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Clara Ortegón Salas  
University of Greifswald, Institute for Medical Biochemistry and Molecular Biology

Yana Bodnar  
University of Greifswald, Institute for Medical Biochemistry and Molecular Biology

Dennis Uhlenkamp  
University of Greifswald, Institute for Medical Biochemistry and Molecular Biology

Katharina Schneider  
University of Greifswald, Institute for Medical Biochemistry and Molecular Biology

Lara Knaup  
University of Greifswald, Institute for Medical Biochemistry and Molecular Biology

Marcel Deponte  
University of Kaiserslautern  https://orcid.org/0000-0003-2141-917X

Carsten Berndt  
Heinrich Heine University Düsseldorf

Christopher Lillig  
University of Greifswald

Manuela Gellert  (✉ gellertm@uni-greifswald.de)  
University of Greifswald  https://orcid.org/0000-0002-2545-7125

Article

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NADPH-dependent oxidation of CRMP2 through a MICAL1-Prx1 redox relay controls neurite outgrowth

Clara Ortegón Salas, Yana Bodnar, Dennis Uhlenkamp, Katharina Schneider, Lara Knaup, Marcel Deponte, Carsten Berndt, Christopher Horst Lillig, and Manuela Gellert

From the Institute for Medical Biochemistry and Molecular Biology, University Medicine Greifswald, Germany (1), the Department of Chemistry, Technical University, Kaiserslautern, Germany (2), and the Department of Neurology, Medical Faculty, Heinrich Heine University Düsseldorf, Germany, (3).

* Corresponding authors: Manuela Gellert and Christopher Horst Lillig, Institute for Medical Biochemistry and Molecular Biology, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße, DE-17475 Greifswald, Germany, Tel: +49 3834 865407, Fax: +49 3834 865402, E-mails: manuela@gellert.org, horst@lillig.de

Running title: The MICAL1-Prx1-CRMP2 redox relay
Abstract

CRMP2/DPYL2 is an effector protein in the semaphorin signaling pathway that controls cytoskeletal dynamics, linking extracellular signals to the formation of axonal networks. CRMP2 is regulated by post-translational modifications including a dithiol-disulfide redox switch. The mechanisms of reduction of this switch were established, the signal-induced oxidation, however, remained unclear. Here, we show that CRMP2 is oxidized through a redox relay involving the flavin-mooxygenase MICAL1 and the peroxidase Prx1 as specific signal transducers. Using molecular oxygen and electrons provided by NADPH, MICAL produces hydrogen peroxide and specifically oxidizes Prx1 through direct interactions between the proteins. Subsequently, Prx1 oxidizes CRMP2. The lack of any components of this redox relay dysregulates neurite outgrowth. Consequently, both oxidation and reduction of CRMP2 require reducing equivalents in the form of NADPH.

Keywords: MICAL, CRMP2, DPYL2, redox relay, redox regulation, thiol switch, neurite outgrowth
Introduction

The semaphorin (Sem) signaling pathway contributes to the regulation of axonal outgrowth and neuronal connectivity. Activation of the Sem3A pathway induces growth cone collapse and retraction of the outgrowths by controlling the de-polymerization of the cytoskeleton that subsequently allows the axons to change their direction. Sem3A acts via a hetero-dimeric trans-membrane receptor composed of neuropilin 1 (NP1) and a plexin A (PlexA) family member \(^1\)\(^2\). Effector proteins include the kinases CDK5 and GSK3\(\beta\) that sequentially phosphorylate collapsin response mediator protein 2 (CRMP2, gene: DPYSL2) \(^3\). CRMP2 was reported to regulate microtubuli dynamics by binding to \(\alpha\)-\(\beta\)-tubulin dimers. MICAL (molecule interacting with CasL) proteins bind to the cytoplasmic domain of PlexAs \(^4\). The human genome encodes three MICAL proteins. All contain an N-terminal FAD-dependent monooxygenase (MO) domain, followed by a calponin homology (CH) and a LIM domain. Human MICAL 1 and 3 contain an additional C-terminal Rab-binding domain (RBD) \(^5\). MICALs can produce \(\text{H}_2\text{O}_2\) upon activation of the Sem3A pathway \(^6\). Furthermore, MICALs were characterized as specific oxidases of actin methionyl residues \(^7\)\(^8\), thus regulating microfilament dynamics.

In general, redox-mediated signal transduction occurs by both reversible oxidation and reduction of key molecules. The side chains most vulnerable to redox modifications are cysteinyl and methionyl residues. Redox modifications occur – often rapidly – under physiological conditions and they are highly specific with respect to both, the molecules involved and the nature of the redox modification; reversible oxidation and reduction are key mechanisms in cell signaling \(^9\)\(^10\). While the specificity of the reduction of cysteinyl and methionyl residues has always been attributed to catalysis by enzymes of the thioredoxin (Trx) family \(^11\), the oxidation reactions were often attributed to rather unspecific reactions of the proteins with ‘reactive’ species, most of all \(\text{H}_2\text{O}_2\). This model has been increasingly challenged, in particular by the low reaction rates of the modified side chains with \(\text{H}_2\text{O}_2\). Cellular peroxidases, \textit{i.e.} the glutathione peroxidases and peroxiredoxins (Prxs) display
≈10^6-fold higher rate constants and are generally high abundant proteins in mammalian cells. It was thus suggested that these peroxidases may function as receptors and transducers of redox signaling, e.g. 12. CRMP2 has been reported to be regulated by a dithiol-disulfide redox switch of two cysteiny1 residues in the homo-tetrameric quaternary complex, that is essential for neurite outgrowth and axonal connectivity 6,13. In vivo, the reduction of this disulfide appears to be specifically regulated by the cytosolic isoform of glutaredoxin 2 (Grx2c), a process linked to both formation of a neuronal network and the progression of cancer cells 13–15. MICAL-produced H_2O_2 was suggested as potential source of CRMP2 oxidation 14, however, the rate constant of the direct reaction of CRMP2 with H_2O_2 is also too low to be of significance in vivo 16. In fact, two studies provided evidence for the involvement of Prx1 in the oxidation of CRMP2 17,18. We thus hypothesized a signal-induced redox relay in the semaphorin signaling pathway that involves both MICAL1 and Prx1 as signal transducers and CRMP2 as effector protein.
**Results**

We hypothesized that Sem3A signaling induces a redox relay involving a FAD-dependent monooxygenase of the MICAL family, one of the cytosolic Prxs, and CRMP2. The latter should then affect axonal outgrowth by binding to tubulin and actin, thus controlling cytoskeletal dynamics. We have used purified recombinant human CRMP2 to perform affinity capture assays in cell extracts. Using Western blotting, we confirmed the interaction between CRMP2 and both actin and tubulin (Fig. 1a). Moreover, also MICAL1 and Prx1 were captured in this assay; their release from the complex required reducing equivalents, indicating redox-dependent associations.

