

Redox signaling modulates Rho activity and tissue contractility in the *Caenorhabditis elegans* spermatheca

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ABSTRACT Actomyosin-based contractility in smooth muscle and nonmuscle cells is regulated by signaling through the small GTPase Rho and by calcium-activated pathways. We use the myoepithelial cells of the *Caenorhabditis elegans* spermatheca to study the mechanisms of coordinated myosin activation in vivo. Here, we show that redox signaling modulates RHO-1/Rho activity in this contractile tissue. Exogenously added as well as endogenously generated hydrogen peroxide decreases spermathecal contractility by inhibition of RHO-1, which depends on a conserved cysteine in its nucleotide binding site (C20). Further, we identify an endogenous gradient of H₂O₂ across the spermathecal tissue, which depends on the activity of cytosolic superoxide dismutase, SOD-1. Collectively, we show that SOD-1-mediated H₂O₂ production regulates the redox environment and fine tunes Rho activity across the spermatheca through oxidation of RHO-1 C20.

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INTRODUCTION

Coordinated cellular contractility is important for many biological processes, including shaping of tissues during morphogenesis (Mason *et al.*, 2013; Siedlik and Nelson, 2015) and proper function of contractile tissues, including those found in the intestine (Citalán-Madrid *et al.*, 2017; Hartl *et al.*, 2019), cardiovascular system (Smiesko and Johnson, 1993; Wettschurek and Offermanns, 2002; Pedrosa Nunes *et al.*, 2010), and respiratory systems (Gunst *et al.*, 2003; Smith *et al.*, 2003; Doeing and Solway, 2013). To study regulation of cell contractility in vivo, we use the *Caenorhabditis elegans* spermatheca, which is part of the hermaphroditic somatic gonad, as a model system. The somatic gonad is made up of two U-shaped gonad arms, in which germ cells and oocytes are surrounded by smooth muscle-like sheath cells (Strome, 1986; McCarter *et al.*, 1997; Hubbard and Greenstein, 2000). The spermatheca, the site of

fertilization, consists of 24 myoepithelial cells and is connected to the uterus by a four-cell syncytium called the spermatheca–uterine (sp-ut) valve (Strome, 1986; McCarter *et al.*, 1997, 1999; Hubbard and Greenstein, 2000; Gissendanner *et al.*, 2008).

Successful ovulation into, and transit through, the spermatheca requires the coordination of spermathecal contractility. Upon oocyte entry into the spermatheca, the distal cells of the spermatheca and sp-ut valve contract to keep the newly fertilized embryo in the spermatheca (Tan and Zaidel-Bar, 2015; Kelley *et al.*, 2018) while the eggshell forms (Maruyama *et al.*, 2007). After a regulated period of time, the distal spermatheca contracts while the sp-ut valve relaxes, resulting in expulsion of the embryo into the uterus (McCarter *et al.*, 1999). When this process is misregulated, embryos can become trapped in the spermatheca (Bui and Sternberg, 2002; Kariya *et al.*, 2004; Kovacevic and Cram, 2010; Kovacevic *et al.*, 2013; Wirshing and Cram, 2017, 2018; Kelley *et al.*, 2018) or be pushed prematurely into the uterus and become misshapen or damaged (Norman *et al.*, 2005; Kovacevic and Cram, 2010; Tan and Zaidel-Bar, 2015; Kelley *et al.*, 2018; Bouffard *et al.*, 2019).

As in smooth muscle and nonmuscle cells, phosphorylation of myosin drives contraction of the spermatheca (Sellers, 1981; Conti and Adelstein, 2008; Vicente-Manzanares *et al.*, 2009; Wirshing and Cram, 2017). In the spermatheca, two parallel pathways act to activate myosin. In one, the phospholipase PLC-1/PLC ϵ (Kariya *et al.*, 2004; Kovacevic *et al.*, 2013) triggers ITR-1/IP₃ receptor-dependent calcium release and activation of the myosin light-chain kinase

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Abbreviations used: F-actin, filamentous actin; GFP, green fluorescent protein; RNAi, RNA interference; ROCK, Rho kinase; ROS, reactive oxygen species; sp-ut, spermatheca–uterine valve.

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MLCK-1 (Pilipiuk et al., 2009; Kovacevic et al., 2013; Kelley et al., 2018). In the second, the RhoGAP SPV-1 is displaced from the membrane upon oocyte entry, allowing activation of the small GTPase RHO-1/Rho (Tan and Zaidel-Bar, 2015). This results in activation of LET-502/ROCK (Wissmann et al., 1997, 1999; Tan and Zaidel-Bar, 2015), which, in addition to phosphorylating myosin directly (Amano et al., 1997; Totsukawa et al., 2004; Gally et al., 2009; Beach et al., 2017), inactivates the myosin phosphatase, MEL-11 (Kimura et al., 1996; Wissmann et al., 1999). While both of these pathways increase cell contractility, spatial differences have been observed. MLCK-1 is expressed throughout the spermathecal bag (Kelley et al., 2018). LET-502/ROCK is expressed at higher levels in the distal cells of the spermatheca and sp-ut valve than in the central cells of the spermatheca and plays an important role in coordinating the entry of the oocyte and the onset of the exit of the fertilized embryo (Tan and Zaidel-Bar, 2015; Kelley et al., 2018).

Although reactive oxygen species (ROS) can damage cells through covalent modification of lipids, proteins, and DNA (Birben et al., 2012; Schieber and Chandel, 2014), ROS, in the form of hydrogen peroxide (H_2O_2), can also play important regulatory roles at physiological levels (Biswas et al., 2006; Finkel, 2011; Xu et al., 2017), including regulating contractility (Heo and Campbell, 2005; Clempus and Griendling, 2006; Aghajanian et al., 2009; San Martin and Griendling, 2010; Xu and Chisholm, 2014; Horn et al., 2017; Xu et al., 2017). Intracellular ROS are produced primarily by complexes I and III of the mitochondrial electron transport chain (Grivennikova and Vinogradov, 2006; Bleier and Dröse, 2013; Wong et al., 2017) and by NADPH oxidases (NOX; Bedard et al., 2007) in the form of superoxide. Superoxide dismutases (SOD) catalyze the conversion of superoxide to hydrogen peroxide (H_2O_2 ; Fukai and Ushio-Fukai, 2011), a more long-lived oxidant and the second messenger in redox signaling (Dickinson and Chang, 2011). *C. elegans* expresses two cytosolic superoxide dismutases, SOD-1 and SOD-5 (Braeckman et al., 2016). SOD-1 is more widely expressed in the adult than SOD-5 and acts in the cytosol and the mitochondrial intermembrane space (Doonan et al., 2008; Yanase et al., 2009; Braeckman et al., 2016).

H_2O_2 can signal by reversible modification of specific solvent-exposed cysteines. Cysteine thiols (R-SH) that are deprotonated to thiolate anions (R-S⁻) can be oxidized to a sulfenic group (R-SOH), which can condense with a nearby cysteine thiol to form a disulfide (R-S-S-R), reduced back to the original state, or further oxidized, which tends to be damaging (Biswas et al., 2006; Finkel, 2011). Several in vitro (Heo and Campbell, 2005; Heo et al., 2006) and in vivo (Gerhard et al., 2003; Aghajanian et al., 2009; Xu and Chisholm, 2014; Horn et al., 2017) studies have shown that RhoA is redox-sensitive. Oxidation of Rho can lead to increased activity, either by enhancing nucleotide exchange or by alteration of GAP and GEF activity (Heo and Campbell, 2005; Aghajanian et al., 2009; Horn et al., 2017). Other studies have shown that oxidation of the conserved cysteine (C20) results in disulfide bond formation with the adjacent cysteine (C16), thereby blocking GTP loading (Gerhard et al., 2003; Heo et al., 2006; Mitchell et al., 2013; Xu and Chisholm, 2014) and deactivating Rho. It is likely that whether oxidation of Rho leads to its activation or inhibition depends on the select oxidant used (Heo and Campbell, 2005; Heo et al., 2006).

