LETTERS

Molecular basis of the copulatory plug polymorphism in *Caenorhabditis elegans*

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Heritable variation is the raw material for evolutionary change, and understanding its genetic basis is one of the central problems in modern biology. We investigated the genetic basis of a classic phenotypic dimorphism in the nematode *Caenorhabditis elegans*. Males from many natural isolates deposit a copulatory plug after mating, whereas males from other natural isolates—including the standard wild-type strain (N2 Bristol) that is used in most research laboratories—do not deposit plugs[1]. The copulatory plug is a gelatinous mass that covers the hermaphrodite vulva, and its deposition decreases the mating success of subsequent males[2]. We show that the plugging polymorphism results from the insertion of a retrotransposon into an exon of a novel mucin-like gene, *plg-1*, whose product is a major structural component of the copulatory plug. The gene is expressed in a subset of secretory cells of the male somatic gonad, and its loss has no evident effects beyond the loss of male mate-guarding. Although *C. elegans* descends from an obligate-outcrossing, male–female ancestor[3,4], it occurs primarily as self-fertilizing hermaphrodites[5–7]. The reduced selection on male–male competition associated with the origin of hermaphroditism may have permitted the global spread of a loss-of-function mutation with restricted pleiotropy.

The synthesis of evolutionary and developmental genetics demands empirical data on the structure of heritable variation in populations. The required data include the identities of the genes that underlie variation, the mutations that affect those genes, and the positions of the genes within the networks of molecular interactions that shape phenotypes. *C. elegans* is a promising system for the discovery of the molecular basis of variation. Although most studies of *C. elegans* have employed a single genetic background, natural isolates exhibit a wide range of phenotypes[1,8]. Some of this phenotypic variation may result from the change in selective environment associated with the recent origin of hermaphroditism in *C. elegans*. Males arise through rare non-disjunction events and contribute at a low rate to outcrossing[5–7]. Because their relatives in the *Caenorhabditis* clade produce copulatory plugs, the plugging strains of *C. elegans* probably retain an ancestral trait that confers significant advantages in male–male competition for matings (Supplementary Information and Supplementary Fig. 1). The failure of some strains of *C. elegans* to produce plugs may represent a loss-of-function mutation with minimal impact on fitness due to the diminished competition among males. Hodgkin and Doniach[1] showed that plugging exhibits Mendelian inheritance attributable to a single locus, *plg-1*. The plugging phenotype is dominant, consistent with a model of evolutionary and molecular loss of function in the N2 strain. We tested this model and characterized the molecular basis for heritable variation in copulatory plugging by identifying the gene and molecular lesion underlying *plg-1*.

Hodgkin and Doniach localized *plg-1* to a large region of chromosome III. We employed genetic mapping in crosses between the non-plugging strain N2 and a plugging natural isolate from Palo Alto, CB4855, to refine the location of *plg-1* to a 74-kilobase (kb) interval (Supplementary Methods and Supplementary Figs 2 and 3). Independently, we mapped *plg-1* to a coincident interval in a panel of recombinant inbred lines between N2 and a plugging natural isolate from Hawaii, CB4856 (Supplementary Fig. 4). A search for PCR-product length polymorphism showed that a retrotransposon present in the interval in the non-plugging strain N2 is absent in the plugging strains. Sequence analysis revealed that the retrotransposon and its long terminal repeats (LTRs) interrupt a novel, unannotated protein-coding gene whose predicted product has similarity to canonical mucins (Fig. 1a). Mucins are large glycoproteins that are often secreted to form gelatinous substances, including mucus, in diverse metazoans[9]; a mucin is therefore a natural candidate for a major structural component of the plug. The protein is predicted to contain proline-, threonine- and serine-rich (PTS) repeats, characteristic of highly *O*-glyclosylated mucoproteins[10,11] (Fig. 1b); each PTS repeat contains 15 predicted *O*-glycosylation sites[12]. The non-repetitive amino and carboxy termini of the predicted protein are evolutionarily conserved among *Caenorhabditis* species, and the N terminus includes a predicted 21-amino-acid secretory signal peptide[13] (Fig. 1c). Each class of PTS repeats is much more similar within species than between species, consistent with standard models of concerted evolution of repetitive sequences[14] (Fig. 1d).

