SeLR reverses Mical-mediated oxidation of actin to regulate F-actin dynamics

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Actin’s polymerization properties are markedly altered by oxidation of its conserved Met 44 residue. Mediating this effect is a specific oxidation–reduction (redox) enzyme, Mical, that works with Semaphorin repulsive guidance cues and selectively oxidizes Met 44. We now find that this actin-regulatory process is reversible. Employing a genetic approach, we identified a specific methionine sulfoxide reductase (MsrB) enzyme SeLR that opposes Mical redox activity and Semaphorin–Plexin repulsion to direct multiple actin-dependent cellular behaviours in vivo. SeLR specifically catalyses the reduction of the R isomer of methionine sulfoxide (methionine-R-sulfoxide) to methionine, and we found that SeLR directly reduced Mical-oxidized actin, restoring its normal polymerization properties. These results indicate that Mical oxidizes actin stereospecifically to generate actin Met-44-R-sulfoxide (actinMet(R)O–44), and also implicate the interconversion of specific Met/Met(R)O residues as a precise means to modulate protein function. Our results therefore uncover a specific reversible redox actin regulatory system that controls cell and developmental biology.

Identifying the factors that shape the actin cytoskeleton, the basic building blocks of cellular form and function, is a critical biomedical goal1-2. Interestingly, actin is susceptible to post-translational modification of its amino acid residues but the physiological importance of these covalent modifications is still poorly understood3. Recently, we found that actin’s polymerization properties are altered by specific oxidation of its conserved Met 44 residue on the pointed-end of actin subunits4. These observations raise issues of the susceptibility of this residue to pathological modification3, but we have also identified a specific redox enzyme, Mical, that selectively oxidizes Met 44 to disassemble actin filaments (F-actin) and impair actin polymerization5,6. Our results reveal that Mical uses F-actin as a direct substrate, employing an oxidation-dependent post-translational mechanism to regulate filament dynamics4.

MICAL family proteins, which include one Drosophila Mical and three mammalian MICALs, regulate numerous cellular events in different tissues, including morphology, motility, navigation, exocytosis and survival (reviewed in refs 6-9). At least some of these effects occur through the ability of MICALs to regulate actin cytoskeletal organization5,10-12. Interestingly, MICALs also directly link one of the largest families of extracellular guidance cues, the Semaphorins and their Plexin cell surface receptors, to changes in the actin cytoskeleton5,13. Semaphorins are the largest family of repulsive guidance cues14,15 and have been characterized for their ability to disassemble F-actin and ‘collapse’ the actin cytoskeleton of multiple different cell types6,16. MICALs directly bind to the Semaphorin receptor Plexin through their carboxy termini13,17 and employ their actin-binding/regulatory redox domain to mediate the destabilizing effects of Semaphorins–Plexins on the actin cytoskeleton5. These effects include a loss of F-actin, the decreased ability to polymerize new F-actin, a decrease in the number of F-actin bundles, and the regulation of F-actin-rich filopodia/branches6.

We now find a specific methionine sulfoxide reductase enzyme SeLR/MsRB that selectively reverses this Mical-mediated oxidation of actin. SeLR counteracts Mical in vivo to direct multiple actin-dependent cellular processes including axon guidance, synaptogenesis, muscle organization and mechanosensory development. SeLR also neutralizes Semaphorin–Plexin repulsion. Thus, Mical and SeLR comprise a reversible redox cellular signalling system that orchestrates proper cytoskeletal-mediated physiology.

RESULTS
SeLR counteracts Mical-mediated F-actin alterations in vivo
Mical directs the organization of actin in a number of different cell types4,5,10-12, including within developing bristle processes, which are akin to mammalian mechanotransducing inner ear hair cells that detect sound18,19. Bristles have also long served as a simple, single-cell model for characterizing actin-dependent events in vivo6,20,21. Raising the
levels of Mical specifically in bristle cells using the GAL4–UAS (ref. 22) system (bristle-specific GAL4/UAS:Mical) results in F-actin disassembly and bristle branching (compare Fig. 1a with Fig. 1b) that is dependent on Mical’s redox activity and the Met 44 residue of actin. Thus, to better characterize Mical-mediated F-actin alterations, we have initiated a large-scale genetic screen to look for enhancers and suppressors.

Figure 1 SelR counteracts Mical-mediated actin-dependent changes in vivo. (a,b) Wild-type Drosophila bristles are unbranched (a) but become branched (b; arrowhead) when Mical is overexpressed specifically within them using the GAL4–UAS system (B11-GAL4/+; UAS:Mical/+). (c,d) A dominant genetic screen identifies that SelR (SelR[EY22443] and SelR[EP3340]), but not other specific reductase enzymes, strongly decreases/suppresses Mical-induced bristle branching. Txl, thioredoxin-like; Trx-2, thioredoxin-2; Dhd, deadhead/thioredoxin-like. All genotypes are heterozygous (B11-GAL4, UAS:Mical and mutations/C). n = 20 animals per genotype. Mean ± standard error of the mean (s.e.m.). Replicated in at least two independent experiments (separate crosses) per genotype. (e) Both the EY22443 and EP3340 mutations contain UAS sequences that are directed towards SelR. (f) Bristle-specific expression of SelR localizes with Mical (middle, GFP–SelR and mCherry–Mical) and F-actin (phalloidin), suppresses Mical-induced bristle branching (left and upper graph; n = 40 bristles assessed in 10 animals per genotype; mean ± s.e.m.), and generates wild-type appearing bristles (left and lower graph; n = 40 bristles assessed in 10 animals per genotype). Likewise, the F-actin alterations (right), including areas of decreased F-actin (arrowhead) and actin-rich branches (arrow) that occur on bristle specific expression of Mical, are suppressed by co-expression of SelR. n = 30 bristles assessed in 8 animals per genotype. All quantitative data in f were replicated in at least 2 independent experiments (separate crosses) per genotype.
of Mical-mediated bristle branching. One of the mutations that we identified in our genetic screen, the transposable element mutation EY22443, strongly suppressed Mical-induced actin-dependent bristle branching (Fig. 1b–d). Molecular analysis revealed that the EY22443 transposable element mutation was situated within the Drosophila SelR gene (Fig. 1e). SelR codes for a methionine sulfoxide reductase (MsrB) family enzyme, which has been characterized for its ability to reduce oxidized methionine residues. In light of our observations that Mical oxidizes methionine residues on actin, we wondered whether SelR might play a role in modulating Mical’s effects on actin.

The EY22443 transposable element mutation situated in SelR contains a UAS promoter (Fig. 1e), thereby suggesting that this mutation might be abnormally inducing SelR expression to suppress GAL4/UAS:Mical-dependent bristle branching. To test this hypothesis, we generated transgenic flies expressing SelR directly under the UAS promoter. Consistent with our results with EY22443 (Fig. 1c,d) and another UAS-containing mutation within SelR, EP3340 (Fig. 1d), multiple transgenic lines revealed that raising the levels of SelR specifically in bristles strongly suppressed Mical-induced bristle branching and even generated normal appearing bristles (Fig. 1f).

Moreover, elevating the levels of SelR in a wild-type background generated abnormally bent bristles that resembled Mical−/− mutant bristles (Supplementary Fig. 1 and ref. 5); and these effects of SelR were genetically enhanced by decreasing the levels of Mical (Supplementary Fig. 1). Further analysis revealed that SelR localized with Mical at the tips of bristles and suppressed Mical-mediated F-actin disassembly and reorganization (Fig. 1f). Therefore, SelR counteracts the effects of Mical on actin reorganization in vivo.

SelR restores the polymerization of Mical-treated actin

To better understand the role of SelR in counteracting Mical-mediated actin reorganization, we purified recombinant Drosophila SelR protein (Supplementary Fig. 2). Using in vitro actin biochemical and imaging assays, we previously observed that purified Mical protein in the presence of its coenzyme NADPH disrupts actin polymerization and induces F-actin disassembly. Strikingly, we found that purified SelR protein rescued the ability of Mical-treated actin to polymerize (Fig. 2a). This Mical/SelR-treated actin re-polymerized to an extent that was indistinguishable from normal untreated actin (Fig. 2b). Moreover, whereas Mical-treated actin failed to polymerize even after removal of Mical and NADPH (Fig. 2c and ref. 4), SelR induced the polymerization of this purified Mical-treated actin in a dosage-dependent manner (Fig. 2c). Thus, SelR restores the polymerization properties of Mical-treated actin.

