

RESEARCH ARTICLE

A novel small molecule that disrupts a key event during the oocyte-to-embryo transition in *C. elegans*

Steven E. Weicksel¹, Assaf Mahadav^{1,2}, Mark Moyle³, Patricia G. Cipriani², Michelle Kudron¹, Zachary Pincus⁴, Shirin Bahmanyar⁵, Laura Abriola⁶, Janie Merkel⁶, Michelle Gutwein², Anita G. Fernandez⁷, Fabio Piano^{2,8,*}, Kristin C. Gunsalus^{2,8,*} and Valerie Reinke^{1,*}

ABSTRACT

The complex cellular events that occur in response to fertilization are essential for mediating the oocyte-to-embryo transition. Here, we describe a comprehensive small-molecule screen focused on identifying compounds that affect early embryonic events in *Caenorhabditis elegans*. We identify a single novel compound that disrupts early embryogenesis with remarkable stage and species specificity. The compound, named C22, primarily impairs eggshell integrity, leading to osmotic sensitivity and embryonic lethality. The C22-induced phenotype is dependent upon the upregulation of the LET-607/CREBH transcription factor and its candidate target genes, which primarily encode factors involved in diverse aspects of protein trafficking. Together, our data suggest that in the presence of C22, one or more key components of the eggshell are inappropriately processed, leading to permeable, inviable embryos. The remarkable specificity and reversibility of this compound will facilitate further investigation into the role and regulation of protein trafficking in the early embryo, as well as serve as a tool for manipulating the life cycle for other studies such as those involving aging.

KEY WORDS: Small molecule, Oocyte-to-embryo transition, Secretory pathway, *C. elegans*

INTRODUCTION

The transition from oocyte to embryo (OET) immediately following fertilization is a pivotal point at which parentally provided factors first interact to initiate the zygotic program. Correct coordination of this transition is essential to launch the development of a new, and often genetically unique, individual. During OET, many important molecular events rely on precise spatial and temporal deployment to properly activate the zygotic genome and elaborate the basic aspects of the body plan. Because of its importance during the life cycle, the OET is also very highly selected upon evolutionarily and many key differences between species are established at this very early event (Shu et al., 2015).

Many aspects of OET have been well studied, particularly in *C. elegans* (Robertson and Lin, 2015; Rose and Gönczy, 2014). These include oocyte maturation, meiosis and fertilization, as well as molecular processes such as the merging of parental haploid genomes, activation of zygotic transcription and the regulation of maternally provided mRNA and proteins. One key aspect of the OET is the formation of the eggshell, a multi-layer structure that assembles immediately upon fertilization by sperm. Its assembly requires substantial coordinated reorganization of diverse protein and lipid structures within a very short time frame, a process that is only partially understood. A properly formed eggshell prevents polyspermy, facilitates passage through the spermatheca, is required for successful progression through meiotic divisions and establishing embryonic polarity (reviewed in Johnston and Dennis, 2012). Thus, the eggshell is an early and essential mediator of several key events in the OET.

To uncover previously unidentified regulatory mechanisms that are important during early embryogenesis, we conducted an unbiased small-molecule screen in *C. elegans*, which provides a multicellular developmental system in which to monitor small-molecule effects. In particular, a relatively sophisticated and detailed understanding of the cell biological processes that occur during the oocyte-to-embryo transition offers a context in which resulting phenotypes can be interpreted. Finally, the identification of a specific molecule affecting OET in *C. elegans* could potentially identify a class of related molecules that might disrupt the life cycle of other types of nematodes, such as parasitic species that have negative impacts on agricultural industries and human health.

This study focuses on a single compound from the screen, which we call C22. C22 had a striking and specific effect on embryogenesis without altering any other aspect of the *C. elegans* life cycle. Treatment with C22 resulted in complete embryonic lethality even at low concentrations; by contrast, development, overall stress resistance, aging, and fertility are unaffected in larvae and adults. Closer examination of the embryonic lethality revealed defects in proper formation of the eggshell, leading to osmotically sensitive embryos that never proceed beyond the ~100 cell stage. This phenotype is both inducible and reversible; moreover, the effects are limited to *C. elegans*: all tested strains of *C. elegans* are sensitive to C22, but other nematode species are resistant. A genetic suppressor screen identified the LET-607/CREBH transcription factor as a key mediator of this lethality. We demonstrate that *let-607* levels are induced in response to C22, and that C22 exposure leads to misregulation of key ER-to-Golgi trafficking genes in a *let-607*-dependent manner. We therefore propose that C22 disrupts normal trafficking of key components of eggshell development, and as such provides an important tool to investigate how this process functions during the oocyte-to-embryo transition.

¹Dept. of Genetics, Yale University School of Medicine, New Haven, CT 06520, USA. ²Center for Genomics and Systems Biology, Dept. of Biology, New York University, New York, NY 10003, USA. ³Dept. of Cell Biology, Yale University School of Medicine, New Haven, CT 06520, USA. ⁴Dept. of Developmental Biology and Dept. of Genetics, Washington University in St. Louis, St. Louis, MO 63110, USA. ⁵Dept. of Molecular Cell and Developmental Biology, Yale University, New Haven, CT 06520, USA. ⁶Yale Center for Molecular Discovery, West Haven, CT 06516, USA. ⁷Dept. of Biology, Fairfield University, Fairfield, CT 06824, USA. ⁸Division of Biological Sciences, New York University Abu Dhabi, Abu Dhabi, United Arab Emirates.

*Authors for correspondence (fp1@nyu.edu; kcg1@nyu.edu; valerie.reinke@yale.edu)

 V.R., 0000-0002-8967-2756

RESULTS

Screen for small molecules that disrupt *C. elegans* embryogenesis

We screened ~37,000 compounds from five different libraries (see Materials and Methods) for small molecules that disrupted early embryogenesis as outlined in Fig. 1A. Approximately 10 animals at the second stage of larval development (L2) were placed in individual wells of 384-well plates containing culture medium, *E. coli* as a food source and 16 μM of compound. After a 5 day incubation to allow for maturation and progeny production, we added 4-methylumbelliferyl-B-D-N,N',N''-triacylchito-trioside, a substrate that fluoresces upon exposure to chitinase (Robbins et al., 1988). We measured the absorbance at 620 nm to determine bacterial concentration (food uptake) as a proxy for growth, and the fluorescence at 355 nm excitation/460 nm emission to determine whether chitinase was released into the medium, as a measure of the number of hatching embryos. To identify compounds that specifically disrupt embryogenesis without affecting larval growth, we selected wells in which both measurements decreased significantly (see Materials and Methods). This primary screen identified 43 compounds that strongly reduced the hatching rate without affecting food uptake.

