injury, superoxide dismutase attenuated neointima formation by inhibiting VSMC migration and proliferation [392, 393], potentially through down-regulation of the actin polymerization protein, mDia1 [392]. In a hypercholesterolemic rabbit model, changes in actin microfilaments initially promoted endothelial–substratum adhesion, but eventually led to a reduction of stress fibers and dysfunctional adhesion, with accumulation of macrophages and atherosclerotic plaque formation [394]. Several studies indicated that inhibition of actin filament polymerization interferes with vasodilator signaling in human coronary arterioles and the pulmonary circulation, also suggesting a pivotal role for endothelial cytoskeleton integrity in modulating endothelial-dependent vasodilator signal transduction [206, 395, 396]. However, none of these studies considered the potential role of redox regulation of the actin cytoskeleton in these outcomes, even though in every case ROS have been implicated in the response.

The redox-sensitive Rho family GTPases also play an important role in hypertension and atherosclerosis. Alterations in activity of RhoA/ROCK signaling have been proposed to contribute to the increased peripheral vascular resistance in hypertension [397–402]. Uehata et al [403] first reported a reduction of smooth muscle contraction and correction of hypertension in several hypertensive rat models by the specific ROCK inhibitor Y-27632. Recent studies showed that ROCK inhibition also prevented hypertrophic remodeling of coronary arterioles in spontaneously hypertensive rats [400, 404]. In addition, ROCK has been shown to participate in the pathogenesis of atherosclerosis [405], since long-term inhibition of ROCK can significantly reduce atherosclerotic lesion both in LDLr<sup>-/-</sup> mice and porcine models [406–408]. However, these studies did not explore the role of ROS in ROCK activation. Only Jin et al. [153] reported that Y-27632 relaxed rat aortic segments made to contract in response to experimental protocols that generate ROS. Because oxidative stress characterizes many models of vascular diseases [26, 409, 410], the intersection between ROS and the Rho/ROCK pathway should be further explored.

## CONCLUSIONS AND FUTURE DIRECTIONS

While ROS regulate cell motility and proliferation, and direct oxidation of many of the actin/actin-regulatory proteins involved in these responses has been described, the precise role of

oxidation of specific proteins in these critical cellular functions largely remains to be determined. Measurement of oxidation of specific cytoskeletal proteins during migration, proliferation and contraction is warranted. As mentioned above, different concentrations and durations of ROS signaling can have very different effects [342–344], and ROS are transient signaling molecules that must be generated at the correct time and place to regulate cellular processes. This suggests that measurement of oxidation of cytoskeletal proteins must be considered over time and in specific subcellular localizations. Tools are just now being developed that allow us to determine the subcellular localization of specific ROS generated throughout the cell cycle and during migration (for example organelle-targeted HyPer for H<sub>2</sub>O<sub>2</sub> [411] or DCP-Bio to detect oxidized proteins in situ [412]). Additionally, it will be necessary to synchronize cells or use single cells to determine if each of the proteins described above is oxidized at specific times during migration, contraction and cell division. Oxidation-resistant mutants of these proteins should be tested for their ability to impair these processes in order to confirm a role for oxidation. Although many contributing factors lead to the development of vascular diseases [413], the potential role of oxidative modification of actin/actin-regulatory proteins appears to be a ripe area for investigation.

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## ABBREVIATIONS

<b>α</b>	alpha
<b>β</b>	beta
<b>γ</b>	gamma
<b>G-actin</b>	globular actin
<b>F-actin</b>	filamentous actin
<b>ROS</b>	reactive oxygen species
<b>O<sub>2</sub><sup>•-</sup></b>	superoxide
<b>H<sub>2</sub>O<sub>2</sub></b>	hydrogen peroxide
<b>HO•</b>	hydroxyl radical
<b>RNS</b>	reactive nitrogen species
<b>NO</b>	nitric oxide
<b>•NO<sub>2</sub></b>	nitrogen dioxide
<b>ONOO-</b>	peroxynitrite
<b>NOXes</b>	NADPH oxidases
<b>SOD</b>	superoxide dismutase