Here, we asked if H_2O_2 plays a role in the coordinated contractility of the *C. elegans* spermatheca. We found that exogenous H_2O_2 inhibits myosin activation through oxidation of RHO-1, leading to a subsequent loss of contractility in the spermatheca. Furthermore, we show that there are spatial differences in H_2O_2 across the tissue, and that this gradient is mediated in part by SOD-1, a cytosolic su-

peroxide dismutase. Together, our data suggest that enzymes that regulate H_2O_2 can modulate the spatial redox environment and contractility across a tissue.

RESULTS

Exogenous hydrogen peroxide inhibits spermathecal contractility

Ovulation of an oocyte into the spermatheca and subsequent expulsion of the fertilized embryo into the uterus require coordination of the contractility of cells between the sheath, spermatheca, and sp-ut valve (Tan and Zaidel-Bar, 2015; Wirshing and Cram, 2017; Kelley et al., 2018; Kelley and Cram, 2019). Entry requires coordination between the sheath cells and the distal spermathecal cells (McCarter et al., 1997; Lints and Hall, 2009) and begins when the distal neck cells open. We refer to the time from distal neck dilation until entry of the oocyte into the spermatheca and neck closure as the “entry time” (Figure 1A). Upon entry, the oocyte is fertilized, and the egg-shell begins to form. We refer to the time during which the embryo is completely surrounded by the spermathecal tissue as the “dwell time.” During the dwell time, the distal neck cells and sp-ut valve contract to prevent both reflux of the embryo into the oviduct and premature exit of the embryo into the uterus. During embryo exit, the sp-ut valve relaxes, while the distal and central cells of the spermatheca contract to expel the embryo into the uterus. We refer to the time from the moment the sp-ut valve begins to open until full expulsion of the embryo into the uterus as the “exit time” (Figure 1A).

To determine if redox signaling might play a role in regulating ovulation or transit of embryos through the spermatheca, we first treated animals with exogenous H_2O_2 and examined overall tissue response using time-lapse DIC microscopy. *C. elegans* are fed *Escherichia coli*. However, *E. coli* metabolize H_2O_2 , resulting in a reduction in H_2O_2 concentration. To prevent this, we used a modified *E. coli* strain JI377 (Δ ahpCF katG katE) and compared it with the otherwise isogenic strain MG1655 (wild-type *E. coli*; Seaver and Imlay, 2001). MG1655 expresses catalases and peroxidases and rapidly degrades H_2O_2 . JI377 lacks two catalases and an alkyl hydroperoxide reductase and does not degrade H_2O_2 (Seaver and Imlay, 2001). We placed nematodes on plates seeded with either of the two strains, supplemented or not with 1 mM H_2O_2 and monitored ovulations after 45 min. While entry time was not affected by H_2O_2 treatment (Figure 1B, comparing 1 mM H_2O_2 + JI377 with the other three conditions), animals exposed to H_2O_2 had shorter dwell times and longer exit times than those not treated with H_2O_2 (Figure 1, C and D, comparing 1 mM H_2O_2 + JI377 with the other three conditions). Because no significant differences were observed between unsupplemented plates with either bacterial strain, for subsequent experiments we used MG1655 on plain NGM plates as our untreated control (referred to as untreated or – H_2O_2). We also took time-lapse movies of wild-type animals treated with a more stable peroxide analog, *tert*-butyl hydroperoxide (An et al., 2005). Similarly to the effects seen with H_2O_2 treatment, 45-min pretreatment with 1 mM *tert*-butyl hydroperoxide results in shorter dwell times and longer exit times, while there was no significant difference in entry time (Figure 1, E–G). These results suggest that oxidation results in a shortened time the embryo spends in the spermatheca.

To determine if exogenously applied H_2O_2 leads to increased levels of H_2O_2 within the spermatheca, we expressed the ratiometric GFP-based probe roGFP2::TSA2 Δ C_R, localized to the outer mitochondrial membrane via the targeting sequence from TOMM-20 (Wiedemann et al., 2004; Gutscher et al., 2009; Morgan et al., 2016). This sensor is specifically oxidized by H_2O_2 and is similar to a