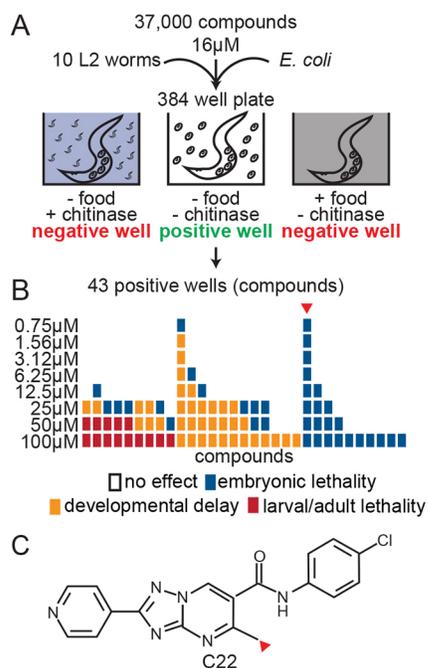


Fig. 1. Small-molecule screen for embryonic lethality identifies C22.

(A) A high-throughput screen was performed with a library of ~37,000 small molecules, with a phenotypic readout determined by the concentration of bacterial food (represented by gray, right well) and chitinase release (represented by blue, left well) during hatching. To define positive wells, we required both low food and chitinase readings (represented by clear middle well), as an indicator that treated worms were capable of feeding but incapable of producing viable embryos. (B) Worms were treated with titrated concentrations of the 31 positive compounds identified in the primary screen (false positives not depicted). Compound treatments were visually monitored and scored for reduction of growth rate (yellow), adult/larval lethality (red), and embryonic lethality (blue) or no effect (white). The compound ChemBridge 934555, called C22, displayed only embryonic lethality at all concentrations tested (red arrowhead). (C) The structure of C22. Red arrowhead indicates functionally important methyl group identified by structure-function activity assays.

We re-tested these 43 compounds at multiple concentrations (0.75-100 μM) in liquid culture, visually monitoring growth rates and larval and embryonic lethality. Of these, 22 resulted in extensive embryonic lethality at one or more concentrations, whereas nine exhibited larval growth delay or lethality prior to embryo production and 12 were false positives (Fig. 1B). Twelve of the 22 were set aside because they exhibited increasing severity of phenotypes as compound concentration increased, a trend that could be attributable to general toxicity. Ten compounds produced only embryonic lethality at any concentration. One compound in particular, called C22, produced complete embryonic lethality without other defects at all doses tested (Fig. 1B, red arrowhead). Because of this striking stage and phenotypic specificity, we focused on C22 for further characterization.

The chemical composition of C22 is $\text{C}_{18}\text{H}_{13}\text{N}_6\text{OCl}$, and the compound is arranged with a pyridine ring attached to a heterocyclic core, and an anilide group with a chlorine moiety (Fig. 1C). Structure-activity tests demonstrated that one key structural element is the adjacency of the methyl group on the heterocyclic core to the non-cyclic nitrogen group nearby (Fig. S1; red arrowhead in Fig. 1C). The active molecule does not have any obvious structural relationship to compounds with any effect in our screen, or any compounds with known activity.

C22 exposure rapidly and specifically results in embryonic lethality without affecting lifecycle and longevity

We tested C22 efficacy within solid medium at various doses, and found that a final concentration as low as 0.6 μM produced extensive embryonic lethality in wild-type worms (Fig. 2A). Subsequently, we used 5 μM as the standard concentration at which we perform assays on solid agar unless otherwise specified. We then measured the effects of C22 exposure on various features of the *C. elegans* lifecycle, including growth rates, longevity, stress response and egg production, and found these essentially unchanged (Fig. S2A-C). These observations suggest that C22 does not cause general cellular toxicity or organismal stress in somatic tissues. Moreover, microscopic examination of dissected gonads did not reveal any major defects in germ cell organization and morphology (Fig. S2D). Oocyte development and ovulation rates also appeared grossly normal.

We also investigated the effects of C22 on the related *Caenorhabditis* species *C. remanei*, *C. briggsae* and *C. brennerei*. Strikingly, all of these species were resistant to C22 at both 10 and 50 μM , whereas every one of 12 strains of *C. elegans* tested was sensitive to the compound and exhibited complete embryonic lethality (Fig. S3). Possibly, the compound is not taken up by these species; alternatively, these species have evolved such that the target or some downstream component is not present.

Based on the phenotype, C22 appears to act within a developmental window or event that focuses around the early embryo. This specificity led us to determine whether the effects of C22 were inducible and/or reversible (Fig. 2B,C). *C. elegans* exposed to C22 from larval stages to adulthood were moved to untreated plates and the production of live offspring (hatchlings) was assessed at 4 h intervals. Within 12 h, embryos began to hatch, and by 16 h, the lethality was completely reversed. Conversely, introducing C22 to adults after the onset of embryo production induced significant embryonic lethality by 12 h and reached 100% by 16 h. We conclude that C22 must act rapidly after exposure, because lethality corresponds to the amount of time it takes for an embryo to go from fertilization to hatching (12 h under these

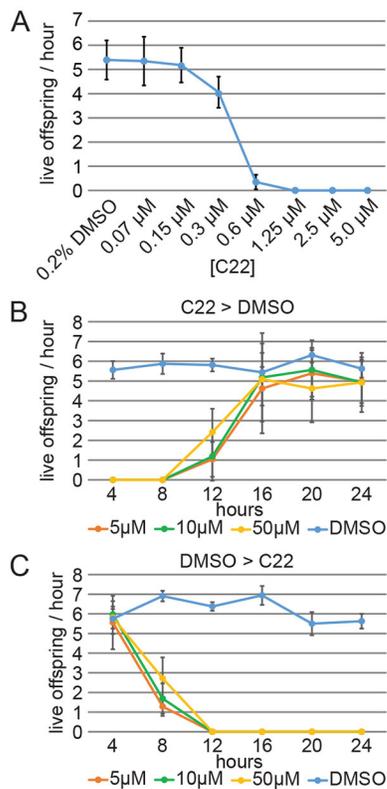


Fig. 2. C22 rapidly and reversibly induces embryonic lethality at low doses. (A) Dosage curve measuring the live offspring produced per hour from WT hermaphrodite worms at concentrations of C22 indicated on the x-axis. Error is measured as s.d. from the mean ($N=4$). (B) WT L1 larvae were grown to young adult (YA) stage on plates containing either 5 μM (orange), 10 μM (green), 50 μM (yellow) C22 or a 0.2% DMSO control (blue) and then transferred to plates with 0.2% DMSO at 4 h intervals and embryonic viability was monitored. Embryonic viability is observed on all plates around 12 h post transfer and 100% viability at 16 h post transfer. Error bars represent s.d. ($N=8$). (C) WT L1 larvae were grown to young adult (YA) stage on plates containing 0.2% DMSO and then transferred to plates with 5 μM (orange), 10 μM (green), 50 μM (yellow) of C22 or 0.2% DMSO (blue) at 4 h intervals and embryonic viability was monitored. Embryonic lethality is observed on all plates containing C22 around 8 h post transfer and 100% C22-induced lethality is observed at 12 h post transfer. Error bars represent s.d. ($N=8$).

conditions), and conversely, is turned over rapidly, as its effects do not persist.

