Spectrin regulates cell contractility through production and maintenance of actin bundles in the Caenorhabditis elegans spermatheca

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ABSTRACT Disruption to the contractility of cells, including smooth muscle cells of the cardiovascular system and myoepithelial cells of the glandular epithelium, contributes to the pathophysiology of contractile tissue diseases, including asthma, hypertension, and primary Sjögren’s syndrome. Cell contractility is determined by myosin activity and actomyosin network organization and is mediated by hundreds of protein–protein interactions, many directly involving actin. Here we use a candidate RNA interference screen of more than 100 Caenorhabditis elegans genes with predicted actin-binding and regulatory domains to identify genes that contribute to the contractility of the somatic gonad. We identify the spectrin cytoskeleton composed of SPC-1/α-spectrin, UNC-70/β-spectrin, and SMA-1/β heavy-spectrin as required for contractility and actin organization in the myoepithelial cells of the C. elegans spermatheca. We use imaging of fixed and live animals as well as tissue- and developmental-stage-specific disruption of the spectrin cytoskeleton to show that spectrin regulates the production of prominent central actin bundles and is required for maintenance of central actin bundles throughout successive rounds of stretch and contraction. We conclude that the spectrin cytoskeleton contributes to spermathecal contractility by promoting maintenance of the robust actomyosin bundles that drive contraction.

INTRODUCTION Actin and the actin motor protein myosin form contractile actomyosin networks involved in the perception and production of forces in contractile nonmuscle cells (Burridge and Wittchen, 2013; Zaidel-Bar et al., 2015; Svitkina, 2018). These include smooth muscle cells of the cardiovascular and gastrointestinal systems as well as myoepithelial cells of the glandular epithelium. Disruption to actomyosin network organization and myosin activity contributes to the pathophysiology of contractile tissue diseases, including asthma (Ammit et al., 2000; Flores et al., 2007; Gao et al., 2007; Lavoie et al., 2009; Leguilliette et al., 2009), hypertension (Uehata et al., 1997), and primary Sjögren’s syndrome, a disease affecting the lacrimal and salivary glands resulting in dry mouth and eyes (Sisto et al., 2018). Understanding the mechanisms that regulate actomyosin networks is complicated by the fact that actin is involved in more protein–protein interactions than any other protein (Dominguez, 2004) and has more than 100 different binding partners (Dos Remedios et al., 2003). Contraction in nonmuscle cells alone is regulated by 230 specific interactions and counting (Zaidel-Bar et al., 2015). To identify actin-binding and regulatory proteins involved in actomyosin network organization and tissue contractility in vivo, we used a candidate RNA interference (RNAi) screen approach to identify actin-binding and regulatory genes required for contractility of the myoepithelial cells of the Caenorhabditis elegans spermatheca. The spermatheca is an organ of the C. elegans somatic gonad composed of a monolayer of 24 myoepithelial cells that surround and contain the sperm (Hirsh et al., 1976; Hubbard and Greenstein, 2000). Caenorhabditis elegans is hermaphroditic, producing both eggs and sperm in two symmetrical gonad arms that connect...
to a common uterus (Figure 1). Each gonad arm contains contractile sheath cells and the spermatheca. During ovulation, sheath cells surrounding the developing oocytes contract to push the proximal oocyte into the spermatheca; the spermatheca is stretched by the incoming oocyte, the oocyte resides in the spermatheca while fertilization occurs and the eggshell develops, and then coordinated spermathecal cell contractions expel the embryo through the spermathecal-uterine (SP-UT) valve and into the uterus (Figure 1) (Hirsh et al., 1976; Ward and Carrel, 1979; Hubbard and Greenstein, 2000). This process occurs ~150 times per gonad arm, requiring robust regulation of spermathecal contractility. We and others have shown that the actin cytoskeleton plays a central role in spermathecal contractility (Deng et al., 2007; Kovacevic and Cram, 2010; Hegsted et al., 2016; Wrishing and Cram, 2017). The most prominent spermathecal actin cytoskeletal features are stress-fiber-like actomyosin bundles that are oriented along the long axis of each cell (Wrishing and Cram, 2017). These bundles develop concomitantly with contraction during the first ovulation and are maintained throughout adulthood (Wrishing and Cram, 2017). Myosin activity is required for bundle development (Wrishing and Cram, 2017; Kelley et al., 2018). However, myosin is clearly not the only actin interacting protein involved in regulating spermathecal actin organization (Deng et al., 2007; Kovacevic and Cram, 2010; Hegsted et al., 2016) and the molecular mechanisms allowing for bundle maintenance during successive rounds of ovulation are largely unknown.

In our candidate RNAi screen of 102 genes with predicted actin-binding and regulatory domains we identified unc-70 and sma-1, encoding the C. elegans β- and β heavy-spectrin, respectively, as required for spermathecal contractility. Spectrin was first discovered in erythrocytes (Yu et al., 1973) where it functions to provide mechanical durability to the erythrocyte membrane (Greenquist et al., 1978; Tse et al., 1990). Since its discovery in erythrocytes, it has been shown that nonerythroid isoforms of spectrin are broadly expressed in different cell types and have diverse functions (Bennett and Baines, 2001; Zhang et al., 2013; Machnicka et al., 2014). In addition to providing mechanical durability, the spectrin cytoskeleton is involved in vesicle transport (Holleran et al., 1996; Devarajan et al., 1997; Stankewich et al., 1998; Holleran et al., 2001; Muresan et al., 2001; Johansson et al., 2007), establishment or maintenance of cell polarity (Nelson et al., 1990; Dubreuil et al., 1997, 2000; Piepenhagen and Nelson, 1998), and regulation of the actomyosin cytoskeleton (Benz et al., 2008; Metral et al., 2009; Ponceau et al., 2015; Wong et al., 2015). Spectrin forms heterodimers composed of one α and one β subunit aligned in an anti-parallel orientation. Through head-to-head interactions, these dimers form tetramers that are capable of binding and cross-linking short actin filaments to form the spectrin-actin cytoskeleton (Li et al., 2008, 2010). Spectrin heterotetramers form a meshwork at the cell plasma membrane and have also been observed associated with organelles including the Golgi (Devarajan et al., 1997; Stankewich et al., 1998) endoplasmic reticulum (ER) (Lencesova et al., 2004), and nucleus (McKeown et al., 1999).

In vertebrates, there are two α- and five β-spectrins. The α and β isoforms are erythrocyte specific, while αII and βI are broadly expressed (Moon and McMahon, 1990; Cianci et al., 1999). Caenorhabditis elegans has one α-, SPC-1/α, and two β-spectrins, a conventional β-spectrin, UNC-70/β, and a β-heavy-spectrin, SMA-1/βH. β-heavy-spectrin has additional spectrin repeats, an SRC homology 3 domain (SH3) protein–protein interaction domain, and unique binding partners and functions (Médina et al., 2002; Lee et al., 2010b). UNC-70/β and SMA-1/βH have distinct expression patterns and subcellular localizations with the expression and function of SMA-1/βH restricted to a specific subset of tissues (McKeown et al., 1998; Buechner et al., 1999; Norman and Moerman, 2002; Ferrier et al., 2011). However, mutations in SMA-1/βH enhance the UNC-70/β loss of function phenotype, indicating that the two β isoforms have some overlapping functions (Norman and Moerman, 2002). In C. elegans, spectrin is required for cell shape changes during embryonic morphogenesis (McKeown et al., 1998; Moorthy et al., 2000; Norman and Moerman, 2002; Praitis et al., 2005), actin organization within the body wall muscles and hypodermis (Hammarlund et al., 2000; Moorthy et al., 2000; Norman and Moerman, 2002; Praitis et al., 2005), and the mechanical durability of neurons (Hammarlund et al., 2007, Krieg et al., 2014). While spectrin has been shown to contribute to C. elegans reproduction through regulating egg-laying and embryogenesis (McKeown et al., 1998; Moorthy et al., 2000; Norman and Moerman, 2002; Praitis et al., 2005), the role of spectrin in the spermatheca was unknown. Here we show that SPC-1/α, UNC-70/β, and SMA-1/βH are expressed in the spermatheca and the SP-UT valve. We find that loss of the spectrin reduces spermathecal contractility and alters actomyosin network organization. We use auxin inducible decay (AID) to deplete spectrin postdevelopment and show a role for spectrin beyond morphogenesis. Additionally, we use time-lapse confocal microscopy of live animals to show that alterations to the spectrin cytoskeleton influence actin dynamics. This work contributes to our understanding of the diverse roles of the spectrin cytoskeleton and demonstrates a role for spectrin outside of morphogenesis or the nervous system in C. elegans. We provide evidence that spectrin influences spermathecal contractility through regulating the organization and dynamics of the stress-fiber-like actomyosin bundles.