We proposed the production of hydrogen peroxide by a MICAL protein that leads to the oxidation of a Prx. Subsequently, this Prx may oxidize the Cys504 redox switch in tetrameric CRMP2. We cloned, recombinantly expressed, and purified the two cytosolic Prxs (Prx1-2) and the monooxygenase domains of MICAL1-3, henceforth named MICAL(1-3)-MO. All three recombinant MICAL-MO domains contained oxidized FAD, indicated by additional absorption bands at approx. 370 and 450 nm (Fig. 1b). For the comparison of spectra of both liberated and MICAL bound FAD, see Fig. S1. These proteins oxidized NADPH in the presence of O2, a reaction that yields H2O2, proving their activity as monooxygenases (Fig. 1c). Next, we incubated both Prx1 and Prx2 with the three MICAL-MO domains in a one-to-one stoichiometry (one Prx dimer to one MICAL-MO) in the presence of NADPH. In this setting, all MICAL-MOs led to the oxidation of the two Prxs, *i.e.* the formation of the catalytic inter-molecular disulfide between the peroxidatic and resolving cysteiny1 residues of two Prx monomers (Fig. 1d). When a mutant of the resolving cysteiny1 residue of the Prxs was used, dimer formation could not be observed. As a control, we have also confirmed that MICALs (in a 1:1 stoichiometry in the presence of oxygen and NADPH) do not directly oxidize CRMP2 (Fig. S2).
To analyze the mechanisms of the MICAL reaction and the oxidation of the Prxs, we have turned to stopped-flow kinetics. As seen before, MICAL1-MO oxidized NADPH in a concentration-dependent manner in the presence of O$_2$ at pH 7.4 and 298 K (Fig. 2a-b, reaction 1).

**reaction 1:** \[ \text{NADPH/H}^+ + \text{O}_2 \leftrightarrow \text{NADP}^+ + \text{H}_2\text{O}_2 \]

Using linearized integrated second order kinetics, we determined the rate constant of this reaction to be $137\pm14$ M$^{-1}\cdot$s$^{-1}$ (Fig. 2c, Supplementary information). The exemplary difference spectra shown in Fig. 2d demonstrate a decrease in absorbance between 340-350 nm (NADPH oxidation), but not at 450 nm (FAD reduction), indicating that FAD is oxidized during all phases of this reaction.

Noteworthy, decrease in pH (to 6.0) and temperature (to 277 K) did not significantly affect the rate constant of reaction 1: $126\pm7$ M$^{-1}\cdot$s$^{-1}$ (Fig. 2e-f). To analyze the oxidation of the MICAL1-MO (reaction 2), we incubated the protein with exactly the amount of NADPH required to fully reduce the FAD to FADH$_2$, i.e. the concentration that equaled the sum of the MICAL-MO and O$_2$ concentrations in the reaction mix (Fig. S3). The reaction was started by mixing this protein solution with an oxygen-containing solution in the stopped-flow chamber.

**reaction 2:** \[ \text{FADH}_2 + \text{O}_2 \leftrightarrow \text{FAD} + \text{H}_2\text{O}_2 \]

The concentration of O$_2$ was determined from the amount of (excess) NADPH oxidized by the enzyme in a separate control experiment, see Fig. S3. The oxidation of MICAL1-MO occurred fast, equilibrium was reached in less than a second (Fig. 2g-h). When we analyzed this reaction applying linearized integrated second order kinetics, we found the reaction to be bi-phasic (Fig. 2i, Supplementary information). We determined the rate constant of the first reaction to be $\geq 1.9\pm0.2\cdot10^5$ M$^{-1}\cdot$s$^{-1}$, the rate constant of the second phase to be $2.4\pm0.3\cdot10^4$ M$^{-1}\cdot$s$^{-1}$. The spectra recorded during these reactions did not provide any evidence for the formation of any form of stable intermediate. The difference spectra (Fig. 2j) display a decrease at 462 nm and, to a lesser degree at 378 nm, typical for the oxidation of FADH$_2$ to FAD. We thus concluded that the reaction of the
FADH$_2$ with O$_2$ occurs via an unstable peroxy-flavin intermediate (reaction 3), that immediately decays to oxidized FAD and H$_2$O$_2$ (reaction 4), for details see Fig. S4.

reaction 3: $\text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD-OOH} + \text{H}^+$

reaction 4: $\text{FAD-OOH} + \text{H}^+ \rightarrow \text{FAD} + \text{H}_2\text{O}_2$

We have also performed the oxidation of MICAL-MO in the presence of Prxs. The remaining spectra, however, did not suggest any other intermediates, e.g. a charge transfer complex between the FAD and the Prx. The reaction remained bi-phasic and the rate constants were in the range of those determined for the reaction in the absence of a Prx (Fig. 2k-l, Table 1). We thus conclude that the Prx is oxidized by H$_2$O$_2$ produced by the MICAL-MO domain and not by direct transfer from the FAD to the peroxidatic cysteinyl residue.

Next, we have analyzed the oxidation of Prx1-2 by catalytic amounts of the MICAL(1-3)-MOs. As shown in Fig. 3a and Fig. S5, 60 nmol·l$^{-1}$ of the MICAL-MOs efficiently oxidize 10 µmol·l$^{-1}$ of either Prx when NADPH was present. This reaction was in no way inhibited by the presence of excess catalase (> 1000 U), suggesting that the H$_2$O$_2$ produced by the MICAL-MOs is not released into the solution to be turned over by the Prxs. Although all MICAL-MOs were able to oxidize the Prxs, the time course of these reactions (Fig. 3b) evinced a clear preference of MICAL1-MO for the oxidation of either Prx. We have thus focused, from here on, on MICAL1. Michaelis-Menten kinetics of the Prxs’ oxidation by the MICAL1-MO suggested that the Prxs are in fact substrates of the monooxygenase. Both Prxs were oxidized at similar rates (Fig. 3c, Table 1, Fig. S6). We also analyzed this reaction using a MICAL1-MO variant elongated by the calponin homology domain of the native protein (MICAL1-MO-CH, Fig. S6). As reported for the oxidation of actin by MICAL, this enhanced the catalytic efficiency, here approx. 3-fold (Fig. 3d, Table1). Together, these results support the formation of an enzyme-substrate complex between the two proteins in which the H$_2$O$_2$ produced by MICAL is directly shuttled to the peroxidatic cysteinyl residue of the Prx. We have performed all atomistic molecular dynamics simulation of this proposed complex between MICAL1

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and dimeric Prx1. Using a start structure obtained by molecular docking, we have analyzed the
dynamics of the complex over the time course of 250 ns (Fig. 4). Within 50 ns, the simulation
reached an equilibrium (Fig. 4d-e). In this complex, one of the peroxidatic cysteiny1 residues of the
Prx-dimer is placed at the exit of a channel in MICAL1 that connects to the FAD/FADH₂ active site
of the enzyme (Fig. 4f-g), the side chains of both the peroxidatic and resolving cysteiny1 were at the
low end of the spectrum (Fig. 4h), suggesting a rather stable conformation.