As expected, direct exposure of embryos to C22 had no effect on viability, indicating that C22 is acting through the parent at some point prior to eggshell formation. We therefore tested whether the compound might enter through the vulva and act directly on mature gametes at or just before fertilization, by exposing *let-23(n1045)* null mutant animals, which are Vul (vulvaless), to C22. Like the wild type, *let-23* mutants were sensitive to C22 and displayed 100% embryonic lethality (Fig. S4). This observation indicates that the compound is likely to be taken up by the adult, but through some other path besides the vulva, and is presumably ingested with the bacterial food source. In sum, the effect of C22 on progeny is mediated through the parent, but does not have any apparent effect on parental physiology.

C22 disrupts eggshell morphology and function

We next characterized the defect underlying the embryonic lethal phenotype. Embryos from C22-treated animals do not proceed beyond the ~100 cell stage. Imaging of early embryonic

development under standard mounting conditions (i.e. mounted with a coverslip) resulted in embryos with variable cell division defects (Movie 1). These defects were more severe if the embryos were dissected from the parent, and less severe if filmed *in utero*, indicating that the embryos were sensitive to pressure, osmolarity, or some other condition of imaging. To determine if imaging conditions were the cause of the cell division defects, we imaged under minimal pressure in osmotically balanced medium, and found that both meiosis and the initial cell divisions proceed normally in C22-affected embryos under these conditions (Movie 2). By contrast, imaging in hypo- or hypertonic solution resulted in swelling or shrinking, respectively, of C22-treated but not DMSO-treated control embryos (Fig. S5A). This phenotype often occurs if eggshell assembly or function is defective (Carvalho et al., 2011; Edgar, 1995; Johnston et al., 2006; Olson et al., 2006; Parry et al., 2009). We therefore examined eggshell integrity using DAPI incorporation, which is normally excluded from live embryos (Rappleye et al., 1999; Fig. 3A). Embryos from C22-treated parents readily incorporated DAPI at various stages of development, whereas controls remain unstained. In this analysis, the C22-treated embryonic blastomeres were also demonstrably swollen relative to control blastomeres, as often occurs with compromised eggshells.

The eggshell has multiple layers that must form properly as soon as fertilization occurs, including an outer chitin-rich layer, two layers each marked with the related but distinct chondroitin proteoglycans CPG-1 and CPG-2, and an inner, poorly characterized permeability layer, all of which are separated from the embryo proper by the peri-embryonic space (Olson et al., 2012). CPG-1 and CPG-2 have very different localization patterns within their domains: CPG-1 forms a thin ring around the embryo, whereas CPG-2 fills much of the peri-embryonic space but is still held apart from the embryo by the permeability layer (Olson et al., 2012; Fig. 3B, WT). We examined the localization of these two proteins in embryos from C22-treated adults using diSPIM microscopy optimized for imaging *C. elegans* embryos under pressure-free conditions (Kumar et al., 2014). Strains carrying mCherry-tagged CPG-1 or CPG-2 along with a GFP-tagged cell membrane marker were treated with C22, and imaged at the one-cell stage via diSPIM microscopy (Fig. 3B). C22 exposure altered the localization of CPG-2::mCherry, but not CPG-1::mCherry. Specifically, CPG-2::mCherry now filled the peri-embryonic space and was not excluded from regions near the cell membrane (Fig. 3B, compare white arrows and red arrows), indicating that the permeability barrier had not formed or was not functioning properly. Taken together, these observations suggest that defective eggshell formation, specifically improper or incomplete development of the permeability barrier, prevents the embryo from maintaining the correct osmolarity and resistance to pressure. Notably, osmotic sensitivity is often accompanied by polarity defects (Rappleye et al., 1999). We investigated whether C22 disrupted polarity by examining localization of anterior and posterior polarity markers (PAR-2 and PAR-6, respectively), as well as P granule segregation. Somewhat surprisingly, all these markers were normally distributed in C22-treated embryos (Fig. S5A,B). Thus, whether additional defects ultimately contribute to C22-induced embryonic lethality is not known at this time.

The CREBH-related transcription factor LET-607 is required for the embryonic lethal phenotype of C22

To find potential target molecules or pathways through which C22 acts, we performed a forward genetic screen to recover suppressors of the embryonic lethality in the presence of C22. We screened

