A Strategy To Isolate Modifiers of Caenorhabditis elegans Lethal Mutations: Investigating the Endoderm Specifying Ability of the Intestinal Differentiation GATA Factor ELT-2

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ABSTRACT The ELT-2 GATA factor normally functions in differentiation of the C. elegans endoderm, downstream of endoderm specification. We have previously shown that, if ELT-2 is expressed sufficiently early, it is also able to specify the endoderm and to replace all other members of the core GATA-factor transcriptional cascade (END-1, END-3, ELT-7). However, such rescue requires multiple copies (and presumably overexpression) of the end-1p::elt-2 cDNA transgene; a single copy of the transgene does not rescue. We have made this observation the basis of a genetic screen to search for genetic modifiers that allow a single copy of the end-1p::elt-2 cDNA transgene to rescue the lethality of the end-1 end-3 double mutant. We performed this screen on a strain that has a single copy insertion of the transgene in an end-1 end-3 background. These animals are kept alive by virtue of an extrachromosomal array containing multiple copies of the rescuing transgene; the extrachromosomal array also contains a toxin under heat shock control to counterselect for mutagenized survivors that have been able to lose the rescuing array. A screen of /C24 produced 17 independent surviving strains. Whole genome sequencing was performed to identify genes that incurred independent mutations in more than one surviving strain. The C. elegans gene tasp-1 was mutated in four independent strains. tasp-1 encodes the C. elegans homolog of Taspase, a threonine-aspartic acid protease that has been found, in both mammals and insects, to cleave several proteins involved in transcription, in particular MLL1/trithorax and TFIIA. A second gene, pqn-82, was mutated in two independent strains and encodes a glutamine-asparagine rich protein. tasp-1 and pqn-82 were verified as loss-of-function modifiers of the end-1p::elt-2 transgene by RNAi and by CRISPR/Cas9-induced mutations. In both cases, gene loss leads to modest increases in the level of ELT-2 protein in the early endoderm although ELT-2 levels do not strictly correlate with rescue. We suggest that tasp-1 and pqn-82 represent a class of genes acting in the early embryo to modulate levels of critical transcription factors or to modulate the responsiveness of critical target genes. The screen’s design, rescuing lethality with an extrachromosomal transgene followed by counterselection, has a background survival rate of <10^-4 without mutagenesis and should be readily adapted to the general problem of identifying suppressors of C. elegans lethal mutations.

KEYWORDS C. elegans intestine endoderm specification ELT-2 GATA factor tasp-1 taspase pqn-82 Mutant Screen Report

The classical Waddington landscape (Figure 1) provides a metaphor for the decisions made by a developing embryo. Waddington envisioned that the precise shape of this landscape reflected genes acting “beneath the surface”, determining whether the walls of the different valleys or
cell trajectories are steep (stable robust pathways) or shallow (those more easily perturbed) (see Figure 5, Chapter 2 of Waddington 1957). For the well-defined case of the transcription factor cascade driving development of the \textit{C. elegans} endoderm, we wish to identify background functions that contribute to the overall behavior and robustness of the regulatory network, thereby preventing an intestinal precursor from adopting incorrect cell fates because of random noise or environmental fluctuations. These background functions ensure that each transcription factor appears at the correct time, at the correct level, and with the appropriate interacting partners, thereby—to continue the metaphor—producing a “deep valley” in the Waddington landscape. The present study will describe a genetic screen aimed at identifying such background or secondary factors.