SelR converts methionine sulfoxide (MetO) to methionine, requiring a redox-active cysteine (Cys124) residue and also using reducing agents to cycle back to its reduced form. In some cases methionine oxidation is also reversed by general reducing agents, so we wondered whether Mical-treated actin was specifically reversed by SelR. In contrast to SelR, neither chemical reducing agents such as dithiothreitol (DTT; Fig. 2a) nor other reducing enzymes including thioredoxins/thioredoxin reductases altered Mical-mediated effects on actin in vitro (Supplementary Fig. 3) or in vivo (Fig. 1d). Furthermore, SelR did not restore the normal polymerization properties of other oxidized forms of actin (for example, H2O2-treated actin; Supplementary Fig. 3), indicating that SelR selectively affects Mical-modified actin. Mutating the critical catalytic cysteine of SelR (Cys124) to generate an enzymatically dead SelR (SelRΔCys124; Fig. 2c and ref. 25) abolished the effects of SelR on Mical-treated actin in vitro (Fig. 2b,f) and in vivo (Fig. 2g). Moreover, consistent with such a role for the reductase activity of SelR in counteracting Mical’s oxidative effects on actin, elevating the levels of wild-type SelR not only phenocopied the in vivo effects of disrupting Mical’s monooxygenase (redox) domain (Supplementary Figs 1 and 4), but it also reversed the severe bristle/F-actin alterations that result from hyperactive Mical redox signalling (Supplementary Fig. 4; MicalΔredoxCys1S). Thus, SelR specifically employs its catalytic activity to restore Mical-treated actin polymerization and counteract the in vivo effects of Mical.

SelR reverses Mical-mediated actin oxidation

In many organisms, including Drosophila and mammals, two main types of methionine sulfoxide reductase have been identified: SelR (MsrB family proteins) and Drosophila Eip71CD (MsrA; Fig. 3a and ref. 27). Interestingly, SelR and MsrA/Eip71CD are both methionine sulfoxide reductases, but they do not exhibit similarity in their sequence, domain organization or substrate specificity. Interestingly, SelR and MsrA/Eip71CD are both methionine sulfoxide reductases, but they do not exhibit similarity in their sequence, domain organization or substrate specificity. Interestingly, SelR and MsrA/Eip71CD are both methionine sulfoxide reductases, but they do not exhibit similarity in their sequence, domain organization or substrate specificity.

SelR reverses Mical-mediated actin oxidation

Mical oxidizes actin on its Met 44 and Met 47 residues, although it is the oxidation of the Met 44 residue through which Mical induces F-actin disassembly. Thus, we wondered whether SelR directly reverses Mical-mediated oxidation of actin. Previously, we determined the conditions for purifying Mical-treated actin, which is polymerization impaired and exhibits a mass increase of two oxygens (32 daltons). SelR, but not the enzymatically dead SelRΔCys124 protein, restored the polymerization properties of purified Mical-treated actin (Supplementary Fig. 3), an effect that was maintained even after removal of SelR (Fig. 3d). Subjecting both purified Mical/SelR-treated and Mical/SelRΔCys124-treated actin to mass spectrometry revealed that SelR, but not the enzymatically dead SelRΔCys124 protein, eliminated the Mical-catalysed two-oxygen (32 dalton) mass increase on actin.

Mical’s ability to effect actin in vitro and in vivo is dependent on the presence of the Met 44 residue of actin. To further examine a physiological role for SelR in reducing Mical-mediated oxidation of Met 44, we turned to in vivo assays. We first noted that overexpression of either a non-Mical oxidizable M44L version of actin or wild-type...
Figure 2 SelR restores the polymerization properties of Mical-treated actin. (a) Pyrene–actin assays, where the fluorescence is higher in the polymerized state, reveal that SelR (green dots) restores the polymerization of Mical-treated (600 nM Mical, 100 µM NADPH; refs 4,5) actin (1.15 µM actin), whereas buffer alone (blue dots, containing 20 mM of DTT) does not. n.u., normalized units between the two graphs. (b) Mical-treated actin polymerizes to a normal extent following addition of SelR, but not the enzymatically inactive SelR\text{C124S}. Sedimentation assay and Coomassie-stained gel. Actin monomers/G-actin is in the supernatant (S); actin polymers/F-actin is in the pellet (P). Right, quantification of pelleted actin from \(n\) = 2 separate experiments per condition. See also Supplementary Fig. 3e for the uncropped gel. (c) SelR (0.3–2.4 µM) restores the polymerization of purified Mical-treated actin in a concentration-dependent manner. a.u., arbitrary units. (d) SelR/MsrB family proteins use the conserved cysteine (Cys 124) residue to reduce MetO to Met (refs 23,25). (e) Catalytically inactive Cys (C) to serine (S) mutation (SelR\text{C124S}; ref. 25). Hs, human; Dm, Drosophila. (f,g) Unlike wild-type SelR, SelR\text{C124S} does not restore Mical-treated actin polymerization in vitro (f) or suppress Mical-induced actin reorganization/bristle branching (g). (g) Note that in contrast to bristle overexpression of SelR (SelR\text{+++}), which suppresses bristle branching owing to bristle overexpression of Mical (Mical\text{++++}), bristle-specific expression of SelR\text{C124S} (SelR\text{C124S + + + +}) enhances Mical-dependent bristle branching (increasing both the number and length of branches). Mutating Mical’s substrate residue on actin, the Met 44 residue, and expressing this mutant actin in bristles (Actin\text{M44L} + + + +), suppresses the effects of SelR\text{C124S} on Mical. \(n\) = 40 bristles assessed in 10 animals per genotype. Replicated in at least two independent experiments (separate crosses) per genotype. SelR generated the same effects: suppression of Mical-mediated actin/bristle morphology and Mical loss-of-function-like defects\(^4\) (Figs 1f and 2g and Supplementary Figs 1 and 4). Furthermore, we found that actin\text{M44L} worked in combination with SelR to generate Mical loss-of-function-like bristle defects (Supplementary Fig. 4). Moreover, actin\text{M44L} prevented the enhanced Mical-mediated bristle
Figure 3 SelR/MsrB reverses Mical-mediated actin\[^{Met\ 44}\] oxidation.
(a) SelR/MsrB and Eip71CD/MsrA family proteins including catalytically active cysteine (CxxS) and PMSR (peptide methionine sulfoxide reductase) motifs. (b) Methionine-R-sulfoxide (top) and methionine-S-sulfoxide (bottom) are reduced by SelR/MsrB family proteins and Eip71CD/MsrA, respectively. (c) MsrA (purple dots) does not restore polymerization of Mical-treated actin (pyrene–actin assay). (d) Mical-oxidized actin was treated with SelR or SelR\[^{C124S}\] (left) and then purified to reveal that SelR-treated, but not SelR\[^{C124S}\]-treated, Mical-oxidized actin polymerizes (right; pellet (P)). Coomassie-stained gel. See also Supplementary Fig. 3f for the uncropped gel. (e) Mass spectrometry of Mical/SelR-treated purified actin reveals that SelR, but not SelR\[^{C124S}\], reverses the Mical-catalysed 32 dalton (two oxygen) increase\[^{4}\] in the mass of actin. Note that the different peaks are different modified versions of actins that have been purified from rabbit. (f) Mical oxidizes the Met 44 residue of actin and SelR reverses this Mical-catalysed Met 44 oxidation (compare arrowheads).

branching/actin reorganization that occurred with expression of the reductase dead SelR\[^{C124S}\] (Fig. 2g). Thus, SelR reverses Mical-mediated oxidation of actin, including using its catalytic activity to directly reduce Mical-induced MetO 44 actin to Met 44 actin (Fig. 3f)—and these observations with purified proteins are supported by our \textit{in vivo} genetic assays.

The Mical/SelR system regulates actin organization in multiple cell types

In addition to bristle cells, Mical regulates the organization of actin in multiple other cell types including mammalian cells \textit{in vitro} and muscles \textit{in vivo}\[^{4,6,10,11}\]. Thus, we wondered whether SelR could also counteract the effects of Mical on actin in these other cellular systems. Our initial examination revealed that as in bristle cells, SelR rescued Mical-dependent changes in morphology and actin organization in cultured cells (Fig. 4a). Further examination revealed that overexpression of SelR in muscles \textit{in vivo} phenocopied the muscle actin defects found in \textit{Mical}~\textsuperscript{−/−} mutants\[^{10}\] (Fig. 4b). Moreover, SelR could even rescue the lethality and changes in actin organization associated with overexpression of Mical in muscles (Fig. 4c)—as well as the lethality that results when Mical is broadly expressed using an actin promoter (Fig. 4c).

