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.

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 (Citálan-Madrid et al., 2017; Hartl et al., 2019), cardiovascular system (Smiesko and Johnson, 1993; Wettschureck 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; Winshield 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; Winshield and Cram, 2017). In the spermatheca, two parallel pathways act to activate myosin. In one, the phospholipase PLC-1/PLCe (Kariya et al., 2004; Kovacevic et al., 2013) triggers ITR-1/IP₃ receptor-dependent calcium release and activation of the myosin light-chain kinase.
peroxide dismutase. Together, our data suggest that enzymes that regulate H$_2$O$_2$ 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 (McCarte 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 eggshell 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$_2$O$_2$ and examined overall tissue response using time-lapse DIC microscopy. C. elegans are fed Escherichia coli. However, E. coli metabolize H$_2$O$_2$, resulting in a reduction in H$_2$O$_2$ concentration. To prevent this, we used a modified E. coli strain JI377 (ΔaspcF 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$_2$O$_2$. JI377 lacks two catalases and an alkyl hydroperoxide reductase and does not degrade H$_2$O$_2$ (Seaver and Imlay, 2001). We placed nematodes on plates seeded with either of the two strains, supplemented or not with 1 mM H$_2$O$_2$ and monitored ovulations after 45 min. While entry time was not affected by H$_2$O$_2$ treatment (Figure 1B, comparing 1 mM H$_2$O$_2$ + JI377 with the other three conditions), animals exposed to H$_2$O$_2$ had shorter dwell times and longer exit times than those not treated with H$_2$O$_2$ (Figure 1, C and D, comparing 1 mM H$_2$O$_2$ + 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$_2$O$_2$). 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$_2$O$_2$ 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$_2$O$_2$ leads to increased levels of H$_2$O$_2$ within the spermatheca, we expressed the ratiometric GFP-based probe roGFP2::TSA2ΔC$_{86}$, localized to the outer mitochondrial membrane via the targeting sequence from TOMM-20 (Wiedemann et al., 2004; Gutschler et al., 2009; Morgan et al., 2016). This sensor is specifically oxidized by H$_2$O$_2$ 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$_2$O$_2$ treatment (+H$_2$O$_2$ seeded with JI377 bacteria, –H$_2$O$_2$ seeded with K12, -H$_2$O$_2$ seeded with JI377, or + H$_2$O$_2$ seeded with MG1655). Treatment with H$_2$O$_2$ (+H$_2$O$_2$ 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. Scale bar 20 μm. (J) Quantification of 405/488 ratio in live animals imaged either with no H$_2$O$_2$ or on slides supplemented with 1 mM H$_2$O$_2$. Error bars are SEM. Unpaired t-test: * $p \leq 0.05$. 

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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.

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.
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
two cytosolic superoxide dismutases, are responsible for converting superoxide radicals to H$_2$O$_2$ 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 the sheath, the spermathecal bag cells (labeled s), and the sp-ut valve (labeled v). Scale bar is 10 μm. (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 $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$. (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$_2$O$_2$-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 $p > 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
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 \( \text{H}_2\text{O}_2 \)-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 (Figure 5, A and B). If RHO-1 activity is decreased when it is oxidized by \( \text{H}_2\text{O}_2 \), 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).

**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 \( \text{H}_2\text{O}_2 \). We observed an \( \text{H}_2\text{O}_2 \) 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 Montellano, 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 (Cuanoal-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 (Cuanoal-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 msex171[st-1::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 HRB63 let-502(sb106) (Pieky et al., 2000) with UN1502 bxs1502[ACT-1::GFP; pRF4(rol-6(su1006))] (Wirshing and Crum, 2017).

See Supplemental Table 1 for a complete list of strains.

RNAi treatment

RNAi feeding was performed as described previously (Kovačević and Crum, 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-1::GFP::sod-1::fln-1 3’utr. pUN615 was generated by amplifying the sod-1 gene from genomic DNA (gDNA) with primers (F 5′-aa description of primers and products) and using the following primers that amplified the region of interest: F 5′-aa2 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 Montellano, 2009).

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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 pJJR50 (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 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 H$_2$O$_2$, we supplemented NGM plates with 1 mM H$_2$O$_2$. 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 H$_2$O$_2$ 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 KH$_2$PO$_4$, 42 mM NaHPO$_4$, 86 mM NaCl, and 1 mM MgSO$_4$; 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 ovaitions were imaged using a Nikon Eclipse 80i microscope with a 60x oil-immersion lens, a charge-coupled device camera, and SPOT R3 software. Ovaitions 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 (H$_2$O$_2$). 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 H$_2$O$_2$ 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 H$_2$O$_2$ (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 H$_2$O$_2$ (1:1:1.5 ratio of 0.01% tetramisole and 0.1% tricaine solution in M9 buffer, 3.5 mM H$_2$O$_2$ 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. Animals 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 (Wirsching 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 (Wirsching 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 (Gutsch 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 5E1), 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 FibriTool (Boudaoud et al., 2014; Wrshing 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 same regions or different groups. 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 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 ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001

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