The \textit{C. elegans} endoderm (intestinal or E-lineage) forms as a simple clonal lineage under control of a cascade of GATA-type transcription factors (Figure 2A) (reviewed in (McGhee 2007; Maduro 2010; McGhee 2013; Maduro 2015, 2017)). The endoderm is normally specified by the redundant action of the GATA transcription factors \textit{end-1} and \textit{end-3}, acting within the single E blastomere of the eight-cell embryo (Zhu et al. 1997; Maduro et al. 2005; Maduro et al. 2007; Owraghi et al. 2009; Boeck et al. 2011; Maduro et al. 2013). Expression of \textit{end-1} and \textit{end-3} is controlled by a spatially patterned set of transcription factors, including the maternal \textit{SKN-1} (aided by its transient zygotic targets \textit{MED-1/2}) (Bowerman et al. 1993; Maduro et al. 2015) and maternal \textit{POP-1} (whose levels and interacting co-factors are determined by intercellular signaling within the four-cell embryo) (Goldstein 1992, 1993; Lin et al. 1998; Maduro et al. 2005; Shetty et al. 2005). When there are two cells in the E lineage (2E cell stage; Figure 2A), \textit{end-1} and \textit{end-3} activate the gene encoding the GATA factor \textit{ELT-7} (Sommermann et al. 2010; Dineen et al. 2018). \textit{ELT-1}, \textit{END-3} and \textit{ELT-7} then combine to activate the gene encoding the GATA factor \textit{ELT-2} at the early 4E cell stage (Wiesenfahrt 2015; Dineen et al. 2018). Expression of \textit{end-1} and \textit{end-3} is transient and ceases by the 4-8E cell stage (Raj et al. 2010).

ELT-2 normally controls genes associated with intestinal structure and function, e.g., genes encoding digestive enzymes (Fukushige et al. 1998; McGhee et al. 2007; McGhee et al. 2009; Wiesenfahrt et al. 2016; Dineen et al. 2018). ELT-2 is not normally involved with the earlier events of endoderm specification. However, when ELT-2 is expressed earlier than normal (i.e., under control of a transgenic \textit{end-1} promoter introduced into an \textit{elt-2(+)} background), ELT-2 is able to replace all other members of the core endoderm transcription factor hierarchy (i.e., \textit{END-1}, \textit{END-3} and \textit{ELT-7}) to specify the \textit{C. elegans} endoderm as well as drive intestinal differentiation (Wiesenfahrt et al. 2016). This ELT-2-only rescue is successful if the \textit{end-1p::elt-2} cDNA construct is present in multiple copies (either as an integrated-chromosomal or a non-integrated-extrachromosomal transgenic array; Figure 2B). In contrast, a similar \textit{end-1p::elt-2} cDNA construct does not rescue if it is present as a single copy, i.e., a MosSCI insertion into a site that has been shown to be a permissive environment for early transcription (Frokjaer-Jensen et al. 2012; Maduro et al. 2015; Wiesenfahrt et al. 2016). We hypothesize that successful rescue requires higher levels of \textit{ELT-2} protein than are provided by a single copy transgene. We further hypothesize that mutation in some “modifier” gene might increase levels of \textit{ELT-2} and/or increase the sensitivity of key \textit{ELT-2} target genes, thereby allowing the survival of an \textit{end-1 end-3} double mutant animal harboring the single copy \textit{end-1p::elt-2} transgene. As will be described in this paper, a genetic screen was indeed able to identify genes that, when mutated, increase survival of \textit{end-1 end-3} embryos carrying the single copy \textit{end-1p::elt-2} transgene and that could be viewed as altering the topography of the Waddington landscape. Two of these genes were identified by whole genome sequencing, without prior out-crossing, mapping or complementation testing. Mutations in both genes were indeed found to modestly increase levels of \textit{ELT-2} in the early embryo but increased \textit{ELT-2} levels may not be sufficient for rescue. We describe several straightforward ways in which the screen could be optimized to more fully map the developmental landscape of the \textit{C. elegans} endoderm. The approach of rescuing a lethal mutation with an extrachromosomal transgene, followed by mutagenesis and counterselection against the extrachromosomal array, appears to be generally suited to identifying modifiers of lethal mutations in \textit{C. elegans}. Fay and co-workers (Joseph et al. 2017) have recently described a conceptually similar screen to identify suppressors of a synthetic lethal moulting defect.