RESULTS
A screen of genes for actin-binding and regulatory proteins in C. elegans reveals that spectrin is involved in spermathecal contractility
To identify actin-binding and regulatory proteins required for spermathecal contractile and actin organization, we used a candidate RNAi screen approach. The Ontology Browser on WormBase

![FIGURE 1: Diagram of the C. elegans somatic gonad. (A) Brightfield image of an adult hermaphrodite false colored to indicate the sheath cells (yellow), spermathecae (green), uterus (blue), and gut (red). (B) Diagram of the area indicated by a black box in A during an ovulation. First panel: sheath cell contractions begin to push the proximal oocyte (white). Second panel: sheath contractions force the oocyte into the spermatheca, where it is fertilized. Third panel: the spermathecal-uterine valve opens as the spermathecal bag contracts to expel the fertilized embryo into the uterus. Insert in B shows a magnified cross-section of the spermatheca indicating that the apical surface faces the lumen. Scale bars, 50 μm in A and 20 μm in B.](image-url)
Spectrin regulates spermathecal actin

A screen of C. elegans genes encoding actin-binding proteins reveals spectrin is required for spermathecal contractility. (A) Images of whole animals expressing GFP::ACT-1 in the spermatheca treated with control RNAi, and RNAi against nmy-2 and nmy-1. Note the lack of embryos in the uteri of nmy-2(RNAi) animals and the increase in occupied spermathecae in nmy-1(RNAi) animals. (A) A schematic of the C. elegans reproductive system with the spermathecae highlighted in green above a magnified insert of the area indicated by a dotted line in A, showing one occupied and one empty spermatheca in the same animal. (B) A summary of the somatic gonad contractility phenotypes observed for the 102 genes encoding actin-binding proteins screened. Genes that disrupt the sheath result in exit defects. For an example, see nmy-2(RNAi) in C. Genes that disrupt the spermatheca result in exit defects. For an example, see nmy-1(RNAi) in C. (C) Scoring results for animals treated with the indicated RNAi. Spermathecae undergoing ovulation that are occupied by an oocyte and appear distended are scored as occupied, and spermathecae between ovulations that contain only sperm and appear compact are scored as empty. Animals with occupied spermatheca but no embryos in the uterus are scored as “occupied on first,” and animals with empty spermatheca and no embryos in the uterus are scored as “no entry.” (D) Schematic of a spectrin heterotetramer composed of α-spectrin and β-spectrin subunits. UNC-70/β has two tandem, N-terminal, actin-binding, calponin homology domains (CH), 17 spectrin repeats (β-SPEC), and a C-terminal pleckstrin homology domain (PH). SPC-1/α has 20 spectrin repeats (α-SPEC), an SH3 within the ninth spectrin repeat, two tandem Ca2+-binding EF-hand domains (EFh), and a C-terminal Ca2+-insensitive domain (EFh Ca2+ insen.). Scale bars, 50 μm and 20 in the insert. Chi-square test, p value: ****p ≤ 0.0001, n = number of animals scored.

(wormbase.org) was used to identify genes that encode proteins with conserved domains for actin-binding and actin filament organization. In total, 102 genes were screened for spermathecal contractility defects in a line expressing actin labeled with green fluorescent protein (GFP) in the spermatheca (Supplemental Figure 1 and Supplemental Table 1). The GFP allows for easy visualization of the spermatheca under the dissection scope. With this line, we can distinguish spermathecae undergoing ovulation that are occupied by an oocyte and appear distended, termed “occupied,” from spermathecae between ovulations that contain only sperm and appear compact, termed “empty” (Figure 2A). In wild-type (WT) animals, spermatheca as the spermatheca fails to contract and push the embryo into the uterus (Kovacevic and Cram, 2010; Kovacevic et al., 2013; Wirshing and Cram, 2017). Entry defects are also seen when disrupted sheath contractions prevents oocyte entry into the spermatheca, and the oocyte remains in the gonad arm (Myers et al., 1996; Ono and Ono, 2004, 2016; Ono et al., 2007; Ono and Ono, 2004, 2016; Ono et al., 2007; Obinata et al., 2010). For example, the nonmuscle myosin II isoform, NMY-2, is part of the contractile machinery of the sheath (Ono and Ono, 2016) and also regulates germline morphology (Green et al., 2011). RNAi of this gene produces an entry defect (Figure 2, A and C). This is distinct from the phenotype of animals with functional germlines and sheath cells but disrupted spermathecal contractions. In these animals, the oocyte enters but becomes trapped within the ovulation occurs over ∼10 min, and the spermatheca spends most of the time unoccupied between ovulations (Ward and Carrel, 1979). Consistent with this, we find that 71.7 ± 7.9% of animals fed control RNAi have two empty spermathecae, 25.4 ± 7.0% have one occupied and one empty spermatheca, and only 2.8 ± 1.3% have two occupied spermathecae (mean ± SD, n = 1753 animals, 11 experiments). These percentages are highly reproducible between experiments, giving us confidence that deviation from this likely indicates a role for the RNAi candidate in spermathecal contractility (Supplemental Figure 2).

In addition to spermathecal contractions, sheath cell contractions and a functional germine that produces oocytes are also required for successful ovulation (Myers et al., 1996; Ono and Ono, 2004, 2014, 2016; Ono et al., 2007; Obinata et al., 2010). Oocytes are produced from proliferative germ cells in the distal gonad that enter meiosis as they move proximally. The proliferative germ line and the transition to meiosis is regulated by somatic germ cell signaling (Mccarter et al., 1997, 1999). These cells then mature into large oocytes in the proximal gonad through actin-dependent cytoplasmic streaming (Gibert et al., 1984; Wolke et al., 2007). At the start of ovulation, the most proximal oocyte is driven into the spermatheca by sheath cell contractions. Disruption of genes required for oogenesis results in an entry defect where no oocyte enters and expands the spermatheca (Cechetelli et al., 2016; Wirshing and Cram, 2017). Entry defects are also seen when disrupted sheath contractions prevents oocyte entry into the spermatheca, and the oocyte remains in the gonad arm (Myers et al., 1996; Ono and Ono, 2004, 2016; Ono et al., 2007; Obinata et al., 2010). For example, the nonmuscle myosin II isoform, NMY-2, is part of the contractile machinery of the sheath (Ono and Ono, 2016) and also regulates germline morphology (Green et al., 2011). RNAi of this gene produces an entry defect (Figure 2, A and C). This is distinct from the phenotype of animals with functional germ lines and sheath cells but disrupted spermathecal contractions. In these animals, the oocyte enters but becomes trapped within the
genes results in entry defects (Supplemental Figure 1 and Supplemental Table 1). However, we are unable to determine whether spermathecal contractility is also impacted. This suggests some of the genes we have identified as resulting in an entry defect may have functions in the spermatheca in addition to potential functions in other somatic and germline cells of the reproductive system.