\textit{Drosophila} SelR, like Mical, is broadly expressed\[^{5,10,13,25,28–31}\] (Supplementary Fig. 5) and thus to better examine these Mical-SelR interactions and their physiological effects on actin, we characterized SelR~\textsuperscript{−/−}~ mutants (Supplementary Fig. 6). Strikingly, loss of SelR
Figure 4 SelR opposes Mical-mediated effects in different cell types. (a) SelR-mediated rescue of Mical-induced morphology and F-actin defects in 3T3 cells. Expression of Mical (GFP–MicalredoxCH) in 3T3 cells results in a loss of F-actin stress fibres and generates an abnormal rounded cell morphology. SelR localizes together with Mical when it is co-expressed with Mical (GFP–MicalredoxCH + mCherry–SelR) and significantly rescues this Mical-mediated rounded cell morphology. Note also the localization of GFP–MicalredoxCH and mCherry–SelR with F-actin. ***P < 0.0001; one-way analysis of variance with multiple comparison correction; n = 79 cells assessed from two independent experiments including a total of four different transfected plates per condition. Mean ± s.e.m. (b) Muscle SelR phenocopies Mical mutant muscles. The percentage of muscles exhibiting abnormal accumulations of actin is shown (n = 24 muscles assessed in 9 animals per genotype). Replicated in at least two independent experiments (separate crosses) per genotype. Mical (G56/MicalI666). (c) SelR-mediated rescue of Mical-induced lethality and muscle actin defects. Mical overexpression (Mical++) using either an actin promoter (Actin5C-GAL4) or a muscle-specific promoter (24BGAL4) is lethal (graph). SelR (SelR++) co-expression completely rescues this Mical-induced lethality (n = 100 animals examined per cross) and also rescues the changes in actin organization that result from Mical overexpression in muscles (n = 24 muscles assessed in 9 animals per genotype). Both experiments were replicated in at least two independent experiments (separate crosses) per genotype.

generated bristle and muscle defects that resembled overexpression of Mical (Fig. 5a,e). Moreover, loss of SelR specifically enhanced Mical-mediated effects on actin organization/bristle morphology (Fig. 5b,c and Supplementary Fig. 4) and phenocopied overexpression of the SelRC124S reductase mutant protein (Fig. 5d). Thus, SelR, like Mical, plays both important and selective roles in regulating actin organization in vivo in different cell types. Likewise, an equilibrium between Mical and SelR activities underlies normal actin-directed cell biology.
SelR neutralizes Semaphorin–Plexin–Mical repulsive signalling

Besides its redox region that Mical uses to oxidize actin, Mical has several other domains and protein interaction motifs including a region that interacts with the cytoplasmic portion of Plexin (Fig. 6a and refs 13,17). Plexins are receptors for Semaphorin guidance cues and play critical roles in regulating multiple actin-dependent events in vivo6,32,33. Semaphorins–Plexins signal through Mical to induce changes in bristle morphology and F-actin disassembly5, so we wondered whether SelR also counteracted the effects of Semaphorin–Plexin–Mical signalling. Employing loss- and gain-of-function genetics in the bristle system, we found that similar to our results with Mical, SelR counteracted Semaphorin–Plexin effects on actin-dependent bristle morphology (Supplementary Fig. 1). Next, we turned to in vivo axon guidance assays, where Semaphorins–Plexins have been characterized as repulsive axon guidance molecules15 and were first linked to MICAL family proteins13. Interestingly, one of the SelR mutants that we found in our screen (EP3340, Fig. 1d,e) recently emerged from a genetic screen as an uncharacterized regulator of axon guidance34.
Figure 6 SelR counteracts Semaphorin–Plexin–Mical repulsive signalling. (a) Drosophila and mammalian MICAL proteins are characterized by multiple domains including their actin-regulatory redox domain and Plexin receptor interacting C terminus (Plexin IR), CH, Calponin homology domain; LIM, LIM domain; PxxPs, proline (P)-rich motifs. (b) Neuronal SelR phenocopies Mical+/− axon guidance and synaptogenic defects. Top row: neuronal overexpression of SelR generates Semaphorin-1a+/− (ref. 45), PlexinA+/− (ref. 35), and Mical+/−-like intersegmental nerve b (ISNb) axon guidance defects that are characterized by decreased axonal defasciculation/repulsion and a failure of axons to reach their correct targets (closed arrowheads). Wild-type innervation (open arrowheads). Similar, percentage of the neuronal SelR–/– ISNb defects that are significantly rescued by neuronal (ELAV–GAL4) expression of SelR. Both SelR+/− ISNb (green bar) and central nervous system (CNS; see image) axon guidance defects resemble the increased axonal defasciculation/repulsion seen with neuronal Mical overexpression. Note motor axons projecting into abnormal areas (filled arrowheads), discontinuous, thin or missing CNS longitudinal connectives (arrows), and CNS axons abnormally crossing the midline (open arrowheads). See Supplementary Fig. 6d for the ISNb guidance defects from this neuronal Mical–/– image. χ² test; ***P < 0.0001; n = 94 hemisegments assessed in 10 animals per genotype. (c) Neuronal SelR generates Mical+/−-like synaptogenesis defects, with a decreased length of synaptic innervation. One-way analysis of variance with correction for multiple comparisons; ***P < 0.0001; mean ± s.e.m.; n = 20 synapses assessed in seven animals per genotype. Mical+/−, Mical+/−(ref K1496) and Mical+/−(DfExel7305); (d) SelR−/− (SelR+/−; ref. K1496/DfExel7305) mutants exhibit ISNb and segmental nerve a (SNa) axon guidance defects that are significantly rescued by neuronal (ELAV–GAL4) expression of SelR. Both SelR+/− ISNb (green bar) and central nervous system (CNS; see image) axon guidance defects resemble the increased axonal defasciculation/repulsion seen with neuronal Mical overexpression. Note motor axons projecting into abnormal areas (filled arrowheads), discontinuous, thin or missing CNS longitudinal connectives (arrows), and CNS axons abnormally crossing the midline (open arrowheads). These PlexA axon guidance defects are dependent on both Semaphorin-1a and Mical+/−, and raising the levels of SelR in neurons (neuronal PlexA + neuronal SelR) significantly rescues these Semaphorin-1a–PlexA–Mical-dependent axon guidance defects. χ² test; ***P < 0.0001; n = 47 animals per genotype.
Employing our SelR transgenic lines, we found that overexpression of SelR generated axon guidance and synaptogenic defects that phenocopy Mical*/* mutants (Fig. 6b and Supplementary Fig. 6). Furthermore, SelR*/* mutants generated axon guidance defects that phenocopy increased Semaphorin–Plexin–Mical-mediated repulsive axon guidance (Fig. 6c and Supplementary Fig. 6). Moreover, increasing the levels of SelR rescued these Semaphorin–Plexin–Mical-triggered repulsive axon guidance defects (Fig. 6d and Supplementary Fig. 6). Thus, SelR also plays critical roles in axon guidance and synaptogenesis and counters the effects of Semaphorin–Plexin–Mical repulsive signalling in vivo.

**DISCUSSION**

Our results reveal that Mical-mediated actin alterations—a selective means to post-translationally regulate F-actin dynamics and cellular behaviours—are reversible. This Mical-catalysed reaction is directly reversed by a specific methionine sulfoxide reductase enzyme, SelR/MsrB, which also selectively controls actin–dependent cellular events in vivo and regulates specific neuronal, muscular and mechanosensory developmental processes. We also find that SelR counteracts Semaphorins, which are one of the largest families of extracellular guidance cues and play a critical role in the formation and function of multiple tissues. Thus, our results demonstrate an important role for these methionine sulfoxide reductases—enzymes thought to function primarily in the repair of oxidatively ‘damaged’ methionine residues—in modulating normal signalling events. Moreover, our genetic data, which reveal that SelR and Mical loss- and gain-of-function phenotypes are opposite in appearance, indicate that SelR has a specific, primary, and regulated role in counteracting Mical during development.

The Mical substrate Met 44 residue of actin is conserved in all actin family members from yeast to humans and a dominant (heterozygous) mutation in the Met 44 residue (M44T) of skeletal muscle actin underlies a human musculoskeletal disease associated with actin accumulation and aggregation (nemaline myopathy). This Met 44 mutant version of human skeletal muscle actin would be predicted to prevent Mical from having effects on skeletal muscle actin—and generally phenocopies both Mical*/* and SelR muscle overexpression. However, the Met 44 residue is well conserved and is at a subunit interface in filaments and thus mutating it may influence F-actin organization for reasons other than that it is non-oxidizable. It should be noted, however, that our previous results indicate that Met 44 mutant actin (M44L) seems to polymerize normally in vitro and in vivo, but is resistant to Mical-mediated F-actin disassembly.

It is also interesting to note the differences in the cellular localization we see between SelR and different forms of Mical. For example, in bristles, SelR shows overlapping localization with Mical, but is more broadly distributed than full-length Mical, which strongly localizes to bristle tips (Fig. 1f). The broader cellular localization of SelR is similar to that seen when the hyperactive MicalredoxCH is expressed in bristles and other cells (Fig. 4a). One of the differences between full-length Mical and the hyperactive MicalredoxCH is the presence of the Plexin-interacting region (Fig. 6a). Our results indicate that full-length Mical is susceptible to regulation by Plexin, whereas the MicalredoxCH protein (which does not have the Plexin-interacting region) is not regulated by Plexin (see also refs 11,17). Interestingly, the MICALs express multiple different transcripts, including versions that may be similar to MicalredoxCH (ref. 6). Thus, there may be roles for both endogenous Semaphorin–Plexin-regulated and, perhaps, non-Semaphorin–Plexin-regulated forms of Mical (which seem to be more generally localized in cells). In any case, it should be noted that we find that SelR rescues both the lethality and F-actin defects associated with overexpression of either full-length Mical or MicalredoxCH. Likewise, we find that SelR counteracts Semaphorin–Plexin effects in vivo.