**MATERIALS AND METHODS**

**Strain maintenance, production and manipulation**

Genotypes of the \textit{C. elegans} strains used in this study are listed in Table 1. All strains were propagated on OP50-seeded NGM plates by standard methods (Brenner 1974). We note that several strains (e.g., JM246, JM274) were deleted for the \textit{elt-4} gene (allele \textit{ca16}); loss of \textit{elt-4} causes no detectable phenotype (Fukushige et al. 2003) and, for the purposes of the current study, we made no distinction between strains that were \textit{elt-4(+)} or \textit{elt-4(-)}. The \textit{end-1 end-3} rescued strains in our previous
study were null for elt-7 (Wiesenfahrt et al. 2016) but strain JM246 on which the current genetic screen was performed is elt-7(+). Based on the known properties of ELT-7 (Sommermann et al. 2010; Riddle et al. 2013; Riddle et al. 2016; Dineen et al. 2018), we surmised that enhanced ELT-7 expression could provide a potential class of modifier mutations.

In the event, no candidate elt-7 mutations were detected; we also tested that all other candidates remained viable following elt-7 RNAi; (a strain that depended on ELT-7 function provided a positive control that the elt-7 RNAi had worked as expected).

Multicopy extrachromosomal transgenic arrays were produced by standard gonadal injection (Mello et al. 1991). In particular, the extrachromosomal array (caEx10) that provides the basis of the current genetic screen was created by co-injecting three plasmids: pJM513 at 10 μg/ml (2162 bp of the end-1 promoter fused to elt-2 cDNA + 3’-UTR); pMA122 at 10 μg/ml (peel-1 toxin under control of the heat shock promoter (Frokjaer-Jensen et al. 2012)), and; pJM473 at 80 μg/ml (2.3 kb of the myo-3 promoter fused to tdTomato). The single copy insert of the end-1p::elt-2 cDNA (caSi2 II (Wiesenfahrt et al. 2016)) was produced by MosSCI as described by Frokjaer-Jensen et al. (2012); the transforming plasmid was pJM661 (1996 bp of the end-1 promoter fused to elt-2 cDNA + 3’-UTR) inserted into pCFJ350 injected into strain EG6699 (Frokjaer-Jensen et al. 2012). Single copy MosSCI insertions were also produced on Chromosome I (allele caSi5 inserted into the oxi185 locus in strain EG8078) and Chromosome III (allele caSi6 inserted into the oxi444 locus in strain EG8080). These three single copy insertions were combined by standard genetic crosses, monitoring the presence of the different alleles by PCR; strains were not subsequently checked for the presence of unc-19. Expression from the triple MosSCI strain appeared unstable and was eventually extinguished; measurements of ELT-2 levels shown in Figure 3A, row 7, as well as rescuing ability, were made immediately after the strain was produced.

RNAi by feeding was performed essentially as described by (Kamath and Ahringer 2003). Birefringent gut granules were scored using standard polarization optics. Survival rates (at 20°C) for the various strains were measured as follows. Individual L4 larvae were placed on separate plates and transferred to a new plate every day for four days. For each plate from each original animal, total progeny were counted immediately after the adult was transferred to a new plate, dead/unhatched embryos were counted one day later and surviving adults were counted four days later. Dead/arrested larvae were estimated by subtracting the number of dead embryos and surviving adults from the number of total progeny. Overall survival rates were calculated by combining data from 3-5 original L4 larvae.