Next, we aimed to confirm the proposed redox relay in an established human cellular model of
neuronal differentiation. SH-SY5Y neuroblastoma cells can be differentiated into mature neurons
through a variety of different mechanisms including treatment with retinoic acid (RA) ¹⁹. At first,
we have treated the cells in the neuroblast state with 1µg·ml⁻¹ Sem3A and analyzed the oxidation of
Prx1 and CRMP2. Both proteins form a homodimeric inter-molecular disulfide that can be
identified after non-reducing Western blotting. We found an increase in both oxidized Prx1 and
CRMP2 between 5 to 10 minutes after addition of the ligand (Fig. 5a). Since we proposed a
Sem3A-induced redox-relay from MICAL1 to Prx1 to CRMP2, we repeated the experiment with
cells silenced for the expression of MICAL1 (Fig. 5b-c). In the presence of MICAL1, we confirmed
the oxidation of CRMP2 within minutes following Sem3A treatment (Fig. 5c). With silenced
MICAL1 expression, however, Prx1 oxidation (Fig. 5b, analyzed by diagonal electrophoresis, non-
reducing vs. reducing dimension, see Fig. S7), as well as CRMP2 oxidation (Fig. 5c) were
prevented. In the following, we have induced the differentiation from the neuroblast state to the
neuron-like cells by treatment with RA over the time course of 7 days. Oxidized CRMP2 started to
appear 48 hours after induction and reached a maximum at day 5 (Fig. 5d-e), confirming earlier
results ¹³. With silenced expression of MICAL1 in this model, Prx1 (Fig. 5f-h), but not Prx2 (Fig.
5j-l), was significantly less oxidized 5 days after induction of differentiation. The lack of increase in
the oxidized dimeric form of the Prx was not the result of an over-oxidation of the monomeric form
(Fig. 5i). With silenced expression of Prx1, oxidation of CRMP2 did not occur (Fig. 5m-n). Next,
we investigated neurite outgrowth as physiological function of the characterized redox relay.

Compared to controls, SH-SY5Y cells with silenced expression of either MICAL1, Prx1, or CRMP2 featured significantly longer neurites (Fig. 5o-u). The average neurite length of cells lacking MICAL1 and Prx1 increased 1.6-fold at day 5, the lack of CRMP2 led to a 1.4-fold increase at day 3 following the beginning of RA treatment.
**Discussion**

The semaphorin signaling pathways are part of the regulatory networks controlling the processes that shape the developing nervous system, *i.e.* axon guidance, synaptic plasticity, and neuronal migration. Both MICAL and CRMP2 are required for the Sem signaling pathway and interact with the cytosolic domain of the Sem receptor plexinA. The activity of the MICAL monooxygenases is required for their function in the pathway, for instance MICALs directly oxidize actin and promote growth cone collapse and cellular repulsion. CRMP2 contains a dithiol-disulfide redox switch that is operated in the SH-SY5Y model of neuronal differentiation. Previous studies suggested MICAL-produced $\text{H}_2\text{O}_2$ as source of the oxidation equivalents, however, this was put into question by the low rate constant of the reaction, *i.e.* $0.82 \text{ M}^{-1} \text{s}^{-1}$. In fact, more recent studies pointed to 2-Cys Prxs as mediators of CRMP2 oxidation.

Here, we propose the following pathway and mechanism of CRMP2 oxidation: MICAL1 is activated in response to Sem3A signaling, presumably by direct interaction with the Sem3A receptor. Next, reduced MICAL1 ($\text{FADH}_2$) reacts fast with molecular oxygen yielding a peroxyflavin intermediate ($\text{FAD-OO}^-$). This immediately decays to $\text{H}_2\text{O}_2$ and oxidized MICAL (FAD). The efficient oxidation of the Prxs in the presence of excess catalase (Fig. 1d) suggests that the $\text{H}_2\text{O}_2$ is not released but rather directly channeled to the peroxidatic cysteinyl residue of Prx1, oxidizing it to the sulfinic acid intermediate. This induces the attack of the resolving cysteinyl residue of the second Prx monomer, resulting in partial unfolding, conformational changes and disulfide formation, characteristic for the 2-Cys Prx reaction mechanism, see *e.g.*. This ought to induce to the dissociation of the MICAL-Prx complex. MICAL is re-reduced by NADPH and Prx1 can transfer the disulfide to CRMP2 in dithiol-disulfide exchange reaction.

Various stages of development in the central nervous system appear to be associated with changes in the intra-cellular redox milieu. This has been demonstrated for the production of $\text{H}_2\text{O}_2$. 


that participates, for instance, in axonal path finding. MICAL proteins can produce $H_2O_2$, which made them prime candidates as oxidases in redox signaling pathways.

MICAL family proteins are conserved from insects to vertebrates. The proteins are $O_2$- and NADPH-dependent monooxygenases. The domain structure of the three human isoforms, however, differs. All three MICALs contain the monooxygenase domain (MO) with FAD as prosthetic group at the N-terminus followed by calponin homology and LIM domains. MICAL1 and 3 contain an additional C-terminal Rab-binding domain. The C-terminal region of MICAL is involved in the modulation of its activity and mediates the interaction between MICAL and plexin. This interaction was reported to release the auto-inhibition of MICALs by their C-terminal domain upon the binding of SemA to its receptor. This release of MICALs is required for their redox activity.

Unlike Rab1, CasL, or Vimentin, CRMP2 doesn't seem to bind to the C-terminal region but closer to the N-terminus including the LIM domain. It remains unclear how the interaction of MICALs with the Sem receptor protein plexinA affects the binding of CRMP2. MICALs catalyze the stereo-specific oxidation of two methionyl residues (M44 and M47) in actin filaments to methionyl-R-sulfoxides by the production of $H_2O_2$, thereby facilitating actin de-polymerization. These modifications are reversible through the action of methionine sulfoxide reductases.

These redox modifications of actin were implied in axonal outgrowth, neuronal plasticity, cell division, and cancer progression. The oxidation of actin by MICAL-MO was enhanced when the following CH domain was included in the recombinant protein. We confirm the higher catalytic efficiency of this construct for the oxidation of the Prxs as well (Fig. 3c-d, Table 1).