Our results herein, coupled with our previous observations, also indicate that unlike diffusible oxidants that induce random protein modifications, Mical-mediated oxidation is substrate-, residue- and stereo-specific. Our results indicate that Mical oxidizes the Met 44 residue of actin stereospecifically to generate actin methionine-44-R-sulfoxide (actinMet(R)O−44) to alter F-actin dynamics. These observations contend that the enzyme-driven interconversion of specific Met/Meth(R)O residues, similar to the reversible phosphorylation of specific serine, threonine and tyrosine residues, provides a selective means to precisely modulate protein function. Along these lines, our results are supported by recent biochemical experiments demonstrating that one of the mammalian SelRs/MsrBs (MsrB1) reverses MICAL-mediated F-actin disassembly. Moreover, in contrast to a view that oxidation simply plays a destructive role in cell health and protein function, our results indicate that the site-specific and reversible oxidation of proteins is critical for proper cellular physiology. Thus, together, our results uncover a specific reversible redox cellular signalling system that dynamically regulates multiple actin cytoskeletal-mediated events and controls Semaphorin–Plexin repulsion.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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1. Pollard, T. D. & Cooper, J. A. Actin, a central player in cell shape and movement. Science 326, 1208–1212 (2009).
2. Fletcher, D. A. & Mullins, R. D. Cell mechanics and the cytoskeleton. Nature 463, 485–492 (2010).
3. Terman, J. R. & Kashina, A. Post-translational modification and regulation of actin. Curr. Opin. Cell Biol. 25, 30–38 (2013).
4. Hung, R. J., Pak, C. W. & Terman, J. R. Direct redox regulation of F-actin assembly and disassembly by Mical. Science 334, 1710–1713 (2011).
ARTICLES

5. Hung, R. J. et al. Mical links semaphorins to F-actin disassembly. *Nature* **463**, 823–827 (2010).

6. Hung, R. J. & Terman, J. R. Extracellular inhibitors, repellents, and semaphorin/plexin/MICAL-mediated actin filament disassembly. *Cytoskeleton* **68**, 415–433 (2011).

7. Zhou, Y., Gunput, R. A., Adolfs, Y. & Pasterkamp, R. J. MICALs in control of the cytoskeleton, exocytosis, and cell death. *Cell Mol. Life Sci.* **68**, 4033–4044 (2011).

8. Caplan, S. & Girdharan, S. S. MICAL-family proteins: complex regulators of the actin cytoskeleton. *Antioxid. Redox Signal.* [http://dx.doi.org/10.1089/ars.2013.5487] (2013).

9. Vanoni, M. A., Vitali, T. & Zucchini, D. MICAL, the flavoenzyme participating in cytoskeleton dynamics. *Int. J. Mol. Sci.* **14**, 6920–6959 (2013).

10. Beuchle, D., Schwarz, H., Langegger, M., Koch, I. & Aberle, H. *Drosophila* MICAL regulates myofilament organization and synaptic structure. *Mech. Dev.* **124**, 390–406 (2007).

11. Girdharan, S. S., Rohn, J. L., Naslavsky, N. & Caplan, S. Differential regulation of actin microfilaments by human MICAL proteins. *J. Cell Sci.* **125**, 614–624 (2012).

12. Morinaka, A. et al. Thioredoxin mediates oxidation-dependent phosphorylation of CRMP2 and growth cone collapse. *Sci. Signal.* **4**, ra26 (2011).

13. Terman, J. R., Mao, T., Pasterkamp, R. J., Yu, H. H. & Kolodkin, A. L. MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. *Cell* **109**, 887–900 (2002).

14. Kolodkin, A. L. et al. Fasciclin IV sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron* **9**, 831–835 (1992).

15. Kolodkin, A. L. & Tessier-Lavigne, M. Mechanisms and molecules of neuronal wiring: a primer. *Cold Spring Harb. Perspect. Biol.* **3**, a001727 (2011).

16. Luo, Y., Raible, D. & Raper, J. A. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **139**, 1081–1121 (2009).

17. Schmidt, E. F., Shim, S. D. & Strittmatter, S. M. Release of MICAL autoinhibition by semaphorin-plexin signaling promotes interaction with collapsin response mediator protein. *J. Neurosci.* **28**, 2287–2297 (2008).

18. Gillespie, P. G. & Walker, R. G. Molecular basis of mechanosensory transduction. *Nature* **413**, 194–202 (2001).

19. Gillespie, P. G. & Muller, U. Mechanotransduction by hair cells: models, molecules, and mechanisms. *Cell* **139**, 33–44 (2009).

20. Tilney, L. G. & DeRosier, D. J. How to make a curved *Drosophila* bristle using straight actin bundles. *Proc. Natl Acad. Sci. USA* **102**, 18785–18792 (2005).

21. Sutherland, J. D. & Witke, W. Molecular genetic approaches to understanding the actin cytoskeleton. *Curr. Opin. Cell Biol.* **11**, 142–151 (1999).

22. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fate and generating dominant phenotypes. *Development* **118**, 401–415 (1993).

23. Kryukov, G. V., Kumar, R. A., Koc, A., Sun, Z. & Gladyshev, V. N. Selenoprotein N is a zinc-containing stereo-specific methionine sulfoxide reductase. *Proc. Natl Acad. Sci. USA* **99**, 4245–4250 (2002).

24. Kim, H. Y. & Gladyshev, V. N. Methionine sulfoxide reductases: selenoprotein forms and roles in antioxidant protein repair in mammals. *Biochem. J.* **407**, 321–329 (2007).

25. Kumar, R. A., Koc, A., Cerny, R. L. & Gladyshev, V. N. Reaction mechanism, evolutionary analysis, and role of zinc in *Drosophila* methionine-R-sulfoxide reductase. *J. Biol. Chem.* **277**, 37527–37535 (2002).

26. Lundblad, R. L. Chemical Reagents for Protein Modification 3rd edn 205–211 (CRC Press, 2005).

27. Lee, B. C. & Gladyshev, V. N. The biological significance of methionine sulfoxide stereochemistry. *Free Radic. Biol. Med.* **50**, 221–227 (2011).

28. Graveley, B. R. et al. The developmental transcriptome of *Drosophila melanogaster*. *Nature* **471**, 473–479 (2011).

29. Robinson, S. W., Herzyk, P., Dow, J. A. & Leader, D. P. FlyAtlas: database of gene expression in the tissues of *Drosophila melanogaster*. *Nucleic Acids Res.* **41**, D744–750 (2013).

30. Cherbas, L. et al. The transcriptional diversity of 25 *Drosophila* cell lines. *Genome Res.* **21**, 301–314 (2011).

31. Knowles-Barity, S., Longair, M. & Armstrong, J. D. BrainTrap: a database of 3D protein expression patterns in the *Drosophila* brain. *Database (Oxford)* **2010**, baq005 (2010).

32. Tran, T. S., Kolodkin, A. L. & Bharadwaj, R. Semaphorin regulation of cellular morphology. *Annu. Rev. Cell Dev. Biol.* **23**, 263–292 (2007).

33. Dent, E. W., Gupton, S. L. & Gertler, F. B. The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harb. Perspect. Biol.* **3**, a001800 (2011).

34. Kraut, R., Menon, K. & Zinn, K. A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in *Drosophila*. *Curr. Biol.* **11**, 417–430 (2001).

35. Winberg, M. L. et al. Plexin A is a neuronal semaphorin receptor that controls axon guidance. *Cell* **95**, 903–916 (1998).

36. Ayoob, J. C., Yu, H. H., Terman, J. R. & Kolodkin, A. L. The Drosophila receptor guanylyl cyclase Gyc76C is required for semaphorin-1a-plexin A-mediated axonal repulsion. *J. Neurosci.* **24**, 6639–6649 (2004).

37. Yang, T. & Terman, J. R. 14-3-3 epsilon couples protein kinase A to semaphorin signaling and silences plexin RasGAP-mediated axonal repulsion. *Neuron* **74**, 108–121 (2012).

38. Stadtmann, E. R., Moskovitz, J. & Levine, R. L. Oxidation of methionine residues of proteins: biological consequences. *Antioxid. Redox Signal.* **5**, 577–582 (2003).

39. Laing, N. G. et al. Mutations and polymorphisms of the skeletal muscle alpha-actin gene (ACTA1). *Hum. Mutat.* **30**, 1267–1277 (2009).

40. Sheterline, P., Clayton, J. & Sparrow, J. C. Actin structure and function. *Annu. Rev. Biophys.* **38**, 107–126 (2009).