Mutagenesis and screening

We performed three separate mutagenesis protocols on L4/young adults of strain JM246: 1) 50 P0 animals were treated with 50 mM EMS; 2) 50 P0 animals were treated with a combination of 50 mM ENU and 25 mM EMS; and 3) ~300 worms were treated with 25 mM EMS. All treatments were performed in M9 buffer for 4h on a rotator at room temperature. Mutagenized animals were then washed three times with M9 by centrifugation and allowed to recover on an OP50-seeded NGM plate, either for three hours or overnight. Three to five mutagenized animals were then transferred to fresh OP50-seeded 100 mm plates and incubated at 20°C; plates were cleared of food in ~two weeks (3-5 generations), which should provide adequate time for a modifier mutation to become homozygous and the hsp-peel-1 counterselection plasmid in the extrachromosomal array to be lost. Plates were then incubated at 34°C for 2 hr to induce the PEEL-1 toxin as described in Frokjaer-Jensen et al. (2012), killing essentially all worms that still contained the rescuing array. 100% of elt-7(-) end-1(-) end-3(-); elt-2(+); elt-7(-) end-1(-) end-3(-); elt-2(+); and elt-7(-) end-1(-) end-3(-); elt-2(-) survive (see also Owraghi et al. 2009) unless they are transgenic for multiple copies of an end-1p::elt-2 cDNA transgene. They do not survive if the transgene is present as a single copy.
Table 1 Genotypes of strains used

| Strain | Genotype |
|--------|----------|
| JM229  | caSi2 II Pqn-82 gk3768 III E141.1 A depletion in the K01G5.9/C176 gene was performed by the Targetfinder method of Norris et al. (2015) to target Chromosome III: 1850135 to 1851020, using the following sequences http://genome.sfu.ca/crispr/. Dpy and Rol worms were individualized using the CRISPR/Cas9 mutagenesis method of Norris et al. (2014). Guide RNA (CCTTGGACATAGCAATTTGG) was selected using the website: http://genome.sfu.ca/crispr/. Dpy and Rol worms were individualized and analyzed by PCR for deletions using primers flanking the guide RNA target site (Primers: oJM815: CCTTGCACATAGCAATTGG). The deletion of the pqn-82 gene was performed by the C. elegans Gene Knock Out Lab using the method of Norris et al. (2015) to target Chromosome III: 1850135 to 1851020, using the following sequences flanking the deletion:

**Immunohistochemistry**

ELT-2 protein levels in embryos were measured as described previously (Van Fürden et al. 2004; Wiesenfahrt et al. 2016), using the anti-ELT-2 monoclonal antibody 455-2A4. Images were recorded with a Hamamatsu Orca ER camera attached to a Zeiss Axioskop 2 fluorescent microscope (40X objective); images for comparing different strains were collected with constant microscope/camera parameters. Total fluorescent intensity in the intestinal primordium (2E or 8E stage embryo) was measured (in arbitrary units) using ImageJ and corrected for background intensity measured elsewhere in the embryo. The probability that ELT-2 levels measured in one set of embryos have the same distribution of intensities as measured in JM247 embryos (single copy end-1 elt-2 transgene) was calculated by the Wilcoxon-Mann-Whitney rank test (Marx et al. 2016) implemented online (https://cch-compute2.cs.uni-saarland.de/wtest/).

**Single molecule fluorescent in situ hybridization (smFISH)**

Custom Stellaris FISH Probes were designed against end-1 (oEO221) and set-3 (oDMP123) mRNA using the Stellaris FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearch.com/stellarisdesigner. Probes were labeled with CalFluor610 and Quasar670 fluorophores. Probe sequences are listed in Table S2.

N2 and pqn-82(–/–) worms were grown at 20°C to gravidity on NGM plates, bleached for embryos, re-suspended in -20°C methanol, freeze cracked in liquid nitrogen, and fixed at -20°C for 24-48 hr. A protocol was performed that combined (Li and van Oudenaarden 2012; Shaffer et al. 2013) and Stellaris RNA FISH protocol for C. elegans (available online at www.biosearchtech.com/stellarisprotocols). Briefly, embryos were equilibrated in Stellaris Wash Buffer A followed by hybridization in Stellaris Hybridization Buffer containing 50 pmol of each primer set (37°C, overnight). Hybridization was followed by a wash in Wash Buffer A (37°C, 30 min). DAPI staining in Wash Buffer A (37°C, 30 min), a wash in Stellaris Wash Buffer B (room temp, 5 min), and final resuspension in N-propyl gallate mounting media. Embryos were mounted as described in (Li and van Oudenaarden 2012) using either N-propyl gallate or VectaShield Diamond anti-fade to prevent photobleaching.