The crystal structures available for MICAL-MO domains imply a conformational change of the FAD from an ‘out’ conformation in the oxidized state to an ‘in’ conformation in the reduced state. This may be the explanation why the reduction of the FAD by NADPH is the rate limiting step in the MICAL monooxygenase reaction mechanism (Fig. S4). It may also explain why the rate constant of this fully reversible reaction step appeared to be independent of temperature or pH (Fig. 11/39).
2a-f). The rate was likely not limited by the electron transfer reaction itself, but rather by the
following conformational changes. Reduced FAD immediately reacted with molecular oxygen. The
apparently bi-phasic reaction likely resulted from the formation of an unstable peroxy-flavin, which
decays to H$_2$O$_2$ and the oxidized flavin (Fig. S4) $^{36}$. The rate constant of the first step was too high
for us to spectroscopically verify the peroxy-flavin intermediate (Fig. 2g-l). No spectroscopic
evidence, e.g. charge transfer complex, was detected that could point to a direct reaction of Prx’s
peroxidatic Cys$_p$ with a peroxy-flavin. Despite the high homology between the three human
MICALs $^4$, their catalytic efficiency towards the oxidation of Prx1 and Prx2 differ distinctly (Fig.
3c-d, Table 1) with MICAL1 being the most efficient. Our structural models suggest large
differences in the electrostatic properties that may be key to their diverse enzymatic activities (Fig.
S8) $^{45}$. Our molecular dynamic simulations suggest an area of contact between dimeric Prx1 and the
MICAL1-MO domain that complies with both the geometric restraints and complementary
electrostatic surfaces (Fig. 4a-c, Fig. S8). Such a binding event brings the peroxidatic cysteinyln residue of one monomer of the dimeric Prx in close proximity to the active site opening in the
MICAL1-MO domain where the FAD is bound (Fig. 4f-g), the H$_2$O$_2$ produced may then be directly
channeled to the Prx, which is supported by the finding that catalase does not inhibit Prx oxidation.
(Fig. 3). The oxidation of CRMP2 by Prx1 was demonstrated before. However, it seems that this
reaction requires at least one additional factor that may act as scaffold bringing Prx1 and CRMP2
into close enough proximity for the reaction to take place $^{17,31}$.

Different studies suggested an involvement of the oxidized form of CRMP2 in the semaphorin
signaling pathway before although without clarifying the mechanism of oxidation nor a sufficient
explanation for the specific reduction. The use of a mutant thioredoxin in this enzymatic reaction
leads to the formation of an artificial disulfide $^{6,36}$.

CRMP2 is subject to a variety of post-translational modifications. Five phosphorylation sites are
located at the C-terminus $^{3,46-49}$, following the redox-sensitive cysteinyln residue 504 $^{13}$. The
accessibility of the phosphorylation sites may depend on the redox state of Cys504, implying a
cross talk between redox- and phosphorylation signaling\textsuperscript{16}. Oxidation of the CRMP2 redox switch
appears to be required for growth cone retraction of outgrowing neurites. We have analyzed the
proposed redox relay in an established model of neuronal differentiation (Fig. 5). Treatment of the
cells with Sem3A led to a fast increase in oxidation of both Prx1 and CRMP2. Induction of
differentiation with retinoic acid triggers the transcriptional induction of class 3 semaphorins\textsuperscript{13,50–52},
as well as CRMP2 oxidation\textsuperscript{13}. Using gene silencing in the differentiation model, we have shown
that the oxidation of Prx1 depends on the presence of MICAL1 and the oxidation of CRMP2 on the
presence of MICAL1 and Prx1. In contrast, we did not find evidence for the involvement of Prx2.

As demonstrated here, the silencing of any of the components of the oxidation redox relay results in
a 1.6-fold increase in average neurite length in the differentiation model (Fig. 5o-u). CRMP2’s
redox switch is reduced by the cytosolic isoform of glutaredoxin 2 and its operation was implied in
neuronal development and the control of cell migration and invasion\textsuperscript{14,15}. Collaboratively, over-
expression of Grx2c leads to an increase in average neurite length\textsuperscript{14}. Silencing of the expression of
Grx2c in zebrafish embryos results in an increase in oxidized CRMP2 in the developing brain. In
consequence, outgrowing axons are 4-fold shorter, fail to establish an axonal scaffold, and virtually
all neurons perish apoptotically\textsuperscript{14}.

We propose receptor-induced redox relays for both the oxidation and reduction of CRMP2 (Fig.
6) that control these proteins functions, \textit{e.g.} the regulation of neurite outgrowth and neuronal
connectivity. Activation of the NP1/PlexA receptor activates MICAL1. Using molecular oxygen and
electrons provided by NADPH, MICAL specifically oxidizes Prx1, that in turn oxidizes the
regulatory CRMP2 disulfide, thereby affecting its biological activity. This is reverted by reduction
of the switch catalyzed by Grx2c\textsuperscript{13} or Trx1\textsuperscript{16}. These oxidoreductases are reduced by GSH and
glutathione reductase and thioredoxin reductase, respectively, both at the expense of NADPH. One
of the most surprising conclusions of this study may thus be that both the oxidation and the
reduction of the CRMP2 thiol switch require reducing equivalents provided in the form of NADPH (Fig. 6).
**Material and Methods**

**Materials** – Chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis MO, USA), unless otherwise stated, and were of analytical grade or better. The antibodies used in this work were as follows: MICAL1 (14818-1-AP, Proteintech, Manchester, UK), Prx1 (LF-MA0214, Abfrontier, Liestal, Switzerland), Prx2 \( ^{53} \), PrxSO\( _{2/3} \) (ab16830, abcam, Cambridge, UK), CRMP2 (ab129082, abcam), actin (sc-47778, Santa Cruz, Heidelberg, Germany), tubulin (T9026, Sigma-Aldrich), horseradish peroxidase conjugated anti-rabbit and anti-mouse IgGs (Bio-Rad, Hercules, CA, USA).

