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Regulated methionine oxidation by monooxygenases

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Abstract

Protein function can be regulated via post-translational modifications by numerous enzymatic and non-enzymatic mechanisms, including oxidation of cysteine and methionine residues. Redox-dependent regulatory mechanisms have been identified for nearly every cellular process, but the major paradigm has been that cellular components are oxidized (damaged) by reactive oxygen species (ROS) in a relatively unspecific way, and then reduced (repaired) by designated reductases. While this scheme may work with cysteine, it cannot be ascribed to other residues, such as methionine, whose reaction with ROS is too slow to be biologically relevant. However, methionine is clearly oxidized *in vivo* and enzymes for its stereoselective reduction are present in all three domains of life. Here, we revisit the chemistry and biology of methionine oxidation, with emphasis on its generation by enzymes from the monooxygenase family. Particular attention is placed on MICALs, a recently discovered family of proteins that harbor an unusual flavin-monooxygenase domain with an NADPH-dependent methionine sulfoxidase activity. Based on the structural and kinetic information we provide a rational framework to explain MICAL mechanism, inhibition, and regulation. Methionine residues that are targeted by MICALs are reduced back by methionine sulfoxide reductases, suggesting that reversible methionine oxidation may be a general mechanism analogous to the regulation by phosphorylation by kinases/phosphatases. The identification of new enzymes that catalyze the oxidation of methionine will open a new area of research at the forefront of redox signaling.

Keywords

MICAL; methionine; methionine oxidation; methionine sulfoxide; methionine sulfoxide reductase; actin; monooxygenase; DUF3585; multidomain proteins; scaffolding proteins; redox signaling

1. Novel mechanism of redox regulation

Oxidative and nitrosative changes of amino acid residue side chains are considered as a major route for protein posttranslational modifications, with relevance to both physiology and pathology. Naturally formed oxidants such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are no longer viewed as primarily "toxic" molecules and instead function as "signaling" metabolites[1–3]. While cysteine oxidation and reduction is a part of well-established reversible signaling and regulatory systems including "receptor proteins" (e.g. peroxiredoxins) and "transducer proteins" (e.g. thiol-disulfide oxidoreductases)[4–6], the signaling cascades involving oxidative modifications of groups other than thiols are far less characterized. In this regard, methionine oxidation represents a paradigmatic case: formation

of methionine sulfoxide (MetO) is a posttranslational modification that is known to happen *in vivo* under both normal or stress conditions[7,8]. MetO is reduced by a group of proteins collectively known as methionine sulfoxide reductases (Msr) that have been extensively reviewed in the last few years and will not be covered in detail here (for representative references see [9–13]). There are major questions that remained unsolved: *how is methionine sulfoxide formed in vivo under physiological conditions*? *Is methionine oxidized by the direct reaction with reactive oxygen species (ROS) or via an enzymatic system*? This review provides an overview of methionine oxidation, with special attention to MICAL proteins, a recently discovered family of flavin-dependent monooxygenases that catalyze the oxidation of methionine residues in actin. The targeted stereospecific oxidation and reduction of methionine has emerged as a novel molecular mechanism for regulation of cellular functions, and here we propose a model for methionine sulfoxide-dependent signaling[10,14].

2. Oxidation of methionine: where, when and how?

There is a large body of evidence showing that oxidation of methionine can affect protein function both *in vitro* and *in vivo*, with calmodulin (CaM) and Ca^{2+}/CaM-dependent protein kinase II (CaMKII) being the best studied cases (reviewed in [15,16]). Methionine oxidation has also been shown to severely impair cytoskeleton organization, affecting actin[17–19], myosin[20] and cofilin[21] functions. Over the last decade, a major paradigm has been that both free methionine and methionine residues are oxidized ("damaged") by oxidants such as H_2O_2, hypochlorous acid (HOCl) and chloramines in a relatively unspecific way, and are reduced (repaired) by Msrs (Figure 1A). This model where the oxidation is a *chemical step* and the reduction is an *enzymatic step* has been largely influenced by the widely accepted cysteine-dependent redox signaling. Thiol groups in cysteine residues can be highly reactive with H_2O_2 (ref.[22]), nitrogen oxides[23,24] or chloramines[25], leading to the formation of sulfenic acids that can subsequently progress to disulfides or higher oxidation states. Intrinsic thiolate nucleophilicity contributes to making the reaction between cysteine and electrophiles feasible under physiological conditions, at least for some protein-bound cysteines and low-molecular-weight thiol compounds such as glutathione[22,26]. Moreover, the physicochemical environment surrounding a cysteine can greatly influence its reactivity towards specific oxidants transforming this chemical step into a catalytically-driven reaction[22,27–29]. By analogy, we are used to reading that due to the "high susceptibility of methionine to oxidation", formation of MetO in response to H_2O_2 is a physiological outcome of oxidative stress. However, experimental evidence does not support this notion. The thioether of methionine side chain has lower nucleophilicity compared to cysteine and no acid-based equilibrium in the range of physiological pH. The reaction between Met and oxidants is a standard nucleophilic substitution, wherein the sulfur atom of methionine acts as a nucleophile attacking, for example, the electrophilic H_2O_2 (Figure 1B). The amino acid methionine is not a chiral molecule *per se* and the tetrahedral intermediate will progress to a racemic mixture of diastereomers known as Met-(S)-O and Met-(R)-O. This can be considered a pure chemical case, but Msrs are stereospecific, suggesting that this isomeric speciation is biologically relevant[12]. Additionally, free and protein-based methionine reacts with H_2O_2 with rate constants ~10^{-2} $M^{-1}s^{-1}$ (refs.[30–34]) that are too low to explain its formation *in vivo* and certainly do not support competition with proficient peroxidases such

as peroxiredoxins or glutathione peroxidases that react 10^8–10^{10} times faster[27,35]. Therefore, only a very small fraction of methionines can be oxidized inside the cells, even under conditions of oxidative stress. While physiological Met oxidation offers sufficient selection in the origin and evolution of Msrs, quantitative and even significant oxidation of Met by oxidants such as H_2O_2 is difficult to achieve in the intracellular milieu.

In addition, not every methionine in a cell will be equally susceptible to oxidation. A recent bioinformatics analysis shows that, as expected, methionines located on solvent-exposed and flexible structures are more prone to oxidation in comparison with buried methionines. However, oxidation of buried methionines can be promoted by the oxidation of neighboring methionines that are more exposed and/or on flexible structures[36]. Solvent accessibility is a relevant factor for the propensity of methionine to react with oxidants, but not the only one. The protein environment within which the methionine is located can affect its reactivity. Contrary to the case of cysteine, where a set of defined interactions in the protein environment are known to promote its reactivity with H_2O_2 (refs.[22,27–29]), no similar analysis has been done for methionine. The only established pattern known to affect methionine reactivity is the interaction of its sulfur atom with the aromatic ring of tyrosine and other aromatic amino acids[37,38], reducing its propensity to oxidation[39]. Additionally, a recent report provided a bioinformatics evidence that methionine oxidation is enriched in the proximity of phosphorylation sites[40]. Identification of methionines oxidized *in vivo* lags behind the research on cysteine oxidation. While dozens of proteomic papers reported on the identification and quantification of sulfenic acids, disulfides and persulfides in proteins[41–45], few reports targeted formation of MetO *in vivo* and, if they did, studies focused on cells subjected to high (non-physiological) concentrations of H_2O_2 or chloramines[19,46]. In this regard, future studies may benefit from the use of novel highly sensitive proteomic techniques[47] as well as the utilization of genetically-encoded biosensors for MetO (MetROX and MetSOX)[48,49].

Several reports established that MetO is formed *in vitro* and *in vivo* even when no oxidative insult is applied[48,50–53]; in these cases, the source of oxidant is unknown. It was found that *in vivo* CaM oxidation is dependent on aldosterone-induced NAPDH oxidase (NOX) activation, suggesting that $O_2^{\bullet-}/H_2O_2$ is the endogenous oxidizing agent[54]. However, CaM is stereospecifically oxidized to Met-(S)-O, which is unlikely to occur if the oxidation is the consequence of an uncatalyzed chemical reaction (standard nucleophilic substitution between H_2O_2 and the thioether of methionine, Figure 1B). Another source of MetO are heme-dependent peroxidases[55], but so far this has been demonstrated only in bacteria. Considering chemical reactivity with natural and endogenous oxidants, random oxidation of Met by reactive oxygen species is unlikely and, as stated by Elfrara and coworkers a decade ago “[…] this suggests that there are specialized oxidases/reductases that are responsible for the oxidation of specific protein targets”[56].