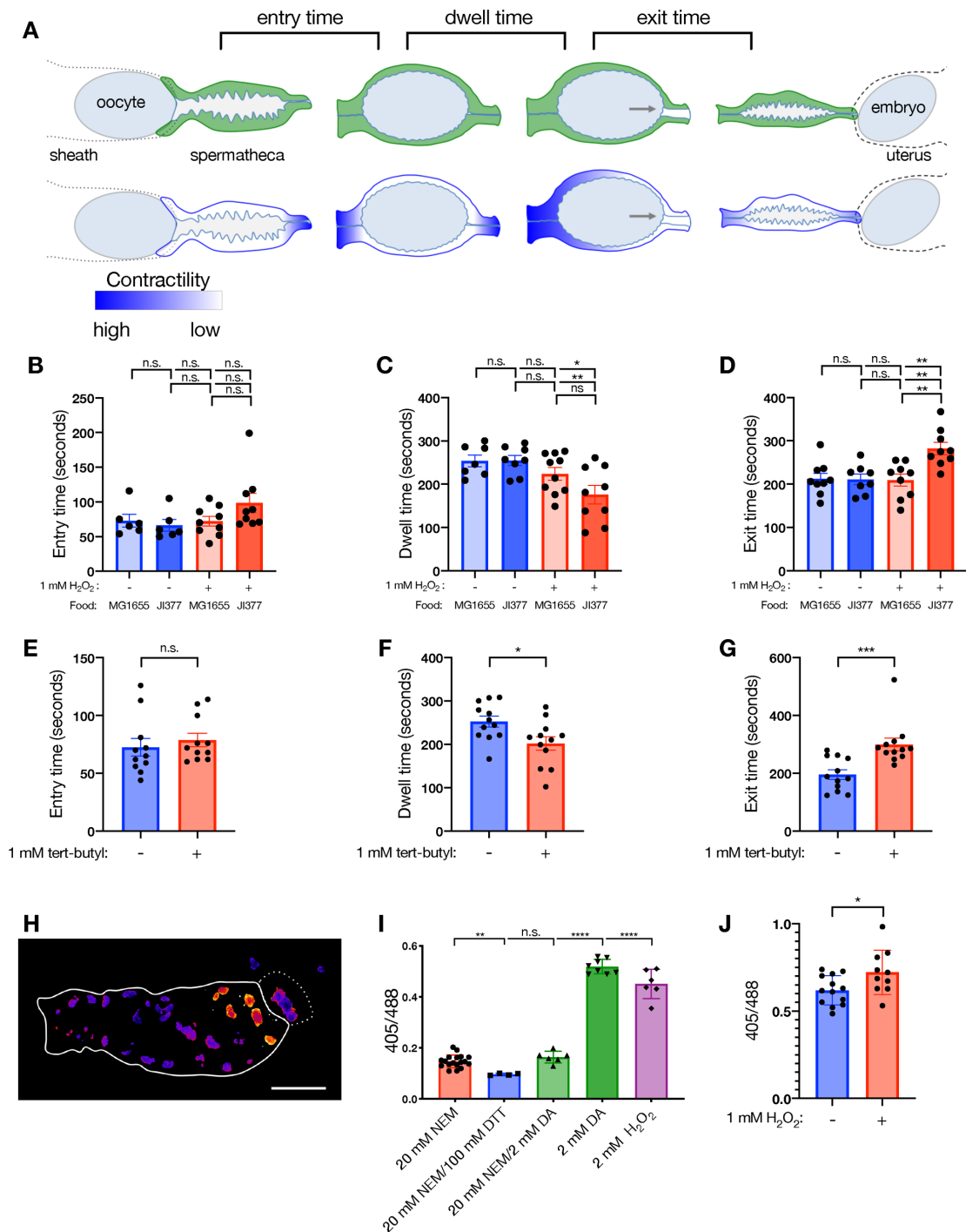


FIGURE 1: Exogenous hydrogen peroxide treatment affects spermatheca tissue contractility and oocyte transit. (A) Diagram of oocyte transit through the spermatheca, illustrating the transit metrics (top panel) and which regions of the spermatheca must contract to allow proper timing and direction of ovulation (bottom panel). (B) No significant change in entry time is observed with or without H_2O_2 treatment (+ H_2O_2 seeded with JI377 bacteria, - H_2O_2 seeded with K12, - H_2O_2 seeded with JI377, or + H_2O_2 seeded with MG1655). Treatment with H_2O_2 (+ H_2O_2 seeded with JI377 bacteria) results in shorter dwell times (C) and slower exit times (D) than all other conditions. Each point represents a time measurement taken from a single first ovulation. Error bars are SEM. One-way ANOVA with Tukey's multiple comparisons: ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$. Treatment with 1 mM tert-butyl hydroperoxide before imaging results in no difference in entry time (E), shorter dwell times (F) and longer exit times (G). Each point represents a time measurement calculated from a single, first ovulation. Error bars are SEM. Unpaired t test: * $p \leq 0.05$. (H) Representative image of a dissected spermatheca treated with NEM before fixation and imaging. Scale bar 20 μm . (I) Quantification of 405/488 ratio for animals treated with NEM or oxidants or reducing agents. Each point represents the average 405/488 of an entire spermatheca treated with the given agent before fixation and imaging. Error bars are SEM. Unpaired t test: ns $p > 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. (J) Quantification of 405/488 ratio in live animals imaged either with no H_2O_2 or on slides supplemented with 1 mM H_2O_2 . Error bars are SEM. Unpaired t test: * $p \leq 0.05$.

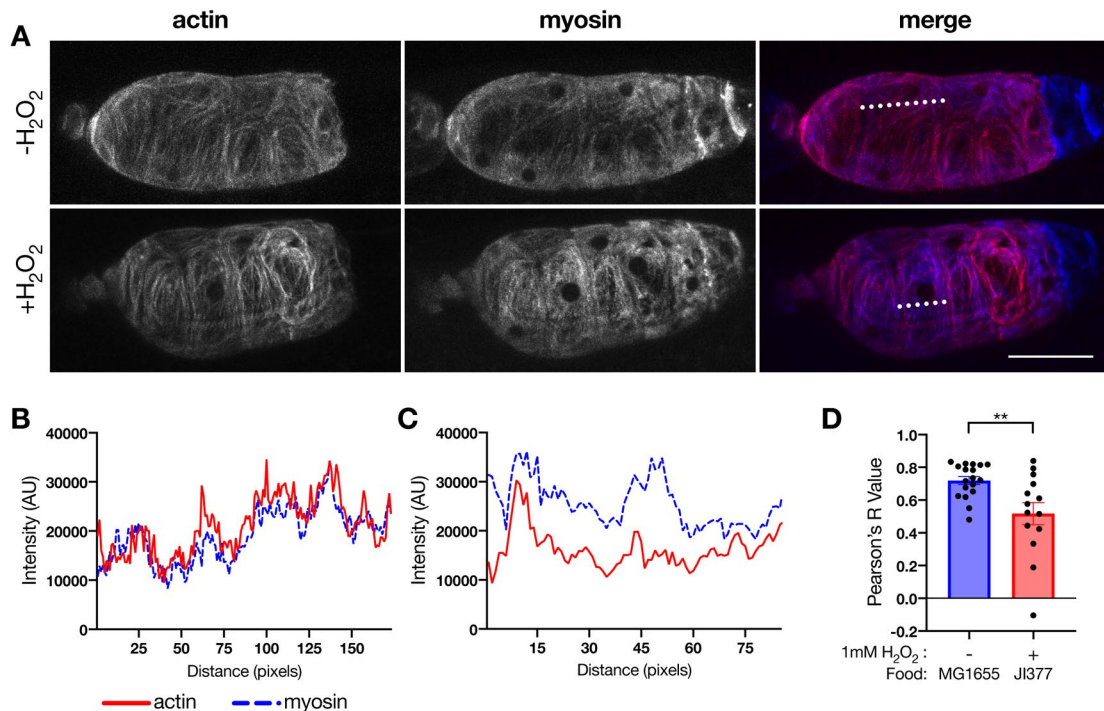


FIGURE 2: Exogenous hydrogen peroxide treatment reduces actomyosin colocalization. (A) Representative confocal maximum-intensity projections of spermathecae labeled with moeABD::mCherry and GFP::NMY-1 and either untreated (NGM+MG1655) or treated (1 mM H₂O₂ +JI377) for 45 min before imaging. Plotted fluorescence intensities across a 10 pixel-wide line (dotted line in A) for either untreated (B) or H₂O₂-treated (C) cells. Pearson's *R* coefficient is plotted for each cell measured. Untreated, *n* = 18 cells (5 animals); 1 mM H₂O₂, *n* = 14 (4 animals). Error bars are SEM. Unpaired *t* test: * *p* ≤ 0.05.

previous-generation sensor that has been used in *C. elegans*, roGFP::ORP1, but with increased sensitivity to H₂O₂ oxidation (Gutscher *et al.*, 2009; De Henau *et al.*, 2015; Morgan *et al.*, 2016). We validated the sensitivity and measured the dynamic range of the sensor using excised gonads treated chemically with either oxidizing or reducing agents, or as a control with N-ethylmaleimide (NEM), a compound that blocks free thiols from further oxidation (Hansen and Winther, 2009; Figure 1, H and I). We observed that without treatment with exogenous H₂O₂, an H₂O₂ gradient is observed across the spermatheca, where the more distal cells and the sp-ut valve displayed lower levels of H₂O₂, while the more central and proximal bag cells had higher levels (Figure 1H). Treatment of live animals with 1 mM exogenous H₂O₂ resulted in further oxidation of the sensor within the spermatheca (Figure 1J). Together, these results suggest that endogenous H₂O₂ is not uniformly distributed over the spermatheca and that exogenous H₂O₂ treatment further enhances H₂O₂ levels. Differential H₂O₂ levels could contribute to altered redox signaling through local oxidation of client proteins (Stöcker *et al.*, 2018).

Exogenous addition of H₂O₂ results in the embryo spending less time in the spermatheca. This change could be due to increased contractility in the spermathecal bag, which could force the embryo out prematurely (Tan and Zaidel-Bar, 2015), or, conversely, could be the result of premature opening of a less contractile sp-ut valve (Kelley *et al.*, 2018). If the spermathecal bag cells were more contractile, we would expect to see faster exit times to accompany the shorter dwell times. However, we observed slower exit times with H₂O₂ treatment (see Figure 1D), suggesting both the bag and the valve cells are less contractile. To test the idea that the spermathecal tissue is less contractile when treated with H₂O₂, we observed the

actomyosin cytoskeleton in treated and untreated animals. We have shown previously that oocyte entry triggers myosin activation, resulting in organization of the actin network into bundles and an increase in actomyosin colocalization (Wirshing and Cram, 2017; Kelley *et al.*, 2018). We treated animals colabeled with GFP::NMY-1 and the F-actin binding protein moeABD::mCherry on H₂O₂ plates as described above. Treated animals exhibited less colocalization of actin and myosin than untreated animals (Figure 2, A–D). This suggests that H₂O₂ treatment results in reduced myosin activity, consistent with the reduced contractility and the altered transit time of the ovulations observed after H₂O₂ treatment.

Exogenous hydrogen peroxide inhibits Rho activity

RHO-1/Rho and its downstream effector, LET-502/ROCK, drive spermathecal contractility (Wissmann *et al.*, 1999; Tan and Zaidel-Bar, 2015; Kelley *et al.*, 2018). RHO-1/Rho has two conserved cysteines adjacent to the active site in the GTP-binding pocket of the protein (Ihara *et al.*, 1998; Figure 3A). Formation of a disulfide bond between these cysteines blocks GTP binding and inhibits Rho activity (Gerhard *et al.*, 2003; Mitchell *et al.*, 2013; Xu and Chisholm, 2014). To determine if the observed differences in ovulation timing were dependent on the oxidation of RHO-1, we used animals in which the endogenous cysteine 20 had been replaced with alanine (RHO-1(C20A)) using CRISPR, rendering it less sensitive to oxidizing conditions. Unlike wild-type transits, which responded to H₂O₂ treatment, transit parameters and actomyosin colocalization in RHO-1(C20A) animals treated with H₂O₂ exhibited no differences from those in untreated animals (Figure 3, B–D). These data suggest that cysteine oxidation of RHO-1 plays an important role in the response of the spermatheca to oxidizing conditions.