<b>Cys</b>	cysteine
<b>Met</b>	methionine
<b>-SOH</b>	sulfenic acid
<b>GSH</b>	glutathione
<b>GSSG</b>	glutathione disulfide
<b>RS-SR'</b>	disulfide bond
<b>SNO</b>	S-nitrosylation
<b>SO<sub>2</sub>H</b>	sulfinic acid
<b>SO<sub>3</sub>H</b>	sulfonic acid
<b>NF-<math>\kappa</math>B</b>	nuclear factor- $\kappa$ B
<b>AP-1</b>	activator protein-1
<b>Hic-5</b>	hydrogen peroxide-inducible clone-5
<b>MICALs</b>	molecule interacting with CasL
<b>NM myosin II</b>	non-muscle myosin II
<b>VSMCs</b>	vascular smooth muscle cells
<b>MHC</b>	myosin heavy chain
<b>MLCK</b>	myosin light chain kinase
<b>MLCP</b>	myosin light chain phosphatase
<b>ROCK</b>	Rho kinase
<b>ECM</b>	extracellular matrix
<b>NAC</b>	N-acetyl-cysteine
<b>GEFs</b>	guanine nucleotide exchange factors
<b>GAPs</b>	GTPase activating proteins
<b>GDI</b>	guanine nucleotide dissociation inhibitors
<b>PAO</b>	phenylarsine oxide
<b>PTPs</b>	protein tyrosine phosphatases
<b>LMW-PTP</b>	low-molecular-weight protein tyrosine phosphatase
<b>VEGF</b>	vascular endothelial growth factor
<b>PDGF</b>	platelet-derived growth factor

<b>FAK</b>	focal adhesion kinase
<b>CSK</b>	C-terminal Src-kinase
<b>PKC</b>	protein kinase C
<b>DAG</b>	diacylglycerol
<b>LTCC</b>	L-type voltage-gated Ca <sup>2+</sup> channels
<b>IP<sub>3</sub>R</b>	inositol 1,4,5-trisphosphate receptor
<b>SR</b>	sarcoplasmic reticulum
<b>SERCA</b>	sarco-/endoplasmic reticulum Ca <sup>2+</sup> -ATPase
<b>NCX</b>	sodium-calcium exchanger
<b>CaM</b>	calmodulin
<b>CAMKII</b>	calmodulin-dependent protein kinase II
<b>RTKs</b>	receptor tyrosine kinases
<b>GPCRs</b>	G-protein-coupled receptors
<b>GPx</b>	glutathione peroxidase
<b>bFGF</b>	basic fibroblast growth factor
<b>WASP</b>	Wiskott–Aldrich Syndrome protein
<b>SSH1L</b>	slingshot1L
<b>PAK</b>	p21-activated kinase
<b>MAPK</b>	mitogen-activated protein kinase
<b>PLC</b>	phospholipase C
<b>PKG</b>	protein kinase G
<b>SRF</b>	serum response factor
<b>MRTF-A</b>	myocardin-related transcription factor
<b>YAP</b>	yes-associated protein
<b>TAZ</b>	transcriptional co-activator with PDZ-binding motif

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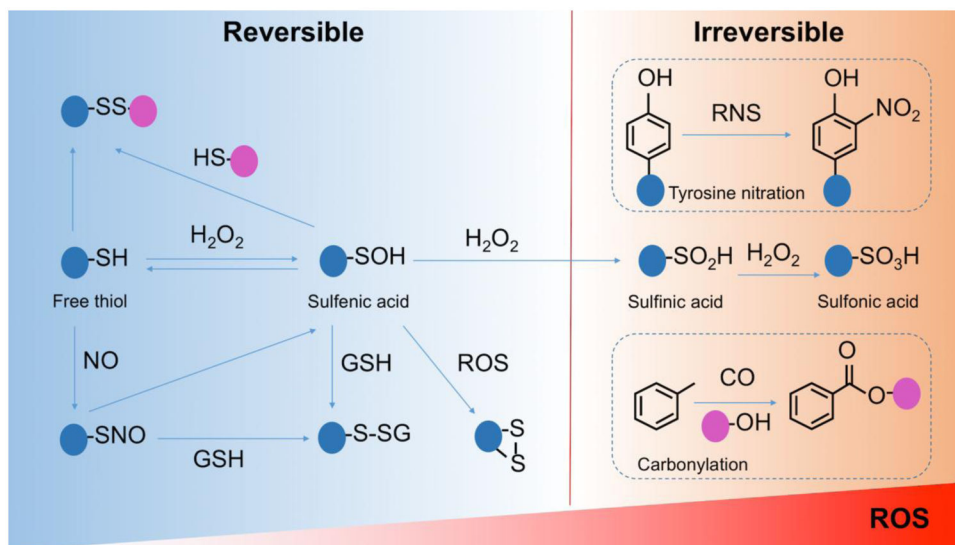
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### Highlights