41. Kraut, R., Menon, K. & Zinn, K. A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in *Drosophila*. *Curr. Biol.* **11**, 417–430 (2001).

42. Shacter, E. Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **32**, 307–326 (2000).

43. Walsh, C. T. Posttranslational Modification of Proteins. *Expanding Nature’s Inventory* (Roberts Company, 2006).

44. Lee, B. C. et al. MsrB1 and MICALs regulate actin assembly and macrophage function via reversible stereoselective methionine oxidation. *Mol. Cell* **51**, 397–404 (2013).

45. Yu, H. H., Araji, H. H., Ralls, S. A. & Kolodkin, A. L. The transmembrane Semaphorin Sema I is required in *Drosophila* for embryonic motor and CNS axon guidance. *Neuron* **20**, 207–220 (1998).

46. He, H., Yang, T., Terman, J. R. & Zhang, X. Crystal structure of the plexin A3 intracellular region reveals an autoinhibited conformation through active site sequestration. *Proc. Natl Acad. Sci. USA* **106**, 15610–15615 (2009).
METHODS

Mical-dependent genetic screen. Increasing the levels of Mical in bristle cells generates actin-rich bristle branches that result from F-actin disassembly and reorganization and are dependent on Semaphorin-1 and PlexinA (refs 5,6). Thus, a fly line containing a UAS:SelR transgene under the bristle-specific B11-GAL4 driver20,47-51 was constructed and is being used as the basis for a genome-wide dominant enhancer-suppressor genetic screen (that is, we are crossing flies available for deficiencies, transposable element lines, and EMS-generated mutants to this bristle Mical (UAS:Mical, B11-GAL4/*+) transgenic line). This screen therefore generates adult flies expressing one copy of Mical specifically in bristles in a background heterozygous for different mutations. In our previous studies examining genetic interactions with Mical52, we quantified the number of branches per bristle so as to characterize genes (for example, Semaphorin–Plexins, actin5C53,54), that enhanced (increased) or suppressed (decreased) the number of Mical-dependent bristle branches. This Mical-dependent bristle branching using our constructed transgenic line is highly similar/reproducible from animal-to-animal—with the branch being localized at the tip of the bristle process, oriented in the same direction, at the same angle, and of the same length (Fig. 1b, present paper; and Fig. S13a (×1 bristle Mical) from ref. 5). Thus, to make our enhancer-suppressor genetic screening assay even more sensitive, we searched for genes/mutations that either increased the number or length of branches (enhancers) or decreased the number or length of branches (suppressors). The bristles from flies that emerged from our screen were quantified on a 7-point scale. Specifically, all crosses were performed to genotype where the bristles of both male and female adult offspring were scored relative to normal Mical-induced branching. The offspring of the correct genotype were scored (in comparison with normal Mical-induced branching) as either normal Mical-induced branching (score 0), weak enhanced branching (score 1+1), enhanced branching (score 2+2), strong enhanced branching (score 3+3), weak suppressed branching (score 4+1), suppressed branching (score 5+2), or strong suppressed branching (score 6+3). n = 20 flies per genotype.

Adult bristle characterization. Bristles were examined, imaged and drawn as described previously5. The four scutellar bristles were quantified in each animal from young, recently emerged male and female adults5, allowing for precise comparison from animal to animal because the bristle morphology of the same four cells could be quantitatively assessed5.

Drosophila pupa characterization. Male and female pupae were staged and dissected as described previously5-7. For Mcherry–Mical and GFP–SelR localization in bristles, pupae were placed in depression-well slides and imaged. For phalloidin staining, pupae were placed in depression-well slides and imaged. For mCherry localization, pupae without their cases were placed on double-sided tape and submerged in bristles, pupae were placed in depression-well slides and imaged. For phalloidin staining, pupae without their cases were placed on double-sided tape and submerged in bristles. For mCherry localization, pupae without their cases were placed on double-sided tape and submerged in bristles. For phalloidin staining, pupae without their cases were placed on double-sided tape and submerged in bristles. For mCherry localization, pupae without their cases were placed on double-sided tape and submerged in bristles.

Actin polymerization and sedimentation assays. Actin polymerization assays were performed as described previously using standard approaches47-54. Briefly, purified rabbit skeletal muscle actin (pyrene-labeled; supplied at 0.4–0.6 pyrene dyes per actin monomer; Cytoskeleton) was used to monitor actin polymerization. G-actin was resuspended to 100 μM in a G-actin buffer (5 mM Tris-HCl at pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP and 1 mM DTT) and incubated on ice for 1 h. After all experiments, the solution of G-actin was centrifuged for 1 h at 100,000 g at 4 °C to remove residual actin nucleating centres. After ultracentrifugation, the actin was further diluted to 2 μM using G-actin buffer. Multiple independent experiments were performed for each condition such that Drosophila Mical23,51,55,56,57,58,59 (ref. 5), Drosophila SelR, Drosophila SelR23,51,55,56,57,58,59, Drosophila MsrA or hydrogen peroxide (EMD Chemicals) was added to the actin, and polymerization was initiated at 25 °C by adding an equal volume of 2× polymerization buffer (10 mM Tris-HCl at pH 7.5, 100 mM KCl, 4 mM MgCl2, 2 mM EGTA, 1 mM DTT and 0.4 mM ATP) to generate a final concentration of 1.15 μM actin. Fluorescence intensity was immediately monitored at 407 nm with excitation at 365 nm by a fluorescence spectrophotometer (Spectra max M2, Molecular Devices).

To examine the ability of SelR to induce Mical-oxidized actin to polymerize into filaments, multiple independent experiments were performed. In particular, purified rabbit muscle actin (Cytoskeleton) or Drosophila actin SC (generated as described previously4) was resuspended in G-actin buffer to 2.3 μM (as with other assays). The resuspended actin was then polymerized with 2× polymerization buffer in the presence of 600 nM Mical51,55 and 100 μM NADPH and actin was treated with this Mical/NADPH for 1 h. The Mical-treated actin was then either treated with SelR, SelR23,54 or MsrA, in a buffer containing the 10 mM MgCl2 and 20 mM DTT for 1 h at 37 °C (that is, using conditions that are standardly used with these enzymes5,55). In some cases, the NADPH or Mical51,54 were removed.
from Mical-treated actin as described previously4 (using a centrifugal filter (Amicon Ultra, Ultracel-10K, Millipore) for removal of NADPH and a Mono Q 5/50 GL ion exchange column (GE Healthcare) for removal of Mical) before treating with SelR.

To examine the effects of thioredoxin (Trx), thioredoxin reductase (TrxR), SelR and Mical oxidizing Mical-oxidized actin to polymerize into filaments, Mical-oxidized actin was generated and purified as described previously4. The activity of TrxR was confirmed as previously described19. Purified Mical-oxidized actins at 2.3 μM were treated with 5 μM of Trx (T9010, Sigma), 85 nM of TrxR (T7915, Sigma) or 1.2 μM of SelR in a buffer containing 5 mM MgCl2 and 10 mM DTT for 1 h at 37 °C and were induced to polymerize with 10% polymerization buffer at room temperature for 1 h. The mixtures were ultracentrifuged at 150,000g for 20 min at 25 °C. Supernatant and pellet fractions were adjusted to the same volumes, subjected to SDS-PAGE and stained with Coomassie blue.

**Generation, purification and utilization of SelR-treated Mical-oxidized actin.** Rabbit muscle actin at concentrations of 1.1 μM was treated with 600 nM of Mical5.13,37 and 1000 μM NADPH for 90 min at room temperature in 1× polymerization buffer to generate Mical-oxidized actin (Mical-actin-O). Mical-actin-O was then either treated with 2.4 μM of SelR or SelRC124S in buffer containing 10 mM MgCl2 and 10 mM DTT for 1 h at 37 °C. Samples were then exchanged several times with G-actin buffer using a centrifugal filter (MWCO 10K, Millipore), concentrated, and loaded into a Mono Q 5/50 GL ion exchange column (GE Healthcare) equilibrated with buffer A1 (20 mM Tris-HCl at pH 7.0, 0.2 mM CaCl2, 0.1 mM ATP and 0.1 mM DTT). The actin was then eluted with a linear gradient of 0–0.5 M NaCl in buffer A1. The purest fraction was then collected, exchanged to G-actin buffer and concentrated. The actin concentration was determined by a Bradford assay. Each actin sample was then directly subjected to mass spectrometry for analysis or frozen with liquid nitrogen and stored at −80 °C. To examine the ability of SelR-treated Mical-actin-O to re-polymerize (that is, with no SelR or Mical protein present), the purified SelR-treated Mical-actin-O was resuspended to 2.3 μM in G-actin buffer, and polymerization was initiated as described above with 2× polymerization buffer (final concentration of actin was 1.15 μM). The ability of actin to re-polymerize was determined as described by either monitoring the fluorescence intensity or through a sedimentation assay.