All smFISH images were acquired using a Cool Snap HQ2 camera on a DeltaVision Elite System (a modified, inverted IX71 Olympus microscope), with a 60x objective (NA 1.42) and SoftWorx software (Applied Precision) using fixed exposure and acquisition conditions and z-stacks at 0.25 μm thickness. Images were deconvoluted (Applied Precision). Quantitation was performed on stacks using FISH-Quant (Mueller et al. 2013). The ratio of end-1 mRNA molecules per set-3 mRNA molecules was calculated and the significance of differences between N2 and pqn-82 embryos was assessed using the Student’s two-tailed t-test.
Upstream: TATCTTTTCTGGGCAAAGTTCACAGAAGTTTTTAAAGAAAACTGGGTCAAG
Downstream: AGGAGCAATTGGCTACGACCATCACAGCACACGACACGATGCTCAAA

Whole genome sequencing
Candidate populations (together with a population of the unmutagenized starting strain JM246) were grown from single animals. Genomic DNA was prepared using a standard protocol and resuspended in distilled water. Sequencing libraries were constructed using the Illumina Nexteraxt library preparation kit and sequenced on either an Illumina HiSeq 2 × 100 Rapid Run or MiSeq platform. Paired sequence reads were mapped to the C. elegans reference genome version WS230 (www.wormbase.org) using the short-read aligner BWA (Li and Durbin 2010). Single-nucleotide variants (SNVs) were identified and filtered with the help of the SAMtools tool (Li et al. 2009). Variant calls also present in the parental strain were eliminated, each SNV was annotated with a custom Perl script, and gene information downloaded from WormBase version WS230.

Data availability
The raw sequence data from this study have been submitted to the NCBI BioProject (http://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA433018 and can be accessed from the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra) with accession number SRP132234. Table S1 (summary of coding sequence variants) and Table S2 (probe sequences and raw counts for smFISH) are available at Figshare: https://doi.org/10.25387/g3.5972902.

RESULTS AND DISCUSSION
Rationale for the modifier screen
We previously found that multiple copies of an end-1p:elt-2 cDNA construct are able to rescue the lethality of an end-1 end-3 double mutant, whereas a single copy of a similar construct is unable to rescue (Figure 2B) (Wiesenfarth et al. 2016). A possible cause of this differential rescue is overexpression of ELT-2 from the multicopy transgene in the early endoderm lineage. We therefore measured ELT-2 levels at the 2E cell stage using an anti-ELT-2 monoclonal antibody. ELT-2 protein can be detected in ~1% of wildtype embryos at the late 2E cell stage and in 100% of wildtype embryos at the early 4E cell stage (Fukushige et al. 1998; Raj et al. 2010; Wiesenfarth et al. 2016). In contrast, ELT-2 protein can be detected at the 2E cell stage (when the end genes are normally expressed) in 100% of embryos from either strain JM247 (single caSi2 copy MosSCI insertion of the end-1p:elt-2 cDNA transgene) or from strain JM229 (the integrated multicopy array ca185 of a similar end-1p:elt-2 cDNA transgene) (Figure 3A rows 1 and 2, respectively). (Genotypes of key strains are summarized in Table 1.) As we suspected, ELT-2 levels at the 2E cell stage produced by the multicopy array are, on average, ~six fold (5.5+/− SD = 3.8) higher than levels produced by the single copy MosSCI insertion (p~10−16). The large variability in fluorescence intensity is commonly seen when immunostaining C. elegans embryos by standard procedures, either because of variable permeabilization of the eggshell, inability to select for a precise time point within the cell cycle, or intrinsic variability in gene expression. Nonetheless, we are confident of at least semi-quantitative interpretations. To support this claim, Figure 3B (rows 1 and 2) shows the results of measurements on the same two strains performed one year later than the measurements of Figure 3A; the ratio of ELT-2 levels was estimated at 4.7+/− SD = 3.6, (P < 10−11).