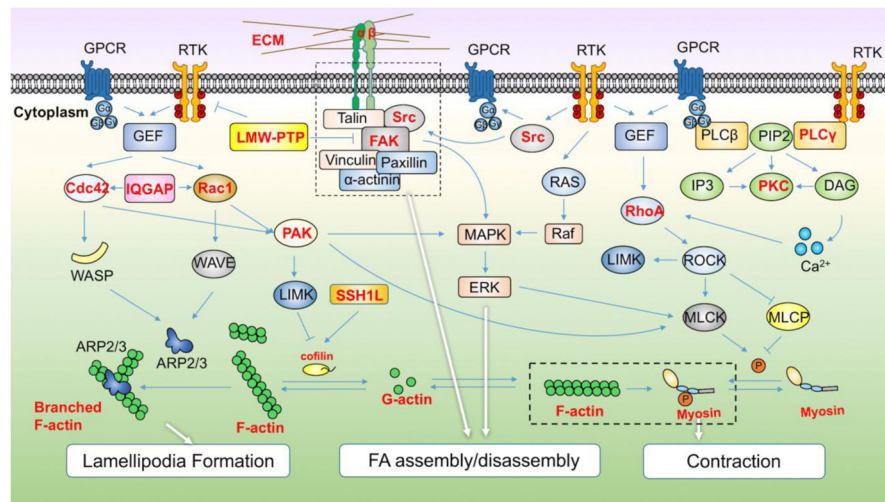
- The actin cytoskeleton serves structural and signaling functions in vascular cells.
- Actin, its associated proteins and upstream signaling molecules can be oxidized by reactive oxygen species induced by physiological or pathophysiological stimuli.
- Redox-regulation of the actin signaling network is involved in cell migration, contraction and proliferation.
- Redox modification of actin cytoskeletal proteins may be important in the development of vascular diseases





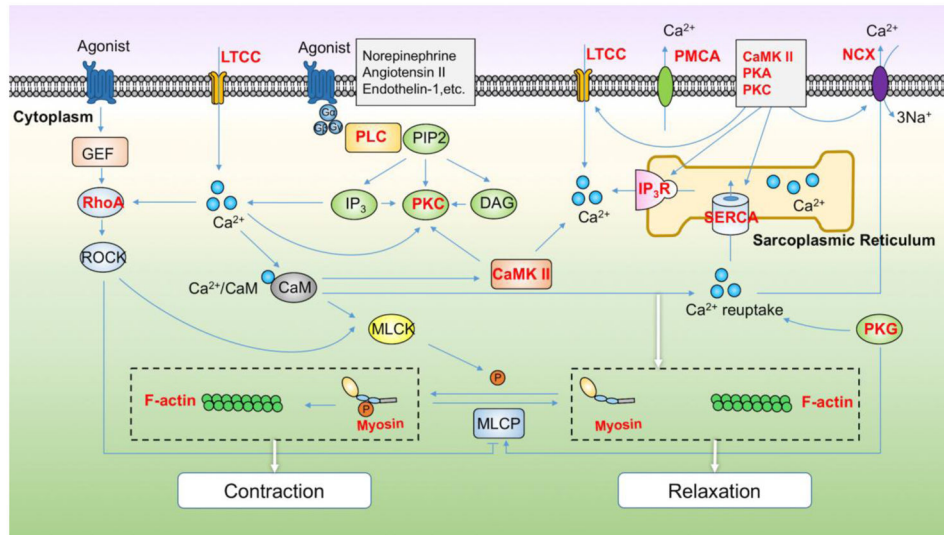
**Figure 1. The major mechanisms of protein oxidative modifications**