**Mass spectrometry.** The molecular weight of intact protein was measured by an ABI QStar XL mass spectrometer with a nano-electrospray ionization source. Both purified SelR-treated Mical-oxidized actin and purified SelRC124S-treated Mical-oxidized actin in G-actin buffer (1 mg ml−1) were diluted 10 times with 1% formic acid in acetonitrile/H2O (50:50, v/v), and then infused into the mass spectrometer without desalting. The molecular weight of this purified SelR-treated Mical-oxidized actin is consistent with previous reports of unmodified actin47.

**Drosophila embryonic axon guidance assays and guidance defects.** Embryos (including the SelR trap line CPTD01015) were collected, processed, staged, dissected and analysed as previously described15,15,17. All embryos (including SelR+/− mutants) were assessed using standard approaches15,17, and did not exhibit any gross abnormalities or defects in the attachment of muscles. All assessment of axon guidance defects was also done using standard approaches15,17, an antibody to Fasciclin II (1:4, 1D4 supernatant, Developmental Studies Hybridoma Bank), a Zeiss Axiosmager microscope equipped with differential interference contrast optics, a Zeiss Axioimager HR camera, and Zeiss Axiovision software, which allows for imaging and reconstruction of multiple different planes of the embryo15,15,17. The detailed criteria used to characterize the axon guidance defects are as previously reported15,15,17,18. Plexin repulsive CNS guidance defects were as described previously15,17,18, where embryos were examined for defects in CNS axonal pathfinding including discontinuous or missing first or second CNS longitudinal connectives and/or axons crossing the midline. For all axon guidance assays, at least 2 independent experiments (separate crosses) were performed per genotype with similar results. SelR (EST clone LD07760) in situ hybridization was done using previously described protocols19,20 with sense and anti-sense probes.

**Drosophila larval neuromuscular innervation, muscles and adult lethality.** Male and female wandering third instar larvae were dissected as described previously20. Briefly, larvae were pinned using insect pins in a Sylgard dish (Dow Corning) containing PBS and cut longitudinally between the tracheal tubes on the ventral surface using a razor blade tip and blade holder (Fine Science Tools). Inner organs and fat were removed except for the brain, and the larvae were filleted using dissecting pins. For the SelR trap line CPTD01015, the larval CNS was immediately viewed and imaged on a Zeiss Discovery M2 Bio stereomicroscope. For histochemistry, larvae were fixed in methanol and incubated with either rhodamine-conjugated (1:500) or Alexa-Fluor 635-conjugated (1:1000) phalloidin (Life Technologies) or a GFP antibody (AlexaFluor 488-conjugated rabbit anti-GFP; Life Technologies; A21311; 1:1,000) in PBS for 1–2 h at room temperature. Larvae were then washed in PBS and mounted on glass slides. Larval muscle and synaptic images were taken on a Zeiss LSM510 confocal microscope using a ×40 oil objective. Slices of muscle were taken at a thickness of 0.67 μm. Neuronal muscle innervation was visualized with the aid of CDB-6-GFP-Shaker (ref. 10). Synaptic length measurements were made in ImageJ by drawing a straight longitudinal line along muscle 6/7 that started at one end of the synapse and ended at the other (as described in ref. 60). At least two independent experiments (separate crosses) were performed per genotype with similar results. One copy of the ELAV–GALA driver was employed for neuronal expression. One copy of the Me2–GALA or the 24B–GALA driver was employed for muscle analysis as described previously21. Both Mical-actin-O (one copy) or full-length Mical (one or two copies)22 were employed with similar results. All lethality studies were done with either one copy of the Actin5C–GALA (Bloomington Drosophila Stock Center #3954) or the 24B–GALA (muscle expression) fly lines, and one copy of MicalD124S (ref. 5). Lethality and rescue of lethality was also seen when two copies of full-length Mical and one copy of SelR were used, respectively, with the same GALA drivers. The loss of F-actin in muscles was quantified by analysing muscles for a decrease in phalloidin staining, and the areas with decreased F-actin staining were measured and presented as a percentage of the total muscle area. It should be noted that superficial layers of muscles showed phalloidin staining in all genotypes, whereas internal areas of the muscles showed differences between genotypes. Thus, the staining of the superficial layers with phalloidin could be used as a control for the success of the muscle staining. Statistical analysis was performed in GraphPad Prism.

**Generation of SelRΔ/Delta mutants and characterization of other SelR mutant fly lines.** Transposable elements containing FRT sites were employed to generate a small FLP-recombine-induced deletion of SelR (using the procedure in ref. 61; see Supplementary Fig. 6a). Eight separate white-eyed deletion lines were identified, one of which was verified by sequencing, and on the basis of complementation analysis they all showed similar semi-lethality and bristle defects. We attempted to make homozygous stocks from SelR−/− mutant escorter adults but with multiple attempts we did not get offspring/embryos. Two of these lines, (SelRΔ2/w and SelRΔ2/Δ2), were chosen for further study and showed similar results. The SelR RNAi line KK108939 and Eip71CD/Ms/Mr RNAi line KK109010 (Vienna Drosophila RNAi Center) were also employed, as were other transposable element insertions within the SelR locus.

**Morphology and F-actin organization of mammalian 3T3 cells.** Cell culture assays with 3T3 cells (a gift from M. Rosen, UT Southwestern, USA) were as described previously4. Drosophila SelR was inserted into a mammalian expression vector pmCherry–C1 (Clontech) and tagged N-terminally with mCherry. Drosophila GFP–Micalactin5 was as described previously4. 3T3 cells were subpassaged to 6 × 105 cells ml−1 in 35-mm uncoated Mattek dishes. After 24 h, cells were transfected with GFP, GFP–Micalactin5, mCherry or mCherry–SelR plasmids using Lipofectamine 2000 (Life Technologies). After 24 h, cells were fixed with 4% paraformaldehyde, washed with PBS and permeabilized with 0.1% Triton X-100. Cells were stained with Alexa-Fluor 635 phalloidin (1:50 dilution; Life Technologies), mounted with VectaShield, and imaged with a Zeiss LSM510 confocal microscope using a ×63 oil objective.

**Repeatability of experiments.** For each representative image, gel or graph the experiments were repeated at least two separate independent times. At least two different preparations were also examined for the immunofluorescence/reaction product experiments. In all cases, there were no limitations in repeatability. For in vivo experiments, at least two independent experiments (separate crosses per examined genotype) were performed with similar results. For cell culture assays, the same-embryon experiments (with duplicate transfections in each were performed with similar results. At least two independent protein purifications and multiple independent actin biochemical experiments were performed with similar results. No statistical method was used to predetermine the sample size. The sample size was

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chosen on the basis of what is standardly done in the field in published manuscripts. For the animal studies, differences between experimental and control conditions were large, with little variability—and so the sample size was larger than what was needed to ensure adequate power to detect an effect. All animal studies were based on pre-established criteria to compare against age-matched animals. The animal experiments were not randomized. Animals of the correct genotype were determined and all animals that were collected of that genotype were included as data. All genetic screening was done blinded during the experiment and when assessing the outcome. For other genetic experiments, in which the genotype needed to be determined on the basis of different Drosophila genetic/chromosome markers, blinding was not employed. The figure legends list the sample size for each experiment. Statistical tests have been employed on the basis of discussions with professional statisticians on faculty at UT Southwestern. To the best of our knowledge the statistical tests are justified as appropriate.

47. Tilney, L. G. et al. The role actin filaments play in providing the characteristic curved form of Drosophila bristles. Mol. Biol. Cell 15, 5481–5491 (2004).
48. Tilney, L. G., Connelly, P. S., Ruggiero, L., Vranich, K. A. & Guild, G. M. Actin filament turnover regulated by cross-linking accounts for the size, shape, location, and number of actin bundles in Drosophila bristles. Mol. Biol. Cell 14, 3953–3966 (2003).
49. Guild, G. M., Connelly, P. S., Ruggiero, L., Vranich, K. A. & Tilney, L. G. Long continuous actin bundles in Drosophila bristles are constructed by overlapping short filaments. J. Cell Biol. 162, 1069–1077 (2003).
50. De la Cova, C., Abril, M., Bellota, P., Gallant, P. & Johnston, L. A. Drosophila myc regulates organ size by inducing cell competition. Cell 117, 107–116 (2004).
51. Hopmann, R. & Miller, K. G. A balance of capping protein and profilin functions is required to regulate actin polymerization in Drosophila bristle. Mol. Biol. Cell 14, 118–128 (2003).
52. Tilney, L. G., Connelly, P., Smith, S. & Guild, G. M. F-actin bundles in Drosophila bristles are assembled from modules composed of short filaments. J. Cell Biol. 135, 1291–1308 (1996).
53. Grimaud, R. et al. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. J. Biol. Chem. 276, 48915–48920 (2001).
54. Cooper, J. A. The Cytoskeleton: A Practical Approach 47–71 (The Practical Approach Series, Oxford Univ. Press, 1992).
55. Moskovitz, J., Weissbach, H. & Brot, N. Cloning the expression of a mammalian gene involved in the reduction of methionine sulfoxide residues in proteins. Proc. Natl Acad. Sci. USA 93, 2095–2099 (1996).
56. Amer, E. S., Zhong, L. & Holmgren, A. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. Methods Enzymol. 300, 226–239 (1999).
57. Bergen, H. R. 3rd, Ajtai, K., Burghardt, T. P., Nepomuceno, A. I. & Muddiman, D. C. Mass spectral determination of skeletal/cardiac actin isoform ratios in cardiac muscle. Rapid Commun. Mass Spectrom. 17, 1467–1471 (2003).
58. Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. & Goodman, C. S. Genes that control neuromuscular specificity in Drosophila. Cell 73, 1137–1153 (1993).
59. Budnik, V., Gorczyca, M. & Prokop, A. Selected methods for the anatomical study of Drosophila embryonic and larval neuromuscular junctions. Int. Rev. Neurobiol. 75, 323–365 (2006).
60. Kisiela, M., Majumdar, D., Campbell, S. & Stewart, B. A. Myosin VI contributes to synaptic transmission and development at the Drosophila neuromuscular junction. BMC Neurosci. 12, 65–65 (2011).
61. Parks, A. L. et al. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat. Genet. 36, 288–292 (2004).
**SUPPLEMENTARY INFORMATION**