3. Flavin-dependent monooxygenases and methionine oxidation

Flavin-dependent enzymes are classified into two families known as “oxidases” and “monooxygenases”. Monooxygenases (FMO, E.C. 1.14.13.8) are the second largest group of enzymes involved in phase I metabolism of drugs and xenobiotics[57]; they use flavin adenine

dinucleotide (FAD) cofactor and molecular oxygen (O_2) for various oxygenation reactions[58]. The FAD cofactor is reduced by NADH or NADPH (Figure 2, step a) and the resulting $FADH_2$ reacts with O_2 forming a hydroperoxide on the C4a position (step b). This "reactive" intermediate can then undergo productive oxygen transfer (steps c, d), introducing one oxygen atom into the substrate and reducing the second one to water, or eliminate H_2O_2 (ref [59]). The reactions catalyzed by these oxygenating enzymes often feature an outstanding degree of chemo-, regio- and/or stereospecificity[60]. A classification based on sequence, structural features, electron donor and type of oxygenation reaction identified six classes of FMOs[60], but those relevant to mammals are class A and B. The human genome codes for five class B FMOs (FMO1–5, historically known as "microsomal monooxygenases") that participate mainly in oxidative xenobiotic metabolism[61]. These five FMOs are monomeric enzymes with a topology similar to the Rossmann fold and highly conserved residues for FAD and NADPH cofactor binding[62]. Mammalian FMOs use the electrophilic nature of a flavin-bound hydroperoxide to oxygenate a wide variety of carbon-bound nucleophilic nitrogen, sulfur, and halide species[58,59]. Class A monooxygenases are mechanistically similar to class B but act mostly on hydroxyl-containing substrates. The prototypical example of this group is *para*-hydroxybenzoate hydroxylase (pHBH, E.C. 1.14.13.2) from *Pseudomonas fluorescen*, a key enzyme in the degradation of aromatic compounds[63]. pHBH is the most extensively studied FMO, and its catalytic cycle has been reviewed elsewhere[64]. Two features of class A FMOs require special attention: they catalyze stereospecific reactions, leading to *ortho*- or *para*-hydroxylated compounds and that the binding of the acceptor substrate strongly stimulates the reduction of the FAD by NADPH[60]. The mechanism of class A and B FMOs implies a stoichiometric consumption of one equivalent of NADPH and molecular oxygen per catalytic cycle, resulting in a hydroxylated substrate, $NADP^+$ and water. However, most of the monooxygenases can produce H_2O_2 under "uncoupling" conditions, *i.e.* the nonproductive decay of the hydroperoxideintermediate[59,65–68] (Figure 2, step e). While mammalian FMOs have been extensively characterized as detoxifying enzymes, it has been shown that FMO1, FMO2, and FMO3 possess catalytic activity toward methionine, generating MetO[56,69–72]. Interestingly, the MetO formed in liver, kidney, and lung microsomal fraction shows enrichment for the R isomer, pointing to a catalytic mechanism rather than a direct reaction with H_2O_2. While the role of FMOs in Met metabolism has been largely overlooked, free and protein-based MetO has, however, long been identified in urine and plasma of man or animals, both in normal and pathological conditions[73–75]. How this MetO is produced and/or excreted requires further studies, but the available literature suggests that mammalian FMOs may indeed be involved in this process. S-oxidation by FMO has been described for several organic compounds, including thioanisoles and thioureas[58,66,76]. A recent paper identified an FMO as the protein responsible for the inactivation of the transcription factor NirA in *Aspergilus nidulans* via the catalytic oxidation of a Met residue in its nuclear export signal[77], suggesting that this mechanism may be more common than originally thought. Indeed, yeast FMO can oxidize thiol-compounds like glutathione and cysteine, but not methionine[78].

4. FMO in metabolic regulation and aging

During the last decade, mammalian FMOs have gained attention with roles beyond xenobiotic detoxification, in particular, FMO3, an abundant liver enzyme that catalyzes a variety of oxygenation reactions of nitrogen- and sulfur-containing xenobiotics, including the oxidation of trimethylamine (TMA) to trimethylamine N-oxide (TMAO). In humans, TMA is mostly produced by the gut microbiota from phosphatidylcholine and/or carnitine, found in large quantities in red meat and certain vegetables[79,80]. Loss-of-function mutations in the FMO3 gene cause the inherited disorder trimethylaminuria (TMAU) or “fish odor syndrome” due to the excretion of TMA in urine and sweat. Besides TMAU, a number of other diseases are associated with abnormal levels of TMA, particularly metabolic and cardiovascular diseases. Several reports suggest that FMO3 and TMAO levels strongly correlate with atherosclerosis, cardiovascular disease risk and cholesterol metabolism[79,81–83], and this has been attributed to a pro-atherogenic role of TMAO inducing the activation of nuclear factor-κB (NFκB) and the induction of an inflammatory response in vascular endothelia[84]. However, FMO3 and TMAO have also been linked with longevity. FMO3 expression varies with age and sex: adult males have lower expression than females, and this down-regulation of FMO3 is related to androgen production after puberty [85]. Accordingly, young animals excrete TMAO in the urine, and its concentration is reduced during aging[86]. Interestingly, FMO3 is strongly upregulated in long-lived mouse models such as Snell and Ames Dwarfs and in mice subjected to a calorie restriction diet or treated with calorie-restriction “mimetics” such as metformin, glipizide or rosiglitazone[87,88]. Accordingly, CR results in an increase of TMAO in plasma and urine of mice, rats and dogs[75,89,90]. Moreover, it was shown that amine catabolism positively correlates with lifespan[91] across mammals and that TMAO levels positively correlate with longevity[92]. Finally, a recent report showed that the single flavin monooxygenase gene is necessary for dietary restriction-mediated lifespan extension in *Caenorhabditis elegans*[93]. How can TMAO be a toxic compound with a detrimental effect on the cardiovascular system and, at the same time, be a signature of longevity? Future studies should address this question.

5. MICAL

5.1. Discovery of a new family of monooxygenases that oxidize methionine residues

MICAL proteins are a family of large (~120 kDa) cytoplasmic, actin-binding proteins that gained attention in the last few years as redox-dependent actin regulators[94,95]. MICALs were discovered in 2002 almost simultaneously by Hirai[96] and Kolodkin[97] groups. Suzuki *et al.*[96] first reported the identification, in non-neuronal human cell lines, of a multidomain protein that interacts with the SH3 domain of CasL (currently named NEDD9) and named it “MICAL” for *M*olecule *I*nteracting with *CasL*. CasL/NEDD9 belongs to the p130 family of proteins that link actomyosin cytoskeleton to adhesion complexes[98]. They also proved that this new protein interacts with vimentin, an essential component of intermediate filaments in mesenchymal cells[99]. Based on a yeast-two-hybrid assay, Terman *et al.* identified MICAL as an interactor of the cytoplasmic domain of plexin A receptor required for semaphorin-mediated repulsive axon guidance in fruit flies[97]. Semaphorin signal triggers a rapid collapse of the nerve growth cone accompanied by the depolymerization of filamentous (F)-actin[100].

This publication was the first to address the complex domain organization of MICAL proteins, with its unique combination of a non-canonical FMO domain followed by a calponin homology (CH) domain, a LIM domain and a coiled-coil C-terminal domain (CTD) of unknown function (Figure 3). Soon after, Barkenow's group reported that human MICAL1 (orthologous of *Drosophila melanogaster* single MICAL gene) interacts with Rab1[101], the observation that was later extended to other MICALs and Rabs[102,103]. Interestingly, all the interactions that led to the identification of MICAL docked to the CTD, which provided no clue as to how MICALs exert their functions. Although Terman *et al.* did not formally prove that MICAL functions as a monooxygenase, they showed that pharmacological inhibitors of FMO suppress semaphorin signaling in an *in vitro* growth cone repulsion assay (see section 5.4.2). Ventura and Pelicci then put the pieces together and proposed that MICAL proteins function as recruiting factors downstream of several signaling pathways and exert their actions via the "production of reactive oxygen species or direct oxidation on downstream targets" [100]. While indispensability of the monooxygenase activity for MICAL function was confirmed in a screen for flies with abnormally structured neuromuscular junctions[104], it was not until a breakthrough report from Terman's group was published that proposed a mechanism for MICALs. Hung *et al.*[105] showed that homozygous MICAL$^{-/-}$ adult mutant flies had abnormally shaped bristles. Like axon guidance, bristle development is an actin-dependent process, and the authors proved that MICAL was involved in binding and modifying the actin cytoskeleton via the oxidation of two conserved methionine residues in actin, altering its polymerization properties[105]. These findings were later confirmed *in vitro* and in several cell types[106–109] launching MICAL proteins as the *first and only redox-dependent F-actin disassembling factor* described so far. The Met residues targeted by MICAL are highly conserved among actins and actin-related proteins and belong to the DNAseI binding loop (D-loop, for short) a flexible structure on actin subdomain II that is involved in globular (G-)actin subunit interaction during polymerization[110,111]. The subsequent key contribution to firmly position MICAL proteins in the field of redox signaling and regulation[112,113] was the discovery that MICALs stereospecifically oxidize two Met in actin to Met-(R)-O and that these oxidized residues are reduced by methionine sulfoxide reductase B1 (MsrB1), a cytoplasmic selenocysteine-dependent methionine sulfoxide reductase specific for the Met-(R)-SO diastereomer[114], defining MICAL/MSRB1 as an enzymatic couple for reversible Met oxidation that regulates protein function (F-actin assembly and disassembly)[112].

5.2. Biological roles of MICALs and MICAL-like proteins

MICAL-dependent depolymerization of actin has been involved in processes as diverse as axonal guidance[97,105], phagocytosis by macrophages[112], vesicle trafficking[102], epithelial to mesenchymal transition (EMT)[115–117], and cancer invasion and metastasis[115,116,118,119]. Three paralogous genes were identified in mammals, named MICAL1–3[97], with different distribution patterns in embryonic and adult tissues[95,109,120]. While all three MICALs can depolymerize actin[120], they differ in their functions. MICAL1 is not essential for embryonic development (its knockout causes a mild neurological phenotype[121]) in mice, while MICAL3 is mostly associated with vesicle trafficking in non-neuronal cells[102]. MICAL2, on the contrary, was identified as one of 44 essential genes needed for EMT based on a gene expression analysis done in several cancer cell lines[122]. EMT is an essential process for

embryo formation and development, but also for metastasis (for a review see [123]). At the same time, MICAL2 was reported as a key protein for cell migration during metastasis in prostate and breast cancer models[115,119,124] and also during changes in endothelial permeability mediated by vascular endothelial growth factor receptor 1 (VEGFR1) in response to Sem3A treatment both in cell culture and *in vivo*[125]. The three MICAL genes have different subcellular localization and a different pattern of expression in cells and mammalian tissues, with MICAL1 being mostly cytosolic and MICAL2 and 3 enriched in the nuclear fraction, probably due to the presence of a bipartite nuclear localization signal (NLS) downstream of the LIM domain[109,120,121] (Figure 3). Expression patterns of MICALs during mammalian development were examined in rat[126] and mouse[121] hippocampus. In rats, MICAL1 and MICAL3 are abundant in early stages of development and remain expressed through adulthood, whereas MICAL2 is expressed largely in adult animals. Based on ribosome profiling data, our lab has found that the three MICAL genes are translated in various tissues of adult mice, showing several splice variants and large differences in abundance (Gerashchenko and Gladyshev, unpublished). With regard to MICAL gene regulation, there is only one report stating that *D. melanogaster* MICAL is a major downstream target of transcription factor Sox14 during nerve pruning[127]. MICALs can also affect gene expression, as reported for MICAL2, whose redox activity on nuclear F-actin reduces G-actin availability increasing nuclear translocation of myocardin-related transcription factor-A (MRTF-A) factor in response to serum (SRF) pathway[120].