Of the 102 genes screened, 49.0% result in ovulation defects highlighting the importance of the actin cytoskeleton in C. elegans reproduction. Of these genes, 30.4% result in entry defects (Figure 2B, Supplemental Figure 1, and Supplemental Table 1). Many of the genes resulting in severe entry defects have been described previously in the sheath: tln-1 (Cram et al., 2003), nmy-2 (Ono and Ono, 2016), unc-60 (Ono et al., 2008), unc-54, deb-1, and lev-11 (Ono et al., 2007). Two, ctn-1 and nru-3, are potential novel regulators of C. elegans reproduction identified in our screen and may contribute to sheath development and contractility, germline development, or oogenesis (Supplemental Figure 1 and Supplemental Table 1). We also identified genes required for the spermathecal contractions that drive exit of the embryo from the spermatheca into the uterus including 6 (5.8%) genes that result in severe spermathecal exit defects (Figure 2B, Supplemental Figure 1, and Supplemental Table 1). Of these genes, we have previously characterized two, nmy-1 and ftn-1, encoding nonmuscle myosin II and filamin, respectively, as required for spermathecal contractility and actin organization (Kovacevic et al., 2013; Wirshing and Cram, 2017). One, unc-87, encodes an actin-binding protein with some structural similarity to calponin but with no orthologues outside of nematodes (Kranewitter et al., 2001). Another, unc-115, encodes a LIM domain containing protein homologous to abLIM involved in axon path finding (Lundquist et al., 1998; Struckhoff and Lundquist, 2003; Yang and Lundquist, 2005; Shakir et al., 2008; Demarco and Lundquist, 2010). The other two, unc-70 and sma-1, encode β- and β-heavy spectrin, respectively, indicating spectrin is required for spermathecal contractility. Because two of our top hits impact the spectrin-actin cytoskeleton, we decided to characterize the role of spectrin in spermathecal contractility.

The spectrin-actin cytoskeleton is composed of heterotetramers formed from two α- and two β-spectrin subunits that bind through head-to-head interactions producing a rodlike spectrin tetramer with an actin-binding domain at each end (Li et al., 2008, 2010). The interaction between α- and β-spectrin is required to form tetramers that are capable of cross-linking actin and producing the spectrin-actin cytoskeleton (Figure 2D). However, in Drosophila epithelial cells β-spectrin has α-spectrin independent functions (Dubreuil et al., 2000). SPC-1/α was not initially included in our screen because it does not directly bind actin. To determine whether SPC-1/α is also required for spermathecal contractility, we scored animals as described above fed RNAi against spc-1. Depletion of SPC-1/α results in a more severe disruption to ovulation than depletion of either UNC-70/β or SMA-1/βH. We frequently observe animals with occupied spermathecae but no embryos in the uterus, indicating the oocyte is able to enter the spermatheca but not pass through the valve (Figure 2C). This is distinct from the phenotype observed with nmy-1 depletion where the spermathecal bag and valve remain flaccid and sheath contractions continue to push oocytes through the distended spermathecal bag and valve (Kovacevic et al., 2013). It may be that loss of SPC-1/α influences sheath function or the germline in addition to spermathecal contractility or disrupts the valve preventing embryo transit through the valve.

To confirm that the RNAi phenotypes observed are due to a specific loss of spectrin, we scored unlabeled WT, N2, and animals expressing the null allele of UNC-70/β, unc-70(ts1502) (Johnsen and Bailie, 1991; Hammarlund et al., 2007), as well as two alleles that result in single-amino-acid substitutions between spectrin repeats 16 and 17 of UNC-70/β, unc-70(e542), and unc-70(n493) (Krieg et al., 2014). Spectrin repeats 16 and 17 of β-spectrin are involved in the interaction between β- and α-spectrin (Li et al., 2008, 2010), and the two alleles are predicted to disrupt this interaction (Krieg et al., 2014). We find that depletion of UNC-70/β using the null allele or RNAi results in significant disruption to spermathecal contractility. Both perturbations increase the percentage of spermathecae occupied by an intact or pinched oocyte compared with WT animals fed control RNAi (Figure 3).

FIGURE 3: Disruption of the spectrin-based membrane skeleton (SBMS) results in ovulation defects. (A) Scoring of gonad contractility defects in animals with disrupted SBMS either by RNAi or the use of alleles. unc-70(ts1502) is a null allele and unc-70(e524) and unc-70(n493) are point mutations predicted to disrupt the ability of UNC-70/β to bind SPC-1/α. Example spermathecae that are empty, occupied with an intact oocyte/embryo, occupied with a pinched oocyte, and one gonad with abnormal oocytes are indicated (asterisk). (B) Gonad scored for the EMO phenotype, which was detected using DAPI to label DNA. The spermatheca is indicated by a yellow dashed line in each image. Animals with an EMO phenotype have abnormal, highly polyploid oocytes (yellow arrow). Scale bar, 20 μm. (A) Chi-square test, (B) Fisher’s exact test p value: **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. n = number of gonads scored.
Milder defects were observed for unc-70(n493), whereas unc70(e542) did not differ statistically from WT (Figure 3). We did not detect any defect in sma-1(RNAi) animals. This suggests that expression of GFP labeled actin may partially sensitize the spermatheca to disruption of spectrin. However, depletion of SPC-1/α does result in more profound ovulation defects than depletion of either UNC-70/β or SMA-1/βH alone (Figure 3, A and B). In these animals, we frequently observe the accumulation of abnormal oocytes in the proximal gonad (Figure 3A). This phenotype is not observed for depletion of UNC-70/β or SMA-1/βH (Figure 3A). Staining with 4',6-diamidino-2-phenylindole (DAPI) suggests that these abnormal oocytes are endomitotic oocytes (EMO) with large abnormal nuclei (Figure 3B). The EMO phenotype occurs when spermathecal entry defects lead to retention of mature oocytes in the proximal gonad where they undergo multiple rounds of abnormal mitosis becoming highly polyploid with large nuclei (Iwasaki et al., 1996). Overall, these findings show that spectrin is required for robust spermathecal contractions that reliably drive exit of embryo through the SP-UT valve and into the uterus ~150 times throughout the adult life of the animal during successive rounds of ovulation.

**UNC-70/β- and SMA-1/β heavy-spectrin have unique expression patterns and complementary roles in the spermatheca and SP-UT valve**

To better understand the complementary roles of the two β-spectrin isoforms and to determine the subcellular localization of spectrin in the spermatheca, we used transgenic lines expressing labeled SPC-1/α, UNC-70/β, or SMA-1/βH. Prominent UNC-70 expression in the spermatheca has been described previously using an UNC-70/β-specific antibody (Moorthy et al., 2000). However, a translational reporter failed to reproduce this expression pattern (Krieg et al., 2014). The unc-70 gene is alternatively spliced to generate four isoforms, a–d. The translational reporter described previously (Krieg et al., 2014) reports the expression of isoforms a, b, and d only. To characterize spermathecal expression, and to monitor unc-70 expression during spermathecal development, we designed a construct that reports the expression of all four unc-70 isoforms (Supplemental Figure 3). This construct results in an expression pattern (Figure 4A) that closely matches the antibody results reported previously (Moorthy et al., 2000). Strong expression of UNC-70/β in the spermatheca and SP-UT valve begins early in spermathecal development during larval stage 4 (L4) and continues into adulthood (Figure 4B). This suggests isoform UNC-70c is the dominant isoform expressed in the spermatheca. UNC-70c is predicted to use an alternative start codon resulting in the expression of a protein with a unique 103-amino-acid extension just N-terminal to the CH domains (Supplemental Figure 3).