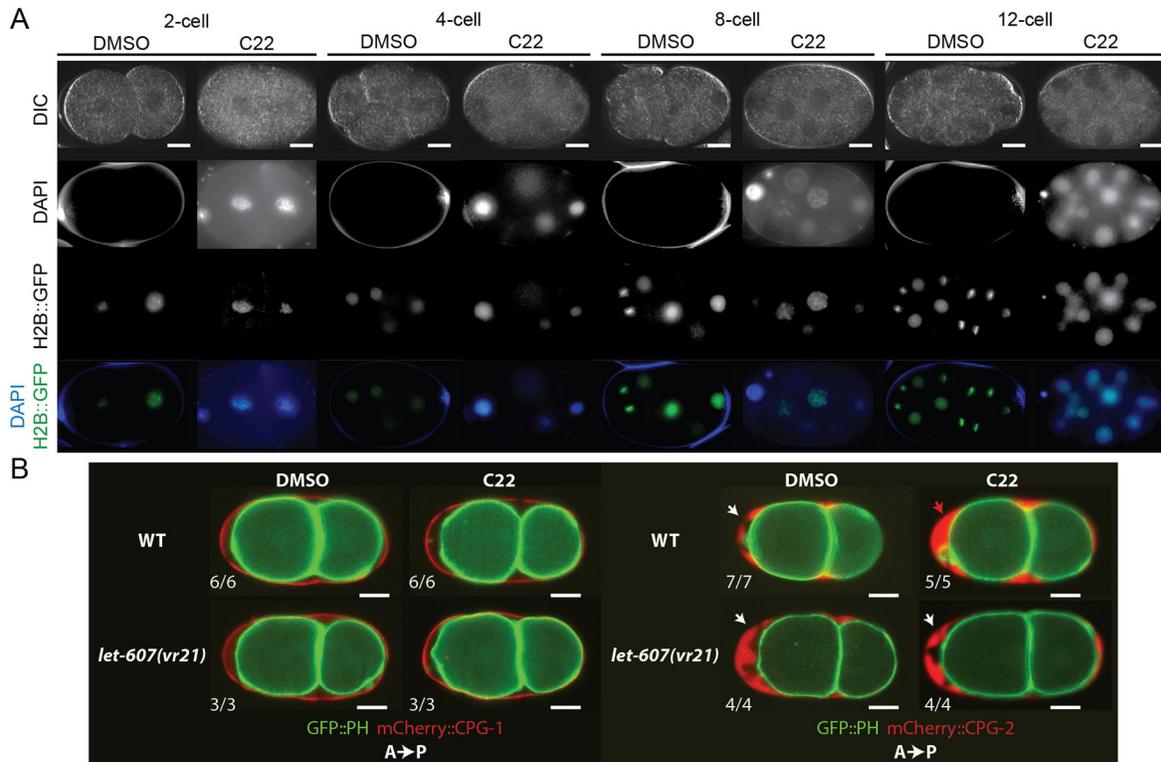


Fig. 3. C22 exposure results in impaired eggshell integrity and CPG-2 mislocalization. (A) GFP::H2B embryos at the 2-, 4-, 8- and 12-cell stages dissected from YA worms grown in the presence of 0.2% DMSO or 5 μ M C22 ($N > 50$ per stage/condition). First row, DIC; second row, DAPI; third row, GFP::H2B; fourth row, GFP::H2B merged with DAPI images. (B) GFP::PH; mCherry::CPG-1 or GFP::PH; mCherry::CPG-2 embryos dissected from YA worms grown in the presence of 0.2% DMSO or 5 μ M C22 carrying either WT *let-607* or *let-607(vr21)*. White arrow indicates peri-embryonic space in WT embryos; red arrow indicates sites where the peri-embryonic space is lost after C22 treatment. Numbers indicate embryos displaying this phenotype/number of embryos assayed. Scale bars: 10 μ m.

pools of mutagenized F2 worms exposed to C22 and selected live, hatched progeny (Materials and Methods). To date, we have characterized one mutation in detail. This mutation, *vr21*, completely suppresses the phenotype of embryonic lethality and results in viable, slow-growing worms with impaired fertility (Fig. S6). Genome-wide sequencing identified a mutation in the noncoding sequence upstream of the short isoform of *let-607*, which encodes a CREBH-related transcription factor (Fig. 4A). We confirmed that loss of *let-607* activity rescued the C22 phenotype using an independent genetic assay. A null allele of *let-607*, *h402*, is embryonic and early larval lethal, which prevents its use in a rescue experiment. We therefore performed a partial knockdown of *let-607* using RNAi (at 25% strength) and substantially rescued C22-induced embryonic lethality, with viability exceeding 80% (Fig. 4B). After hatching, the *let-607(RNAi)* animals exhibit a slow growth phenotype similar to the *let-607(vr21)* allele. Finally, CPG-2::GFP localization in this background was returned to the wild-type pattern, with gaps between CPG-2::GFP and the cell membrane, suggesting that the permeability barrier was intact (Fig. 3B). Together, these data indicate that a reduction of *let-607* activity is indeed responsible for the suppression of C22-induced embryonic lethality.

The mutation upstream of the short isoform of *let-607*, along with suppression of C22-induced embryonic lethality by *let-607(RNAi)*, suggests that *let-607* levels might be altered in response to C22. Indeed, upon C22 treatment, *let-607* expression is upregulated in wild-type but not mutant adults, a response that is even greater in embryos (Fig. 4C). The continued presence of some *let-607* transcript in the *vr21* background suggests that this mutation

primarily prevents induction in response to C22, but does not abolish basal *let-607* expression.

let-607 expression was not induced by C22 treatment in either *C. briggsae* or *C. remanei* (Fig. 4D). Moreover, these genomes lack an obvious orthologous site encompassing the *vr21* mutation (Fig. S7A,B), despite existing within a genomic region that shows conserved organization overall, with multiple related sequences in the same order and spacing relative to the start site of *let-607* as found in *C. elegans* (Fig. S7C). These differences are consistent with the lack of a phenotype in these species, although we cannot rule out a failure to take up the compound at this point.

From published genomic expression assays, *let-607* appears to be broadly expressed, and is present in both the germ line and soma. To determine in which tissue(s) *let-607* is acting in response to C22 treatment, we performed *let-607* RNAi in the *rff-1(pk1417)* mutant background, which restricts RNAi to the germ line (Sijen et al., 2001). Even when RNAi was inactive in the soma, we still observed rescue of C22-induced embryonic lethality by *let-607* RNAi (Fig. 4E). This observation indicates that the major site of action for *let-607* in this response is likely to be the parental germ line. In sum, *let-607(vr21)* potentially disrupts regulation of *let-607* in response to C22 in *C. elegans*, and the data suggest that upregulation of *let-607* in the germ line upon C22 exposure contributes to the embryonic lethal phenotype.

LET-607 does not act through the UPR to mediate the C22-induced phenotype

LET-607 is an ortholog of mammalian CREBH, which is involved in ER stress responses, including the unfolded protein response (UPR)