Left panel indicates the conventional model of reversible oxidative modifications of protein on cysteine thiol groups. These reversible modifications of sulfenic acid residues (-SOH) include formation of glutathione disulfide (GSSG), intra- or extra-molecular disulfide bonds (RS-SR'), S-glutathionylated proteins (R-S-SG) and S-nitrosylation (SNO). The modifications can be reversed by, for example, thioredoxin (Trx) and/or glutathione (GSH). When levels of ROS increase, sulfenic acids undergo further oxidation to sulfinic (SO<sub>2</sub>H) and/or sulfonic acid (SO<sub>3</sub>H), which are irreversible. These and two other major irreversible oxidative modifications of proteins (tyrosine nitration and carbonylation) are shown on the right panel.



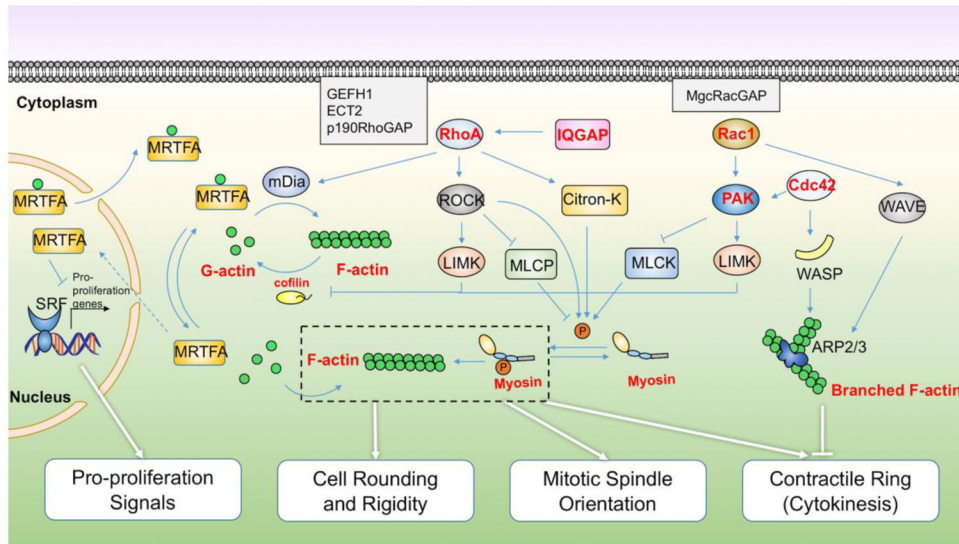
**Figure 2. The actin cytoskeleton signaling network controlling cell motility and its redox regulation**

Cell migration consists of cycles of lamellipodia formation, focal adhesion assembly at the leading edge, contraction of the cell body and de-adhesion and retraction at the rear edge. The signaling pathways that have been implicated in cell adhesion and migration are shown., including cell division control protein 42 homolog, Cdc42; Wiskott-Aldrich syndrome protein, WASP; WASp family verprolin-homologous protein, WAVE; actin-related protein 2/3, ARP2/3; protein kinase C, PKC; Rho-associated protein kinase, ROCK; LIM domain kinase, LIMK; myosin light chain kinase, MLCK; p21-activated kinase, PAK; protein tyrosine phosphatases, slingshot-1L phosphatase, SSH1L, low molecular weight PTPs, LMW-PTPs, myosin light chain phosphatase, MLCP; Rho GTPases and guanine nucleotide exchange factors (GEFs); phospholipase C  $\beta/\gamma$  (PLC  $\beta/\gamma$ ); phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>); inositol 1,4,5-trisphosphate (IP<sub>3</sub>); diacylglycerol (DAG). In this diagram, directly oxidized proteins are indicated by bold in red.