**Electrophoresis, Western blotting, and densiometric analysis** – Protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA, USA). All one-dimensional SDS-PAGE were run using precasted mini-Protean TGX Stain-free gels (4-20%, Bio-Rad, Hercules, CA, USA). In two-dimensional diagonal SDS-PAGE, proteins from cell lysates were denatured in sample buffer (1% SDS, 50% glycerol, 60 mM Tris/HCl pH 6.8, 2 mM EDTA, Bromphenol blue) and separated in the first dimension under non-reducing conditions. Gel slices were incubated in 250 mM DTT (AppliChem, Darmstadt, Germany) and 100 mM pH neutral TCEP in sample buffer for 30 minutes at 65°C. After washing with sample buffer, the gel slices were incubated for 20 minutes at room temperature and mild agitation with 100 mM NEM (Thermo Scientific, Weltham, MA, USA) in sample buffer. After a washing step with sample buffer, every single gel lane was placed horizontally onto a pre-casted mini-Protean TGX Stain-free gels (4-20%, 7 cm IPG, Bio-Rad, Hercules, CA, USA). Western blots were carried out using Trans-Blot Turbo system and the RTA Mini PVDF Transfer Kit according to manufacturer’s instructions (Bio-Rad, Hercules, CA, USA). Blots were developed by enhanced chemiluminescence staining and documented using a ChemiDoc XRS+ documentation system (Bio-Rad, Hercules, CA, USA). Densiometric analyses of Western blots were performed with the ImageJ software (version 1.53e). All values were normalized to the total amount of protein on the blot, determined using the stain-free technology and the...
Cloning of expression constructs – MICALs monooxygenase (MO) domains and MICAL1-monoxygenase and calponin homology (MO-CH) domains were amplified by PCR from human cDNA using described oligonucleotides, ligated into pGEM-T vector (Promega, Madison, WI, USA), excised with restriction endonucleases NdeI and BglII (New England Biolabs, Ipswich, MA, USA), and ligated into the NdeI and BamHI restricted pET15b vector (Merck, Darmstadt, Germany). Correct sequences were confirmed by sequencing (SeqLab, Göttingen, Germany). The oligonucleotides are listed in Table S1.

Recombinant expression and purification – For recombinant protein expression, 400 ml medium containing the selective antibiotics were inoculated with 2% of an overnight culture of cells harboring the dedicated expression construct, and incubated at 37°C under agitation. At OD$_{600}$ = 0.6, expression was induced by addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Carl Roth, Kaiserslautern, Germany) and the temperature was lowered (to 20°C for Prxs, to 15°C for MICALs, or to 10°C for CRMP2). Cells were harvested after 18-24 hours by centrifugation at 1500 x g for 30 minutes at 4°C. Bacteria were lysed by incubation with lysis buffer for 20 minutes at room temperature and mild agitation, and subsequent ultrasonic treatment with 2 cycles of 2.5 minutes, 70% intensity, 0.5 s cycle time (50% cycling) using a Sonoplus HD2070 ultrasonic homogenizer (Bandelin, Berlin, Germany). Extracted lysate was clarified by centrifugation at 17000 x g for 30 minutes at 4°C. Recombinant His-tagged proteins were purified by immobilized metal affinity chromatography at 4°C using an Äkta FPLC system according to manufacturer’s protocol (GE Healthcare, Buckinghamshire, UK).

Cells used for the expression: *E. coli* BL21 (DE3) pRII (Life Technologies, Paisley, UK) strain was used for Prxs and CRMP2, *E. coli* Rosetta 2 (DE3) pLysS (Merck, Darmstadt, Germany) strain was used for MICALs. The following media were used: LB-Medium (Carl Roth, Kaiserslautern,
Germany) for Prxs and CRMP2, SOC medium (2% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7) for MICALs. Lysis buffers: 1 mg/ml lysozyme and 0.05 mg/ml DNase I in a 50 mM NaP buffer containing 140 mM NaCl, 20 mM Imidazole, pH 8 for Prxs and CRMP2, 1 mg/ml lysozyme, 0.05 mg/ml DNase I, 1 mM DTT, and 0.2% Tween 20 in a 50 mM Tris buffer containing 140 mM NaCl, 20 mM Imidazole, pH 8 for MICALs. The following buffers were used for the purification: 50 mM NaP, 300 mM NaCl, 20 mM Imidazole, pH 8, as washing buffer, and 50 mM NaP, 300 mM NaCl, 250 mM Imidazole, pH 8, as elution buffer for recombinant Prxs and CRMP2; for recombinant MICALs, a 50 mM Tris washing buffer containing 140 mM NaCl, 20 mM Imidazole, pH 8, and a 50 mM Tris elution buffer containing 140 mM NaCl, 300 mM Imidazole, pH 8, were used.

**Electrophoretic mobility shift assays** – Protein concentrations were determined using NanoDrop 2000c Spectrophotometer (VWR, Radnor, PA, USA), and calculated extinction coefficients (Prx1 $\varepsilon_{280} = 18450$ M⁻¹·cm⁻¹, Prx2 $\varepsilon_{280} = 21430$ M⁻¹·cm⁻¹, and MICALs-FAD $\varepsilon_{450} = 11300$ M⁻¹·cm⁻¹).

Purified Prxs were incubated with 20 mM DTT (AppliChem, Darmstadt, Germany) and 10 mM TCEP pH 7 for 30 minutes at room temperature and mild agitation. Excess of reductants were removed by gel filtration using NAP5 columns according to manufacturer’s instructions (GE Healthcare, Buckinghamshire, UK) in 50 mM Tris buffer, 100 mM NaCl, pH 8. The reaction was started by the addition of indicated FAD containing MICALs to the reduced Prxs in presence of NADPH, and absence or presence of catalase, and stopped by denaturation with sample buffer. The reaction was performed at room temperature for 15 minutes, unless otherwise stated.

**Stopped-flow kinetics** – Purified MICAL1-MO and reduced Prxs were rebuffered in 50 mM Tris, 140 mM NaCl, pH 7.4 buffer using PD-10 or NAP5 columns, respectively, according to manufacturer’s instructions (GE Healthcare, Buckinghamshire, UK). Flavin-containing MICAL concentration was determined at 450 nm in a Jasco instrument. The kinetics of flavin NADPH consumption, flavin reduction, as well as flavin oxidation, were measured in a stopped-flow.
spectrophotometer (SX20; Applied Photophysics, Leatherhead, UK) with a mixing time of less than 2 milliseconds. The NADPH consumption was analyzed following its absorbance at 340 nm, while the reduction of 10 µM flavin was followed by the absorbance decrease at 450 nm in the presence of different NADPH concentrations at indicated temperature; upon flavin reduction with excess of NADPH for 20 minutes, flavin oxidation was analyzed following the absorbance increase at 450 nm after mixing with oxygenated buffer, in presence or absence of 40 µM reduced Prx2. All data were collected and analyzed using the Pro-Data SX and Pro-Data Viewer software, respectively.