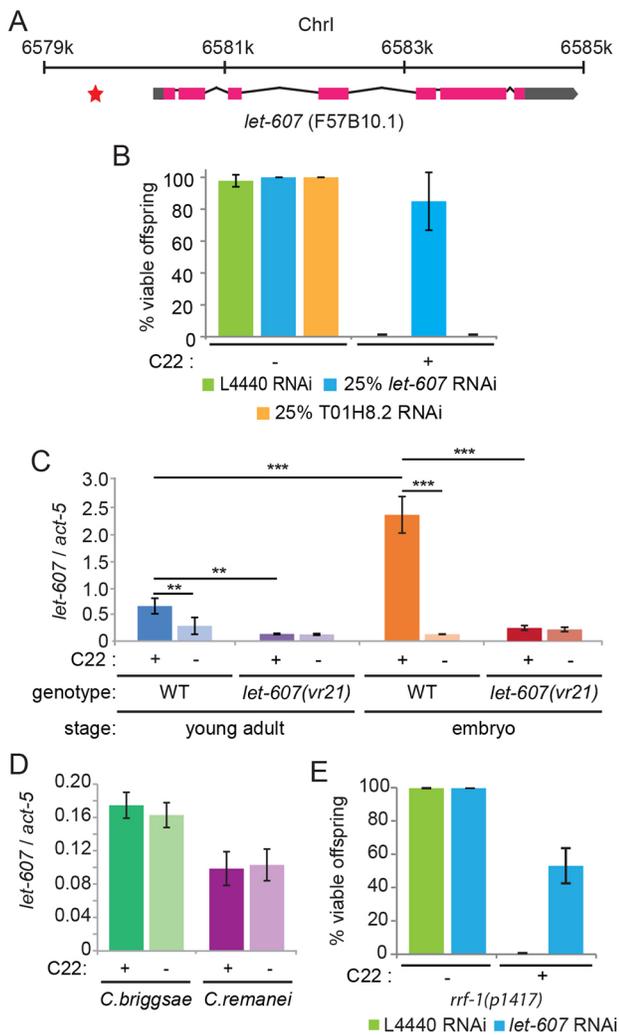


Fig. 4. C22-induced embryonic lethality is mediated by the CREBH transcription factor LET-607. (A) *C. elegans let-607* gene structure. Annotated UTRs are labeled in gray, exons in pink and introns as lines. The *vr21* mutation is located upstream, indicated by the red star. (B) Percentage of viable offspring produced from WT worms grown in the presence of 0.2% DMSO or 5 μ M C22 and treated with RNAi for the empty vector L4440 (green), *let-607* (blue) or a random target T01H8.2 (orange) ($N \geq 19$). (C) *let-607* transcript levels relative to *act-5* transcript levels (*let-607/act-5*) detected by RT-qPCR from WT (blue) and *let-607(vr21)* (purple) young adult (YA) worms, and embryos from WT (orange) and *let-607(vr21)* worms grown in the presence of 0.2% DMSO or 5 μ M C22 ($N=3$ biological replicates). (D) Species-specific *let-607* transcript levels relative to *act-5* (*let-607/act-5*) detected by RT-qPCR from YA *C. briggsae* (green) and *C. remanei* (purple) worms grown in presence of 0.2% DMSO or 5 μ M C22 ($N=3$ biological replicates). (E) Percentage of viable offspring produced from *rrf-1(pk1417)* worms grown in the presence of 0.2% DMSO or 5 μ M C22 and treated with RNAi for the empty vector L4440 (green) or *let-607* (blue) ($N \geq 8$). Error bars represent s.d. Statistical significance is indicated by star over the compared samples (** $P < 0.01$; *** $P < 0.0005$).

(Shen et al., 2005; Silva et al., 2011). CREBH is typically localized in the ER membrane, and upon ER stress, relocates to the nucleus and activates stress-related genes, including those that encode UPR factors, as well as many that function in protein trafficking (Barbosa et al., 2013), suggesting that either or both of these processes may be involved in C22-mediated embryonic lethality.

Previous studies indicate that LET-607 regulates the UPR in *C. elegans*, and can both be activated by the UPR and also boost the

UPR (Shen et al., 2005). We therefore examined whether C22 treatment phenocopied the UPR response and vice versa. C22 treatment led to the induction of known UPR factors *atf-6*, *ire-1* and *pek-1* in wild-type but not *let-607(vr21)* mutant animals (Fig. 5A), suggesting that the UPR is induced by C22 via *let-607*. However, loss of the activity of UPR components *atf-6*, *ire-1*, *pek-1* or *xbp-1* did not rescue the C22-mediated lethality, indicating that the UPR is not essential for the embryonic lethality. Moreover, treatment with tunicamycin, which induces the UPR and causes embryonic lethality, does not result in the same eggshell defects as seen with C22 treatment, as monitored by DAPI incorporation (Fig. 5B). Finally, tunicamycin-induced embryonic lethality cannot be rescued in the *let-607(vr21)* mutant background (Fig. 5C). Altogether, these results indicate that *let-607* is not acting primarily through the UPR to induce embryonic lethality in response to C22 exposure.

LET-607 activates genes involved in protein trafficking

Because LET-607 is predicted to be a transcription factor, we performed CHIP-seq in young adults (YA) to identify candidate LET-607 target genes in an unbiased manner (Materials and Methods) (Fig. 6A). At the peak significance cut-off defined by the modENCODE project (Boyle et al., 2014), we identified 1259 coding genes with LET-607 binding sites within 1.5 kb of the transcription start site (Gene Expression Omnibus, accession number GSE84419). Gene Ontology (GO) analysis of the subset of these candidates with both 100 \times binding enrichment and a binding site within 500 bp (GEO, GSE84419) identified several

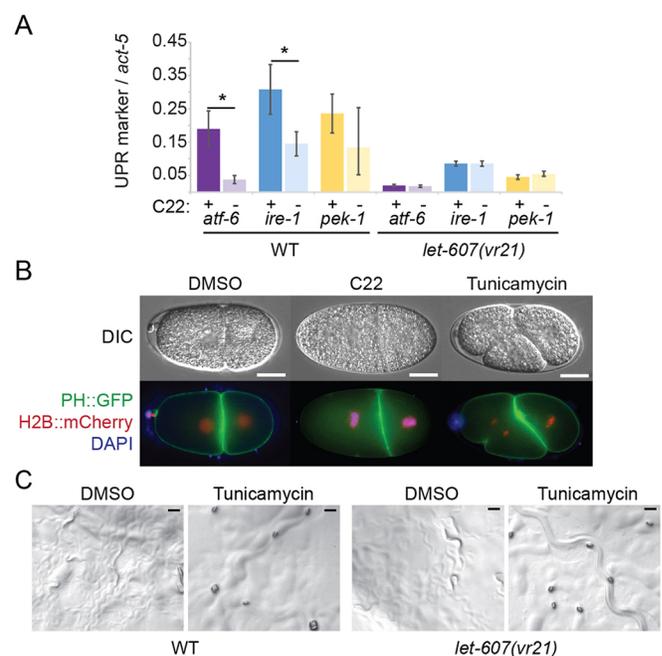


Fig. 5. The UPR is not essential for C22-induced embryonic lethality. (A) Transcript levels for UPR markers *atf-6* (purple), *ire-1* (blue) and *pek-1* (yellow) relative to *act-5* abundance detected by RT-qPCR from young adult WT and *let-607(vr21)* worms grown in the presence of 0.2% DMSO or 5 μ M C22. Error bars represent s.d. ($N=3$ biological replicates). * $P < 0.05$. (B) PH::GFP; H2B::GFP embryos dissected from YA worms grown in the presence of either 0.2% DMSO, 5 μ M C22 or 50 μ g ml $^{-1}$ tunicamycin then stained with DAPI to determine the effect on UPR induction during eggshell formation. DIC and merged image are shown ($N > 50$). (C) WT and *let-607(vr21)* worms treated with either 50 μ g ml $^{-1}$ tunicamycin or 0.2% DMSO. Offspring viability was recorded 48 h after egg laying began. Scale bars: 10 μ m (B) and 50 μ m (C).