The important work by Terman *et al.* also identified a family of conserved proteins related to MICAL but lacking the FMO domains and thus named “MICAL-like” (MICAL-L) proteins. Mammals have two such proteins, MICAL-L1 and MICAL-L2[97] that, besides their names, evolved from MICAL1 by duplication and loss of the FMO domain, followed by neofunctionalization (Mariotti, Manta and Gladyshev, unpublished). MICAL-L proteins are unable to modify actin directly, but they can bind and recruit additional proteins to actin filaments[128]. Another difference between MICAL-L and MICAL is the presence of one or two conserved NPF (asparagine-proline-phenylalanine) motifs in the former. This motif is typically involved in the interaction with Eps15 homology (EH) domains[129]. MICAL-L1 localizes to tubular recycling endosomes and recruits Rab and EH domain-containing proteins[129,130]. Interestingly, the localization of MICAL-L1 to the endosomes depends on its CTD domain rather than on the canonical binding of CH or LIM-binding domains[103]. Another interacting partner of MICAL-L1 is collapsin response mediator protein 2 (CRMP2), providing a connection between endosomes and the microtubule cytoskeleton[131]. While MICAL-L1 is mostly associated with endosomal recycling, MICAL-L2 (also known as “Junctional Rab13-binding”, JRAB) has been described as a major regulatory protein in the coordination of collective cell migration[132]. MICAL-L2/JRAB is recruited to cell-cell junctions and cell periphery via interactions with small GTPases like Rab13[133–137], actin-binding protein (ABP) like actinin-4[138] or nucleotide exchange factors like DENND2B[139]. Contrary to MICAL-L1, MICAL-L2 directly binds to F-actin, but, paradoxically, it contributes to actin bundling and filament stability[134]. Binding of MICAL-L2 to F-actin is inhibited by its own CTD [134], a mechanism conserved with MICAL1 (see below)[109,140]. MICAL-L2 was also identified as a key element in promoting cell migration in ovarian cancer cell lines[118]. Overall, besides the absence of the active FMO domain in MICAL-L

proteins, they can still regulate actin dynamics and, taking into account that they share interaction domains and regulatory sites, a direct interaction with MICAL proteins cannot be excluded and may be an interesting avenue for future studies.

We further review biochemical and evolutionary features of MICAL proteins, including their structures, enzymology, and mechanisms. A particular attention is placed on the role of MICAL proteins in reversible methionine oxidation. Some aspects of MICAL biology have been reviewed in the last few years and these will not be covered here (see references [94,95,128,141]).

5.3. Unique domain organization of MICAL proteins

Organization of the three mammalian MICALs is similar, which harbor FMO, CH and LIM domains (Figure 3). Every domain is present in a single copy and the order is conserved, generating a unique combination that is not found in any other protein. Domain length is also conserved, whereas the interdomain regions exhibit variability. MICAL-like proteins are somewhat similar, with the exception that they lack the FMO domain. The most significant difference among the three MICALs is the C-terminal part of the proteins, wherein MICAL1 and 3 possess an unusual coiled-coil and MICAL2 lacks it. Individual domains are further discussed in detail.

5.3.1. FMO domain—The FMO domain of all MICALs comprises the initial ~500 amino acids and is highly conserved[142]. The crystallographic structure of mouse MICAL1 FMO was reported before it was found that MICAL proteins directly target actin[143,144]. Based on the well-accepted classification of Dym and Eisenberg for FAD-binding proteins[62], the MICAL FMO belongs to the subfamily 2 of glutathione reductase (GR) structural superfamily. All family members adopt the Rossmann fold with the characteristic GxGxxG ("dinucleotide-binding motif") that supports the docking of the phosphate group of the FAD cofactor, and a GD motif that makes contacts with the ribose moiety of FAD (Figure 3). The mouse MICAL1 FMO can be viewed as a combination of two structural subdomains linked by two β-strands (β9 and β15, residues 227–233 and 367–373, respectively) (Figure 4A). The large subdomain (subdomain 1 or FAD-binding domain) comprises residues 1–226 and 373–484 and contains the conserved GxGxxG and GD motifs. Disruption of this motif by mutagenesis abolishes MICAL activity[105]. The small subdomain (subdomain 2, residues 238–366) contains the monooxygenase moiety formed by residues 238–366 and is inserted between β8 and β16 of subdomain 1. The MICAL FMO domain, as other GR2 subfamily members, lacks the extra Rossmann fold present in the GR1 subfamily involved in NADPH binding[62], but present a conserved basic patch surrounding the access to the FAD cofactor that may be involved in NAPDH binding (residues K115, R116, K118, K221, R156, K180, K322 and R356 in mouse MICAL1, Figure 4B)[141]. Based on the structural comparison, Nadella *et al.*[143] proposed that the catalytic core of the mouse MICAL1 FMO is related to pHBH (rmsd 2.9 Å). As in pHBH, the FAD is bound in an extended conformation with the isoalloxazine ring located at the interface between subdomains 1 and 2 (Figure 4A), but, contrary to pHBH, the cofactor is accessible from a large opening on one side of the protein without a defined "substrate binding cavity" for the acceptor substrate (Figure 4B). In MICALs, the FAD position is locked by van der Waals interactions on the *si* face and π

interactions with a conserved tryptophan residue (W400 in mouse MICAL1, Figure 3 and 4A) on the *re* face of oxidized flavin. A broad absorption band that extends up to 700 nm is attributed to a charge transfer interaction between this tryptophan and the isoalloxazine ring in the oxidized conformation[106,145]. The main difference between the mouse MICAL1 FMO and pHBH occurs at the N-terminus (subdomains 1) due to the presence of a four-helix bundle with a highly basic surface represented by several solvent-exposed lysines and histidines (K52, K61, K66, K69, K86, R35, R70, H11, H13 and H49 in mouse MICAL1), conserved among variant and species of MICAL proteins but not in other monooxygenases[141,144] (Figure 3 and 4B).

5.3.2. Classification of MICAL proteins into the monooxygenase family— Classification of MICAL proteins within the monooxygenase family is neither easy nor unequivocal because they share the properties of both class A and B monooxygenases (Figure 1B). Besides the fact that these two groups split at the base of FMO evolution, they share several common features both at the sequence and mechanistic levels[142]. Oxygenation of thioethers and other non-aromatic substrates is a reaction typically catalyzed by class B monooxygenases, generally known as “oxidases”. While the thioether is not a chiral center, the sulfur oxidation leads to the formation of enantioselective products[58], as is the case for MICALs[112]. However, based on sequence comparison and mechanism, MICAL proteins are closer to group A FMO (“hydroxylases”), mostly due to the presence of a single Rossmann fold for FAD binding. Class A FMOs catalyze the oxygenation of aromatic substrates, being very restrictive in terms of substrate specificity[60]. While methionine is clearly not an aromatic substrate, MICALs exhibit narrow substrate specificity, oxidizing exclusively a single pair of methionines in a certain structural environment[112]. Several small molecules containing thioethers or thiols were tested as MICAL substrates with no success (Manta and Gladyshev, unpublished). Another common feature between MICALs and class A FMOs is that flavin reduction is highly stimulated in the presence of the oxygen acceptor substrate[145,146]. If further substrates of MICAL are identified, this will imply a broader substrate specificity, a feature typical of class B enzymes. In conclusion, based on the current knowledge MICALs should be classified as class A FMOs, but further mechanistic studies are needed to shed light on this issue.

5.3.3. CH domain—The calponin homology (CH) domain is a protein module of 100–110 residues, which is often found in signaling and cytoskeletal proteins, particularly in actin-binding proteins (ABPs). They are classified into 3 types according to sequence and their ability to bind to actin. Type 1 (CH1) and 2 (CH2) domains are commonly found together in tandem in cytoskeletal proteins such as dystrophin, spectrin, and filamin[147]. The distribution of CH domains in distantly related eukaryotes suggests that they were present already in their last common ancestor[148]. An isolated CH1 domain is able to bind to actin, but in most cases, a tandem pair of CH domains is required for a fully functional actin-binding protein, and this is usually achieved by combining CH1 and CH2 domains[147,149]. Type 3 CH (CH3) domains are found in proteins that regulate muscle contraction (i.e. calponin) and lack actin-binding properties[150]. Most of the ABPs have two or more CH domains, but the human genome also codes for 30 proteins harboring a single CH in multidomain proteins, including all MICAL and MICAL-like proteins[151]. The CH domain of MICALs is classified as a type

2, as confirmed by sequence comparison and structural evidence[146,152]. MICALs' CH2 domains are highly conserved (compared to other CH2 domains) and contain a hydrophobic core with the $WX_{17–21}GX_{11}P$ signature, an actin-binding region and a phosphatidylinositol-(4,5)-bisphosphate (PIP_2) binding site[146,151] (Figure 5A). In contrast to other CH2 domains, such as those present in α-actinin and microtubule-associated proteins RP/EB (MAPRE)[147], the CH2 domains of MICALs are unable to bind to filamentous actin[134,152]. Several reports showed that, indeed, the CH domain is not needed for MICAL activity on F-actin[105,109,112]. However, the studies on the role of this domain in MICAL protein function and regulation may have been hindered by the complexity of MICAL enzymology. Recent reports suggest that the CH domain cooperates with the MICAL FMO domain in binding actin, improving catalytic efficiency of the monooxygenase reaction[146,153]. The sequence that connects the FMO and CH domains is one of the most variable regions of MICAL, but it is short enough to support a direct physical contact between domains, as demonstrated by a recent crystallographic structure in which a conserved interaction surface between the two domains is clearly defined, providing structural basis for the connection between actin and phosphatidylinositol-4,5-bisphosphate (PIP_2) binding involving the CH domain and the FMO activity[146]. The direct role of PIP_2 in MICAL function, if any, has not been addressed experimentally.