We used RT–PCR (PCR with reverse transcription) to show that the stable transcript of the putative mucin is absent in non-plugging strains. A spliced transcript from the gene is present in RNA from adult males of plugging strains, but is undetectable in adult males of non-plugging strains (Supplementary Fig. 5). The transcript is also undetectable in adult hermaphrodites from plugging strains, consistent with a male-specific function.

To confirm that the disruption of the putative mucin is responsible for the loss of plugging, we used two functional tests. First, we knocked down the expression of the gene with RNAi (RNA interference) in a plugging strain. The resulting males failed to produce copulatory plugs, confirming that the gene is necessary for copulatory plug formation (Fig. 2a). Second, we introduced a transgene carrying the putative mucin into a non-plugging strain. This was sufficient to transform the strain into one that produced plugs (Fig. 2b). We conclude that the putative mucin is the gene underlying the *plg-1* locus, and the absence of the disruptive retrotransposon is the plugging allele *e2001* defined by Hodgkin and Doniach[1].

We next confirmed that the copulatory plug consists of an *O*-glycosylated mucin. We isolated hermaphrodites immediately

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after mating, and stained the resulting copulatory plugs with a fluorescently tagged peanut agglutinin lectin that recognizes Galβ1,3GalNAc-S/T, a carbohydrate modification characteristic of mucins[15,16]. The lectin stained the plugs specifically (Fig. 3a, b), indicating that an important constituent of each plug contains a carbohydrate structure that is typical of mucin glycoproteins.

In order to examine expression of *plg-1*, we introduced a transgenic construct with the evolutionarily conserved 5′ upstream region of *plg-1* driving green fluorescent protein (GFP). We observed strong expression of GFP in 12 cells of the vas deferens (Fig. 3c–f), a tube of 30 secretory cells connecting the seminal vesicle to the cloaca[17]. Expression commences during the fourth larval stage, before the adult moult. In adult males, the cuboidal GFP-expressing cells are arranged in two rows (Fig. 3c). There is left–right asymmetry in the location of the GFP-expressing cells and in the intensity of their expression. Although there seems to be variation among individuals, typically five of the cells are on the right-hand side of the midline, whereas seven are on the left-hand side of the midline; furthermore, three posterior cells express a lower level of GFP, and two of these low-level cells are on the left-hand side (Supplementary Movie 1).

Expression of *plg-1*::GFP provides the first marker for this suite of vas deferens cells, a previously uncharacterized feature of male anatomy, and reveals their dynamic role in mating. Unmated males have a single large vacuole that occupies most of the cell volume in each of the 12 cells (Fig. 3d, e); in contrast, males examined immediately after mating lack these large vacuoles, and the 12 cells exhibit instead multiple smaller vacuoles (Fig. 3f). The vacuoles are present in both plugging and non-plugging genotypes and are probably storage compartments for seminal fluid.

We next assessed the geographic distribution of *plg-1* alleles. We tested 153 natural isolates from around the globe for the ability to complement the N2 loss-of-function allele. Strains (48) from Europe,