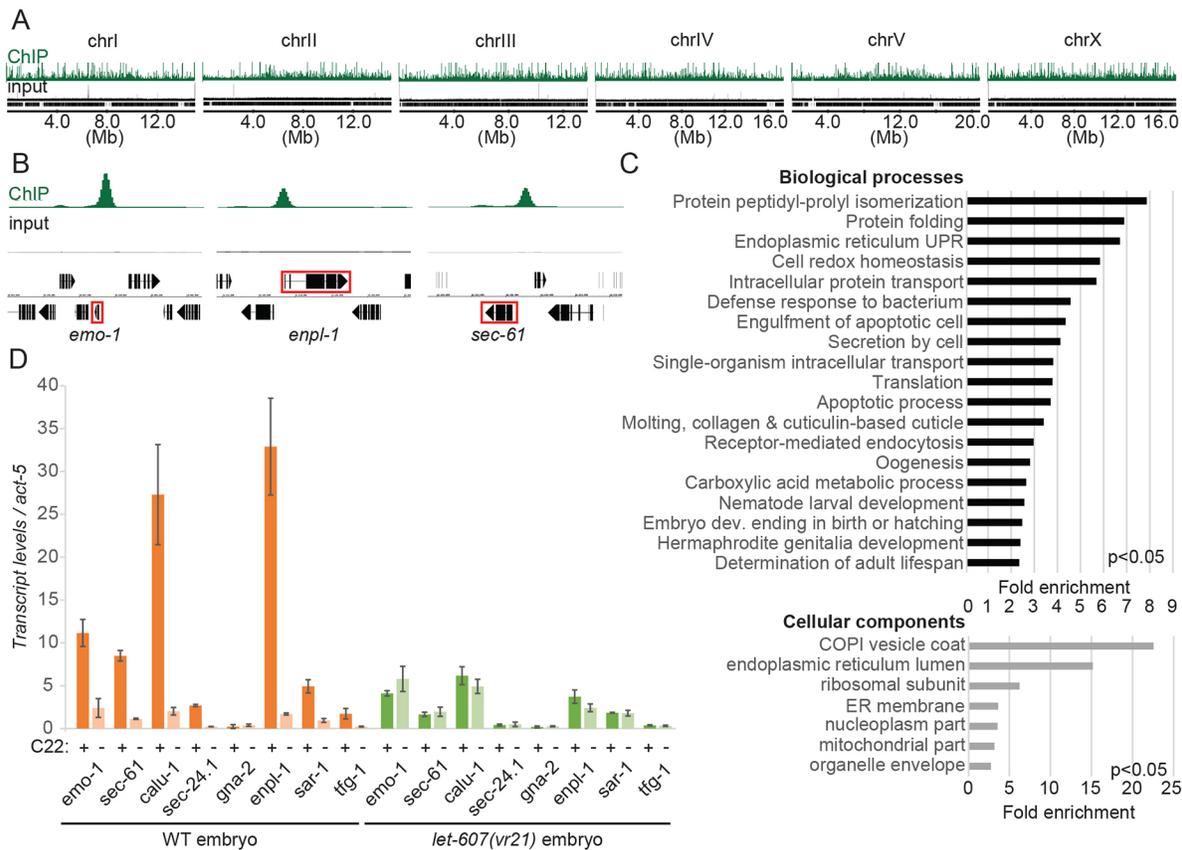


Fig. 6. LET-607 regulates protein trafficking in C22-treated embryos. (A) Normalized sequence read abundance for LET-607::GFP ChIP-seq (green) and an input control (black) over all six *C. elegans* chromosomes (relative chromosome coordinates illustrated for each chromosome below). (B) Representative LET-607 binding at called targets (outlined in red), black arrow indicates direction of transcription. (C) GO analysis of called LET-607 targets for Biological Process and Cellular Component categories. Reported groups express greater than 2-fold enrichment and $P < 0.05$ (Bonferroni corrected). (D) Transcript levels for selected LET-607 targets by RT-qPCR analysis in WT and *let-607(vr21)* embryos from young adult worms grown in the presence of 5 μM C22 (+) or 0.2% DMSO (-). Error bars represent s.d. ($N=3$ biological replicates).

highly enriched categories centering around protein trafficking, including those encoding Golgi and ER components, as well as components involved in protein transport, vesicles and endosomes (Fig. 6B,C). These categories mostly include factors predicted or demonstrated to function at diverse steps during protein trafficking. To highlight just a few, LET-607 binds genes encoding multiple components of the complex that translocates elongating peptides into the ER (SEC-61, EMO-1, and TRAP-1, -2, -3, -4), chaperone proteins in the ER (HSP-3 and -4), factors that form the COPII coat found on vesicles traversing from the ER to Golgi (SEC-23, SEC-24-1, NPP-20, SEC-31 and SAR-1), factors that form the conserved oligomeric Golgi (COG) complex (COGC-2 and COGC-6), factors that form the COPI coat found on retrograde vesicles (COPB-1, COPD-1, COPE-1 and COPZ-1), endocytic components (RAB-11.1, RAB-5, RME-4), as well as a variety of important regulatory factors, including TFG-1, ARF-1 and components of the UPR (PEK-1, XBP-1 and IRE-1).

Notably, mutation of genes encoding trafficking factors often results in embryonic lethality due to osmotic sensitivity, very similar to the phenotype induced by C22 (Green et al., 2011; Hanna et al., 2013; Sato et al., 2006). This association strongly suggests that defects in protein trafficking underlie the C22-induced embryonic lethality. We therefore selected a subset of LET-607 candidate genes known to be involved in trafficking (*emo-1*, *sec-61*, *calu-1*, *sec-24.1*, *enpl-1*, *sar-1* and *tfg-1*) as well as one involved directly in eggshell development (*gna-2*), and tested whether their expression

was affected by C22 exposure in wild-type and *let-607(vr21)* mutant embryos (Fig. 6D). The expression levels of all of the genes involved in trafficking are increased upon C22 treatment in the wild type, but not in the *let-607(vr21)* mutant background, whereas *gna-2* was not affected by C22. Overall, these findings are consistent with aberrant regulation of protein trafficking underlying the embryonic lethality caused by C22. Given the importance of the secretory pathway in eggshell development (Hanna et al., 2013), the data support the concept that one or more components of the eggshell are not successfully transported to or from the embryo surface in a timely fashion or in the proper abundance for a fully developed eggshell.