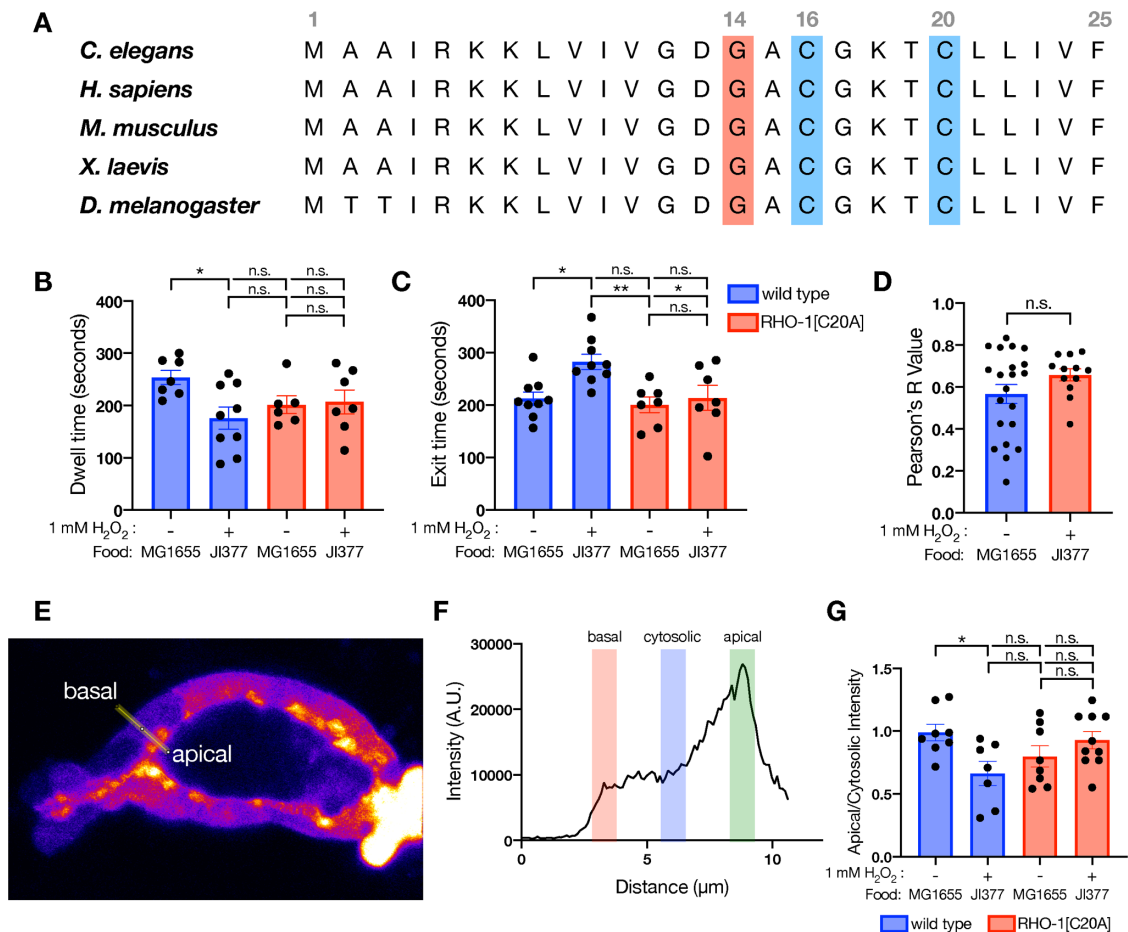


FIGURE 3: RHO-1/Rho is redox-sensitive and hydrogen peroxide treatment reduces its activity. (A) Alignment of residues 1–25 in *C. elegans*, *H. sapiens*, *M. musculus*, *X. laevis*, and *D. melanogaster* Rho shows the conserved active site, G14 (red), and redox-sensitive C20 (blue), with resolving C16 (blue) of RhoA. (B) Dwell time is shorter in wild-type animals treated with H₂O₂ (blue bars). RHO-1(C20A) animals no longer exhibit differences in dwell time with or without H₂O₂ treatment (red bars). (C) H₂O₂ treatment of wild-type animals results in longer exit time than for untreated animals (blue bars), but RHO-1(C20A) animals show no significant difference with H₂O₂ treatment (red bars). Each point represents a time measurement taken from a single first ovulation. Data for wild-type animals treated with 1 mM H₂O₂ or not treated (blue bars) are the same points as in Figure 1, C and D. Error bars are SEM. One-way ANOVA with Tukey's multiple comparisons: ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$. (D) Measurements of individual cells expressing moeABD::mCherry and GFP::NMY-1 with the RHO-1(C20A) mutation indicates no difference in actomyosin colocalization with or without exogenous H₂O₂ treatment. Untreated = 21 cells (six animals); 1 mM H₂O₂, $n = 12$ (four animals). Error bars are SEM. Unpaired t test: ns $p > 0.05$. (E) Representative single z-slice of the AHPH::GFP Rho activity sensor with a 10 pixel-wide line drawn from the basal to the apical surface of the spermatheca, with the average of that line plotted in F. From the fluorescent image, the five most basal and five most apical pixels were annotated, and the five pixels midway between those points are considered the cytosolic intensity. (G). Points represent the apical intensity normalized to the cytosolic intensity. In wild-type animals (blue bars), exogenous H₂O₂ inhibits Rho activity. However, this effect is lost in RHO-1(C20A) animals (red bars). Wild type untreated = eight measurements (four animals); wild type with exogenous H₂O₂ = seven measurements (five animals); C20A untreated = eight measurements (four animals); C20A with exogenous H₂O₂ = 10 measurements (five animals). Error bars are SEM. One-way ANOVA with Tukey's multiple comparisons: ns $p > 0.05$, * $p \leq 0.05$.

To further determine whether RHO-1/Rho activity can be regulated by cysteine oxidation, we used a previously established Rho activity sensor (AHPH::GFP), in which the Rho-binding domain of anillin is fused to GFP (Tse et al., 2012; Tan and Zaidel-Bar, 2015). RHO-1 is predominantly active at the apical membrane, which can be visualized with the Rho activity sensor (Figure 3, E and F; Tan and Zaidel-Bar, 2015). The AHPH::GFP signal at the apical membrane is decreased in H₂O₂-treated animals compared with untreated animals (Figure 3, E–G). In RHO-1(C20A) mutants, no difference in Rho activity in animals treated or not treated with

exogenous H₂O₂ was observed (Figure 3G). These data suggest that exogenous H₂O₂ is capable of inhibiting Rho activity in the spermatheca, and that this inhibition is dependent on the oxidation of C20.