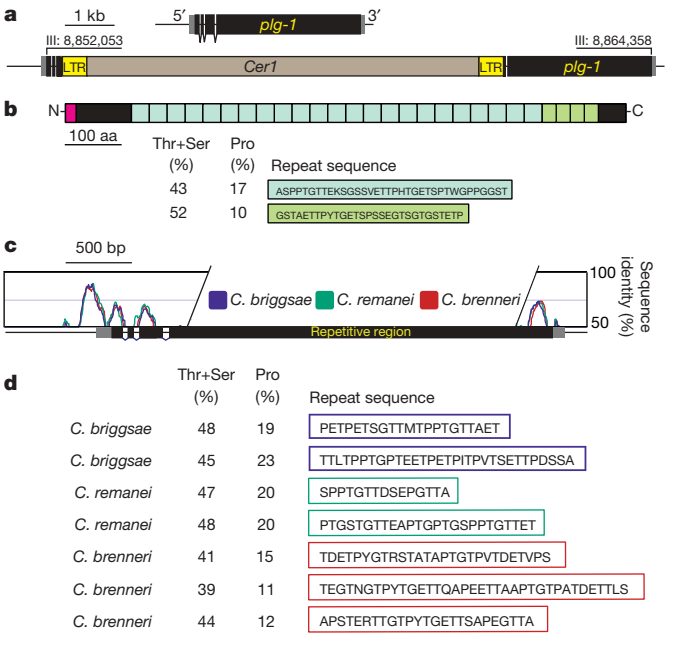

Figure 1 | The *plg-1* gene. **a**, Genomic structure of the *plg-1* gene, showing the exons (black) and UTRs (grey) determined by RACE. *Cer1* (brown) and its LTRs (yellow) disrupt exon 3 in N2, whose genomic organization is shown below. **b**, The PLG-1 protein contains a secretory signal (pink) and unique sequences at the N and C termini (black). The remainder consists of PTS repeats, shown in the coloured boxes. **c**, The non-repetitive regions of the gene and 5′ upstream region are conserved among species. The PTS repeats are not conserved at the sequence level in *C. briggsae* (blue), *C. remanei* (green) and *C. brenneri* (red). Percentage identity to the *C. elegans* sequence is plotted for 100 bp windows of a multiple alignment[30]. **d**, The PTS repeats (boxes coloured as in **c**) exhibit concerted evolution[14].

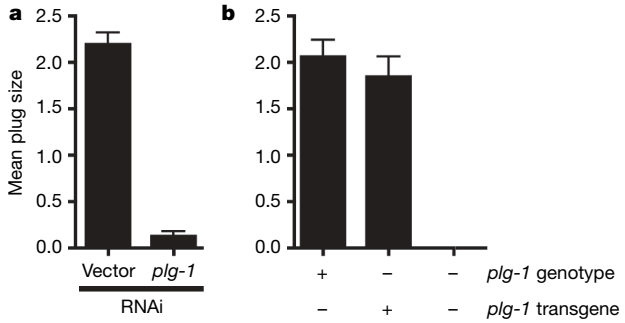

Figure 2 | The mucin gene is *plg-1*. **a**, CB4856 males in which *plg-1* has been knocked down by RNAi produced fewer and smaller plugs than control males (Mann–Whitney *U*-test, $P < 0.0001$; $n = 24$ and 23, respectively). **b**, Transgenic *plg-1* males deposited plugs similar in size and frequency to those produced by males with endogenous *plg-1* and larger than those produced by a strain lacking a functional *plg-1* gene (Mann–Whitney *U*-test, $P < 0.0001$). The genotypes from left to right are CB4856 ($n = 17$), F1 ($n = 19$) from cross of N2 and QX1015 (*plg-1(N2) unc-119(ed3)* III, CB4856 background) carrying the extrachromosomal array [*plg-1(CB4856) unc-119(+)*], and F1 ($n = 18$) from cross of N2 and QX1015 without the array. Plug size was assessed in arbitrary units (0: none, 1: small, 2: medium, 3: large) by an observer blind to treatment. Error bars, s.e.m.

North America, and Australia failed to complement N2, and all carried the retrotransposon insertion in *plg-1*. All 105 strains that complemented N2 lacked the insertion (Fig. 4). As our PCR assay confirms that the location of the insertion and its orientation are shared among the non-plugging strains (Supplementary Information), we infer that the common loss-of-function allele of *plg-1* represents a single mutation that has spread across the globe.