**Figure 3. The actin cytoskeleton signaling network controlling cell contraction and its redox regulation**

Cell contraction is induced when agonists such as norepinephrine or angiotensin II bind to receptors and activate phosphoinositide-specific-phospholipase C (PLC) to catalyze the formation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) from phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>). Meanwhile, Ca<sup>2+</sup> influx induced by voltage-gated Ca<sup>2+</sup> channels (LTCC) along with inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) activation inducing release of Ca<sup>2+</sup> from the endoplasmic reticulum, promotes Ca<sup>2+</sup> / calmodulin (CaM) activation of the actin-myosin complex. Decreased intracellular Ca<sup>2+</sup> concentration achieved by inactivation of LTCC, activation of Ca<sup>2+</sup> reuptake by the sarco/endoplasmic reticulum Ca<sup>2+</sup> -ATPase (SERCA), and activation of Ca<sup>2+</sup> extrusion by the sodium-calcium exchanger (NCX) and plasma membrane Ca<sup>2+</sup>-ATPase (PMCA) results in cell relaxation by reducing Ca<sup>2+</sup> and disrupting actin-myosin interaction. These processes are also regulated by kinases (calmodulin-dependent protein kinase II, CaMKII; Rho-associated protein kinase, ROCK; myosin light chain kinase, MLCK; protein kinase C, PKC; protein kinase A, PKA; protein kinase G, PKG) and phosphatases (myosin light chain phosphatase, MLCP), Rho GTPases and Guanine Nucleotide Exchange Factors (GEFs). In this diagram, directly oxidized proteins are indicated by bold in red.



**Figure 4. The actin cytoskeleton signaling network controlling cell division and its redox regulation**

The ratio of globular to filamentous actin within a cell regulates transcription of anti-proliferative genes; cell rounding at mitosis onset; mitotic spindle orientation and function; and contractile ring formation/cytokinesis completion. These processes are further regulated by transcriptional regulators (serum response factor, SRF; myocardin-related transcription factor, MRTFA), actin regulatory proteins (diaphanous-related formin-1, mDia; Cofilin; cell division control protein 42 homolog, Cdc42; Wiskott-Aldrich syndrome protein, WASP; WASp family verprolin-homologous protein, WAVE; actin-related protein 2/3, ARP2/3), kinases (Rho-associated protein kinase, ROCK; LIM domain kinase, LIMK; myosin light chain kinase, MLCK; citron kinase, Citron-K; p21-activated kinase, PAK), phosphatases (myosin light chain phosphatase, MLCP), Rho GTPases, guanine nucleotide exchange factors (Rho guanine nucleotide exchange factor 2, GEFH1; epithelial cell transforming sequence #2, Ect2), and GTPase activating proteins, of which many can be directly oxidized to regulate their function. In this diagram, directly oxidized proteins are indicated by bold in red.

**Table 1**

Summary of direct oxidation sites on actin, actin binding proteins, and actin regulatory proteins and the consequences of this oxidation.

ACTIN	Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
	Undetermined	Physiologic H <sub>2</sub> O <sub>2</sub> generated in wound migration assay	Increased actin polymerization and endothelial cell migration	[76]
	Cys17*	Glutaredoxin 2 expression	Glutathiolation of actin, correlating with cardiac neural crest cell migration and embryonic heart development	[414]
	Cys217*	X-ray irradiation	Sulfenic, sulfenic, and sulfonic acid oxidation of actin, with decreased actin polymerization and decreased ability of actin to activate myosin II (also observed oxidation of Cys257, Met44, 47, 355, & Trp79, 86 so it is unclear which residue is responsible for functional consequences)	[415]
		Diamide	S-glutathionylation of actin to undetermined endpoint	[416]
		S-nitrosoglutathione	S-nitrosylation of actin to undetermined endpoint	[417]
	Cys257*	X-ray irradiation	Sulfenic, sulfenic, and sulfonic acid oxidation of actin, with decreased actin polymerization and decreased ability of actin to activate myosin II (also observed oxidation of Cys217, Met44, 47, 355, & Trp79, 86 so it is unclear which residue is responsible for functional consequences)	[415]
		7-dimethylamino-4-methyl-(N-maleimidy) coumarin (DACM)	G-actin is oxidized without affecting polymerization; F-actin is not oxidized	[80]
		Glutaredoxin 2 expression	Glutathiolation of actin, correlating with cardiac neural crest cell migration and embryonic heart development	[414]

	Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
		S-nitrosoglutathione	S-nitrosylation of actin to undetermined endpoint	[417]
		Treatment of neutrophils with excessive oxygen to generate physiologic amounts of NO	Nitrosylation of actin, with increased actin polymerization and impaired actin organization (also observed oxidation of Cys272, 285, 374, so it is unclear which residue is responsible for functional consequences); no observed effect on binding of other proteins to actin (mDial, talin, profilin, $\alpha$ -actinin)	[41]
	Cys272* (not in $\alpha$ -actin)	$H_2O_2$ , 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), diamide	Cys272 and 374 oxidation; decreased polymerization and decreased profilin binding to G-actin; fragmentation and depolymerization of F-actin; functional effects are only observed in conditions where both Cys are oxidized	[70]
		Treatment of neutrophils with excessive oxygen to generate physiologic amounts of NO	Nitrosylation of actin, with increased actin polymerization and impaired actin organization (also observed oxidation of Cys257, 285, 374, so it is unclear which residue is responsible for functional consequences); no observed effect on binding of other proteins to actin (mDial, talin, profilin, $\alpha$ -actinin)	[41]
	Cys285*	Excessive $H_2O_2$	F-actin bundles; prevents proliferation at times of oxidative stress to protect yeast from oxidative damage	[9]
		Treatment of neutrophils with excessive oxygen to generate physiologic amounts of NO	Nitrosylation of actin, with increased actin polymerization and impaired actin organization (also observed oxidation of Cys257, 272, 374, so it is unclear which residue is responsible for functional consequences); no observed effect on binding of other	[41]

Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
		proteins to actin (mDial, talin, profilin, $\alpha$ -actinin)	
	S-nitrosoglutathione	S-nitrosylation of actin to undetermined endpoint	[417]
	S-nitrosocysteine	S-nitrosylation of actin correlates with enhanced relaxation and impaired contraction of cardiomyocytes	[82]
	Hypoxia treatment of endothelial cells to generate physiologic amounts of NO	S-nitrosylation of Cys285 to undetermined endpoint	[418]
	Sickle cell disease	Disulfide bond between Cys285 and Cys374 in actin correlates with delayed dissociation of actin and spectrin, impaired actin remodeling and retention of sickle shape	[419]
Cys374 *	H <sub>2</sub> O <sub>2</sub> , 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), diamide	Cys272 and 374 oxidation; decreased polymerization and decreased profilin binding to G-actin; fragmentation and depolymerization of F-actin; functional effects are only observed in conditions where both Cys are oxidized	[70]
	Excessive H <sub>2</sub> O <sub>2</sub>	F-actin bundles; prevents proliferation at times of oxidative stress, to protect yeast from oxidative damage	[9]
	tert-butyl hydroperoxide (t-BH)	Oxidation causes change in actin conformation and results in impaired polymerization	[71]
	Physiologic levels of ROS generated during integrin-mediated cell adhesion	Glutathionylation of actin/Cys374 leading to actin/non-muscle-myosin II disassembly and cell spreading	[98]
	Glutathionylation <i>in vitro</i>	Decreased ability to polymerize (specifically filament elongation is inhibited); increased ATP exchange rate, change in actin conformation,	[420, 421]

Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
	EGF	De-glutathionylation promotes actin polymerization	[72]
	Treatment of neutrophils with excessive oxygen to generate physiologic amounts of NO	Nitrosylation of actin, with increased actin polymerization and impaired actin organization (also observed oxidation of Cys257, 272, 285, so it is unclear which residue is responsible for functional consequences); no observed effect on binding of other proteins to actin (mDial, talin, profilin, $\alpha$ -actinin)	[41]
	S-nitrosoglutathione	S-nitrosylation impairs G-actin polymerization and acts as vasodilator (NO donor)	[417, 422]
	Sickle cell disease	Disulfide bond between Cys285 and Cys374 in actin correlates with delayed dissociation of actin and spectrin, impairing actin remodeling and retention of sickle shape	[419]
	Mutant forms of actin C374A/C374D/C374E	Oxidation-mimetic impaired actin organization, generated less stable actin filaments, promoted actin/non-muscle-myosin II disassembly and cell spreading	[98, 423]
Methionine	X-ray irradiation	Sulfenic, sulfenic, and sulfonic acid oxidation of actin, with decreased actin polymerization and decreased ability of actin to activate myosin II (observed oxidation of Cys217, 259, Met44, 47, 355, & Trp79, 86 so it is unclear which residue is responsible for functional consequences)	[415]
	Chloramine-T (CT)	Met44, 47, 355 are most reactive, but Met176, 190, 227, 269 are also oxidized and Met176, 190, 269	[424]



	Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
<b>ACTIN BINDING PROTEINS</b>	Myosin II		oxidation causes F-actin depolymerization and inhibits G-actin polymerization	
		MICALs (Molecule Interacting with CasL)	Met44, 47 are both oxidized, but Met44 is responsible for decreases in inter-actin contacts leading to fragile F-actin that is quick to disassemble and slow to reassemble; enhances cofilin binding to F-actin; increases SRF/MRTF-A-driven gene expression including cardiac muscle specific genes	[77, 78, 83]
		Integrin engagement (ROS produced by 5- lipoxygenase)	Decreased myosin II:actin binding and increased cell spreading	[98]
	Cofilin	H <sub>2</sub> O <sub>2</sub> (Protista) and oxidized glutathione for muscle-muscle myosin heavy chain  H <sub>2</sub> O <sub>2</sub> , oxidized glutathione, cell migration	Decreased ATPase activity and actin interaction	[96, 97]
<b>ACTIN REGULATORY PROTEINS</b>		Taurine chloramine	Oxidation increases the F-actin:G-actin ratio in the cell by preventing cofilin from binding to and severing actin	[106, 108]
		H <sub>2</sub> O <sub>2</sub>	Unlocking a disulfide bridge	[121]
		O <sub>2</sub> <sup>-</sup> , •NO <sub>2</sub> , or HO•, H <sub>2</sub> O <sub>2</sub> and NO	Cys20 oxidation promotes nucleotide exchange, RhoA activation and stress fiber formation; Cys16 and Cys20 co-oxidation causes disulfide bond formation and RhoA inactivation; S-nitrosylation is inactivating	[138, 144, 146]

	Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
Rac1	Cys18	O <sub>2</sub> <sup>•-</sup> , •NO <sub>2</sub> , or HO•, H <sub>2</sub> O <sub>2</sub> and NO	Oxidation and glutathionylation promote nucleotide exchange and Rac1 activation and lamellipodia formation	[138–142]
Cdc42	Cys18	O <sub>2</sub> <sup>•-</sup> , •NO <sub>2</sub> , or HO•, H <sub>2</sub> O <sub>2</sub> and NO	Oxidation promotes nucleotide exchange and Cdc42 activation	[138]
IQGAP	Unknown cysteine(s)	VEGF stimulation, hindlimb ischemia, H <sub>2</sub> O <sub>2</sub>	Proposed to promote endothelial cell directed migration	[163]
LMW-PTP	Cys12, Cys17	H <sub>2</sub> O <sub>2</sub>	Forms an inactivating disulfide bond	[176]
Src	Undetermined	ROS induced by VEGF	S-glutathionylation; inhibits its phosphorylation and activity	[177]
	Intramolecular sulfhydryl (SH) group	NO or N <sub>2</sub> O <sub>3</sub>	Forms S-S bond; promotes trans-phosphorylation of Tyr416	[186]
PKC	SH <sub>2</sub> domain (Cys245), kinase domain (Cys487)	H <sub>2</sub> O <sub>2</sub>	Further activates an already active Src	[179]
	N-terminal regulatory and the C-terminal catalytic domains	O <sub>2</sub> <sup>•-</sup>	Activation	[212, 213]
LTCC	Zinc finger motif	O <sub>2</sub> <sup>•-</sup>	Activation	[213]
	LTCC: α1C-subunit	HO•, thimerosal, p-chloromercuri-benzene sulphonic acid	Inactivation	[221–223]
IP <sub>3</sub> R	Cys34, Cys42, Cys65	GSH/GSSG oxidizing system	Reduces its activity in line with the conformational changes	[224, 225]
SERCA	Cys674	Nitric oxide, peroxydinitrite	Reversible S-glutathiolation, enhances activity	[226–228]
	Cys674, Tyr294/295	ROS	Sulfenylation of Cys674 or nitration of Tyr294/295 decreases activity	[229]
NCX	undetermined	H <sub>2</sub> O <sub>2</sub> or that produced by xanthine oxidase with hypoxanthine	Activation	[232–234]
	undetermined	Hypochlorite	Disulfide bond formation, activation or inhibition	[235]
CaM	Met36, Met51, Met71, Met72 and Met145	H <sub>2</sub> O <sub>2</sub>	Degradation	[245, 246]