The expression pattern for UNC-70/β closely matches the expression pattern of SPC-1/α (Figure 4, A and B). SPC-1/α expression was determined using a CRISPR generated line that labels the endogenous spc-1 locus with a C-terminal mKate2 tag. Labeled SPC-1/α is functional and animals expressing SPC-1::mKate2 ovulate similarly to WT (Supplemental Figure 4). As with UNC-70/β, SPC-1/α is prominently expressed in both the spermatheca and SP-UT valve beginning in L4 and extending into adulthood (Figure 4B). In adults, SPC-1/α is also faintly expressed in the sheath (Supplemental Figure 5). To determine the expression pattern of SMA-1/βH, we used a low-copy integrated line expressing the first 3.6 kb of sma-1 coding sequence in frame with GFP with the sma-1 promoter and regulatory sequences described previously (Prayit et al., 2001, 2005). SMA-1/βH has a more restricted expression pattern than UNC-70/β (Figure 4, A and B). Beginning in L4, SMA-1/βH is seen in both the spermathecal bag and SP-UT valve with the strongest expression in the four cells most proximal to the SP-UT valve (Figure 4B). Interestingly, this expression pattern changes during spermathecal development, and by adulthood, SMA-1/βH becomes restricted to a ring connecting the spermathecal bag and SP-UT valve (Figure 4B).

To determine the subcellular localization of spectrin, we generated lines coexpressing labeled SPC-1/α and AJM-1::tagRFP, to visualize apical junctions, or GFP::UtrCH, to label F-actin. UNC-70/β has identical subcellular localization to SPC-1/α, so only the results for SPC-1/α are presented here. In adults, the spectrin cytoskeleton, composed of UNC-70/β and SPC-1/α, is most prominent at lateral cell junctions extending from the apical junction marker, AJM-1, to the basal surface (Figure 4C). There is also a thinner layer of spectrin at the basal cell surface that overlaps with the basal, contractile, actomyosin bundles (Figure 4D). This localization pattern is observed...
in young adult animals prior to the first ovulation and is maintained throughout adulthood.

Because the interaction between β- and α-spectrin is required for maintenance of α-spectrin protein expression (Hulsmeier et al., 2007) and the formation of tetramers that cross-link actin (Li et al., 2008, 2010), we hypothesized that the expression level and or localization of α-spectrin is required for robustness and orientation of central actomyosin bundles.

As expected, knockdown of SPC-1/α and UNC-70/β result in similar actin phenotypes (Figure 6, A–C). Both the number and intensity of central actin bundles are reduced in spc-1(RNAi) cells. However, the spc-1(RNAi) actin phenotypes are more variable, possibly due to penetrance of the RNAi (Figure 6, A and B). We occasionally observe defects in actin bundle distribution and orientation in spc-1(RNAi) and unc-70(n493) spermathecae (Figure 6, D and E). In cells with actin bundle orientation defects, the actin bundles within a cell are aligned but run perpendicular to the long cell axis (Figure 6, D and E). Neighboring cells in these spermathecae retain the expected orientation with actin bundles parallel to the long cell axis (Figure 6, D and E). This phenotype is rare and seen only in spermathecal cells with disrupted spectrin that still produce prominent actin bundles (Figure 6, A–C). The unc-70(n493) allele has a stronger actin defect than the unc-70(e524) allele producing fewer actin bundles and bundles that are less prominent (Figure 6, A–C). The unc-70(n493) allele also results in a more severe contractility defect than the unc-70(e524) allele (Figure 3A). This indicates a close relationship between actin organization and spermathecal contractility.

In addition to disruption of actin in the spermathecal bag cells, we also observe defects in actin organization in the SP-UT valve (Figure 4, A–C). In WT animals, the SP-UT valve contains a dense actin network at the apical surface. When the valve is closed, this dense network forms a continuous tube where individual actin bundles are not distinguishable except at the most proximal point of...
Spectrin is required for maintenance of central actin bundles postdevelopment

To determine whether spectrin plays a role in maintaining central actin bundles postdevelopment, we used the auxin-inducible decay (AID) system to deplete SPC-1/α at different time points. AID allows for spatiotemporally regulated degradation of degron-tagged proteins on exposure to the plant phytohormone auxin (Zhang et al., 2015). In the presence of auxin, the plant-derived F-box protein, TIR1, specifically binds to the degron peptide and recruits endogenous E3 ubiquitin ligase complex components leading to ubiquitination and proteasome degradation of the degron-tagged protein (Nishimura et al., 2009). We expressed TIR1 under the control of a spermathecal-specific promoter in the background of the CRISPR-generated line expressing spc-1::degron::mKate2 from the endogenous locus. These animals also express GFP::UtrCH in the spermatheca to label F-actin. TIR1 was fused to a histone mCherry marker with a cleavable F2A peptide allowing us to use mCherry::H2B expression to track expression of the unlabeled TIR1 protein. Expression of TIR1 in the absence of auxin has no effect on spermathecal actin organization and does not result in depletion of SPC-1/α (Figure 8, A–C). The fln-1 promoter driving TIR1::F2A::mCherry::H2B turns on in the spermatheca during early L4 (Kovacevic and Cram, 2010). Therefore, animals grown continuously in the presence of auxin begin to degrade degron-tagged SPC-1/α during L4. By adulthood, SPC-1::degron::mKate2 levels in these animals are below the limit of detection in the spermatheca, and spermathecal central actin bundles are diminished in number and intensity (Figure 8, A–C). This result is similar to what is seen with depletion of spc-1 using RNAi; indicating the AID system effectively depletes SPC-1/α specifically on addition of auxin.
whether myosin recruitment to actomyosin bundles or bundle of prominent parallel bundles coincides with and requires contractile actomyosin bundles in the spermatheca and that the production of central spermathecal actin bundles during contraction throughout successive rounds of ovulation in adults.

To determine the role of SPC-1/α postdevelopment, we exposed TIR1-expressing adult animals to auxin at different time points. Animals were treated with auxin as young adults just before the first ovulation and as mature adults that had undergone approximately two to five ovulations. Animals treated as young adults have completed the cell division and migration events that occur during gonad morphogenesis (Hubbard and Greenstein, 2000) and begin degrading SPC-1/α around the time of the first ovulation when central spermathecal actin bundles are forming (Wirshing and Cram, 2017). The spermathecal cells of these animals have disrupted actin bundles that are diminished in number and intensity compared with untreated controls (Figure 8, A–C). However, the diminished actin bundle phenotype is less severe in animals treated with auxin as young adults compared with those that begin degrading SPC-1/α during L4 (Figure 8, A–C). Animals treated with auxin as mature adults post the first ovulation begin degrading SPC-1/α after development of central spermathecal actin bundles. These animals also show defects in spermathecal actin and have fewer and less prominent central actin bundles (Figure 8, A–C). Overall, these results indicate a role for spectrin in maintaining central actin bundles throughout successive rounds of ovulation in adults.

Spectrin is required for reinforcement of central actin bundles during contraction

We have shown previously that active myosin is recruited to actomyosin bundles in the spermatheca and that the production of prominent parallel bundles coincides with and requires contraction (Wirshing and Cram, 2017; Kelley et al., 2018). To determine whether myosin recruitment to actomyosin bundles or bundle dynamics during contraction is altered by depletion of spectrin, we used fluorescently labeled proteins to visualize actin and myosin and time-lapse confocal microscopy. In the following experiments, we show the results obtained for knockdown of UNC-70/β only, because UNC-70/β and SPC-1/α appear to be mutually required in the spermathecal bag and depletion of either produces similar results.