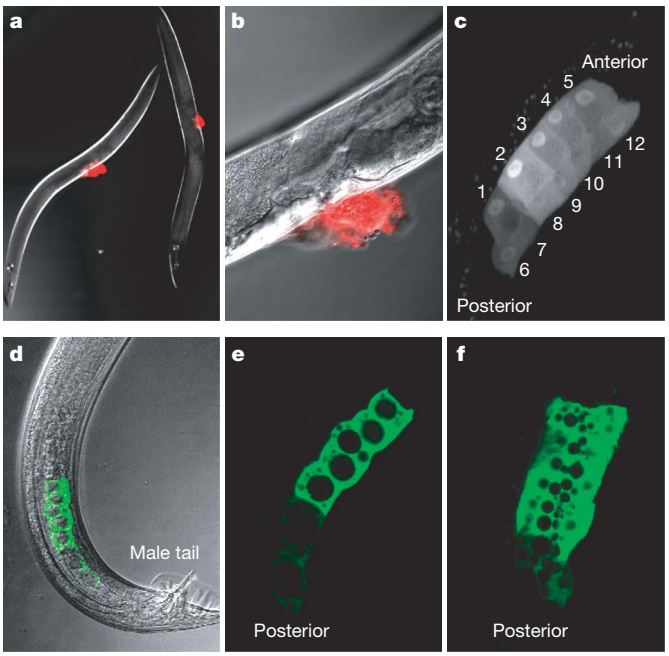

Figure 3 | The copulatory plug contains carbohydrate structures typical of mucins and *plg-1* is expressed in only a small number of male cells. **a**, **b**, Fluorescently labelled lectin that recognizes a carbohydrate modification characteristic of mucins specifically stains copulatory plugs. **c**, The *plg-1* promoter drives GFP expression in 12 vas deferens cells; this image is taken from Supplementary Movie 1. **d**, The GFP-expressing cells are in the central region of the vas deferens, anterior to the mating structures. **e**, In unmated males, each cell contains a large vacuole. **f**, After mating, the large vacuole is replaced by many smaller vacuoles. In **e** and **f**, an optical slice was taken from just left of the midline, so each image includes 5 bright cells anterior and 2 faint cells posterior. Approximate panel sizes: **a**, 0.8 × 1.1 mm; **b**, **d**, 0.13 × 0.19 mm; **c**, **e**, **f**, 0.08 × 0.11 mm.