5.3.4. LIM domain—LIM is an ancient eukaryotic protein domain that originated prior to the last common ancestor of plants, fungi, amoebae, and animals[154]. Its name is an acronym of the first three genes in which it was identified: *Lin-11*, *Isl1* and *Mec-3*[155]. LIM domains are composed of tandem repeats of small (55–60 amino acids) cysteine-rich zinc-finger structures that provide a structural scaffold for protein docking (Figure 5B). They have been ascribed as a docking site for kinases, transcription factors and several cytoskeletal proteins, particularly cytoskeletal complexes such as focal adhesions and adherens junctions[155]. LIM domains are also universally conserved among MICAL and MICAL-L proteins and were identified as the binding site of nuclear Dbf2-related (NDR) kinase[107], a family of kinases related to LATS1/2 kinases and involved in the Hippo pathway that controls organ size and regeneration[156]. LIM domain is also needed for the interaction between MICAL1 and CRMP1, a highly phosphorylated scaffold protein that also participates in axonal branching and guidance (reviewed in [157]). As we comment in the next section, MICAL's LIM domains also interact with the C-terminal region of the same polypeptide[133,135,140]. The structure of the MICAL1 LIM domain (residues 687–755, PDB ID 2CO8) was determined by NMR (Figure 5B), showing high similarity with other LIM domains[155]. While the conservation among MICALs is high at the N-terminal end of the domain, the second half shows significant intra- and interspecies variability. The distance between the CH and LIM domains also varies considerably, with MICAL2 having the largest gap between the two domains[158]. The region downstream LIM domain is unstructured and heavily loaded with linear motifs. It varies in length and composition among different MICALs, being less than 100 amino acids in MICAL2 and over a thousand in MICAL3. A common denominator in this region are proline-rich domains, like the SH3-binding PPKPP motifs found in MICAL1 that support the interaction with Cas and CasL[96] (Figure 3). This unstructured region is also populated with phosphorylation sites and harbor unique composition of aminoacids. For

example, the linker between LIM and CTD domainin MICAL3 is highly enriched in glutamic acid (14%), proline (11%) and serine (14%) residues (Figure 3).

5.3.5. CTD domain—The most intriguing domain of MICAL proteins is the C-terminal domain classified as DUF (domain of unknown function) 3585. It is present in mammalian MICAL1 and 3 genes, but not in MICAL2. This largely uncharacterized DUF3585 domain occurs exclusively in eukaryotes, and in mammals its restricted to 8 proteins: MICAL11 and 3, MICAL-L1 and MICAL-L2 and, two EH-domain-binding-protein (EHBP1 and EHBP1-like1[159,160]) and two recently discovered proteins known as MICAL-C-terminal-like protein (MICAL-CL), also known as Ebitein1[161,162], and C16orf45, also known as MINP[163] (Figure 6). The CTDs of MICAL-L1, MICAL-L2, EHBP1, EHBP1-like1 and MINP are closer in sequence to the MICAL1 CTD, suggesting a common ancestry. MICAL-CL/Ebitein 1 was identified as a downstream interactor of the extracellular signal-regulated kinase (ERK2), also known as p42 mitogen-activated protein kinase, a member of the MAPK superfamily of serine/threonine protein kinases[161,162]. MINP/C16orf45, on the other hand, is more related to MICAL3 CTD (Mariotti, Manta, Gladyshev, unpublished). MINP ("*mi*gration *in*hibitory *p*rotein") received it name because it may inhibit neuron migration during cortex development via a partly characterized tubulin-dependent mechanism[163]. The average identity among the eight DUF3585 domains present in mouse proteins is 34%, but several residues are highly conserved and present a pattern of alternation of charges and hydrophobic residues typical of a coiled-coil structure. In addition, an acidic patch forms in a cleft of MICAL1 and 3 CTDs (Figure 6)[103]. The role of these conserved residues in MICAL function has not been experimentally addressed. MICALs' CTDs are distantly related to the ERM family (for *E*zrin, *R*adixin, and *M*oesin, also known as FERM), a family of eukaryotic proteins involved in linking the plasma membrane to the cortical actin cytoskeleton[164]. Interestingly, ERM proteins share a common auto-inhibitory mechanism based on the binding of N- and C-terminal ends of the protein into a closed conformation, as in the case of focal adhesion kinase[165]. Binding of PIP_2 triggers a conformational change that unmasks membrane- and actin-binding sites[166]. In this regard, the analogy with MICAL autoinhibition and MICAL-L2 concerted conformational change is striking. Due to the ability of the DUF3585 harboring proteins to interacts with Rab proteins and MICALs simultaneously, a recent report suggests to call them collectively "bivalent MICAL/EHBP Rab binding" (bMERB)[103].

5.4. Mechanism and regulation

The multidomain, scaffolding nature of MICAL proteins together with the polymeric nature of their only known substrate (actin) precluded development of assays for detailed enzymatic characterization. Additionally, monooxygenase mechanisms are generally difficult to disclose by steady-state kinetics, especially under standard aerobic conditions routinely used in most labs. Under these conditions, MICAL catalysis can only be tracked by NADPH consumption (due to the decrease in its absorbance at 340 nm) or by F-actin depolymerization[167]. These approaches are not necessarily coupled and are neither fully quantitative, hindering the analysis of stoichiometry and enzymology of MICALs[106,143,145,146,153,168]. MICAL catalysis can be split into two discrete phases (Figure 7): in the reductive phase, NADPH reduces FAD, which subsequently reacts with O_2 to form

the hydroperoxide on the C4a position (see below); in the oxidative phase, this reactive intermediate decomposes to H_2O_2 or directly oxidizes the target substrate. Below, we analyze both possible mechanisms.

5.4.1. MICAL mechanism

A. Reductive phase: Due to the structural similarity between the MICAL FMO domain and pHBH, the MICAL reductive phase has been largely compared with the same phase of the latter, for which a detailed description is available[64]. The flavin ring can adopt two conformations, known as “out” and “in”. The pHBH family is characterized by a conformational change triggered by NADPH binding that moves FAD from an “out” position (not productive for catalysis) to the catalytically active “in” position needed for substrate binding and product release. Similarly, in oxidized MICAL1, FAD is in the “out” conformation and the access to the site of hydroperoxide formation is partly blocked by the lateral chain of a conserved asparagine residue (N123 in mouse MICAL1) (Figure 7). Binding of NADPH and hydride transfer induces a conformational change in the position of the isoalloxazine, which loses planarity, adopts a characteristic butterfly conformation of neutral hydroquinone[169] and opens a channel that protrudes from the surface to the C4a position of $FADH_2$, where the hydroperoxide will be formed (Figure 7, step a). Concordantly, the π-stacking with W400 is displaced in the “in” conformation, as confirmed by the disappearance on the charge transfer band[145]. The binding of NADPH is transient and fast, leading to FAD reduction without formation of detectable NADPH-protein complex[144]. This conformational change is the rate limiting step of the reductive part of the mechanism and probably of the entire catalytic cycle[106,153]. It is important to note that, contrary to other FMOs, the MICAL’s catalytic domain is not fully inhibited in the absence of the acceptor substrate, so the addition of NADPH to a MICAL solution will result in the formation of hydroperoxide on the C4a position of the flavin. In the absence of actin, H_2O_2 will be slowly released. In the case of the mouse MICAL1 FMO domain, this “uncoupled” cycle has a K_M for NADPH ranging from 28 to 222 μM and a catalytic efficiency (k_{cat}) between 3 and 77 s^{-1} (refs[106,143]). The use of stopped-flow spectroscopy combined with anaerobiosis provided a better understanding of the individual steps during NADPH oxidation. In the absence of actin or oxygen, NADPH binds to the protein with the dissociation constant (K_d) in the micromolar range and reduces FAD with the bimolecular reaction constant (k_{red}) ranging from 0.074 ± 0.009 s-1 (mouse MICAL2 FMO domain) to 3.0 ± 0.1 s^{-1} (mouse MICAL1 FMO domain)[106,145]. The difference between mouse MICAL1 and MICAL2 FMOs may be explained by small differences in experimental conditions such as pH or ionic strength, as recently reported by Vitali *et al.*[153]. The k_{red} values (determined by stopped-flow) are in the same order of the k_{cat} values (determined by steady-state experiments), supporting the idea that the reductive part of the cycle limits enzyme turnover. Finally, the k_{cat} and k_{red} values were found to be low, consistent with the behavior of an aromatic hydroxylase in the absence of substrate and supporting the classification of MICALs as class A FMO.

B. Oxidative phase: Once the hydroperoxide is formed on the C4a position of FAD (Figure 7, step b), two possible mechanisms may explain how MICALs oxidize actin’s methionines. First, MICALs may *locally* produce H_2O_2 acting as an “NADPH oxidase” (Figure 7, steps d1, d2). This mechanism is supported by *in vitro* and cell-based analyses that measure an

increase in "reactive oxygen species" once MICALs are activated[106,109,116,143,170]. Additionally, *in vitro* oxidation of actin with H_2O_2 leads to the formation of MetO on the same methionine residues targeted by MICALs[17–19]. While it may be true that under some experimental conditions MICAL proteins produce H_2O_2, the "NADPH oxidase" activity is not supported by the structure or biological function of MICAL. Classical NADPH oxidases are membrane-associated multiproteins composed of several subunits that have no sequence or structural relation with monooxygenases[1]. In addition, the kinetics of actin oxidation by H_2O_2(ref[113]) does not match the kinetics of morphological changes following MICAL activation, in agreement with the very low reactivity between free methionine and hydrogen peroxide commented earlier. However, the idea that MICAL is an "oxidase", albeit misleading, is widely accepted (e.g. the nomenclature used by NCBI and UNIPROT databases for these proteins is "methionine oxidase").