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**Supplementary Figure 1** Additional genetic analyses of SelR and Semaphorin/Plexin/Mical signaling. 

**a-b**, Elevating the levels of SelR in a wild-type background generates abnormally bent bristles (a) that resemble Mical loss-of-function homozygous (Mical-/-) mutant and Mical dominant-negative mutant bristles (b;1). **c**, Bristle specific expression of high levels of SelR in a Mical bristle overexpression background generates bristles that resemble Mical-/- mutant bristles (b). **d**, The bristle defects that result from 1 copy overexpression of SelR in bristles is strongly enhanced by loss of 1 copy of Mical (Mical heterozygous [Mical+/-] mutants). MicalDf(3R)swp2 allele. Chi-Square Test; ***P<0.0001. n=20 animals per genotype. Replicated in at least 2 independent experiments (separate crosses) per genotype. 

**e-g**, SelR counteracts Semaphorin/Plexin A (PlexA) signaling. **e**, Overexpression of PlexA in bristles (x2 Bristle PlexA) results in bristle branching 1. Overexpression of SelR in bristles strongly suppresses these PlexA-dependent defects (x2 Bristle PlexA + x1 Bristle SelR). t-test; ***P<0.0001. n=20 animals per genotype. Mean ± standard error of the mean (SEM). Replicated in at least 2 independent experiments (separate crosses) per genotype. **f**, A loss-of-function/dominant negative mutant of PlexA (PlexA without its cytoplasmic region [PlexADCyto]; 1-4) generates bristle defects when it is expressed in bristles (x1 Bristle PlexADCyto; 1). Bristle overexpression of 1 copy of SelR strongly enhances these PlexADCyto bristle defects (x1 Bristle PlexADCyto + x1 Bristle SelR). In contrast, the catalytically dead SelRC124S does not enhance these PlexADCyto bristle defects (x1 Bristle PlexADCyto + x1 Bristle SelRC124S) but suppresses them. Chi-Square Test; ***P<0.0001. n=8 animals per genotype. Replicated in at least 2 independent experiments (separate crosses) per genotype. 

**g**, A SelR heterozygous loss-of-function mutant (SelRDelta3 mutant/+ ) enhances PlexA-dependent bristle defects. n=20 animals per genotype. Mean ± standard error of the mean (SEM). Replicated in at least 2 independent experiments (separate crosses) per genotype.
Supplementary Figure 2 Purity of Drosophila SelR, SelRC124S, and Eip71CD/MsrA proteins. Standard Nickel affinity (HisTrap FF) and ion-exchange (UNO Q6 and HiTrap Q HP) chromatography procedures were employed for each protein purification. 

a, Purification of Drosophila SelR protein (similar to 5). Coomassie stained gels are shown and the arrowheads point to the SelR protein in both gels. The asterisk (*) indicates the SelR protein that was collected and dialyzed against SelR storage buffer prior to storage and use.

b, Purification of Drosophila SelRC124S protein. Coomassie stained gels are shown and the arrowheads point to the SelRC124S protein in both gels. The asterisk (*) indicates the SelRC124S protein that was collected and dialyzed against SelRC124S storage buffer prior to storage and use.

c, Purification of Drosophila Eip71CD/MsrA protein (similar to 6). Coomassie stained gels are shown and the arrowheads point to the MsrA protein in both gels. The purest eluted fraction of MsrA (*) was collected and dialyzed against MsrA storage buffer prior to storage and use. The molecular weight marker (M) is indicated in each gel.
Supplementary Figure 3 Further characterization of SelR effects on Mical-modified actin. 

a, Schematic of SelR’s reducing activity and catalytic action – and how SelR can be subsequently converted from an oxidized state to a reduced state. SelR converts Met(R)-O in proteins to Met and this reduction reaction generates oxidized SelR. In particular, previous results characterizing SelR have revealed that SelR employs a catalytic cysteine (Cys)-124 thiolate, which directly interacts with methionine sulfoxide, resulting in a methionine–SelR(Cys)-124 sulfinic acid intermediate (SelR-S-OH; 6). A subsequent reaction of this intermediate with SelR(Cys)-69 generates an intramolecular disulfide (SelR-S-S; 6). In vitro (1), DTT can serve as a reducing agent to reduce the oxidized state of SelR 6. In vivo (2), one possible means to regenerate the oxidized state of SelR is through the thioredoxin (Trx)/thioredoxin reductase (TrxR) system 6, 7. Thioredoxins/thioredoxin reductases are also reducing enzymes that have been implicated in regulating the properties of actin in response to oxidation 8-10 and also as being involved in Semaphorin/Plexin signaling 11. Other enzymes also exist that may reduce SelR 7, 12, 13. b, SelR, but not DTT nor thioredoxin (Trx)/thioredoxin reductase (TrxR)/NADPH alone, restores Mical-treated actin polymerization. Consistent with a catalytic requirement for SelR in restoring the polymerization properties of Mical-modified actin, it should also be noted as seen on this gel that SelR utilizes both DTT and thioredoxin/thioredoxin reductase to restore the polymerization properties of Mical-modified actin. Actin monomers/G-actin in supernatant (S); actin polymers/F-actin in pellet (P). Sedimentation/Coomassie staining assay. See also Figure S3g for uncropped gel. c, H₂O₂ alters actin polymerization over time when added in high concentrations (40 mM; 14-16) but neither SelR (2.4 μM; purple dots) nor MsrA (2.4 μM; blue dots) nor both together (1.2 μM of SelR and 1.2 μM of MsrA; grey dots) restores normal polymerization to H₂O₂-treated actin. d, SelR (green dots) but not SelRC124S (blue dots) induces polymerization of pyrene actin that has been treated with Mical/NADPH and purified. Pyrene actin assay. e-g, Uncropped Coomassie-stained gels for Figure 2b (e [red box]), Figure 3d (f), and Figure S3b (g).
Supplementary Figure 4 Mical and SelR Redox requirement and the Met-44 residue of actin in Mical/SelR-mediated bristle/actin reorganization. 

(a) Bristle-specific expression of a Mical transgene with point mutations disrupting Mical’s monooxygenase (Redox) domain (Mical\textsuperscript{D\text{redox}}) in a wild-type background generates bristle defects similar to when the levels of active SelR are increased in wild-type bristles \textsuperscript{1}. 

(b-c) Bristle overexpression of high levels of SelR (x2 bristle SelR) in a wild-type background generates severe bristle defects with multiple bends (b) that resemble bristles present in flies homozygous for a point mutation \textsuperscript{17} disrupting the Redox domain of Mical (c; Mical\textsuperscript{D\text{redox}} mutant [Mical\textsuperscript{I1367}\text{D}]; \textsuperscript{1}).

(d-e) Elevating the levels of wild-type SelR in bristles markedly suppresses the severe bristle/F-actin alterations \textsuperscript{1} that result from hyperactive Mical Redox signaling (Mical\textsuperscript{D\text{redoxCH}}). 

(f) In contrast to its enhancing effects on wild-type Mical in bristles, the SelR mutant (SelR\textsuperscript{Delta3/+}) suppresses the effects of a loss of Mical activity (Mical\textsuperscript{D\text{redox}}) in bristles (x1 Bristle Mical\textsuperscript{D\text{redox}} + SelR\textsuperscript{Delta3/+}).