Colabeling of F-actin and myosin using the moesin actin-binding domain fused to mCherry (MoeABD:mCherry) to label F-actin and myosin tagged with GFP (GFP::NMY-1) shows that actin and myosin colocalize into basal actomyosin bundles (Supplemental Figure 6). In agreement with our phalloidin staining results, these bundles are prominent in WT cells and appear less prominent in unc-70(RNAi) cells (Supplemental Figure 6). As seen in excised and fixed spermathecae, UNC-70/β depletion results in a loss of central actomyosin bundles and more prominent actin at the cell periphery (Supplemental Figure 6). However, in both WT and unc-70(RNAi) cells, actin and myosin colocalize into basal bundles, suggesting spectrin is not required for activation and recruitment of myosin into actomyosin bundles.

Because spectrin is required postdevelopment to maintain actin bundles, we wanted to determine whether cell stretch or contraction during ovulation contribute to diminished actin bundles in cells with disrupted spectrin. We used time-lapse confocal microscopy to monitor spermathecal actin bundles during ovulation in animals expressing GFP labeled actin. In WT cells immediately after oocyte entry, central bundles are less prominent than actin at the cell periphery (Figure 9, A and B, and Supplemental Movie 1). These central bundles become more prominent during contraction (Figures 9, A and B, and Supplemental Movie 1). In agreement with our phalloidin staining results, these bundles are similar to WT at the start of ovulation (Figure 9, A and B, and Supplemental Movie 1). However, in these cells central actin bundles diminish in intensity during ovulation and become less prominent than actin at the cell periphery (Figures 9, A and B, and 10, A and B, Supplemental Movie 1). To determine whether contraction during ovulation is required to drive thinning of the central bundles, we used the null allele of plc-1, plc-1(rx1). PLC-1 is required for the production of Ca²⁺ transients during ovulation (Kovacevic et al., 2013) that activate the myosin light chain kinase, MLCK-1, increase myosin activity, and drive contraction (Kelley et al., 2018). RNAi knockdown of unc-70 in the plc-1(rx1) background attenuates the diminished central actin bundle phenotype (Figure 10, A–C). In these spermathecal cells, there is a slight but not significant reduction in central actin during ovulation (Figure 10C). The plc-1(rx1);unc-70(RNAi) actin phenotype is similar to the plc-1(rx1) actin phenotype (Figure 10, A–C). This suggests actomyosin contraction contributes to bundle thinning in spermathecal cells with disrupted spectrin.

DISCUSSION

Spectrin influences spermathecal contractility and actin network organization

Here we describe a novel screening approach designed to identify actin interacting and regulatory genes required for C. elegans spermathecal contractility. With this approach we identify six genes required for robust spermathecal contractility, including two we have previously characterized in the spermatheca encoding non-muscle myosin II, NMY-1 (Kovacevic et al., 2013; Wirshing and Cram, 2017), and filamin, FLN-1 (Kovacevic and Cram, 2010; Kovacevic et al., 2013), as well as the components of the spectrin cytoskeleton that we characterize in this work. Caenorhabditis elegans has three spectrin genes, spc-1, unc-70, and sma-1, encoding α-spectrin, β-spectrin, and β heavy-spectrin, respectively. While all three genes influence ovulation, the two beta isoforms,
UNC-70β and SMA-1/βH, have distinct expression patterns and roles. SPC-1/α and UNC-70β are broadly expressed in the spermatheca throughout development, while SMA-1/βH expression changes as animals enter adulthood and becomes restricted to a ring joining the spermathecal bag and valve. Similarly, isoform specific expression patterns have been reported in invertebrate (Thomas and Kiehart, 1994; Thomas et al., 1998; Thomas and Williams, 1999; Dubreuil et al., 1997, 2000; McKeown et al., 1998; Moorthy et al., 2000; Médina et al., 2002; Norman and Moerman, 2002) and vertebrate systems (Stankewich et al., 2016), and reorganization of the spectrin cytoskeleton during development and cell response to stress is also seen in Drosophila (Thomas and Kiehart, 1994; Thomas et al., 1998; Thomas and Williams, 1999; Dubreuil et al., 2000; Médina et al., 2002), Xenopus (Ryabova et al., 1994), and vertebrate systems (Dosemeci and Reese, 1995; Faddis et al., 1997; Lee et al., 2001; Kim et al., 2016). These similarities suggest insights into the roles of spectrin in the spermatheca may be broadly applicable to other systems.

To determine whether spectrin influences spermathecal contractility through regulation of the contractile actomyosin machinery, we observed actin structures in WT and spectrin-depleted cells. Consistent with the expression patterns, UNC-70β regulates actin morphology in spermathecal bag cells, while both UNC-70β and SMA-1/βH are required in the valve. In WT spermathecal bag cells, prominent, parallel actin bundles are present on the basal surface of each cell. In contrast, depletion of either SPC-1α or UNC-70β results in thin central actomyosin bundles that are less prominent than the actin bundles at the cell periphery. Depletion of SPC-1α also results in actin in the valve tearing and partially pulling away from the spermathecal bag. Spectrin may function to strengthen actin attachment in the valve through interactions with cell–cell adhesion components as seen in vertebrates (Nelson et al., 1990; Kizhatil et al., 2007) and invertebrates (Zarnescu and Thomas, 1999; Médina et al., 2002). These actin phenotypes are distinct from what we have observed previously in NMY-1/myosin II-depleted spermathecal cells, which fail to develop prominent, parallel, central bundles (Wirshing and Cram, 2017), or with FLN-1/filamin depletion, which results in complete loss of central bundles (Kovacevic and Cram, 2010). These differences may explain our finding that depletion of either NMY-1/myosin II or FLN-1/filamin results in stronger loss of spermathecal contractility compared with depletion of spectrin. We suggest that spectrin
Spectrin is required for production of prominent central actin bundles during ovulation. (A) Confocal maximum intensity projections of spermathecae expressing GFP::ACT-1 to label actin. Images are frames from first ovulation movies at 280-s intervals from oocyte entry (0 s) to after contraction (1120 s) colored to highlight differences in fluorescence intensity. In the WT cell before contraction (0 s), three prominent central bundles are seen (white arrows) that appear less bright than actin bundles at the cell periphery (asterisks). During contraction, central actin bundles become more prominent. After contraction (1120 s), at least five prominent central actin bundles are visible (white arrows) that are nearly as bright as actin bundles at the cell periphery (asterisks). In the unc-70(RNAi) cell before contraction (0 s), there are two prominent central actin bundles (white arrowheads). These central bundles diminish in intensity during contraction while peripheral bundles are maintained and slightly increase in intensity. After contraction (1120 s), the central bundles (white arrowheads) are less prominent than the bright actin bundles at the cell periphery (asterisks). (B) Quantification of fluorescence intensity of a line drawn across the WT cell and the unc-70(RNAi) cell indicated by dashed lines in A. Prominent central bundles are indicated by black arrows and peripheral actin bundles are indicated by black asterisks. Scale bar, 5 μm.