A second mechanism considers MICAL proteins as non-standard "monooxygenases" related to class A FMOs ("hydroxylases"). This mechanism involves a direct oxygen transfer from the FAD-hydroperoxide intermediate to the sulfur atom of Met (Figure 7, step e1,e2) and is supported by the MICAL FMO sequence and structure as well as, more importantly, by the strict enantioselectivity of the reaction product[105,112,113,145]. MICALs oxidize methionine residues in actin exclusively to the R enantiomer[112,113]. As we will comment below, F-actin (but not G-actin or its D-loop peptide) highly increase NADPH consumption by mouse MICAL1 and 2, driven by an increase in k_{cat} [106,145,153]. This observation confirms that MICALs exhibit a typical regulatory mechanism of class A FMOs, wherein oxidation of the electron donor is highly accelerated by the binding on the final acceptor. When F-actin is added, NADPH consumption shows a hyperbolic relation with O_2 concentration, indicating an isomerization step of the *reduced* enzyme from a form that reacts slowly with oxygen ("$FADH_2$-off") to a state that rapidly forms the hydroperoxide on position C4a ("$FADH_2$-on"). So far, no structural evidence of how F-actin induces this retrograde conformational change has been obtained, and numerous attempts to co-crystallize actin or its D-loop with FMO or FMO-CH domains of MICALs have proven unsuccessful[146]. Mechanistically, this is a very unusual property for a monooxygenase that suggests a new level of regulation via a conformational change triggered by F-actin binding[168] (see section 5.4.3). Interestingly, the authors also report that, in these experimental conditions, half of the oxygen is eliminated as H_2O_2 without actin oxygenation. Put together, even the more detailed kinetic analysis failed to demonstrate that MICALs are fully coupled enzymes, suggesting that i) MICALs are naturally leaking, and/or ii) additional regulatory elements are still missing. We suggest that the complex nature of MICAL's substrate requires a new and more complex FMO cycle, including substrate docking, conformational changes and a regulatory mechanism not present in other monooxygenases.

5.4.2. Inhibition—(−)-Epigallocatechin-3-gallate (EGCG) is a polyphenol abundant in green tea that was shown to inhibit certain FMOs such as pHPB with a non-competitive mechanism (K_i = 18 μM [171]). An indirect evidence that EGCG inhibits MICAL was provided by Terman *et al.*[97], based on the analysis of *Drosophila* axon pathways. The same group showed later an effect of EGCG on neuronal regeneration using a rat model of spine cord injury[126]. The first evidence that EGCG was a non-competitive inhibitor of the

MICAL1 NADPH oxidase activity was provided by[143]. The K_i reported varies from 2 μM[143] to 17 μM[106], but these findings require further verification in a substrate-dependent enzymatic assay due to artifacts of the kinetic technique used: EGCG reacts with H_2O_2, and this can lead to false positives in the inhibition assay, because resorufin, a product of the H_2O_2 assay by Amplex Red oxidation, reacts with FAD-containing proteins leading to their inhibition[145]. A recent report shows a marked effect of EGCG on retinal ganglion cell axons (which connect the eye to the brain): EGCG enhanced lopodial stability by stabilizing F-actin within the growth cone[172], but specificity was not examined in detail. Xanthofulvin, a fungal catechin structurally related to EGCG, inhibits Sem3A-dependent axonal regeneration in a model of spinal cord injury[173]. Based on what we know, both inhibitors may act through MICAL inhibition, but this has not been specifically addressed.

Lundquist et al. recently demonstrated that recombinant mouse MICAL2 (FMO domain) is directly inhibited by CCG-1423 with the K_i in the low micromolar range[120]. This is a synthetic compound originally identified as an inhibitor of the small GTPase RhoA[174], but nowadays accepted as an inhibitor of SRF-dependent responses[175–178]. SRF is a transcription factor considered as a master regulator in both cancer and embryonic development[179]. It was found that SRF is regulated by changes in actin dynamics[180,181]. MRTF-A is downstream of SRF signaling and is regulated by binding to nuclear G-actin. The most accepted mechanism for CCG-1423 is the inhibition of the translocation to the nucleus of MRTF-A by inhibiting its interaction with nuclear importins[182]. Under this scenario, if CCG-1423 effectively targets MICAL (and not indirectly by modulating MRTF-A nuclear availability), this would provide a valuable scaffold for further development. Studies to test if CCG-1423 and other compounds from the same family[183–187] are general MICAL inhibitors are ongoing (unpublished). Finally, $NAPD^+$ also acts as a competitive inhibitor with respect to NADPH with the K_i of 77 μM[106] on human MICAL1 (FMO domain).

5.4.3. Regulation of FMO activity of MICALs—By integrating the information provided above, we propose that the regulation of MICAL's FMO activity occurs at two levels: i) enzymatic regulation is provided by substrates of the FMO catalytic domain, and ii) long-range interactions involving C-terminal sequences, particularly LIM and DUF3585 domains, affect the FMO activity via an unknown mechanism.

The enzymatic regulation of MICALs is controlled by substrate binding. The monooxygenase domain of MICALs has a weak NADPH oxidase activity in the presence of NADPH and O_2, producing H_2O_2. F-actin functions as a *non-essential activator*[146], increasing this activity several hundred fold. This requires a specific protein-protein recognition and bidirectional conformational changes: while FAD reduction induces a conformational change that promotes a catalytically active position of the FAD, binding of actin induces a conformational change in the NADPH binding subdomain that speeds up the transition of the FAD from a non-productive to a productive configuration. The substrates of MICALs are two conserved methionines (M46 and M49 in human alpha-skeletal muscle actin) located in the D-loop in the filamentous form of actin (Figure 8). The D-loop is a 51-amino-acid-long flexible segment unstructured in G-actin[188] but engaged in protomer interactions in F-actin[111]. The D-loop of one protomer protrudes to the next one, making salt

bridges and hydrophobic interactions with conserved residues from an adjacent protomer (Figure 8). M46, a more conserved of the two methionines and the only one that has been shown to be universally targeted by MICALs *in vivo*[105,112,113,153], is buried at the protomer interfase[111] and needs to be pulled out to become a MICAL substrate, as suggested by Alqassim *et al.*[146]. The first step is the binding of MICAL to F-actin. Besides the universal presence of CH domains in MICAL and MICAL-L proteins, this domain is not essential for docking of the MICAL catalytic domain to F-actin, as demonstrated by several *in vitro* and cellular experiments with the isolated FMO domain[107,109,140,145,153]. Moreover, CH domain does not change the binding affinity for F-actin (*i.e.* does not affect the K_M[153]), but produces a slight increase in the overall catalytic constant[146]. Additionally, G-actin does not stimulate NADPH oxidation, pointing to the fact that the unstructured D-loop present in G-actin[188] is not a substrate of MICAL[153]. It is important to note that the FMO domain does not compete with fascin or α-actinin binding to F-actin[105], suggesting that a narrow region around the D-loop as the MICAL's recognition site.

How the D-loop is recognized and anchored in the MICAL active site is unknown. The conserved N-terminal charged region (Figure 3) may assist in this binding or participate in F-loop extrusion, but this has not been experimentally probed. The residues responsible for placing the D-loop in the position productive for catalysis at MICALs active site had not been identified, but the highly-conserved residues depicted in Figure 4 are strong candidates. We suggest that oxidation of conserved methionines precludes the D-loop from rebinding the adjacent protomer, weakening the interaction to sustain fibrillar actin. Under *in vitro* conditions, this leads to a rapid disassembly of F-actin, because oxidized G-actin monomers that are formed during the steady-state actin treadmilling are not able to polymerize. This explains the biphasic shape of MICAL-dependent F-actin depolymerization curves *in vitro*[105,112,167]: actin protomers are oxidized *in the filament* and progressively accumulate as oxidized G-actin. Cofilin accelerates this process *in vitro*, severing F-actin once D-loop methionines are oxidized[189]. Oxidized G-actin may have an unexplored signaling function. For example, oxidation of G-actin by MICAL2 determines a depletion of nuclear G-actin with the concomitant accumulation of MRTF-A in the nucleus, leading to SRF/MRTF-A gene expression[120]. Recycling of G-actin requires its reduction, a process that in mammals depends on the selenocysteine-containing MsrB1[112–114]. The *in vivo* conditions are certainly more complex and far from being completely understood. Depending on the cell type, metabolic state or stimuli, actin filaments may be linear or branched but are always highly "decorated" by several proteins that regulate its dynamics[190]. We expect that oxidation of the D-loop methionines under physiological conditions will not have such a dramatic effect as *in vitro*, at least not in the absence of further effectors. Indeed, a recent report demonstrates that cofilin acts cooperatively with MICAL to induce F-actin disassembly *in vivo*[189]. Cofilin is an actin-depolymerizing/severing factor that exerts its effect affecting D-loop conformation[191], whose methionine oxidation increases cofilin-severing properties[189].

The first level of regulation ("enzymatic") implies that MICAL's FMOs will be constitutively active in the presence of F-actin, a ubiquitous component of the cellular cytoskeleton. A second level of regulation is needed to allow MICAL proteins to act only under certain stimuli or in restricted subcellular compartments. This regulation is provided by long-range interactions involving the C-terminal domains, providing an "on-off" switch

that triggers MICAL's redox-dependent activity. The full-length MICAL1 (harboring the four domains) is several times less active in NADPH oxidation that FMO or FMO-CH domains alone both *in vitro* and *in vivo*[109,140,146,153]. This was originally attributed to a direct inhibition of the unusual coiled-coil CTD domain over the FMO domain. However, the CTD and FMO domains do not interact[140]. A molecular mechanism of the inhibition of FMO activity by CTD or LIM-CTD domains is still unknown, but may involve large conformational changes affecting global MICAL structure, as reported for MICAL-L2/JARB[132]. MICALs have also been proposed to bind themselves or other proteins with DUF3585 domains, opening the door to new regulatory mechanisms based on the formation of homo- or heteromeric complexes[103]. This may be particularly important for MICAL2 that lacks its own CTD suggesting that it is either constitutively active or its regulation depends on heterologous interaction with other DUF3585 domains or other proteins.