DISCUSSION

Through an unbiased chemical screen, we identified C22, a small molecule that has a remarkably strong and specific effect on key events in OET in *C. elegans*. The embryonic lethality that results from even relatively low concentrations of C22 is completely penetrant, whereas other aspects of *C. elegans* development and lifespan appear unaffected, even at very high concentrations. The earliest detectable effect in embryos is osmotic sensitivity due to a permeable eggshell. This phenotype often occurs when factors required for the secretory pathway have impaired or reduced function (Hanna et al., 2013); in line with this observation, a genetic suppressor screen identified LET-607, a conserved CREBH-like transcription factor that targets genes encoding key ER and Golgi

proteins, as an essential mediator of the embryonic lethality. Our data suggest that C22 treatment results in induction of *let-607* expression. Higher levels of LET-607 lead to inappropriate upregulation of a variety of target genes involved in protein trafficking in the early embryo. This aberrant regulation presumably causes an imbalance in trafficking that affects at least one component required for proper eggshell assembly, particularly formation of the permeability barrier and ultimately leads to osmotic sensitivity and embryonic lethality.

Mutation of an upstream sequence in the strain *let-607(vr21)* and subsequent lack of *let-607* induction is sufficient to completely suppress the embryonic lethality of C22 treatment, indicating that the upregulation of *let-607* is essential for the phenotype. Currently, the mechanism by which C22 treatment results in activation of *let-607* expression is unclear. A relatively simple explanation is that C22 somehow directly affects the transcriptional regulation of *let-607*, for instance by interacting with a hypothetical regulatory factor. Alternatively, C22 could interfere with protein trafficking and indirectly affect *let-607* expression. Both LET-607 and its mammalian ortholog CREBH (Shen et al., 2005) can be induced by treatment with tunicamycin or DTT, mediators of the unfolded protein response (UPR). The UPR might be triggered if C22 directly targets a secreted protein and blocks its progress through the ER or Golgi, leading to *let-607* induction. Consistent with this possibility, we found that additional components of the UPR, such as *ire-1* and *xbp-1*, are induced by C22 treatment. However, inappropriate induction of the UPR does not explain the C22-mediated embryonic lethal phenotype, as tunicamycin treatment does not cause osmotic sensitivity. Furthermore, mutating other components of the UPR besides *let-607*, such as *xbp-1*, *pek-1*, *ire-1* or *atf-6*, is not sufficient to suppress the C22 phenotype. Together, these observations rule out the UPR as the cause of the embryonic lethality, but it is still possible that the UPR causes the initial induction of *let-607* expression. Additional experiments to identify the direct molecular target of C22 will be necessary to distinguish between these possibilities.

Once C22 induces its expression, LET-607 apparently leads to upregulation of target genes, many of which encode secretory and endocytic pathway components. Disruption of these pathways in *C. elegans* cause pleiotropic effects in many different cell types, including developing germ cells, coelomocytes, intestine cells, epithelia and neurons (e.g. Ackema et al., 2013; Hoover et al., 2014; Patel and Soto, 2013). Given the broad importance of this process, the spatial and temporal specificity of C22-induced embryonic lethality is remarkable. Loss of *let-607* activity in the germ line is sufficient to suppress the effects of C22, indicating that this tissue is the major site of action. C22 does not enter through the vulva, and is probably ingested with food, possibly passing directly through the intestine to the germ line along with yolk proteins. Notably, yolk import from the intestine to oocytes was not noticeably impaired in C22-treated animals (Fig. S8), suggesting that yolk secretion from the intestine to the germline was unaffected. Even though C22 appears to act in the germ line, it shows no significant effect in developing and proliferating germ cells, as would be expected if protein trafficking were disrupted (Ackema et al., 2013). These observations suggest that C22 does not have a global effect on trafficking in most cell types. Instead, we suggest that the interaction of C22 with its direct molecular target occurs in the germ line and/or early embryo, and this interaction, in turn, leads to upregulation of LET-607 and consequent stage-specific disruption of processing or trafficking of some specific component during eggshell development.

One of the earliest and most important events of the OET is the formation of the eggshell immediately upon fertilization. The eggshell is a multi-layered structure and its assembly requires a series of coordinated events in which many different components are brought to and retrieved from the cell membrane through the protein trafficking network in the one-cell embryo. Given the prominent sorting and trafficking of the many protein components of the eggshell layers, C22 might disrupt the progress of one or more of these important cargoes. Notably, C22-treated embryos still retain a refractile eggshell and normal polarity, and at least two important eggshell components, CPG-1 and CPG-2, were successfully trafficked to the cell surface, indicating that much of the eggshell is still assembled. We have not yet identified a specific component whose localization might be affected by C22, but the aberrant CPG-2 pattern we identified indicates that the permeability barrier is not intact. The genes encoding the lipid biosynthetic enzymes and modifiers known to promote formation of the permeability barrier, such as *pod-2*, *fasn-1*, *emb-8*, *cyp-31a2/cyp-31a3*, *perm-1* and *dgr-1* (Olson et al., 2012) are not bound appreciably by LET-607, suggesting that the mechanism of LET-607-mediated repression is not through their regulation.

The complete suppression of C22-induced embryonic lethality by prevention of LET-607 upregulation argues that LET-607 inappropriately triggers a detrimental response in the embryo. Because LET-607 appears to act as a master regulator of many different factors involved in the secretory process, from the earliest stages of ER targeting to exo- and endocytosis, it will be necessary to examine many different steps of trafficking to determine whether any particular step is specifically disrupted in C22-treated embryos. Interestingly, while previous experiments demonstrated that partial reduction of genes involved in protein trafficking results in osmotic defects in the embryo, C22 induces the same defect in the opposite manner, by increasing expression of genes involved in protein trafficking via LET-607. Therefore, overexpression and reduction of protein trafficking components appear to have a similar outcome. The many quality control checks and feedback pathways that occur during trafficking are probably sensitive to any impairment or imbalance in the sorting process.

In the future, C22 treatment and the *let-607* regulatory mutation that we identified will be valuable tools to further dissect mechanisms regulating trafficking in the early embryo. In particular, identification of the direct target of C22 will lead to significant insight into how specificity of the response is achieved. We note that since rapid evolution of the molecules required for fertilization can be an important mechanism for speciation, it is possible that the species-specific action of C22 is due to a target that is fast-evolving or found exclusively in *C. elegans* strains and not in other related nematode species. Importantly, the reversibility and specificity of C22 makes it useful in the laboratory for other studies examining diverse aspects of the OET, as it can be used to stage embryos. Beyond this specific developmental stage, C22 can be used in studies such as aging assays, where it could serve as an alternative to FUDR treatment to block reproduction (Gandhi et al., 1980; Mitchell et al., 1979). FUDR can be problematic as it has been shown to artificially enhance lifespan in several mutant backgrounds (Aitlhadj and Stürzenbaum, 2010; Anderson et al., 2016; Davies et al., 2012; Van Raamsdonk and Hekimi, 2011). C22 has the added advantage of not altering rates of germ cell proliferation, which can also affect lifespan. In sum, we have identified a novel compound that demonstrates extraordinary specificity even while affecting fundamental cell biological processes, opening new opportunities for probing how cells

change in response to specialized developmental events. Ultimately, studies using C22 might point us toward analogous small molecules with similar functions in related, less characterized parasitic nematodes that are relevant in agriculture and human disease.