Deviations of the second order rate constants for the MICAL-catalyzed reduction of O$_2$ by NADPH and the oxidation of MICAL-FADH$_2$ by O$_2$ were calculated as follows:

1.) For the MICAL-catalyzed reduction of O$_2$ by NADPH with the following reaction: NADPH/H$^+$ + O$_2$ $\leftrightarrow$ NADP$^+$ + H$_2$O$_2$, the reaction rate of can be expressed as:

$$
\frac{\delta [NADPH]}{\delta t} = -k \cdot [NADPH] \cdot [O_2]
$$

With the following substitutions,

$$
x = [NADPH]_0 - [NADPH]_t = [O_2]_0 - [O_2]_t
$$

the expression rate law becomes:

$$
\Rightarrow -\frac{\delta x}{\delta t} = -k \cdot ([NADPH]_0 - x) \cdot ([O_2]_0 - x)
$$

$$
\Rightarrow \frac{\delta x}{([NADPH]_0 - x) \cdot ([O_2]_0 - x)} = k \cdot \delta t
$$

Integration:

$$
\int_o^x \frac{\delta x}{([NADPH]_0 - x) \cdot ([O_2]_0 - x)} = k \cdot \int_o^t \delta t
$$

Integration using the method of partial fractions:

$$
\Rightarrow \frac{1}{[O_2]_0 - [NADPH]_0} \cdot \ln \left[ \frac{[NADPH]_0}{[NADPH]_0 - x} \right] - \ln \left[ \frac{[O_2]_0}{[O_2]_0 - x} \right] = k \cdot t
$$

Rearrangement and re-substitution yields:

$$
\Rightarrow \frac{1}{[O_2]_0 - [NADPH]_0} \cdot \ln \left[ \frac{[O_2]_0 \cdot [NADPH]_0}{[NADPH]_0 \cdot [O_2]_0} \right] = k \cdot t
$$

Upon re-arrangement, we obtain the linearized equation of the integrated second order kinetics:
(8) \[ \ln \frac{[O_2]_t [NADPH]_0}{[NADPH]_t [O_2]_0} = k \cdot [O_2]_0 - [NADPH]_0 \cdot t \]

In practice, \([NADPH]_0\) was given and controlled based on its molar absorptivity at 340 nm; \([NADPH]\) was calculated based on its molar absorptivity at 340 nm; \([O_2]_0\) was determined experimentally based on the maximal NADPH consumption in the assay mixture; and \([O_2]\) was calculated as \([O_2]_0 - [NADPH]_0 + [NADPH]\).

2.) For the oxidation of MICAL-FADH\(_2\) by \(O_2\) with the following reaction: \(\text{M-FADH}_2 + O_2 \leftrightarrow \text{M-FAD} + H_2O_2\), the linearized integrated second order kinetics was deduced as outlined above yielding:

(8) \[ \ln \frac{[FADH_2]_t [O_2]_0}{[FADH_2]_0 [O_2]_0} = k \cdot [O_2]_0 - [FADH_2]_0 \cdot t \]

In practice, \([FADH_2]_0\) was given and controlled based on the maximal conversion to FAD in the assay mixture; \([FADH_2]\) was calculated based on its molar absorptivity at 450 nm; \([O_2]_0\) was determined experimentally based on the maximal NADPH consumption in the assay mixture in control experiments; and \([O_2]\) was calculated as \([O_2]_0 - [FADH_2]_0 + [FADH_2]\).

**Molecular Dynamics Simulations** – The starting structure for the molecular dynamics simulation of the MICAL1-Prx1 complex was prepared using HawkDock\(^{55}\). The human MICAL1 and Prx1 models were prepared by homology modeling using pdb entries 6ici and 5hqp, respectively. The simulations were performed in GROMACS 2016.3\(^{56}\), with AMBER-99ff-ILDN force field\(^{57}\). The parametrization of FAD was done with ACPYPE\(^{58}\) script. The protein structure was solvated with TIP3P water in a cubic box under periodic boundary conditions and at least 1 nm away from the edge of the box. \(Na^+\) and \(Cl^-\) ions were added to neutralize the charge of the system. An initial energy minimization was performed using steepest descent algorithm until the system converged to 1000 kJ·mol\(^{-1}\)·nm\(^{-1}\). System equilibration was performed for 100 ps at a constant number of molecules, volume, and temperature 300 K (NVT) and for duration of 100 ps with constant number of molecules, 1 bar pressure, and temperature 300 K (NPT). The duration of the
production simulation was 250 ns (125,000,000 time steps, 2 fs each). The bonded interactions of hydrogens were constrained with LINCS algorithm. The Parrinello-Rahman method was used for pressure coupling and the modified Berendsen thermostat – velocity rescale for the temperature coupling. The Particle Mesh Ewald method was used for the calculation of the long-range electrostatic interactions; for the short-range interactions, Verlet cut-off scheme with 1.5 nm cut-off distance was applied, for both Coulomb- and van-der-Waals interactions. The RMSD and RMSF analysis and calculation of solvent accessible surface area were performed with GROMACS. The representative structure of the simulation was calculated with the linkage algorithm of cluster tool in GROMACS with the distance cut-off of 0.2 nm. The further evaluation including the calculation of the surface of the representative structure was performed in UCSF Chimera.

**Cell culturing** – DMEM, serum (FCS), antibiotics (penicillin, streptomycin), trypsin, and PBS were purchased from PAN-Biotech (Aidenbach, Germany), disposable plastics from Sarstedt (Nümbrecht, Germany), and culture flasks from Greiner (Merck, Darmstadt, Germany). HeLa cells were propagated in Dulbecco modified Eagle Medium (DMEM) with 1 g/l glucose, SH-SY5Y cells in HyClone MEM medium with Earle’s Balanced Salt Solution (EBSS) and 2 mM L-Glutamine (GE Healthcare, Buckinghamshire, UK) each supplemented with 10% (v/v) FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C.

**Affinity capture assay** – Purified recombinant CRMP2 was coupled to a cyano-bromid (CNBr)-activated sepharose 4B matrix using coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3) according to manufacturer’s instructions (GE Healthcare, Buckinghamshire, UK). Immobilized CRMP2 was either oxidized (1 mM H₂O₂) or reduced (5 mM pH-neutralized TCEP) for 30 minutes at room temperature and agitation prior to the incubation with lysates of HeLa WT or HeLa Grx2c⁺ⁱ⁵ cells, respectively (cells were harvested, washed once with PBS and lysed for 20 minutes at room temperature. Lysis buffer: 50 mM Tris, 150 mM NaCl, 1% (w/v) CHAPS, pH 7.5, 1-fold protease and phosphatase inhibitor mixture (Roche Applied Science, Mannheim, Germany)). After 12-16
hours incubation, unbound proteins were removed by washing followed by elution of bound proteins first under denaturing (100 mM acetic acid, pH 2.1) and second under reducing (10 mM pH-neutralized TCEP) conditions. The input, flow-through and eluates were subjected to SDS-PAGE and Western blot analysis.