(g) Expressing a non-Mical oxidizable version of actin in bristles (x1 Bristle Actin\textsuperscript{M44L}) generates bristle defects that are enhanced by SelR (x1 Bristle Actin\textsuperscript{M44L} + x1 Bristle SelR). Chi-Square Test: ***P<0.0001. n=10 animals per genotype. Replicated in at least 2 independent experiments (separate crosses) per genotype.
Supplementary Figure 5 SelR expression patterns and localization. **a**, Embryonic and larval patterns of SelR expression. In situ hybridization using an antisense probe that is specific to SelR reveals staining in Drosophila embryos including in the nervous system (brain and cord). A YFP protein fusion trap Drosophila transgenic line, SelR<sup>CPTI001015</sup> (FlAnnotater [http://www.flyprot.org/stock_report.php?stock_id=17068#]; 18), reveals expression in the embryonic and larval nervous systems in a pattern that is similar to Mical 17, 19 including, as shown here, in the brain and cord, and including, in neurons and axons. Expression in embryonic muscles is also seen in this image (e.g., areas between the brackets). The higher-power inset (from the outlined region) shows SelR<sup>CPTI001015</sup> expression in motor axons. **b**, SelR (CPTI001015) is present in bristles where it is distributed in small punctae (green). SelR overlaps in localization with Mical (Mical antibody 19 staining). The tip of the bristle is indicated with an arrow. **c**, SelR (CPTI001015), like Mical 1, also localizes along the striped pattern of bundled actin filaments (phalloidin staining) in the bristle. **d**, SelR protein localizes to axons as seen in the embryonic (left) and larval (right) CNS, following expression of UAS<sup>GFP</sup>SelR in neurons using the ELAV-GAL4 driver.
Supplementary Figure 6 Characterization of SelR loss-of-function mutants and additional analyses of SelR axon guidance defects. a, Loss-of-function SelR mutations. Several transposable element mutations are situated within SelR. In addition, transposable element mutations that contained FRT sites, e00293 and d04974, were identified as situated in genes that flanked the SelR locus. Using a FLP-FRT strategy, we employed these two transposable element alleles to delete the region between d04974 and e00293 and generate the SelRDelta3 allele. Crosses between SelRDelta3 and DfExel6159 and DfExel7305 are semi-lethal. Crosses between SelRDelta3 and DfExel7306 (which does not remove SelR; see diagram) exhibit mendelian ratios. b-d, Further presentation of axon guidance defects found in SelR neuronal overexpression and SelR-/− mutants. For reference, the nerves projecting into each hemisegment are numbered (1, 2, 3) as are the intersegmental nerve (ISN) axons. b, Further characterization of neuronal overexpression of SelR and the guidance of model axons including those within the Drosophila intersegmental nerve b (ISNb) and segmental nerve a (SNa). In wild-type embryos, ISNb axons correctly innervate their muscle targets (open arrows). In contrast, neuronal overexpression of SelR (Neuronal SelR) generates highly penetrant axon guidance defects in which ISNb axons often fail to innervate their muscle targets (closed arrows). We also observe similar highly penetrant SNa axon guidance defects in which 69% of the hemisegments were affected and 81% of these defects resemble Semaphorin-1a+/−, Plexin A−/−, and Mical−/− mutants. Replicated in at least 2 independent experiments (separate crosses) per genotype. Scale bar in b applies to both images. Genotype of the SelR neuronal overexpression embryos was: Elav-GAL4/+; UAS: SelR/+.

c1, Multiple different axon guidance defects are seen in SelR−/− mutants including a lack of ISNb innervation of muscles 6/7 and 12/13 (arrows), abnormal fasciculation/clumping of ISNb axons (arrowhead), and a paucity of fasciculated/bundled ISNb axons (axons in hemisegment 2). c2, SelR−/− mutants also exhibit CNS axon guidance defects including abnormal midline crossing (open arrowheads). SelR−/− = SelRDelta3/DfExel7305 for these images. d, Increasing the levels of Mical in neurons generates motor (closed arrowheads) and CNS (open arrows and open arrowheads) axon guidance defects (Neuronal Mical image and graph; Figure 6d). The CNS from Figure 6d (Neuronal Mical) is a portion of the same CNS as that presented in Figure 6c. Expressing SelR in neurons in combination with Mical significantly rescues these axon guidance defects (Neuronal Mical + Neuronal SelR image [open arrows] and graph). Chi-Square Test; ***P<0.0001. n=80 hemisegments assessed in 8 animals per genotype. Replicated in at least 2 independent experiments (separate crosses) per genotype. Scale bar in d applies to both images.
Supplementary Notes/References

1. Hung, R.J. et al. Mical links semaphorins to F-actin disassembly. Nature **463**, 823-827 (2010).
2. Takahashi, T. et al. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell **99**, 59-69 (1999).
3. He, H., Yang, T., Terman, J.R. & Zhang, X. Crystal structure of the plexin A3 intracellular region reveals an autoinhibited conformation through active site sequestration. Proc Natl Acad Sci U S A **106**, 15610-15615 (2009).
4. Yu, L., Zhou, Y., Cheng, S. & Rao, Y. Plexin a-semaphorin-1a reverse signaling regulates photoreceptor axon guidance in Drosophila. J Neurosci **30**, 12151-12156 (2010).
5. Kryukov, G.V., Kumar, R.A., Koc, A., Sun, Z. & Gladyshev, V.N. Selenoprotein R is a zinc-containing stereo-specific methionine sulfoxide reductase. Proc Natl Acad Sci U S A **99**, 4245-4250 (2002).
6. Kumar, R.A., Koc, A., Cerny, R.L. & Gladyshev, V.N. Reaction mechanism, evolutionary analysis, and role of zinc in Drosophila methionine-R-sulfoxide reductase. J Biol Chem **277**, 37527-37535 (2002).
7. Kim, H.Y. & Gladyshev, V.N. Methionine sulfoxide reductases: selenoprotein forms and roles in antioxidant protein repair in mammals. Biochem J **407**, 321-329 (2007).
8. Lassing, I. et al. Molecular and structural basis for redox regulation of beta-actin. J Mol Biol **370**, 331-348 (2007).
9. Thom, S.R., Bhopale, V.M., Milovanova, T.N., Yang, M. & Bogush, M. Thioredoxin reductase linked to cytoskeleton by focal adhesion kinase reverses actin S-nitrosylation and restores neutrophil beta(2) integrin function. J Biol Chem **287**, 30346-30357 (2012).
10. Wang, X. et al. Redox regulation of actin by thioredoxin-1 is mediated by the interaction of the proteins via cysteine 62. Antioxid Redox Signal **13**, 565-573 (2010).
11. Morinaka, A. et al. Thioredoxin Mediates Oxidation-Dependent Phosphorylation of CRMP2 and Growth Cone Collapse. Sci Signal **4**, ra26 (2011).
12. Kim, H.Y. Glutaredoxin serves as a reductant for methionine sulfoxide reductases with or without resolving cysteine. Acta Biochim Biophys Sin (Shanghai) **44**, 623-627 (2012).
13. Sagher, D. et al. Thionein can serve as a reducing agent for the methionine sulfoxide reductases. Proc Natl Acad Sci U S A **103**, 8656-8661 (2006).
14. Hung, R.J., Pak, C.W. & Terman, J.R. Direct redox regulation of F-actin assembly and disassembly by Mical. Science **334**, 1710-1713 (2011).
15. Hung, R.J. & Terman, J.R. Extracellular inhibitors, repellents, and semaphorin/plexin/MICAL-mediated actin filament disassembly. Cytoskeleton (Hoboken) **68**, 415-433 (2011).
16. Terman, J.R. & Kashina, A. Post-translational modification and regulation of actin. Curr Opin Cell Biol **25**, 30-38 (2013).
17. Beuchle, D., Schwarz, H., Langegger, M., Koch, I. & Aberle, H. Drosophila MICAL regulates myofilament organization and synaptic structure. Mech Dev **124**, 390-406 (2007).
18. Knowles-Barley, S., Longair, M. & Armstrong, J.D. BrainTrap: a database of 3D protein expression patterns in the Drosophila brain. Database (Oxford) **2010**, baq005 (2010).
19. Terman, J.R., Mao, T., Pasterkamp, R.J., Yu, H.H. & Kolodkin, A.L. MICALs, a family of conserved flavoprotein oxidoreductases, function in plexin-mediated axonal repulsion. Cell **109**, 887-900 (2002).
20. Parks, A.L. et al. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat Genet **36**, 288-292 (2004).
21. Yu, H.H., Araj, H.H., Ralls, S.A. & Kolodkin, A.L. The transmembrane Semaphorin Sema I is required in Drosophila for embryonic motor and CNS axon guidance. Neuron **20**, 207-220. (1998).
22. Winberg, M.L. et al. Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell **95**, 903-916 (1998).
23. Yang, T. & Terman, J.R. 14-3-3-epsilon couples protein kinase A to semaphorin signaling and silences plexin RasGAP-mediated axonal repulsion. Neuron **74**, 108-121 (2012).