SOD-1 regulates the redox environment of the spermatheca

Because we found that the contractility of the spermatheca is sensitive to oxidizing conditions induced by exogenous H₂O₂, we next sought to identify genetic regulators of the spermathecal redox environment under physiological conditions. SOD-1 and SOD-5, the

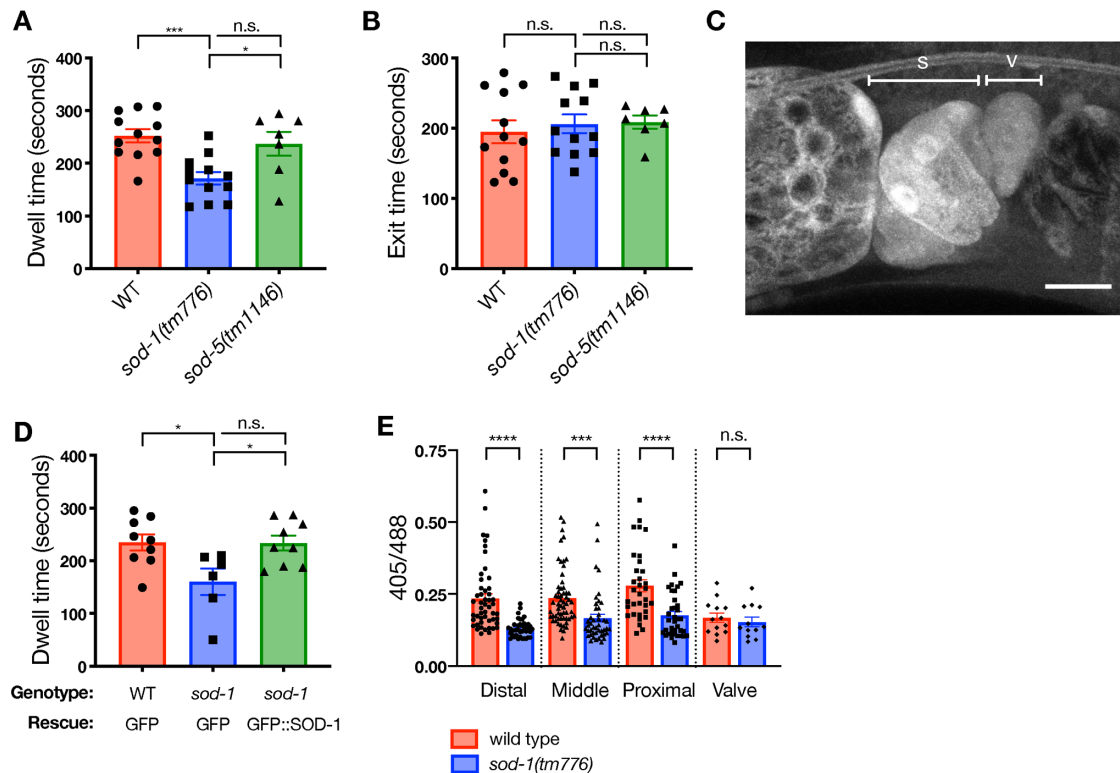


FIGURE 4: SOD-1 regulates hydrogen peroxide-based protein oxidation in the spermathecal bag cells and contractility of the spermatheca. (A) Depletion of *sod-1*, but not *sod-5*, results in shorter dwell times. (B) No significant difference in exit times in *sod-1(tm776)* or *sod-5(tm1146)* animals was observed. Points represent transit measurements from first ovulations. Error bars are SEM. One-way ANOVA with Tukey's multiple comparisons: ns $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$. (C) SOD-1 expression was imaged using a *sod-1p::GFP::sod-1 3'UTR* transcriptional reporter. SOD-1 is expressed in the sheath, the spermathecal bag cells (labeled s), and the sp-ut valve (labeled v). Scale bar is 10 μ m. (D) Tissue-specific rescue of GFP::SOD-1 in *sod-1* animals in the spermatheca and sp-ut valve rescues the short transit times seen in *sod-1* animals to wild-type times. Points represent transit measurements from first ovulations. Error bars are SEM. One-way ANOVA with Tukey's multiple comparisons: ns $p > 0.05$, * $p \leq 0.05$. (E) Quantification of protein oxidation measured using the TOMM-20::roGFP::TSA2 redox sensor in live animals. Each point represents an individual mitochondria/mitochondrial cluster measurement from spermathecae that had already had at least one ovulation, but were not occupied during imaging. Loss of *sod-1* results in a more reduced sensor in all regions of the bag, but there is no difference in H_2O_2 -based oxidation in the sp-ut valve. Wild type (red bars; $n = 10$ animals) *sod-1(tm776)* (blue bars; $n = 6$ animals). Error bars are SEM. Unpaired t test: ns $p > 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

two cytosolic superoxide dismutases, are responsible for converting superoxide radicals to H_2O_2 in the cytosol (Braeckman et al., 2016). DIC time-lapse microscopy of ovulations revealed that mutation of *sod-1*, but not *sod-5*, resulted in shorter dwell times. Neither resulted in a change in exit time from that in wild type (Figure 4, A and B). Analysis of a transcriptional reporter indicates that *sod-1* is expressed in both the spermatheca and sp-ut valve (Figure 4C). To further explore the role of SOD-1 in spermathecal contractility, we performed a tissue-specific rescue experiment. When SOD-1 was expressed in both the bag and valve using the *fln-1* promoter (Kovacevic and Cram, 2010; DeMaso et al., 2011), we observed complete rescue of the transit defects seen in *sod-1* animals (Figure 4D). This suggests that SOD-1 is required within the spermatheca to regulate contractility.

We next asked whether SOD-1 regulates the redox environment of the spermatheca. To further investigate spatial differences across the spermatheca, we used the genetically encoded tomm-20::roGFP2::TSA2 Δ C_R redox sensor (shown in Figure 1H) and digitally sectioned images of each spermatheca from the NEM-treated control by dividing the tissue into thirds plus the sp-ut valve. We then measured individual mitochondrial clusters in each of these regions

and found that the distal regions of the spermatheca and sp-ut valve have lower levels of H_2O_2 -sensor oxidation than the central bag cells and that loss of *sod-1* results in lower levels of oxidation of the sensor throughout the spermatheca, with no change in the sp-ut valve (Figure 4E). Taken together, these results suggest that SOD-1 is required in the spermatheca to produce a gradient of H_2O_2 in the bag cells, and that this can be used to mediate redox signaling.

H_2O_2 modulates the activity of RHO-1 through its redox-sensitive cysteine, C20

We next asked whether the shorter dwell times in *sod-1* animals were due to a change in bag or sp-ut valve contractility, and if so, where SOD-1-derived H_2O_2 might be functioning in the spermathecal contractility signaling pathway. The *sod-1* animals have more organized actin in the spermathecal bag than wild-type animals, suggesting an increase in contractility (Figure 5, A and B). To determine whether this effect was mediated through the Rho pathway, we used animals lacking the phospholipase *plc-1*, which have non-contractile spermathecal cells, and as a result, actin bundles remaining poorly aligned (Wirshing and Cram, 2017). We have