**Transient transfection** – Cells were transiently transfected with 15 µg specific, custom-made siRNA (Eurogentech, Seraing, Belgium) against MICAL1, Prx1, CRMP2 and an unspecific control (see Table S2). 5·10⁶ SH-SY5Y cells were resuspended with the required siRNA in electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM D-glucose, pH 7.15)⁶⁴,⁶⁵. Transfections were performed using the BTX ECM 630 electroporator at 230 V, 1050 µF, 500 Ω. SH-SY5Y cells were transfected three times, 72 hours apart each. 72 hours after the last transfection, cells were harvested, washed with PBS containing 100 mM N-ethyl maleimide (NEM, Thermo Scientific, Weltham, MA, USA), and lysed (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 100 mM NEM, 2% (w/v) CHAPS, 1-fold protease inhibitor mixture (Roche Applied Science, Mannheim, Germany)) for 20 minutes at room temperature.

**Semaphorin treatment** – SH-SY5Y cells were treated with 1 µg/ml recombinant human Semaphorin 3A Fc Chimera Protein (Bio-Techne, Minneapolis, MN, USA) for indicated times. Treatment was stopped by harvesting, washing and lysis of the cells as indicated above.

**Retinoic acid treatment** – To initiate the differentiation into a neuron-like phenotype, SH-SY5Y cells were treated with 10 µM retinoic acid (AppliChem, Darmstadt, Germany) solved in dimethyl sulfoxide (AppliChem, Darmstadt, Germany) for 120 hours, unless otherwise stated. Culturing medium containing retinoic acid was replaced every 48 hours.

**Neurite outgrowth analysis** – Bright field microscopy (EVOS XL core microscope, AMG, Mill Creek, WA, USA) pictures were taken 72 hours after the last transfection of specific siRNA and indicated time of retinoic acid treatment. Analysis of the neurites length was performed using the dendrite length quantifier plug in of ImageJ⁶⁶ and plotted with xmGrace.
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Author contributions

CB, CHL, MD, and MG conceived the study and designed the experiments. COS, DU, KS, LG, and MG performed the experiments, YB the molecular dynamics analysis. All authors analyzed the data. CB, CHL, COS, and MG wrote the paper.

Competing interests

The authors declare that they have no competing interests.
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Figure legends

Figure 1 – Prxs as potential targets of MICALs

(a) Affinity capture assay using immobilized recombinant pre-oxidized and pre-reduced CRMP2 as bait. Lysates of HeLa cells (left) and HeLa-Grx2c\(^{+}\) (right) cells\(^{15}\) were incubated with the protein, and eluted first under denaturing and second under reducing conditions. The input, flow through, and eluates were analyzed by Western blotting for proteins implied in the semaphorin (Sem3A) signaling pathway before. (b) UV-Vis spectra of the recombinantly expressed monooxygenase domains of MICAL1, 2, and 3 demonstrate the presence of (oxidized) FAD in the proteins. (c) Consumption of NADPH measured as decrease in A340 by MICAL1 in the presence of oxygen confirms the monooxygenase activity of the purified protein. (d) Oxidation of 7 µmol·l\(^{-1}\) Prx1 and 2 by 3.5 µmol·l\(^{-1}\) MICAL1, 2, and 3 in the absence and presence of 1150 units catalase per 100 µl assay. MICALs, in the presence of molecular oxygen and 200 µmol·l\(^{-1}\) NADPH were incubated with two-fold molecular excess of wildtype Prx1 and 2 or mutants lacking the resolving cysteinyl residue (C\(_{R}\)→S). The activity of all MICALs led to the oxidation of wildtype Prxs both in the presence and absence of 1150 units (µmol H\(_{2}\)O\(_{2}\) per minute) of catalase.

Figure 2 – Reaction of the MICAL1 flavin monooxygenase domain with NADPH and oxygen

The reaction of the FAD in the MICAL1 monooxygenase domain was analyzed by stopped flow kinetics. (a) Consumption of 150 µmol·l\(^{-1}\) NADPH by 10 µmol·l\(^{-1}\) MICAL1 at pH 7.4 and 298 K over the time course of 6 minutes analyzed by UV-Vis spectroscopy. (b) NADPH-dependence of the reactivity of 10 µmol·l\(^{-1}\) MICAL1 at pH 7.4 and 298 K followed by UV-Vis spectroscopy at 340 nm. (c) Linearized integrated second order kinetics of the data displayed in (b) for the determination of the apparent second order rate constant (see Supplementary information). (d) Differences of the spectra shown in (a) at 311 and 35 s minus the first spectrum taken at 1.78 ms. (e) NADPH-dependence of the reactivity of 10 µmol·l\(^{-1}\) MICAL1 at pH 6.0 and 277 K followed by UV-Vis
spectroscopy at 340 nm. (f) Linearized integrated second order kinetics of the data displayed in (e) for the determination of the apparent second order rate constant (see Supplementary information).

(g) Oxidation of the FAD in 10 μmol·l⁻¹ MICAL1 at pH 7.4 and 298 K over the time course of 1 second analyzed by UV-Vis spectroscopy. (h) Time course of the FAD oxidation followed by UV-Vis spectroscopy at 360, 370, and 450 nm. (i) Linearized integrated second order kinetics of the data displayed in (h, 450 nm) for the determination of the apparent second order rate constants suggest a two-phasic reaction (see Supplementary information).

(j) Differences of the spectra shown in (a) at 1780, 100, and 35.2 ms minus the first spectrum taken at 1.78 ms. (k) Time course of the FAD oxidation in 10 μmol·l⁻¹ MICAL1 in the presence of 20 μmol·l⁻¹ Prx followed by UV-Vis spectroscopy at 360, 370, and 450 nm. (l) Linearized integrated second order kinetics of the data displayed in (k, 450 nm) for the determination of the apparent second order rate constants suggest a two-phasic reaction (see Supplementary information).