6. Concluding comments

MICALs are strictly eukaryotic proteins, formed by a unique combination of domains, most of which are also restricted to eukaryotes. The entire region covering FMO and CH domains is highly conserved across Metazoa. No MICAL or MICAL-like proteins are found in non-animal genomes, while every animal analyzed has at least one MICAL *and* one MICAL-like gene, with the exception Trichoplax (Placozoa), which possesses a single MICAL protein[154]. A single gene, conserved among animals, evolved to three paralogous genes in mammals, carrying almost identical catalytic domains but different regulatory elements. Our analysis (Mariotti, Manta and Gladyshev, unpublished) showed that the ancestral MICAL was already a protein with a DUF domain. We suggest that MICALs originate in multicellular organisms, as was previously proposed for the LIM domain itself[154]. Multicellularity allows the existence of complex organisms, wherein massive actin-dependent cell movements are needed for essential processes such as the establishment of the tri-layered embryo or axon projections.

The biology of MICAL proteins is still barely understood. Most of what we know about MICAL's mechanism has been studied *in vitro* using isolated domains[106,143,145,146,153] or extrapolated from *in vivo* experiments where an indirect read-out is linearly associated with MICAL activity[105,109,140]. Most of MICAL's partners are cell motility effectors or downstream targets of major signaling pathways, but we do not know how these signals converge on MICAL, leading to its activation. The prototypical example is the Semaphorin signaling pathway, whose studies led to MICAL discovery. One of the members of the semaphorin family, Sem3A, inhibits axonal growth, and several reports indicate this is a direct consequence of plexin-dependent MICAL activation[97,105,119,192]. Plexin interacts with the MICAL CTD producing a conformational change in MICAL that exposes/activates F-actin binding sites. Additionally, Sem3A activates glycogen synthase kinase 3 (GSK3) at the leading edge of neuronal growth[192]. GSK3 is a pleiotropic kinase with many possible substrates, including MICAL (MICAL1 in mammals)[193], that is targeted on serine 777 located in the linker between LIM and CTD domains (Figure 3). Both signals, starting from the same extracellular stimuli, converge on MICAL and may enhance or diminish its activation. Another example of MICAL's complex interaction network is its relation with CRMP2, a large scaffolding protein that regulates cytoskeleton dynamics, interacting with

tubulin and myosin II[194]. A recent report shows that CRMP2 is oxidized in conserved cysteine residues when MICAL1 is activated in rat dorsal root ganglion neurons after *in vitro* Sema3A stimulation. Oxidation of CRMP2 leads to the formation of a disulfide-linked intermediate with thioredoxin (Trx) that mediates CRMP2 phosphorylation by GSK3 and thereby growth cone collapse[170]. These results suggest that MICAL activation by the concerted action of Sem3A, plexin and/or GSK3, may end up remodeling tubulin cytoskeleton through CRMP2 oxidation. Whether CRMP2 is directly targeted by MICAL or is indirectly oxidized by locally produced H_2O_2 is unknown.

Several attempts have been made to identify new MICAL substrates, without success. To our knowledge, the actual evidence suggests that MICALs evolve to redox-regulate actin dynamics. MICAL structure and domain organization, together with the conservation of targeted methionines, suggests a very restrictive function of this family of proteins. In line with the importance of these methionine residues for proper actin function, M47T or M47L are naturally occurring mutations responsible for myopathies associated with congenital muscle weakening[195]. Moreover, actin D-loop is also targeted by phosphorylation at Tyr 53, suggesting that this region of F-actin may integrate signals from different pathways[196]. Of course, the discovery of new MICAL targets, if any, may open avenues for characterizing biological processes regulated by methionine oxidation and may contribute to expanding the biological relevance of this oxidative modification. However, a more urgent question is the identification of proteins that regulate the activation of MICALs, particularly in vertebrate models where different MICALs exists and are distributed in different compartments. Identification of upstream signaling pathways that turn “on” MICALs will help to understand how these proteins are regulated and which processes are ultimately dependent on the redox-dependent modification of actin dynamics. Finally, we think reversible redox regulation through methionine oxidation in response to physiological stimuli should open an entirely new direction in the area of redox signaling and regulation (Figure 9). Even if MICAL only targets actin’s methionines, there are other FMO genes with unexplored functions that may expand the horizons of methionine-dependent redox regulation.

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Abbreviations

ABP	actin-binding protein
ABR	actin-binding region
CH	calponin-homology domain
CRMP	collapsin response mediator protein
CTD	C-terminal domain
DUF	domain of unknown function

EH	Eps15 homology domain
EMT	epithelial-to-mesenchymal transition
FAD	Flavin adenine dinucleotide
FMO	Flavin monooxygenase
LIM	Lin-11, Isl1 and Mec-31 domain
MetO	methionine sulfoxide
MICAL	Molecule Interacting with CasL
Msr	methionine sulfoxide reductase
pHBH	*para*-hydroxybenzoate hydroxylase

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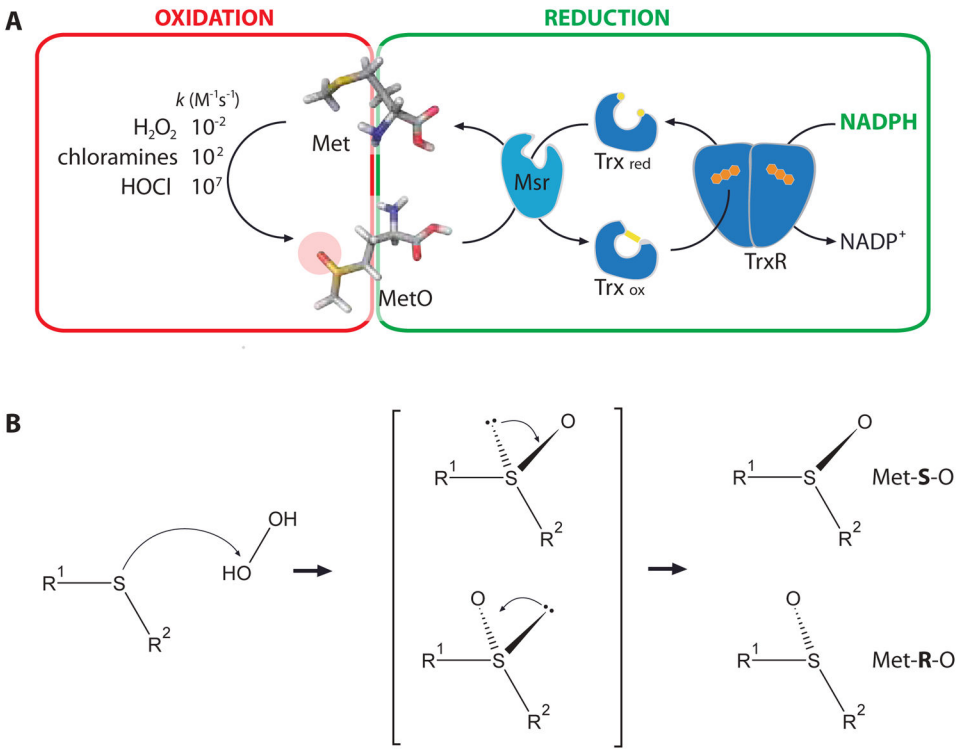

Figure 1. Methionine oxidation

A. Schematic representation of the chemical reaction that leads to methionine oxidation and the NAPDH-dependent reduction system based on Msr, thioredoxin (Trx) and thioredoxin reductase (TR). **B**. Reaction mechanism of a thioether with hydrogen peroxide. The tetrahedral intermediate is chiral, leading to chiral products, represented here as R and S enantiomers of methionine. The figure based on information reported in [25,197,198,30,31,34,32,33,199,10].

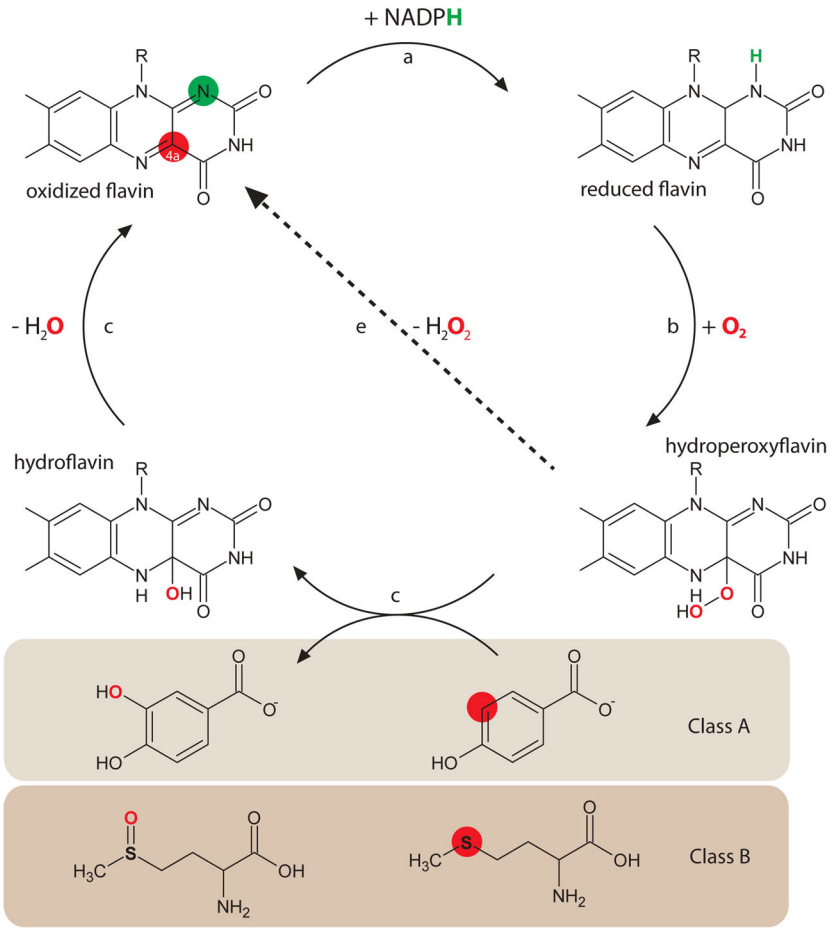

Figure 2. Reaction mechanism of flavin-dependent monooxygenases

Oxidized FAD (represented here by its isoalloxazine ring) reacts with NAPDH forming the neutral hydroquinone (**a**). Molecular oxygen reacts at position C4a (red spot) forming the hydroperoxyflavin (**b**), that can oxidize/hydroxilate substrates (**c** and **d**) or decompose releasing H_2O_2 (**e**, "uncoupled" reaction). Class A FMOs are considered hydroxylases, as depicted here in the reaction of hydroxylation of para-hydroxybenzoate. Class B FMOs may oxidize thioether, as shown in the lower part with methionine as substrate. Adapted from[67,60].