FIGURE 9: Spectrin is required for production of prominent central actin bundles during ovulation. (A) Confocal maximum intensity projections of spermathecae expressing GFP::ACT-1 to label actin. Images are frames from first ovulation movies at 280-s intervals from oocyte entry (0 s) to after contraction (1120 s) colored to highlight differences in fluorescence intensity. In the WT cell before contraction (0 s), three prominent central bundles are seen (white arrows) that appear less bright than actin bundles at the cell periphery (asterisks). During contraction, central actin bundles become more prominent. After contraction (1120 s), at least five prominent central actin bundles are visible (white arrows) that are nearly as bright as actin bundles at the cell periphery (asterisks). In the unc-70(RNAi) cell before contraction (0 s), there are two prominent central actin bundles (white arrowheads). These central bundles diminish in intensity during contraction while peripheral bundles are maintained and slightly increase in intensity. After contraction (1120 s), the central bundles (white arrowheads) are less prominent than the bright actin bundles at the cell periphery (asterisks). (B) Quantification of fluorescence intensity of a line drawn across the WT cell and the unc-70(RNAi) cell indicated by dashed lines in A. Prominent central bundles are indicated by black arrows and peripheral actin bundles are indicated by black asterisks. Scale bar, 5 μm.

actomyosin bundles oriented perpendicular to the long cell axis in spectrin-depleted cells. The spectrin cytoskeleton interacts with mechanosensory components of cell–cell and cell–ECM adhesion complexes (Nelson et al., 1990; Zarnescu and Thomas, 1999; Kizhatil et al., 2007; Metral et al., 2009; Collec et al., 2011; Ponceau et al., 2015), and spectrin itself can act as a mechanotransducer (Krieg et al., 2014; Wu et al., 2017) and contributes to membrane mechanical properties (Krieg et al., 2014; Smith et al., 2018). Cell culture experiments have shown that actin networks align in response to the magnitude and orientation of cell stretch (Kaunas et al., 2005; Lee et al., 2010a; Sears and Kaunas, 2016). Disruption of spectrin may alter the forces produced and experienced by spermathecal cells as has been reported in C. elegans embryos (Vuong-Brender et al., 2017). In support of this hypothesis, the misalignment phenotype is only observed in cells of the main spermathecal bag near the distal neck. We suggest that these cells are more vulnerable to actin bundle misalignment, compared with their central neighbors, due to tissue topography, as this has been shown to regulate actin bundle alignment in Drosophila embryos (Chanet et al., 2017).

Spectrin is required for development and maintenance of robust central actin bundles during contraction

We have shown previously that parallel actin bundles in spermathecal cells develop concomitantly with contraction during the first ovulation and that contraction is required to drive actin bundle production (Wirshing and Cram, 2017; Kelley et al., 2018). These actin bundles are maintained throughout adulthood. However, the mechanism allowing for maintenance of robust actin bundles throughout successive rounds of stretch and contraction remained largely unknown. Here we use AID to deplete spectrin in mature adults with well-developed central actin bundles. Using this approach, we find that central actin bundles become diminished, indicating spectrin is required not only for development but also maintenance of central actin bundles. This expands on previous work in Drosophila (Dubreuil et al., 1998; Wong et al., 2015), C. elegans (Hammarlund et al., 2000; Moorthy et al., 2000; Norman and Moerman, 2002; Pratis et al., 2005), and mouse models (Stankewich et al., 2011; Lim et al., 2014) that has shown that spectrin regulates actin morphology during development and highlights the contribution of spectrin to maintaining actin structures postdevelopment. This role of spectrin may be particularly relevant to the function of spermathecal cells, as these cells withstand numerous cycles of stretch and contraction during ovulation.

To determine when during ovulation central actin bundles become depleted in cells with disrupted SMBS, we use confocal time-lapse microscopy to capture actin dynamics during cell stretch and contraction. We find that central actin bundles in cells with disrupted SMBS become depleted during contraction, while central actin bundles become reinforced in WT cells. Furthermore, we genetically block contraction to show that thinning of central actin bundles in spectrin-depleted cells is driven by contraction. Both cell culture (Medeiros et al., 2006) and in vitro (Molloy et al., 1995; Haviv et al., 2008; Lenz et al., 2012) experiments have shown myosin is capable of producing forces that break and depolymerize F-actin. In other systems, mechanosensory proteins at cell–cell and cell–ECM junctions increase F-actin recruitment and anchorage to strengthen actin networks under myosin-induced tension (Humphries et al., 2007;
Spectrin interacts with cell–cell (Nelson et al., 1990; Zarnescu and Thomas, 1999; Kizhatil et al., 2007) and cell–ECM adhesion complexes (Metral et al., 2009; Ponceau et al., 2015) and may act through these complexes to influence actin bundle maintenance. Additionally, spectrin interacts with tropomyosin (Tang et al., 2003). In C. elegans body wall muscles and sheath cells, tropomyosin/LEV-11 stabilizes F-actin and protects F-actin from cleavage by FIGURE 10: Spectrin maintains central actin bundles during contraction. (A) Confocal maximum intensity projections of spermatothecae expressing GFP::ACT-1 to label actin. Images are frames from first ovulation movies on oocyte entry (0 s) and after contraction (1120 s). Images are colored to highlight differences in fluorescence intensity. In the WT cell (first row), central actin bundles become more prominent after contraction. In the unc-70(RNAi) cell (second row), central actin bundles diminish and actin at the cell periphery appears more prominent. This decrease in central actin fluorescence intensity with RNAi of unc-70 is mitigated in a background with genetically ablated Ca²⁺ signaling, expressing the plc-1 null allele, plc-1(rx1) (third row). In the cell expressing plc-1(rx1) without RNAi knockdown of unc-70 (fourth row), there is no net change in actin fluorescence intensity. (B) Quantification of central actin bundle fluorescence normalized to actin fluorescence at the cell periphery at 70 s intervals during ovulation. (C) Quantification of the change in normalized central actin bundle fluorescence during ovulation. Normalized actin bundle intensity increases in WT cells, decreases in unc-70(RNAi) cells, and remains the same in plc-1(rx1) cells with and without RNAi knockdown of unc-70 during ovulation. The same cells were analyzed in B and C. For WT n = 10 cells (four ovulation movies), for unc-70(RNAi) n = 9 cells (four ovulation movies), for plc-1(rx1);unc-70(RNAi) n = 12 cells (four ovulation movies), and for plc-1(rx1) n = 12 cells (four ovulation movies). No more than three cells were measured from the same movie. Error bars indicate SEM in B and SD in C. Dunnett’s multiple comparison test p value: ns p > 0.05, *p ≤ 0.05, ****p ≤ 0.0001. Scale bar, 5 μm.
ADF/cofilin/UNC-60 (Ono and Ono, 2004; Ono et al., 2007; Yu and Ono, 2006). Tropomyosin/LEV-11 and ADF/cofilin/UNC-60 are also expressed in the spermatheca where they may play similar roles (Ono et al., 2003, 2007).

Conclusion
Work in vivo has elucidated the roles of spectrin in morphogenesis and development both in vertebrates (Tang et al., 2003; Stankewich et al., 2011; Lim et al., 2014) and invertebrates (Thomas and Kiehart, 1994; Thomas et al., 1998; Thomas and Williams, 1999; de Cuevas et al., 1996; Dubreuil et al., 1998; McKeown et al., 1998; Zarnescu and Thomas, 1999; Hammarlund et al., 2000; Moorthy et al., 2000; Médina et al., 2002; Norman and Moerman, 2002; Pratis et al., 2005; Lee et al., 2010b; Wong et al., 2015; Vuong-Brendler et al., 2017). However, much of our insight into the contribution of spectrin to contractile actomyosin networks has come from cell culture work (Tang et al., 2003; Bournier et al., 2006; Metral et al., 2009; Collec et al., 2011; Stankewich et al., 2011), and experiments in live animals are lacking, with one notable exception in Drosophila (Wong et al., 2015). Our work suggests that the C. elegans spectrhematina may serve as a tractable model system for the elucidation of the role of spectrin in the organization and maintenance of contractile systems in vivo. We suggest that the spectrin cytoskeleton is part of a mechanism promoting reinforcement of actin bundles during contraction in the spermatheca.