**Figure 3 – Recombinant Prxs are oxidized by catalytical amounts of MICALs**

Michaelis-Menten kinetics of the Prxs’ oxidation by the MICAL1 monooxygenase domain (MO) and the MICAL1 monooxygenase-calponin homology domains (MO-CH). (a) Analysis of the oxidation of 10 μmol·l⁻¹ μM Prx1 and 2 by 60 nmol·l⁻¹ MICAL1, 2, and 3-MO by SDS-PAGE. The reaction requires NADPH and is not inhibited by the addition of 1150 units (μmol H₂O₂ per minute) of catalase. (b) The time-course of 50 μmol·l⁻¹ Prxs’ oxidation by 60 nmol·l⁻¹ MICALs suggests a preference of MICAL1 for the reaction. (c-d) Michaelis-Menten kinetics of the oxidation of Prx1 and 2 (as indicated) by 60 nmol·l⁻¹ MICAL-MO (c) or 20 nmol·l⁻¹ MICAL-MO-CH (d). All measurements were performed in the presence of 1150 units of catalase per 100 μl assay and 200 μmol·l⁻¹ NADPH.
**Figure 4 – Docking and all atomistic molecular dynamics simulation of a MICAL1-Prx1 complex**

(a) Water accessible surface of the complex in side view (dimeric Prx1 in green, MICAL1-MO in gray, interaction surfaces in yellow). (b-c) Interaction surfaces (in yellow) on Prx1 (B) and MICAL1 (C); the complex was opened by 90° left (Prx1) and 90° right (MICAL1). (d) Root mean square deviation (rmsd) of the peptide backbones in the complex over the course of the simulation (250 ns). (e) Solvent accessible surface area (sasa) of the complex over the course of the simulation. (f-g) One of the peroxidatic cysteiny1 residues of the dimeric Prx1 faces the active site cavity of MICAL1; (g) is a magnification of (f, square). (h) Average side chain fluctuations of the residues in the Prx1 dimer-MICAL1 complex, the peroxidatic (CysP) and the resolving (CysR) are highlighted.

**Figure 5 – The MICAL1-Prx1-CRMP2 redox relay in a model of neuronal differentiation**

(a) Time course of the redox states of CRMP2 and Prx1 in SH-SY5Y cells treated with 1μg·ml⁻¹ Sem3A. (b-c) Redox states of Prx1 (b, 2-D diagonal SDS-PAGE) and CRMP2 (c) in SH-SY5Y cells with silenced expression of MICAL1 and treated with 1μg·ml⁻¹ Sem3A for 150 s; the siRNA control was included in (c). (d-e) Levels of oxidized (d) and reduced (e) CRMP2 in SH-SY5Y cells during their differentiation into neuron-like cells induced by 10 µmol·l⁻¹ retinoic acid (RA) added to the medium; n=3. (f-l) Redox states of Prx1 and Prx2 in RA-treated SH-SY5Y cells depend on MICAL1; n=6. (f, j) Quantification of the redox states analyzed by 2-D diagonal (g) and 1-D gel electrophoresis (h), n=7 and 6 for Prx1, n=9 and 8 for Prx2, respectively. siRNA controls were included in (h) and (l). Silencing of MICAL1 in cells did not induce over-oxidation of Prxs as demonstrated with a Prx-SO₂/³ specific antibody, control cell lysate treated with 1 mmol·l⁻¹ H₂O₂ was used as positive control (i). (m-n) Redox state of CRMP2 in the SH-SY5Y differentiation model depends on Prx1. (m) Quantification of the redox state; n=4. (n) siRNA control and exemplary Western blots. (o-u) Neurite outgrowth during SH-SY5Y differentiation depends on MICAL1, Prx1, and CRMP2. (o) Neurite length in control, MICAL1 and Prx1-depleted cells after
120 hours of differentiation. Each point represents the automated analysis of one picture of n=3 biological replicates. (p) Neurite length in control and CRMP2-depleted cells after 72 hours of differentiation. Each point represents the automated analysis of one picture of n=3 biological replicates. Sample pictures of the cells treated with siControl (q), siMICAL1 (r), siPrx1 (s), siCRMP2 (t, the associated siControl at the 72 hour time point is not shown), and exemplary siRNA controls (u) are shown in the lower row. All box-plots depict the median (line), the 75% quartiles (box) and the minimum and maximum values (whiskers). All densiometric analyses were normalized to the total amount of protein on the respective lane of the blot. The p-values were calculated applying an unpaired T-test analysis.

Figure 6 – NADPH-dependent redox relays in both the oxidation and reduction of the CRMP2 thiol switch control axonal outgrowth
Table 1 – Kinetic analysis of the reaction of MICAL1 with NADPH, molecular oxygen, and Prxs. The apparent rate constants of MICAL1-MO with NADPH and molecular oxygen were determined by stopped-flow kinetics (see Fig. 2). The $K_m$ and $V_{max}$ of MICAL1-MO and MICAL1-MO-CH with Prx1 and 2 as substrates were determined in end-point assays (see Fig. 3).

| Enzyme         | Substrate | 2\textsuperscript{nd} order rate constant ($k_{app}$) | n   | pH | Temperature |
|----------------|-----------|------------------------------------------------------|-----|----|-------------|
|                |           | mol\textsuperscript{-1}\cdot l\cdot s\textsuperscript{-1} |     |    |             |
| MICAL1-MO      | NADPH     | 163.8 ± 13.9                                         | 14  | 7.4| 298         |
| MICAL1-MO      | NADPH     | 140.5 ± 19.2                                         | 8   | 7.4| 277         |
| MICAL1-MO      | NADPH     | 126 ± 6.7                                            | 10  | 6.0| 277         |
| MICAL1-MO      | O\textsubscript{2} (1.) | 1.9 ± 0.2 \cdot 10\textsuperscript{5}       | 5   | 7.4| 298         |
|                |           | (2.) 2.4 ± 0.3 \cdot 10\textsuperscript{4}         |     | 7.4| 298         |
| MICAL1-MO      | O\textsubscript{2} (Prx1 present) (1.) | 2.3 ± 0.1 \cdot 10\textsuperscript{5}       | 7   | 7.4| 298         |
|                |           | (2.) 3.2 ± 0.5 \cdot 10\textsuperscript{4}         |     | 7.4| 298         |

| Substrate | $K_m$ | $V_{max}$ | n   | pH | Temperature |
|-----------|-------|-----------|-----|----|-------------|
|           | \mu mol\textsuperscript{-1} | s\textsuperscript{-1} |     |    |             |
| MICAL1-MO | Prx1  | 30.4 ± 0.8 | 0.28 ± 0.03 | 3   | 7.4 | 298         |
| MICAL1-MO | Prx2  | 22.7 ± 3.1 | 0.31 ± 0.05 | 3   | 7.4 | 298         |
| MICAL1-MO-CH | Prx1 | 23.5 ± 1.7 | 0.72 ± 0.03 | 3   | 7.4 | 298         |
| MICAL1-MO-CH | Prx2 | 14.2 ± 0.8 | 0.57 ± 0.01 | 3   | 7.4 | 298         |
Figure 3
Figure 4
Figure 6

oxidation pathway

NADPH → MICAL1redd → Prx1redd → CRMP2redd → thiol switch → Grx2redd → GSH → GRredd → NADPH

NADP⁺ → MICAL1ox → Prx1ox → CRMP2ox

axon outgrowth / elongation

reduction pathway

NADP⁺ → NADPH

axon outgrowth / change of direction
Supplementary Files

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