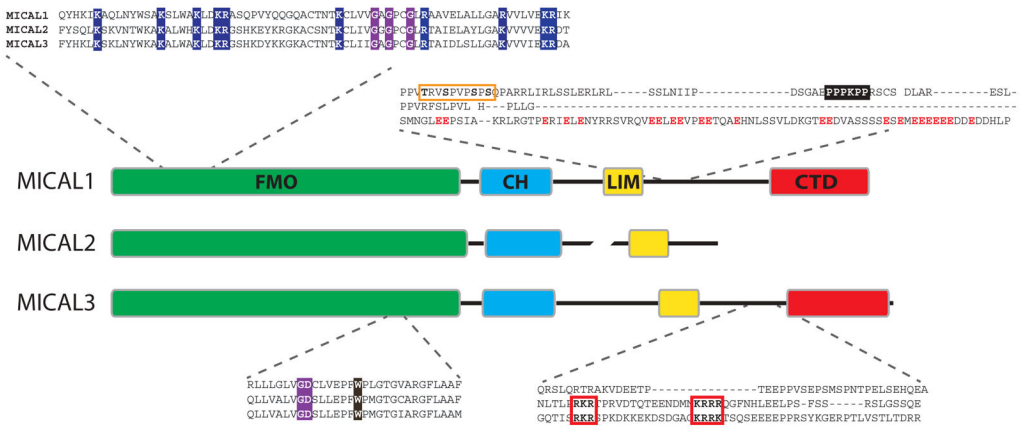

Figure 3. Domains in MICAL proteins

The domain organization of mammalian MICAL1–3 is schematically represented. Each domain is shown in a different color, conserved along the text: green for FMO, light blue for CH, yellow for LIM and red for CTD. The length of each domain is proportional to the number of amino acids in it, so do linkers, unless braked. Inserts show sequence details of mouse MICAL1–3 (NP_612188.1, NP_001180234.1 and NP_001257404.1, respectively). The conserved basic residues in the N-terminal region of the FMO domain are depicted with blue background and the GxGxxG and GD motifs with purple background. The conserved W is shown over dark brown background. Part of the connecting sequence between LIM and CTD domains is highlighted in the upper part, indicating a tandem of phosphorylation sites on MICAL1 (orange box) and the PPPKPP motif (black background), and the enrichment of acidic residues on MICAL3 (red letters). In the lower panel, the bipartite nuclear localization signal present in MICAL2 and 3 is shown with red boxes and bold letters.

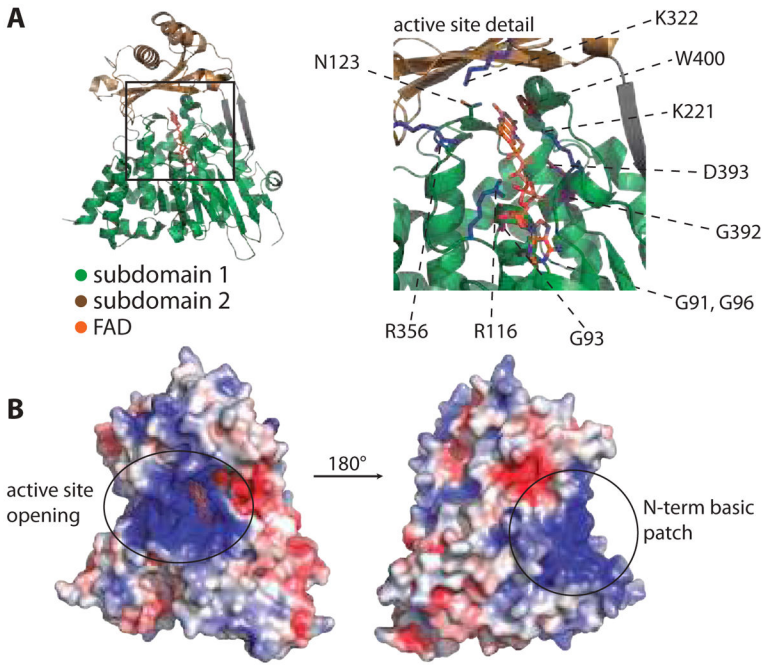

Figure 4. Structure and conserved residues of the FMO domain

A. FMO domain of mouse MICAL1 is represented as green and brown ribbons and the FAD molecule as orange sticks, emphasizing the position of the FAD and active site at the interface between subdomains 1 and 2. A zoomed detail of the conserved residues around FAD is shown on the right side (see text for details). **B**. Electrostatic surface of MICAL1 FMO. The left image is shown in the same orientation as in A, while the right image is turned 180°. Positive areas are shown in blue, negative areas in red, and neutral areas in gray. Figures were done with PyMOL using PDB 2BRY and based on[143,144,141].

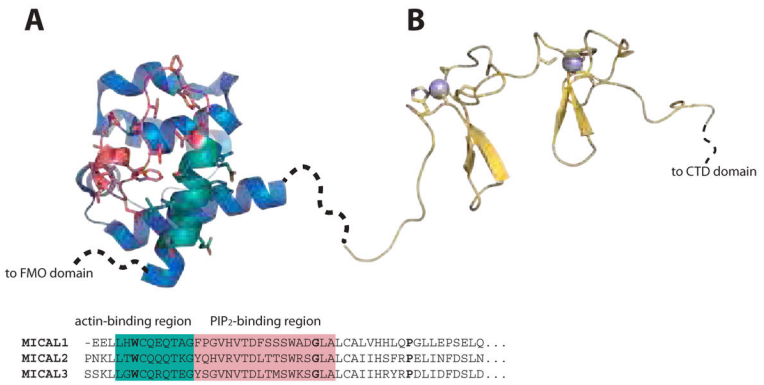

Figure 5. Structures of CH and LIM domains

A. Solution structure of human MICAL1 CH domain (PDB 2DK9[152]) is shown as ribbons, highlighting the actin-binding region (cyan) and PIP2-binding region (pink) with sticks. The sequences of the N-terminal region of CH domains from mouse MICAL1–3 (see Figure 3) are detailed, mapping the conserved regions shown in the structure and the hydrophobic signature residues (bold). **B**. Solution structure of human MICAL1 LIM domain (PDB 2CO8) is shown as yellow ribbons, with cysteines and histidine involved in zinc atoms ligation (cyan spheres) represented as sticks. Figures is prepared with PyMOL, based on[152,146].

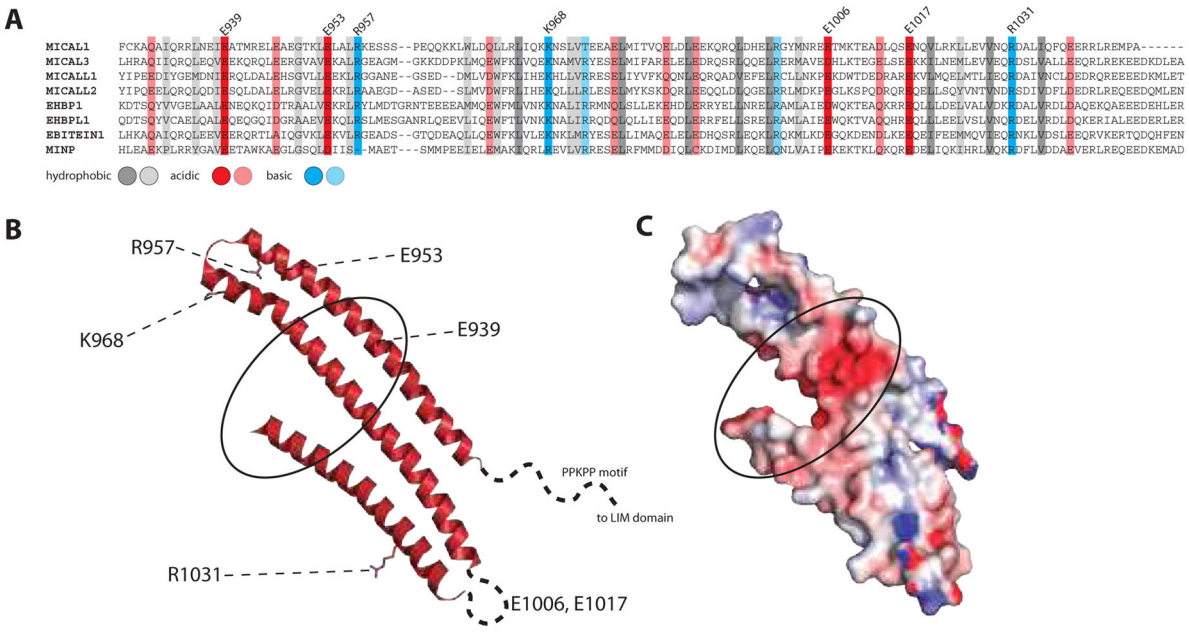

Figure 6. Conservation and structure of DUF3585 domains

A. Alignment of the DUF3585 domains of mouse MICAL1 (NP-612188.1), MICAL3 (NP001257404.1), MICAL-L1 (NP803412.1), MICAL-L2 (NP777275.2), EHBP1 (NP001239444.1), EHBPL1 (NP001108069.1), EBITEIN1 (NP081863.2) and MINP (NP653101.1). Conserved residues are highlighted with red (acidic), blue (basic) or grey (hydrophobic) backgrounds. Full conservation is indicated with plain colors, while translucent colors indicated physicochemical conservation. **B**. The ribbon structure of human MICAL1 DUF3585 domain (PBD 5LPN[103]) is shown on the left side and its electrostatic surface on the right. Conserved residues (numbered accordingly to mouse MICAL1) are highlighted as stick, also indicated on part A (upper line). The conserved acidic patch is encircled. Figure was prepared with PyMOL.