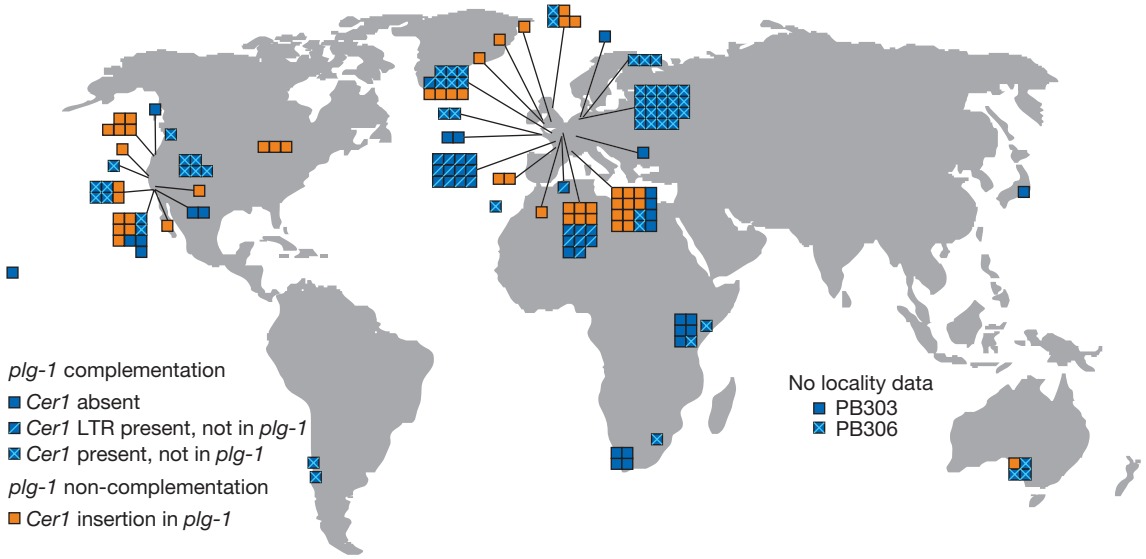

Figure 4 | The *plg-1* loss-of-function allele is globally distributed and *Cer1* has been recently active. Strains that complement the N2 loss-of-function allele of *plg-1* (F1 males produce plugs) are shown as blue boxes, and those that fail to complement (F1 males fail to plug) are shown orange. The distribution of *Cer1* and its LTR in the genomes of plugging strains is indicated with diagonals across the boxes.

Plugging and non-plugging strains co-occur within localities, and the non-plugging genotype persists across years[5,6], suggesting that the loss of plugging incurs little fitness cost in nature.

In the complete genome sequence of N2, the insertion of *Cer1* into *plg-1* is the sole intact copy of the retrotransposon[18]. We assayed natural isolates of *C. elegans* with uninterrupted *plg-1* sequences by PCR for the presence of *Cer1* elsewhere in the genome (Supplementary Table 1). While 25 strains had no evidence of *Cer1* or its LTRs, 59 strains carry the retrotransposon and its LTRs and another 21 carry the LTRs alone, evidence of having had and later lost the retrotransposon. We found by sequence analysis that *Cer1*, a *gypsy/Ty3* type retrotransposon[19], is similar to retrotransposons in the genome sequences of *Caenorhabditis briggsae, Caenorhabditis brenneri* and *Caenorhabditis remanei*, and these mobile genetic elements may be a common source of new mutations in populations of *Caenorhabditis*.

Induced mutations in a large number of genes disrupt male mating and copulatory plug production[20–22], but the naturally occurring allele of *plg-1* pinpoints the terminal gene in the plug-production network, a structural component of the plug itself. Genes upstream of *plg-1* in the male mating network, including genes required for cilium development, neurotransmitter biosynthesis and transmission, and male tail morphogenesis, have extensive pleiotropic roles in *C. elegans* biology[22]. In contrast, the *plg-1* gene has no apparent roles outside plugging. We found that the gene is expressed exclusively in males in a subset of cells with no other known function. Males with the loss-of-function allele of *plg-1* suffer no evident consequences apart from the loss of plugging. This result is consistent with a role for pleiotropy in filtering the mutations that actually contribute to evolutionary change from the much larger set of mutations that possibly could, a principle laid out by Fisher nearly 80 years ago[23]. Mutations with the fewest effects are those least likely to change some traits for the worse and hence are those most likely to contribute to evolutionary divergence[24].

The only other naturally occurring allele known to disrupt male mating is a loss-of-function allele of *mab-23*, a gene required for development of the male copulatory organs, and this allele is known only from a single natural isolate[1]. Although *mab-23* is expressed in both males and hermaphrodites, it exhibits an obvious mutant phenotype only in males[25], consistent with restricted pleiotropy; at the same time, the rarity of the *mab-23* allele suggests that complete loss of male function might be deleterious. Although *C. elegans* reproduces primarily by self-fertilization in hermaphrodites, selection probably acts to preserve male function[26–28], eliminating mutations that disrupt male development, morphology or behaviour. The high frequency of the *plg-1* loss-of-function allele, its co-occurrence with the functional *plg-1* allele, and its temporal persistence all suggest that the frequency of males in typical populations is insufficient to select for mate guarding, which requires not just functional males but competition among males. The evolution of hermaphroditism in *C. elegans* appears to have altered the selective regime of genes required for male–male competitive function. Although it is possible that the loss of *plg-1* is selectively favoured, it may also represent a neutral change drifting to high frequency in the absence of selection against it.

METHODS SUMMARY

Nematode stocks. Nematodes were cultured using standard protocols[29].