MATERIALS AND METHODS
Caenorhabditis elegans strains and culture
All nematode strains were maintained on NGM (0.107 M NaCl, 0.25% wt/vol Peptone [Fisher Science Education], 1.7% wt/vol BD Bacto-Agar, 0.5% Nyastatin [Sigma], 0.1 mM CaCl₂, 0.1 mM MgSO₄, 0.5% wt/vol cholestrol, 2.5 mM KPO₂) agar plates seeded with OP50 Escherichia coli at 23°C (Hope, 1999). Generation of nematode strains for this study was done by standard microinjection (Mello et al., 1991). All constructs were injected at a concentration between 5 and 10 ng/µl with injection markers rol-6 and tx3p::RFP injected at 40 ng/µl and myo-2::GFP injected at 5 ng/µl. Carrier DNA, sheared, denatured salmon sperm DNA (Clontech, Mountain View, CA), was added to bring the final DNA concentration to 100 ng/µl for each injection mix. Extrachromosomal arrays were integrated by UV irradiation essentially as described in Mariol et al. (2013). Nematode observations and manipulations were performed at 23°C unless otherwise noted. Caenorhabditis elegans strains carrying alleles plc-1(rl1), unc-70(n493), and unc-70(e542) were obtained from the Caenorhabditis Genetics Center. The strain EG4492 carries the null allele unc-70(s1502) and transgene oksl59 rescuing unc-70 expression in the hypodermis under the pdi-2 promoter was generously provided by the Jorgensen lab (University of Utah, Salt Lake City, UT), and has been described previously (Hammarlund et al., 2007). Line AZ110 expressing SMA-1::GFP was a kind gift from the Pratis lab (Grinnell College, Grinnell, IA) and has been described previously (Pratis et al., 2001, 2005). Strain COP1720 expressing spc-1::degron::mKate2 at the endogenous locus was generated by NemaMatrix (Eugene, OR), characterized by genomic sequencing, and generously provided by the Zaidel-Bar lab (Tel-Aviv University, Tel-Aviv, Israel). Line COP1720 was backcrossed three times to generate strain UN1823. Transgene qyls198[inf-1p::moeABD::mCherry, unc-119(n)] was obtained from strain NK1069, a kind gift from the Sherwood lab (Duke University, Durham, NC). For a list of strains used in this study, please see Supplemental Table 1.

Expression constructs
Construct fln-1p::GFP::act-1 was generated by restriction dige cloning to insert the fln-1 promoter into pPRRF207 (him-4p::GFP::act-1) provided by the Roy lab (University of Toronto, Toronto, Canada) and has been described previously (Dixon and Roy, 2005). The fin-1p::GFP::UntCH construct was generated by amplifying the mammalian utrophin calponin homology domain (UntCH; actin-binding domain) from GFP-UntCH (addgene #24515) and using restriction dige cloning to insert UntCH into a plasmid backbone with fln-1p::GFP. The use of GFP-UntCH to label F-actin has been described previously (Burkel et al., 2007). Construct fln-1p::ajm-1::GFP was generated by amplifying the first nine exons of ajm-1 from C. elegans gDNA and using restriction dige cloning to insert the ajm-1 coding sequence upstream of GFP. The GFP was then replaced with tagRFP to generate fln-1p::ajm-1::tagRFP. The UNC-70 translational reporter was generated using by using round horn PCR to remove mVenues from plasmid MG319 (unc-70::TSMod; addgene #73367) described previously (Krieg et al., 2014). NEBuilder (NEB, Ipswich, MA) was used to clone the unc-70::mTFP fusion downstream of ~2 kb of the unc-70 promoter region, amplified from C. elegans gDNA, in frame with the third exon of isoform A that is shared by all splice isoforms. The fin-1p::TIR1::F2A::mCherry::h2b construct was generated using NEBuilder (NEB, Ipswich, MA). The TIR1 coding sequence was amplified from the plasmid pLZ31 (addgene #71720) described previously (Zhang et al., 2015). The histone coding sequence (h2b) was amplified from the plasmid pJH4.52 (addgene #26946) previously (Galio et al., 2008) and inserted in frame with an mCherry fluorescent marker separated from the TIR1 coding sequence by the 22-amino-acid F2A peptide sequence, which was ordered as an oligo (Genewiz, South Plainfield, NJ). Use of the F2A peptide to coexpress separate proteins from the same mRNA has been previously validated in C. elegans (Ahier and Jarriault, 2014). Cloning details and plasmid sequences are available on request.

RNA interference
The RNAi feeding protocol was performed essentially as described in Timmons et al. (2001). To prepare seeded NGM—iso-2-propylthio-β-galactoside (IPTG) plates, HT115(DE3) bacteria transformed with the double-stranded RNA (dsRNA) construct of interest were grown overnight at 37°C in Luria broth (LB) supplemented with 40 µg/ml ampicillin. The following day, 150 µl of the culture was seeded on NGM-IPTG agar (NGM supplemented with 25 µg/ml carbenicillin and 1 mM IPTG) and incubated at room temperature for 24–72 h to induce dsRNA expression. Age synchronized animals, prepared as described below, were then transferred to these plates.

Partially synchronized populations were obtained by alkaline lysis procedure (“egg prep”), and tightly synchronized populations were obtained by larval stage 1 (L1) arrest. For egg prep, starved dauer nematodes were allowed to recover for 48 h on NGM plates newly seeded with OP50. This produces young gravid adults for egg collection. Eggs were released using an alkaline hypochlorite solution as described in Hope (1999) and washed three times with filter sterilized M9 buffer (22 mM KH₂PO₄, 42 mM NaHPO₄, 86 mM NaCl, and 1 mM MgSO₄) (Hope, 1999). Clean eggs were then transferred to seeded NGM-IPTG plates. The L1 arrest protocol was adapted from Hope (1999). For L1 arrest, the same alkaline lysis procedure was performed, except clean eggs were transferred onto NGM without bacteria. Eggs were allowed to hatch and develop until L1 at 16°C overnight before being transferred to seeded NGM-IPTG plates. Populations produced by egg prep are ~10 h behind L1 arrest populations developmentally. All RNAi experiments were performed at 23°C. Strains utilized in each RNAi experiment are indicated.
RNAi constructs were obtained from the ORFeome-RNAi v1.1 library or were constructed by PCR amplification of wild-type cDNA and cloned into pPD129.36 (Fire Vector Kit). Empty pPD129.36 vector was used as a negative control in RNAi experiments. All primer sequences and cloning details are available on request.

**RNAi screen of actin-binding and regulatory proteins**

The Ontology Browser on WormBase (wormbase.org) was used to identify genes that encode proteins with conserved actin-binding domains that recognize either filamentous actin (F-actin) or monomeric, globular (G-actin) as well as proteins with conserved domains involved in actin filament bundling and organization (WormBase Gene Ontology IDs: GO:0051015, GO:0003785, GO:0003779, GO:0051017, and GO:0007015). In total, 103 genes were identified using this approach, and 102 genes, excluding vab-10, were scored for spermathecal contractility defects. Knockdown of VAB-10, spectraplakin, resulted in disrupted gonad morphology that precluded scoring (Morrissette et al., 2014). The feeding method was selected to administer dsRNA to allow for continuous dsRNA exposure during post-embryonic development to achieve efficient RNAi in adult animals. However, RNAi efficiencies can differ for specific genes depending on the dsRNA delivery method (Kamath et al., 2001). Therefore, the results of this RNAi screen may differ for specific genes compared with previous screens (Green et al., 2011). RNAi was performed as described above. Partially synchronized populations were obtained by egg prep. Animals were grown for 65 h at 23°C and scored as adults. At this time, animals fed control RNAi have undergone several ovulations and contain embryos within the uterus. Spermathecae in live animals were visualized on the dissection scope using a line that expresses GFP::ACT-1 under a spermatheca specific promoter. This line allows for easy visualization of the spermatheca to distinguish spermathecae undergoing ovulation that are occupied by an oocyte and appear distended, “occupied,” from spermathecae between ovulations that contain only sperm and appear compact, “empty.” Animals with occupied spermathecae but no embryos in the uterus were scored as, “occupied on first,” and animals with empty spermathecae and no embryos in the uterus were scored as, “no entry.” At least 100 animals were scored for each condition (Supplemental Table 1). To ensure the same animal was not counted more than once, animals were removed from the plate by suction using a line that expresses GFP::ACT-1 under a spermatheca specific promoter, “no entry.” At least 100 animals were scored for each condition (Supplemental Table 1). For ovulation movies and observations of live or fixed animals, partially synchronized populations were obtained by egg prep and animals were grown at 23°C for ~50 h, around the time of the first ovulation. Live animals were immobilized with 0.01% tetramisole and 0.1% tricaine in M9 buffer (Kirby et al., 1990; Mccarter et al., 1997) and mounted on 2% agarose pads or with 0.05 µm Polybead Microspheres (Polysciences, Warrington, PA) diluted 1:2 in water and mounted on 5% agarose pads, adapted from Wang and Audhya (2014). Whole animals were fixed by transferring to 1.85% formaldehyde in PBS for 20 min at room temperature. Animals were then rinsed once with PBST and three times with PBS just before being mounted on 2% agarose pads. Confocal microscopy was performed on an LSM 710 confocal microscope (Zeiss) equipped with Zen software (Zeiss) using the Plan-Apochromat 63×/1.40 Oil DIC M27 objective. The 405-nm laser was used to excite DAPI, 488-nm laser was used for GFP, and 561-nm laser was used for mCherry, mKate2, and TexasRed. For movies, 40 z-slices were acquired at 14-s intervals. Illumination of the spermathecae from animals expressing actin labeled with GFP (GFP::ACT-1) with the 488-nm laser for ~5 min prior to oocyte entry frequently caused the valve to remain partially closed during ovulation, increasing oocyte dwell time. For movies of all lines, live animals were imaged for ~30 min total. For still images of live and fixed animals and tissue, z-slices were acquired at 0.38-µm intervals with each slice representing the average of two scans or four scans.