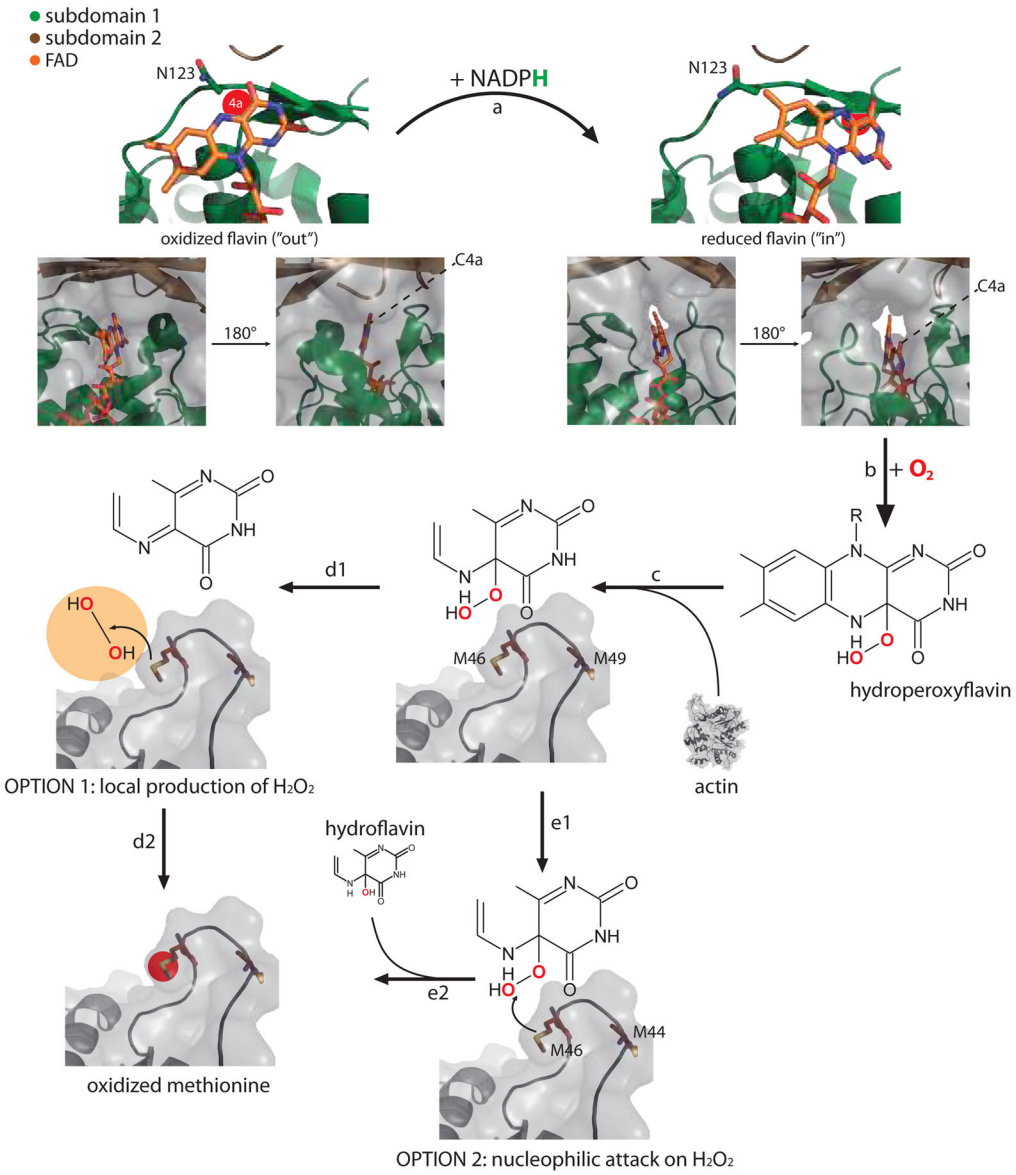

Figure 7. Proposed mechanism of MICAL

Detailed view of the FAD cofactor in oxidized (left) and reduced (right) mouse MICAL1. Colors are as in Figure 4. The conserved N123 is shown in sticks, with lateral chain atoms colored by elements (red for oxygen, blue for nitrogen). The location of C4a position is marked with a red spot. Lower panel shows the surface around FAD in the oxidized (left) and reduced (right) MICAL1, in the same orientation as in Figure 4. A 180° turn is applied to observe both sides of the protein surface. Reduction (**a**) produces a major conformational change that opens a channel to the C4a position. Reaction with oxygen (**b**) leads to the hydroperoxyflavin. Actin binds to reduced MICAL (**c**), and the oxidation proceeds by two alternative pathways. The position of the D-loop (detailed) can favor/induce the release of H_2O_2 (**d1**), leading to methionine oxidation by the nucleophilic attack of the sulfur on the peroxo bond (**d2**). Alternatively, methionine thioether can attack directly on the

hydroperoxyflavin (**e1**), releasing hydroflavin and oxidized methionine (**e2**). Figure was prepared with PyMOL using PDBs 2BRY[143] for oxidized and 2C4C[144] for reduced mouse MICAL1 FMO domain.

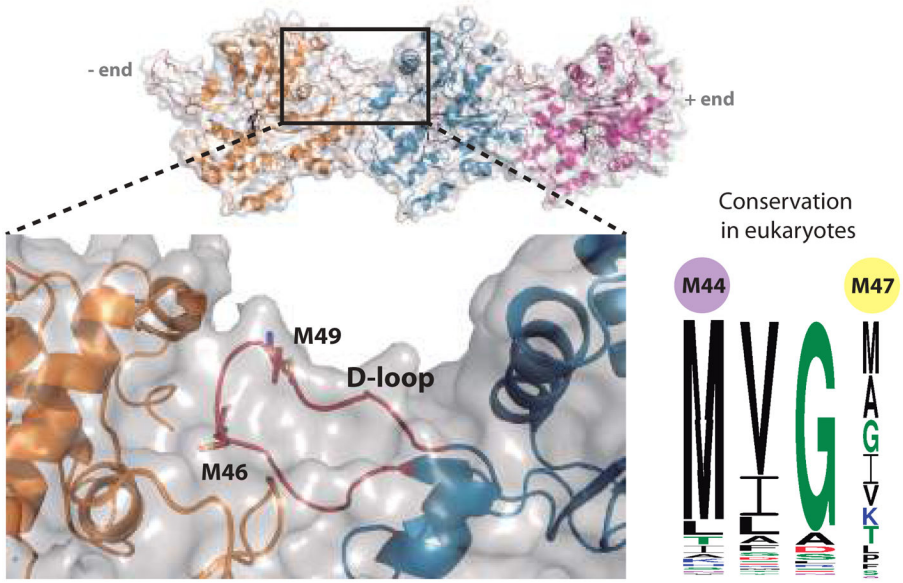

Figure 8. F-actin structure and the role of the D-loop

Three protomers of F-actin are shown in ribbons with grey surface. Details of the D-loop connecting protomers 1 and 2 are shown in the lower panel, with methionines in stick (colored by elements, red for oxygen, yellow for sulfur). M46 is buried, while M49 is exposed to protein surface. Conservation of the D-loop methionines among 485 actin sequences from eukaryotes is shown on the right as a logo. Figure was prepared with PyMOL based on PDB 3J8A[111]

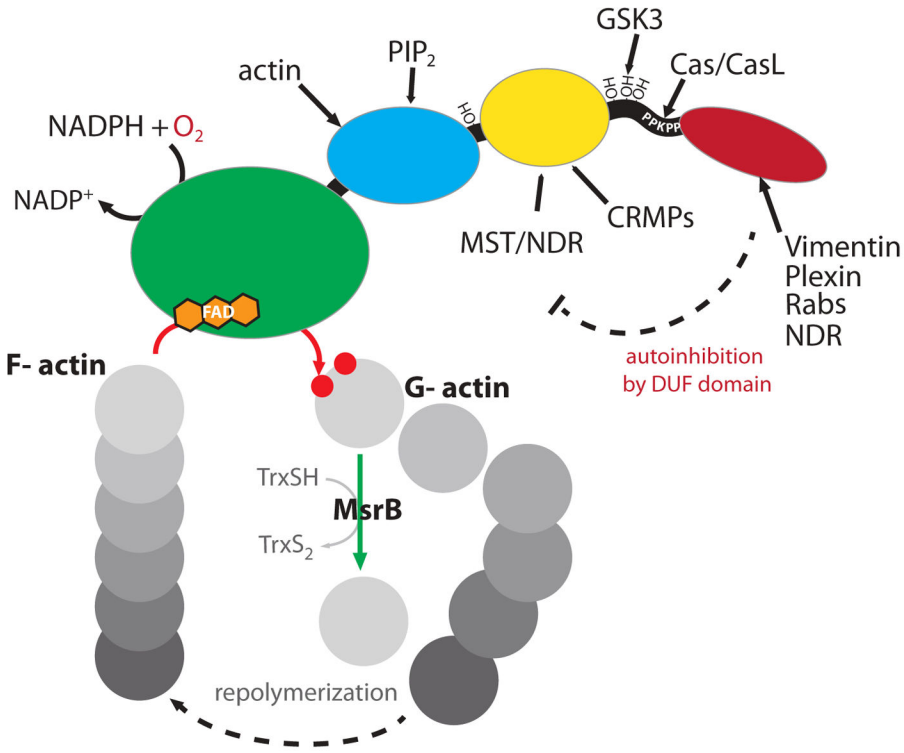

Figure 9. MICALs as redox hubs

Several signals converge on MICAL architecture as shown. Integration on these signals on this multidomain protein will determine, ultimately, its activation. Once activated, MICAL proteins target actin cytoskeleton.