	Site of oxidation	Oxidizing source/stimulus	Consequence	Refs.
	Met144 and Met145	Sulfoxides (CaMox), free-energy simulations, H <sub>2</sub> O <sub>2</sub>	Tertiary structural rearrangements, destabilization and decreased affinity for substrates	[245, 247–249]
CaMK	Cys280/Met281 (α isoform) or Met281/282 (β, γ, and δ isoforms)	H <sub>2</sub> O <sub>2</sub>	Activation	[256]

\* The human β-actin sequence was used for numbering actin residues.

Summary of variables that affect the susceptibility of actin to oxidation and the outcome of this oxidation.

**Table 2**

Variable	Susceptibility/Effect of oxidation			Notes
	High	Medium	Low	
Type of actin	$\alpha$ , $\beta$ , $\gamma$			$\alpha$ -, $\beta$ -, $\gamma$ -actin are all readily oxidized, but $\beta$ -, $\gamma$ -actin have an additional cysteine (272) that can be oxidized which is lacking in $\alpha$ -actin.
Form of actin	Globular		Filamentous	Filamentous actin has been described as less accessible to oxidation [80] and greater amounts of oxidized G-actin have been observed compared to F-actin, yet this could be due to the fact that once actin is oxidized it promotes the globular form (F-actin depolymerizes and G-actin is slow to polymerize) [70].
Cysteine identity	Cys374	Cys217, Cys257, Cys272, Cys285	Cys17	Cys374 is highly reactive and is even oxidized upon air exposure [67]. Cys17 is only oxidized in very specific conditions [70, 79, 414]. In conditions where cysteines other than Cys374 are highly susceptible to oxidation, the functional consequences are not as great. For example, when Cys272 is described to be oxidized before Cys374, it has no effect on polymerization until C374 is oxidized [70]. Similarly, Cys257 is highly reactive with 7-dimethylamino-4-methyl-(N-maleimidy) coumarin, but this doesn't affect polymerization [80].
Methionine identity	Met44	Met47		Actin is oxidized at Met44 and Met47 by a family of proteins called MICALs (Molecule Interacting with CasL), but Met44 oxidation is the key residue for regulation of actin disassembly [77]. Other methionines can also be oxidized under extreme oxidative stress [415, 424].
Divalent ion bound	$Ca^{2+}$		$Mg^{2+}$	Unlike $Ca^{2+}$ -bound G-actin, $Mg^{2+}$ -bound G-actin is resistant to $H_2O_2$ [81].
Calcium concentration	Low $Ca^{2+}$		High $Ca^{2+}$	More residues become available for oxidation at low $Ca^{2+}$ , and high $Ca^{2+}$ (>10 $\mu$ M) shields Cys374 from $H_2O_2$ oxidation [70].
Protein bound	Cys10 of Myosin bound-actin		Cys10 of Myosin free-actin	Actin Cys10 is more reactive when myosin II is bound [79], yet this effect may be specific to this cysteine as others have found that myosin II blocks reactivity of SH groups in actin [425].