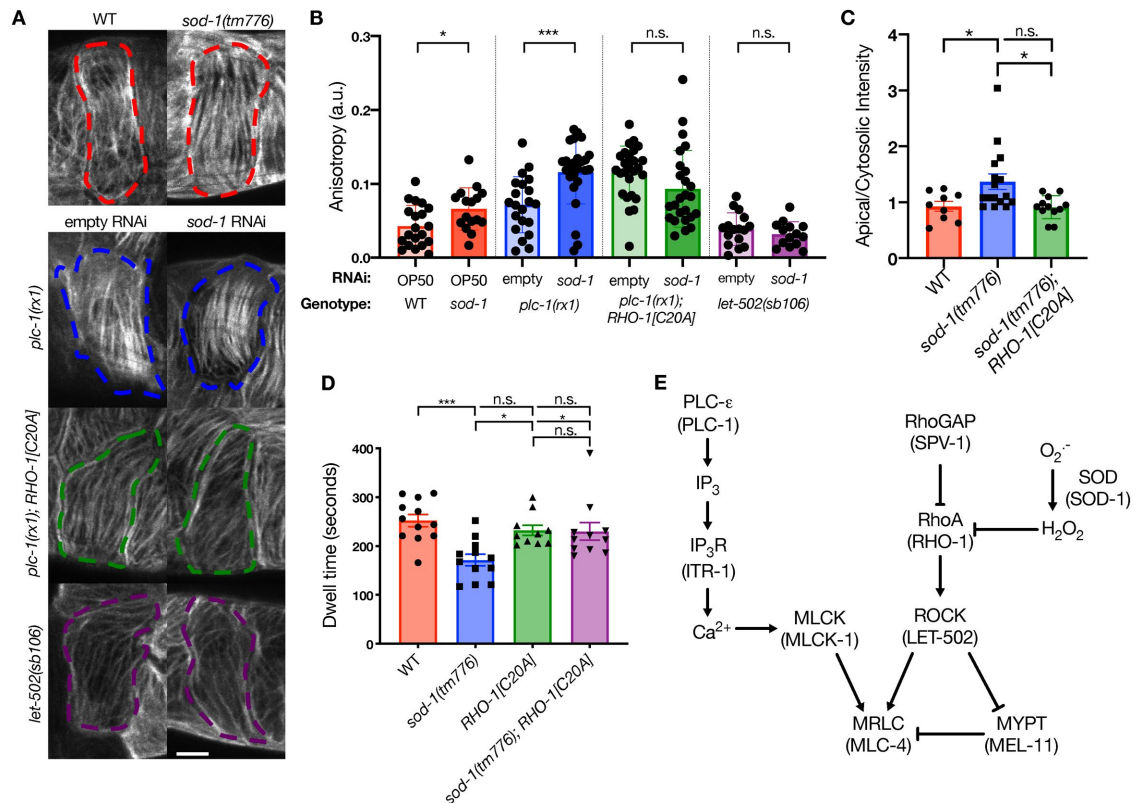


FIGURE 5: SOD-1 increases contractility-dependent actin organization through RHO-1(C20A), independent of calcium signaling and upstream of LET-502/ROCK. (A) Representative cells labeled with ACT-1::GFP. *sod-1(tm776)* animals have more organized actin than wild-type animals on the first ovulation. (B) Loss of *sod-1* can rescue actin organization in *plc-1(rx1)* animals and this rescue is dependent on C20 of RHO-1. However, *sod-1* depletion does not rescue the poor actin organization phenotype in *let-502(sb106)* animals. Points represent anisotropy measured from single cells (examples outlined in A). Error bars are SEM. Unpaired *t* test: ns $p > 0.05$, $* p \leq 0.01$. (C) *sod-1* animals have increased Rho activity when measured as a ratio of the apical to cytosolic AHPH::GFP intensity but there is no difference between wild type and *sod-1(tm776);rho-1(C20A)* double mutants. WT = nine measurements (five animals); *sod-1(tm776)* = 16 measurements (eight animals); *sod-1(tm776);rho-1(C20A)* = 12 measurements (six animals). Error bars are SEM. Unpaired *t* test: ns $p > 0.05$, $* p \leq 0.05$. (D) The short dwell times seen in *sod-1(tm776)* animals are dependent on C20 of RHO-1. Each point represents a dwell time measured from a first ovulation. Error bars are SEM. One-way ANOVA with Tukey's multiple comparisons: ns $p > 0.05$, $* p \leq 0.05$, $*** p \leq 0.001$. (E) Model for the molecular pathway by which SOD-1 modulates spermathecal contractility, where SOD-1 inhibits contractility by producing H_2O_2 , which inhibits RHO-1.

previously shown that increasing activity of the Rho-mediated pathway, which acts in parallel to PLC-1, partially rescues the poor actin fiber alignment of *plc-1* animals (Wirshing and Cram, 2017). We reasoned that if the effect of SOD-1 on spermathecal contractility is mediated through the redox-sensitive inhibition of RHO-1, loss of *sod-1* should also be able to rescue the poor actin alignment of *plc-1* animals through an increase in RHO-1 activity. RNAi of *sod-1* in the *plc-1(rx1)* background significantly rescued alignment and organization of actin compared with controls (Figure 5, A and B). This result suggests that SOD-1 acts in parallel to PLC-1 and likely in the Rho-dependent arm of the pathway. Loss of *sod-1* has no effect on *plc-1(rx1);RHO-1(C20A)* double mutants, suggesting that H_2O_2 -dependent oxidation of C20 is required (Figure 5, A and B). To determine where SOD-1 functions in the Rho/ROCK/LET-502 pathway, we fed *let-502(sb106)* animals *sod-1(RNAi)* and saw no rescue of actin organization as compared with controls, suggesting that SOD-1 indeed acts upstream of ROCK/LET-502 in this pathway (Figure 5, A and B).

If RHO-1 activity is decreased when it is oxidized by H_2O_2 , then in the absence of *sod-1*, RHO-1 should be more active. To test this hypothesis, we measured Rho activity in *sod-1(tm776)* animals. Loss

of *sod-1* resulted in elevated Rho activity at the apical surface comparable to that in wild-type animals, and this elevated Rho activity depended on C20 (Figure 5C). Additionally, we found that the short dwell times seen in *sod-1(tm776)* animals are dependent on C20 of RHO-1 (Figure 5D). This suggests that the effects of *sod-1* on transit times and actin organization depend on the oxidation of C20 and inhibition of RHO-1 (Figure 5E).

DISCUSSION

Contractility in the spermatheca is regulated by Rho and calcium-mediated pathways, which converge on the activation of myosin and are required for the spermathecal bag to contract (Wissmann *et al.*, 1999; Kariya *et al.*, 2004; Kovacevic *et al.*, 2013; Tan and Zaidel-Bar, 2015; Wirshing and Cram, 2017; Kelley *et al.*, 2018). Here, we discovered that contractility in the spermatheca is sensitive to H_2O_2 . We observed an H_2O_2 gradient across the spermatheca, with the highest levels found in the central cells of the spermatheca bag. This gradient is dependent on the cytosolic superoxide dismutase SOD-1 and modulates contractility of the spermatheca through inhibition of RHO-1, independently of calcium signaling and upstream of LET-502/ROCK.

Our results suggest that lowering Rho through oxidation and formation of a disulfide bond between C16 and C20 lead to decreased actomyosin fiber formation and decreased contractility in the spermatheca. While several labs have published that Rho is redox-sensitive, different oxidants appear to yield either increases or decreases in activity (Gerhard *et al.*, 2003; Heo *et al.*, 2006; Aghajanian *et al.*, 2009; Xu and Chisholm, 2014; Horn *et al.*, 2017). Direct oxidation of Rho by 1e⁻ oxidants in vitro results in oxidation of C20 and enhanced nucleotide exchange, due to poor nucleotide binding (Heo and Campbell, 2005; Horn *et al.*, 2017). However compounds that promote formation of a disulfide bond between C16 and C20 are expected to result in Rho inhibition (Gerhard *et al.*, 2003; Heo *et al.*, 2006; Xu and Chisholm, 2014). Our results suggest that exogenous application of H₂O₂ decreases Rho-mediated myosin activity through oxidation of RHO-1(C20). This is consistent with in vitro (Gerhard *et al.*, 2003; Heo *et al.*, 2006) and in vivo (Xu and Chisholm, 2014) data demonstrating that ROS can inhibit Rho activity and the observation that exogenous addition of phenylarsine oxide (PAO), a cross-linker of nearby thiol groups, inhibits Rho, but not other Rho family GTPases that lack the resolving cysteine C16 (Gerhard *et al.*, 2003).

We found that the superoxide dismutase SOD-1 is needed to maintain wild-type H₂O₂-dependent redox signaling in the spermathecal bag. Loss of *sod-1* results in less oxidation of the roGFP2::Tsa2 ΔC_R H₂O₂ sensor, consistent with the role of SOD-1 in converting superoxide to H₂O₂ (Fukai and Ushio-Fukai, 2011). Given that wild-type animals have a relatively high level of H₂O₂, which appears to be generated from an original pool of superoxide anions, it would be interesting to identify the source of superoxide in these cells. Some potential sources could be the mitochondrial electron transport chain (Grivennikova and Vinogradov, 2006; Bleier and Dröse, 2013; Wong *et al.*, 2017) and one of the two DUOX *C. elegans* NADPH oxidases (Edens *et al.*, 2001; Meitzler and Ortiz de Montelano, 2009).