**Auxin-induced decay**

The auxin-induced decay (AID) procedure was adapted from Zhang et al. (2015). Auxin-containing plates were made by adding 400 mM stock of 3-indoleacetic acid (IAA; Sigma-Aldrich I-2886, CAS Number 87-51-4) dissolved in 200-proof ethanol (Fisher) to cooled NGM media just before pouring plates. For control plates, 1% vol/vol 200 proof ethanol (Fisher) was added in place of the IAA stock. For control plates, 1% vol/vol 200 proof ethanol (Fisher) was added in place of the IAA stock. Plates were seeded with concentrated OP50 as described previously (Zhang et al., 2015). The expression level of TIR1 was optimized to minimize background degradation of degron tagged SPC-1 at the endogenous locus in the absence of IAA. Animals expressing the TIR1 construct injected at 20 ng/µl had diminished SPC-1::degron:mKate2 fluorescence and disrupted spermathecal actin (visualized using GFP::UtrCH) on control plates and were excluded from analysis while animals expressing TIR1 injected at 10 ng/µl appeared WT in the absence of IAA. In preliminary experiments, young adult animals were exposed to IAA at concentrations of 0.25, 0.5, 1, and 4 mM for 5 h. Only the 1 and 4 mM IAA-treated animals showed complete reduction in mKate2 fluorescence with the 4 mM grown for 70 h. For quantification of F-actin organization, tightly synchronized populations were obtained by L1 arrest and grown at 23°C for 40 or 45 h for animals carrying the unc-70 alleles. At this time point, N2 control animals have just begun to ovulate. F-actin staining was adapted from Ono et al. (2007). Briefly, animals were dissected using a 25-gauge hypodermic needle in phosphate-buffered saline (PBS), and dissected gonads were fixed in 1.85% formaldehyde in PBS for 25 min at room temperature. Following fixation, gonads were washed twice with PBS, permeabilized for 15 min in PBST (PBS + 0.1% Triton X-100), and then incubated with 0.4 U/ml Texas Red-X phalloidin in PBS (Invitrogen, Carlsbad, CA) overnight at 4°C or 3 h at room temperature. For identification of the Emo phenotype, nuclear stain, DAPI (Sigma-Aldrich, St. Louis, MO), was included at 100 ng/ml. Labeled samples were washed twice with PBS and mounted on 2% agarose pads for observation.
condition producing the most consistent results. Young adults were then exposed to 4 mM IAA and monitored at 1 h intervals. By 4 h SPC-1::degron::mKate2 fluorescence was below detectable levels in the spermatheca. These animals were transferred to control plates and observed 24 h later for SPC-1::degron::mKate2 fluorescence recovery. However, fluorescence levels remained below detectable levels after 24 h.

To determine the role of SPC-1 postdevelopment, animals expressing spc-1::degron::mKate2, Fln-1p::GFP::UtrCH, and Fln-1p::TIR1::F2A::mCherry::H2B were “egg prepped” onto control plates and grown at 23°C for ~58 h. At this time, some animals are just before the first ovulation and many have already begun to ovulate. Approximately 100 animals just before the first ovulation and 100 animals that had undergone approximately two to five ovulations were segregated onto plates containing 4 mM IAA and returned to 23°C for 7 h along with the animals that remained on the control plate. Animals were also directly “egg prepped” onto 4 mM IAA and grown at 23°C for 65 h. The fln-1 promoter driving TIR1::F2A::mCherry::H2B turns on during early L4 and remains on throughout adulthood (Kovacevic and Cram, 2010) therefore animals “egg prepped” onto IAA plates begin degrading degron tagged SPC-1 during L4 when the TIR1 construct is expressed. Animals from all conditions were dissected to excise gonads and fixed as described in the histochemistry section without incubation with phalloidin or DAPI and mounted on 2% agarose pads for observation.

Image analysis
ImageJ software was used for all image analysis. For consistency, all analysis was performed on cells of the main spermathecal bag unless otherwise indicated. For quantification of actin bundle fluorescence intensity in fixed samples, measurements were taken from a single confocal z-slice. A 20-pixel-wide line was drawn across each cell. BAR script Find Peaks was used to identify central and peripheral actin fluorescence peaks. For normalization, the central actin peak intensity measurements of an individual cell were divided by the largest peripheral actin fluorescence measurement of that same cell. For analysis of actin fluorescence intensity in ovulation movies, measurements were taken at 70-s intervals (Five frames) from maximum intensity projections of confocal ovulation movies. As for fixed samples, a 20-pixel-wide line was drawn across each cell. Noise in images acquired using fast acquisition settings to capture actin dynamics in live animals precluded reliable identification of central actin peaks using the BAR script. Actin peaks at the cell periphery were identified using the BAR script, or manually when not possible, and measurement of peripheral actin peak intensity was calculated by taking the average of 10 pixels to the left and right of the identified peripheral peak. Central actin fluorescence was calculated by taking the average fluoresces across the entire 20-pixel-wide line, excluding pixels included in peripheral actin measurement. Normalized measurements were calculated by dividing the central actin measurement of a single cell by the largest peripheral actin measurement for that same cell. OrientationJ (Rezakhaniha et al., 2012), configured using a Gaussian fit with a pixel size of 2, was used to measure the orientation distribution of actin bundles in individual cells and to generate color-coded images where color indicates orientation, hue indicates coherency, and brightness is the brightness of the original image.

Statistical analysis
All statistical analysis was performed with GraphPad Prism software. To compare two unpaired groups, the unpaired t test was used to determine whether the difference between the means of two data sets was significant when data had a normal distribution and Welch’s correction was included if the different treatments were expected to have different standard deviations. To compare three or more unmatched groups, ordinary one-way analysis of variance was performed with either a Dunnett’s multiple comparison or Tukey’s multiple comparison test as necessary. The Mann–Whitney test was used when data did not have a normal distribution. All trapping data were analyzed using the Fisher’s exact test. In all cases, the statistical test used and resulting p values, indicated by symbols ns (p > 0.05), *(p ≤ 0.05), **(p ≤ 0.01), ****(p ≤ 0.001), *****(p ≤ 0.0001), are noted in each figure caption.

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