Sequence analysis. We merged partial gene predictions across *Cer1* in the N2 genome (www.wormbase.org) and then refined the gene model using RACE with RNA from CB4855 males (Clontech SMART RACE kit). Sequences from other species were collected using synteny tracks (www.ucsc.genome.org); we identified N- and C-terminal *plg-1* sequences based on sequence conservation and generated alignments of flanking and intervening sequence using LAGAN[30].

Expression analyses. RT–PCR primers flank intron three of *plg-1* and fall downstream of the *Cer1* insertion site; chimaeric *Cer1:plg-1* transcript should be detected if present. We generated integrated *plg-1::GFP* transgene (allele *qqIs1*) by cloning 311 bp of 5′ upstream sequence into pSM_GFP and co-bombarding it with *unc-119(+)* plasmid pMM016b into QX1015, which carries *unc-119(ed3)* and *plg-1*(N2) introgressed into CB4856. We imaged the GFP using a Zeiss 510 META confocal microscope.

Functional confirmation experiments. We cloned 3 kb of CB4856 *plg-1* exon 4 into RNAi feeding vector L4440 and fed worms *Escherichia coli* HT115(DE3) carrying the vector or L4440 with no insert. Transgenic rescue of plugging employed a construct derived from CB4856 spanning from 686 bp 5′ of the RACE-defined start of transcription to 178 bp 3′ of the RACE-defined 3′ UTR.

Mucin staining. Virgin adults were cleaned of bacteria in PBS, allowed to mate for 30 min on clean agarose plates, then fixed and stained with fluorescently labelled lectin. A labelled antibody that fluoresces at a different wavelength but does not bind to nematodes was included to control for the possibility that the gelatinous plug absorbs probe indiscriminately.

Natural isolates. Each isolate was mated to N2 males and the F1 males tested individually in overnight assays with single N2 hermaphrodites for the ability to produce plugs.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

RT–PCR assays. RNA was isolated using the RNeasy Mini Kit (Qiagen) and amplified using the OneStep RT–PCR Kit (Qiagen) with primers specific for *plg-1* (plgRT125F and plgRT259R, flanking intron 3) and for the male-expressed gene *lov-1* (lovRT497F and lovRT993R), a positive control for male mRNA. Primer sequences for all experiments are provided in Supplementary Information.

RNAi construct and assay. A fragment of the *plg-1* locus was amplified using primers e2.2F1 and plgR12BglII and the product cut with BglII and cloned into the RNAi feeding vector L4440 (Andy Fire Vector Kit, Addgene). The insert is approximately 3 kb and contains almost the entirety of the large repetitive last exon of *plg-1*. The plasmid was then transformed into HT115(DE3) RNase III-deficient bacteria for RNAi feeding experiments, which were conducted using the protocol described in ref. 31. Insertless vector L4440 in HT115 cells served as a negative control. Individual virgin N2 hermaphrodites were allowed to mate overnight with 3–5 CB4856 virgin males that were either exposed to bacteria containing insertless vector (these males should generate normal plugs), or exposed to bacteria producing *plg-1* dsRNA (these males should generate fewer/smaller plugs). The hermaphrodites were scored for plugs the next morning, using an arbitrary plug size rating scale (0: none, 1: small, 2: medium, 3: large), and the observer was blind to treatment. CB4856 exhibits germline RNAi resistance, but it retains sensitivity to systemic RNAi in somatic tissues[32], permitting us to use RNAi to assay gene function in the somatic gonad.

Transgenic complementation construct and transformation. We constructed the *plg-1* rescue transgene pMR5.3 by sequentially cloning the 5′ and 3′ fragments. We cloned the 5′ part of the gene, amplified from CB4856 with primers plgF3 and plgR1 and cut with ApaI and BglII, into pPD34_110 (Andy Fire Vector Kit, Addgene), cut with the same enzymes. The 5′ insert is 1,469 bp and includes 686 bp upstream of the 5′ end of the transcript as determined by RACE. We cloned the 3′ part of the gene, amplified with plgF1 and plgR1 and cut with XbaI and BglII, separately into pPD34_110 cut with those enzymes. This 3′ fragment from CB4856 is ~3.9 kb, versus 2.9 kb in the WormBase N2 genome sequence. We next excised the ApaI–BglII fragment from the first vector and ligated it into the second. We sequenced the resulting construct to the extent possible given the repetitive nature of the last *plg-1* exon and found no differences from the sequence derived from CB4856 genomic DNA.