We observed no differences in H₂O₂ levels in the sp-ut valve in *sod-1* animals, perhaps because of functional redundancy with another enzyme. Additionally, in wild-type animals, H₂O₂-mediated oxidation of the sensor was lower in the sp-ut valve than in the rest of the spermathecal tissue. We have previously shown that the Rho pathway is particularly important in regulating the timing of embryo transits through the spermatheca via its effects on valve contractility (Kelley *et al.*, 2018). This result suggests that in addition to differences in expression, such as the high expression of LET-502/ROCK in the sp-ut valve (Wissmann *et al.*, 1999; Kelley *et al.*, 2018), the reduced valve may provide a permissive environment for increased Rho activity, while the more oxidizing regions decrease Rho activity.

Human cytosolic superoxide dismutase, SOD1, is one of the most commonly mutated proteins in ALS patients, with more than 100 disease-associated alleles in patients (Rosen *et al.*, 1993; Saccon *et al.*, 2013; Pansarasa *et al.*, 2018). Mutations result in altered SOD1 activity, the formation of insoluble toxic aggregates, and subsequent loss of motor neuron function (Cuanalo-Contreras *et al.*, 2013; Saccon *et al.*, 2013; Pansarasa *et al.*, 2018). In addition to motor neuron dysfunction, other manifestations of the disease include altered blood pressure (Cuanalo-Contreras *et al.*, 2013) and hypertension (Lee and Lee, 2012). Several protein kinases control vasoconstriction and blood pressure through redox signaling (Ray *et al.*, 2011; Burgoyne *et al.*, 2013). This work offers new insights into other potential redox-regulated targets that can affect the contractility of vasculature in patients with SOD1 mutations. The *C. elegans* spermatheca may offer a useful tool for studying SOD-1 effects on the contractility of biological tubes to model disease states.

MATERIALS AND METHODS

Strain generation and maintenance of nematodes

Unless otherwise noted, all strains were grown at 23°C on Nematode growth medium (NGM; 0.107 M NaCl, 0.25% wt/vol peptone, 1.7% wt/vol BD Bacto-Agar, 2.5 mM KPO₄, 0.5% Nyastatin, 0.1 mM CaCl₂, 0.1 mM MgSO₄, 0.5% wt/vol cholesterol) seeded with OP50 *E. coli* (Hope, 1999). Extrachromosomal arrays were injected with constructs between 5 and 20 ng/μl with *rol-6(su1006)* as a coinjection marker as previously reported (Mello *et al.*, 1991). UN1513 was made by UV integrating strain RZB171 *msnEx171[sth-1p::AHPH::GFP; pRF4(rol-6(su1006))]* (Tan and Zaidel-Bar, 2015) and then outcrossing the strain three times with N2. UN1757 was generated by crossing HR863 *let-502(sb106)* (Piekny *et al.*, 2000) with UN1502 *xbIs1502[ACT-1::GFP; pRF4(rol-6(su1006))]* (Wirshing and Cram, 2017).

See Supplemental Table 1 for a complete list of strains.

RNAi treatment

RNAi feeding was performed as described previously (Kovacevic and Cram, 2010): HT115(DE3) bacteria containing a double-stranded RNA construct in the T444T backbone (Sturm *et al.*, 2018) were grown in Luria broth (LB) overnight at 37°C in a shaking incubator. Bacteria were seeded onto NGM plates supplemented with 25 μg/ml carbenicillin and 1 mM isopropylthio-β-galactoside (IPTG).

Molecular cloning

pUN615 *fln-1p::GFP::sod-1::fln-1 3'utr*. pUN615 was generated by amplifying the *sod-1* gene from genomic DNA (gDNA) with primers (F 5' aaaaccgggatgtttatgaatcttctcactc 3'; R 5' AAAAGGTACCTgataatgacgagacat 3') that added the restriction sites *Xma*I and *Kpn*I to the 5' and 3' ends of the amplicon, respectively. The PCR product was digested and cloned into pUN236 (*fln-1p::GFP::inx-12::fln-1 3'utr*) in place of the *inx-12* gene.

pUN839 *fln-1p::tomm-20::roGFP2::TSA2ΔC_R::fln-1 3'utr*. The *tomm-20::roGFP2::TSA2ΔC_R* sequence was amplified from the *fbf-1p::tomm-20::roGFP2::TSA2ΔC_R::tbb-2 3' UTR* with primers (F 5' aaaaTCTAGAATGTCGGACACAATTCTTGGT 3'; R 5' aaaaGG-TACCTTAGTTGTTAGCGTTCTTGAAGTA 3') that added *Xba*I and *Kpn*I restriction sites to the 5' and 3' ends, respectively. The PCR product was cloned using restriction cloning into pUN85 (*fln-1p::fln-1 3'UTR*).

pUN1216 *sod-1* in T444T. The *sod-1* sequence used in the Vidal library was cloned into the T444T vector using NEB Builder. The sequence was initially amplified with the following primers that anneal outside the gene-specific sequence: F 5' ctatagggagaccggcagatctgattaatacagactcactataggg 3'; R 5' GGTCGACGGTATCACTCACTATAGGG 3'. This sequence was then cloned into the *Eco*RV site of T444T using NEB Builder.

pUN1241 *sod-1p::GFP::sod-1 3'UTR*. The *sod-1* promoter and 3' UTR were amplified off of gDNA using the following primers: Promoter: F 5' TCACAACGATGGATACGCTAACAAACatataccaa-ctgatcgga 3', R 5' CTTTACTCATTCTTCTACCGGTACcaccgatcta-aaatactga 3'; UTR: F 5' GAACTATACAAATAGCATTCTAGActa-cctgaatcgctctctg 3', R TCTGCTCTGATGCCGCATAGTTAAGaatc-gcccttctcgcag 3'. GFP and the backbone of pUN71 (*fln-1 p::GFP::fln-1 3' UTR* in pPD95_77) were amplified using the following primers: GFP: F 5' GTACCGGTAGAAAAATG 3', R 5' TCTAC-GAATGCTATTGTGA 3'; backbone: F 5' CTAACTATGCGGCAT-CAG 3', R 5' GTTGTAGCGTATCCATCGT 3'. Fragments were purified and assembled using NEB Builder.

Generation of endogenous RHO-1(C20A)

The RHO-1(C20A) strain was generated using a co-CRISPR approach (Ward, 2015). An injection mix containing plasmid pJW1285 that drives expression of Cas9 and *pha-1(e2123)* sgRNA (60 ng/μl, Addgene #61252), the *pha-1(e2123)* PAGE-purified 80mer single-stranded oligodeoxynucleotide (ssODN) HR template (50 ng/μl), two plasmids expressing sgRNAs targeting the *rho-1* locus (50 ng/μl each), and a 100mer ssODN HR template to introduce the desired mutations in *rho-1* (100 ng/μl) was injected into the germline of young adult *pha-1(e2123)* worms (strain GE24). sgRNA plasmids targeting the *rho-1* locus were generated by ligation of annealed oligo pairs into BbsI-digested pJRR50 (Addgene #75026). Sequences of the oligonucleotides used for the generation of *rho-1* mutants can be found in Supplemental Table S2. Injected animals were placed at 25°C and offspring with edited *rho-1* alleles were identified by singling out surviving F1 progeny of injected animals, allowing them to lay eggs followed by lysis with 20 μg/mL Proteinase K. Lysates were used as input in PCR reactions using a *rho-1* F/R primer pair and point mutations were confirmed by Sanger sequencing of the *rho-1* amplicon. This strain (TBD273) was then outcrossed three times with the wild type to generate UN1785, used in all experiments.

Sequence alignment

Sequences for first 25 amino acids of RhoA or the closest ortholog of the species shown were identified from Uniprot (Consortium TU, 2010) and aligned using ClustalOmega (Sievers et al., 2011; McWilliam et al., 2013; Li et al., 2015). Uniprot entry IDs are as follows: *C. elegans* (Q22038), *Homo sapiens* (P61586), *Mus musculus* (Q9QUI0), *Xenopus laevis* (Q9W760), *Drosophila melanogaster* (P48148).