MATERIALS AND METHODS

Strains

N2 Bristol strain was used as WT. Full details of all other transgenic and variant strains are listed in supplemental Materials and Methods.

Compound screen

A total of ~37,000 compounds from the Yale Pilot library, MicroSource Gen-Plus and Natural Products (Gaylordville, CT), ChemBridge (San Diego, CA) Small MW and DIVERSet libraries, and Maybridge (Thermo Fisher Scientific, Waltham, MA) Diversity library were screened. Individual compounds were added uniquely (40 nl of 10 mM compound in DMSO) to each well of a 384-well plate containing 5 μ l S-basal medium, to a final concentration of 16 μ M using the Tecan Aquarius (Durham, NC) with a 384-well pin tool (V&P Scientific, San Diego, CA). A total of 20 μ l of *C. elegans* L2 stage larvae diluted in S-basal medium and *E. coli* (OP50) grown to $A_{620}=0.38$ were added to the assay plates using the Aquarius with wide-bore 200 μ l tips, for a total volume of 25 μ l/well and approximately 10 worms/well. Then 1 μ l of 2.6 mM 5-fluorouracil was added to positive control wells for a final concentration of 100 μ M. A toxicity control was also performed by adding 1.5 μ l DMSO to the well for a final concentration of 5.7%. The A_{620} after mixing was then noted for each well (day 1). After incubation for 4-5 days at 20°C to allow worm growth and reproduction, the A_{620} was read again and compared with Day 1 ($A_{620}=0.38$). The substrate 4-methylumbelliferyl-B-D-N,N',N''-triacetylchito-trioside was then added to a final concentration of 10 μ M and plates were incubated at 37°C and quenched after 1 h with stop solution (1 M glycine/1 M NaOH). Fluorescence was then read at $A_{355\text{ nm}}$ (excitation) and $A_{460\text{ nm}}$ (emission). All values were compared with control wells, which contained 100 μ M 5-fluoro-uracil (5-FU), 5.7% DMSO or 0.27% DMSO. 5-FU prevents embryo production but not larval-to-adult growth and therefore mimics the profile of an effective compound. DMSO at 5.7% kills worms and mimics toxicity by candidate compounds. DMSO at the screening concentration of 0.27% in the absence of compound has minimal impact on growth or reproduction and functions as a negative control. The values from individual wells containing tested compounds were compared with the control values and represented as % effect. For the fluorescence measurements at 355/460 nm, compounds were compared to 5-FU control wells. For A_{620} , differences in absorbance between day 1 and day 5 were compared for 5.7% DMSO and 0.27% DMSO. Little change in bacterial concentration occurs at 5.7% DMSO (set to 100% effect), while the change is significant at 0.27% DMSO (set to 0% effect). C22 (YU134916/ChemBridge 934555) had a percentage effect of 320% ($A_{355/460}$) and 5.6% (A_{620}), from a plate with an average Z' of 0.72.

Genetic suppressor screen

To identify genetic suppressors of the C22-induced embryonic lethality, we performed a screen in which 15,000 L4 larvae (P_0) were exposed to 47 mM EMS for 4 h. These animals were allowed to lay eggs for ~16 h before being washed off plates. The F_1 progeny (~300,000) were grown to gravid adults on 15 cm plates (~20,000 larvae/plate), and allowed to lay eggs for ~1 h. Approximately 600,000 F_2 larvae were plated non-clonally, distributed onto 92 C22-treated plates. Plates with viable F_3 progeny were kept as potentially containing homozygous suppressor mutations. Initial positives were further analyzed by determining whether viable progeny were produced at equivalent rates in the presence or absence of C22; mutants exhibiting roughly similar rates of progeny production in either situation were further analyzed. Complementation analysis defined three groups, of which we followed up on one. One representative candidate mutation, initially called 72, was mapped using standard SNP mapping (Swan et al., 2002) to Chr I, with the highest likelihood between 8 and 10.7 MB. Methods for whole-genome sequencing, verification of the mutation and primers used for qPCR are described in the supplemental Materials and Methods.

Chemical treatment

All C22 treatments subsequent to the chemical screen were performed on nematode growth medium (NGM) agar plates containing 5 μ M C22 and 0.2% DMSO (v/v). Just prior to pouring plates, 200 μ l of 12.5 mM C22 in DMSO was added to 100 ml of autoclaved NGM, cooled to ~50-55°C. Plates were stored at room temperature and protected from light. L1 worms were added and observed at stages noted. For heat shock experiments, uncoordinated worms were defined as worms that did not move forward or backward.

diSPIM microscopy

CPG localization in embryos was determined using OD344, OD367, YL592 and YL593 strains. YA worms were dissected in M9 to access the embryos. Embryos were then transferred to a poly-L-lysine-coated coverslip to immobilize the embryos in a dual-view inverted selective plane illumination microscope (diSPIM) imaging chamber filled with M9 solution. Localization of CPG-1 and -2 in three embryos per genotype and condition was captured on a diSPIM system (Applied Scientific Instrumentation; Kumar et al., 2014) with 40 \times NA 0.8 WD 3.5 mm objectives (Nikon), Orca Flash 4.0 Cameras (Hamamatsu-Model C11440-22CU) and a MLC400B Monolithic Laser Combiner (Agilent Technologies). Micro-Manager 1.4 and the ASI diSPIM Control plugin were used to operate the diSPIM System (Edelstein et al., 2014). CPG-1 and -2 images were captured with a volume of 50 slices with a step size of 1 μ m for both 488 nm and 561 nm excitation wavelengths. Images were analyzed and processed with ImageJ and FIJI (Schindelin et al., 2012).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

V.R. conceived the study; S.E.W., A.M., M.M., P.G.C., M.K., L.A., M.G., Z.P., S.B., A.G.F. performed experiments; S.E.W., A.M., Z.P., J.M., M.G., P.G.C., K.C.G., F.P. and V.R. analyzed data; S.E.W., A.M., P.G.C., K.C.G. and V.R. designed experiments; S.E.W. and V.R. wrote the paper.

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Data availability

LET-607 ChIP-seq data are available in NCBI Gene Expression Omnibus (GEO) under accession number GSE84419 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84419>).

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.140046.supplemental>

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