We introduced pMR5.3 and an *unc-119* rescue plasmid, pMM016B, into strain QX1015, which carries *unc-119(ed3)* in a CB4856 background. Because *unc-119* maps close to *plg-1*, QX1015 retains the N2 allele of *plg-1* and other genes in this region, and N2/QX1015 F1 males fail to plug. We co-bombarded 3 μg of each plasmid into QX1015 using a BioRad gene gun. We recovered several independent transformed lines and selected one with high transmission of the extrachromosomal array, as judged by the scarcity of Unc worms in its progeny. This transformed line was assayed quantitatively for plug formation. Males of three genotypes were compared: CB4856, which is a natural plugging strain; F1 from a cross of N2 to QX1015 (*plg-1(N2) unc-119(ed3)* III, CB4856 background) carrying the extrachromosomal array [*plg-1(CB4856) unc-119(+)*], which are expected to produce plugs; and F1 from a cross of N2 to QX1015 without the array, which are expected to be unable to produce plugs. We allowed 3–5 males to mate overnight with a single N2 virgin hermaphrodite, and plug size was assessed the next morning in arbitrary units (0: none, 1: small, 2: medium, 3: large), with the observer blind to male genotype.

Promoter::GFP construct and transformation. We cloned a CB4856 PCR fragment encompassing the 311 bp 5′ of the start of translation, amplified using primers NotIplgprom471F and NplgproR1, into vector pSM_GFP using a NotI cut site. The plasmid, pMR18K, was introduced into QX1015 by biolistic transformation. We recovered a line with an integrated transgene, assigned allele name *qqIs1*. The transgene maps near the centre of chromosome III, near *plg-1* and *unc-119*, based on linkage mapping with strains MT3751 (*dpy-5* I; *rol-6* II; *unc-32* III), EG1020 (*bli-6* IV; *dpy-11* V; *lon-2* X) and CB1562 (*vab-7* III). We recovered 0/13 Vab GFP worms and 0/50 Unc-32 GFP worms. Several additional lines, in which the transgene is not integrated into the genome, show qualitatively identical GFP expression. Nematodes expressing GFP were imaged with a Zeiss 510 META confocal microscope using a Plan-NEOFLUAR 40X/1.3 oil objective. Nematodes were anesthetized in 10 mM sodium azide and mounted on 5% agar pads for examination. Unmated males (for example, Fig. 3d, e) were collected as L4 larvae and held overnight without access to hermaphrodites. To obtain males soon after mating (for example, Fig. 3f), individual virgin males were placed in the presence of several virgin hermaphrodites and watched until matings were completed, then examined under the microscope immediately afterwards. We corroborated the relationship between mating and vacuole morphology by examining *qqIs1; mab-23* homozygotes, which are incapable of mating. These males exhibited the large vacuole morphology.

Lectin staining. Plugged and unplugged hermaphrodites were fixed in 4% paraformaldehyde in PBS. Fixed hermaphrodites were stained in 1:100 dilutions of the two molecules mentioned below (Invitrogen Molecular Probes), in PBS + 0.1% TritonX, then washed several times in PBS + 0.1% TritonX, and then several times in PBS. (1) Alexa Fluor 488 donkey anti-mouse IgG (A21202); (2) lectin PNA from *Arachis hypogaea* (peanut), Alexa Fluor 594 conjugate (L32459).

Worms were mounted on 5% agar pads and examined under the confocal microscope. Strong lectin signal was observed; negligible signal was observed from the donkey anti-mouse antibody, suggesting that the plug was not simply absorbing probe.

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