Exogenous hydrogen peroxide treatment

To treat animals with exogenous H₂O₂, we supplemented NGM plates with 1 mM H₂O₂. Plates were allowed to dry for 1 day and then seeded with either MG1655 (wild type) or JI377 (ΔahpCF katG katE; Seaver and Imlay, 2001) *E. coli*. Food was allowed to dry for at least 24 h before use, and plates were always made fresh (<5 d) before use. Acute treatment of animals was achieved by transferring adult worms, which had not ovulated yet, to either NGM or 1 mM H₂O₂ NGM plates seeded with either MG1655 or JI377 for 45 min at 23°C. Animals were immediately imaged (described below) for ≤1 h.

Exogenous tert-butyl hydroperoxide treatment

We supplemented NGM plates with 1 mM tert-butyl hydroperoxide. Plates were allowed to dry for 1 day and then seeded with OP50 *E. coli*. Food was dried for at least 1 day, and was always made fresh (<5 d) before use. Animals were grown on plain NGM with OP50 to adulthood and then transferred to the 1 mM tert-butyl plates for 45 min before imaging. Animals were then immediately transferred to a slide and imaged for ≤1 h.

DIC time-lapse imaging

Animals were synchronized using an “egg prep” procedure. Gravid hermaphrodites were lysed using an alkaline hypochlorite solution, and then the embryos were washed in M9 buffer (22 mM KH₂PO₄, 42 mM NaHPO₄, 86 mM NaCl, and 1 mM MgSO₄; Hope, 1999). Animals were grown at 23°C for ~50–54 h. Adults were immobilized on a 5% agarose slide with a 1:1:1.5 ratio of water, M9 buffer, and 0.05-μm Polybead microspheres (Polysciences). First ovulations were imaged using a Nikon Eclipse 80i microscope with a 60× oil-immersion lens, a charge-coupled device camera, and SPOT R3 software. Ovulations were imaged at a rate of 1 Hz and analyzed using ImageJ.

Excising, treating, and fixing redox-sensor gonads

We adapted the protocol below from one originally described for *D. melanogaster* (Albrecht et al., 2011). UN1846 worms were enriched for the redox sensor by putting gravid rollers into 10% sodium hypochlorite in M9 Buffer solution on a fresh NGM plate seeded with OP50. Offspring hatched, and after ~60 h at 23°C gonads were excised using watch glasses and 23-gauge needles and incubated in one of the following solutions for 10 min at room temperature: 20 mM NEM, 2 mM diamide (DA), or 2 or 20 mM hydrogen peroxide (H₂O₂). To fully reduce the sensor, gonads treated with NEM were next treated with 100 mM dithiothreitol (DTT). As a control to ensure that our NEM treatment was sufficiently blocking thiol oxidation, some gonads incubated with NEM were subsequently incubated with 2 mM DA. All solutions were made in phosphate-buffered saline (PBS) at pH 7.0. Animals were rinsed in PBS and then fixed in a 1.6% formaldehyde solution for 25 min at room temperature. Gonads were rinsed twice in PBS and then mounted on a 2% agarose pad on a glass slide, and the coverslip was sealed.

Hydrogen peroxide treatment of live redox sensor-expressing worms

To determine if H₂O₂ treatment resulted in oxidation of proteins within the spermatheca we grew animals on plain NGM plates seeded with JI377 for ~60 h at 23°C. Animals were washed in M9 buffer and then transferred to a slide with a 1.5 × 5-cm 2% agarose pad with either 0 mM H₂O₂ (1:1:1.5 ratio of 0.01% tetramisole and 0.1% tricaine solution in M9 buffer, M9 buffer, and 0.05 μm Polybead microspheres) or 1 mM H₂O₂ (1:1:1.5 ratio of 0.01% tetramisole and 0.1% tricaine solution in M9 buffer, 3.5 mM H₂O₂ in M9 buffer, and 0.05 μm Polybead microspheres). Samples were sealed with a coverslip and imaged immediately.

Confocal imaging

Animals were synchronized using egg prep and imaged as young adults, where only first ovulations were captured. Adults were immobilized on a 5% agarose slide with a 1:1:1.5 ratio of 0.01% tetramisole and 0.1% tricaine solution in M9 buffer, M9 buffer, and 0.05 μm Polybead microspheres. Images were captured using an LSM 710 confocal microscope (Zeiss) with Zen software (Zeiss) and a Plan-Apochromat 63×/1.49 oil DIC M27 objective or 100×/1.4 oil DIC objective. GFP was imaged using a 488-nm laser, and mCherry was imaged using a 561-nm laser. For actomyosin imaging, time-lapse z-stacks of 20 slices were captured at 12-s intervals using the 63× objective similarly to the approach described previously (Wirshing and Cram, 2017). For the AHPH Rho activity sensor, a single 2.1-μm slice was imaged over time to capture a thin sagittal section of the spermatheca at a rate of 1 Hz with the 63× objective. The roGFP2::Tsa2 redox sensor was imaged using sequential excitation of 405- and 488-nm lasers with emission detection at 499–601 nm on the 100× objective. Fixed and live worms were imaged at 0.39-μm intervals. Fixed samples had each slice averaged two times. To image actin alignment, we used methods described previously (Wirshing and Cram, 2017). For *plc-1* worms, images were taken on spermathecae occupied by single-celled embryos with 0.38-μm intervals and averaged two times. For all other genotypes, movies were captured with 40 slices at 15-s intervals.

Image analysis

All image analysis was done using ImageJ software. For analysis of actin and myosin colocalization, a maximum intensity projection from the frame where the sp-ut valve began to open was used. A 10 pixel-wide line was drawn across individual cells from the central

bag region of the spermatheca. For colocalization analysis, intensities across each line were put into GraphPad Prism software and used to measure the Pearson's *R* value of each cell. Rho activity was measured using the single sagittal slice of the frame where the sp-ut valve began to open. A line 10 pixels wide was drawn from the basal to the apical side in the distal region of the spermatheca. The apical/cytosolic intensity plotted is the average of the five most apical pixels divided by the average of five cytosolic pixels. The cytosolic region was determined by finding the midpoint between the most basal and most apical pixels. To quantify the redox sensor in live animals treated with either 0 or 1 mM H₂O₂, we used the sum-intensity projections of the image stacks. Spermathecae were outlined using the polygon selection tool in ImageJ; then the average intensity for the 488 and 405 channels was quantified for each ROI. Only animals that had their spermathecae occupied with an embryo were quantified. To measure the redox sensor in chemically treated excised gonads, the background was first subtracted using the rolling-ball procedure (50 pixels), and then sum-intensity projections were made of the stacks. We measured the average intensity across the entire image in both the 405 and 488 excitation intensities and divided the 405/488 values to plot. To analyze spatial differences in redox sensor measurements, we also subtracted the background using the rolling-ball procedure (50 pixels) and then made a sum-intensity projection of the stacks. Individual mitochondria or mitochondrial clusters were outlined using the freehand tool of ImageJ. Next we measured the mean intensity for that area in both the 405 and 488 channels and plotted the 405/488 values (Gutscher *et al.*, 2009; Albrecht *et al.*, 2011). To determine what region of the spermatheca each measurement fell into, the length of each spermatheca was measured and divided into distal, middle, and proximal thirds or annotated as being in the sp-ut valve. For visualization of the sensor (Figure 5Ei), both channels were initially filtered using a Gaussian blur, the 488 channel was then thresholded, and the 405 image was divided by the 488 image. Actin organization was quantified as previously published using the ImageJ macro FibrilTool (Boudaoud *et al.*, 2014; Wirshing and Cram, 2017). Individual cells were outlined and anisotropy measured using maximum-intensity projections of the images. For movies, the frame where the sp-ut valve began to open is the frame that was measured.

Statistical analysis

We used GraphPad Prism software for all statistical analysis performed. To compare two groups, an unpaired *t* test was used, assuming the data had a normal distribution. To compare three or more groups, ordinary one-way analysis of variance (ANOVA) was performed with a Tukey's multiple comparison test to compare the mean of each group with that of every other group. In all graphs, symbols are represented as follows: $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

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