We designed a genetic screen to identify mutations that would allow end-1 end-3 mutant animals to survive with a single copy of the end-1p:elt-2 cDNA construct. We refer to these putative mutations as “modifiers” rather than enhancers or suppressors, in order to avoid presumptions about mechanisms. The starting strain for the screen, JM246, is caSi2::end-1 end-3 V, caEx10. Because the caSi2 single copy end-1p:elt-2 cDNA construct is insufficient to rescue end-1 end-3 lethality (Figure 2B), strain JM246 survives by virtue of the extrachromosomal multicopy transgenic array caEx10, which is derived from three plasmids: (i) the end-1p:elt-2 cDNA construct able to rescue the end-1 end-3 lethality as multiple copies, and for counterselection;
(ii) a tdTomato fluorescent reporter expressed under control of the body wall myosin myo-3 promoter, and; (iii) a heatshock::peel-1 construct. As described by Frokjaer-Jensen et al. (2012), animals that harbor the heatshock::peel-1 toxin construct can be efficiently killed by brief heat shock; (see also (Seidel et al. 2011; Joseph et al. 2017)). (In principle, a normal genomic copy of the end-1 or end-3 genes could have been used to rescue end-1 end-3 lethality in the starting strain; however, we chose to use an end-1p::elt-2 construct in JM246 to avoid recombination from the multicopy array back into the genome, which would thereby produce false positives; this choice will be discussed in a later section.) We did not observe animals segregating from unmutil- genized JM246 that did not also contain the extrachromosomal transgenic array (i.e., < 1 in 10^4 progeny are Non-Red and Non-Sensitive to heatshock). We noted that ~40% of JM246 progeny lose the rescuing extrachromosomal transgenic array in each generation; however, ~45% of these array-negative (and non-propagating) embryos still express birefringent gut granules, the standard marker of endoderm specification and intestinal differentiation. In other words, the single copy caSi2 insert must be providing some partial level of endoderm specification/differentiation functions and the strain seems optimally poised to detect modifiers. For comparison, the end-1 end-3 double mutant (in the absence of the caSi2 end-1p::elt-2 transgene) shows 0% gut granule expression (Owraghi et al. 2009).

The overall plan of the screen is diagrammed in Figure 4. Young adults from strain JM246 (caSi2 II; end-1 end-3 V; caEx10) were exposed to one of three mutagenic regimes (see below), placed 3-5 individuals per 100 mm plate and incubated at 20° until the bacterial food was exhausted. This took 10-14 days or an estimated 3-4 generations, which should allow either maternal or zygotic modifier mutations to become homozygous, together with or followed by random loss of the rescuing array. Plates were then heatshocked (34° for 2 hr), thereby killing all animals that had not lost the rescuing transgenic array. Array loss was verified by confirming that survivors were Non-Red. Rescued strains were established from a single survivor per plate to assure independence.

We performed three separate rounds of mutagenesis on young adults of strain JM246 (caSi2 II; end-1 end-3 V; caEx10). Round 1 used 50 mM ethyl methane sulfonate (EMS) to mutagenize 50 P0 animals and produced 3 candidate strains; round 2 used 25 mM EMS + 25 mM ethyl-nitrosourea on 50 animals and produced 1 candidate strain; round 3 used 25 mM EMS on 300 adult animals and produced 13 candidate strains. We estimate that we screened 12,000-16,000 haploid genomes from a single survivor per plate and incubated at 20° for 2 hr, thereby killing all animals that had not lost the rescuing transgenic array. Array loss was verified by confirming that survivors were Non-Red. Rescued strains were established from a single survivor per plate to assure independence.

Validation and initial characterization of two candidate modifier genes

K01G5.9/tasp-1: This gene was independently mutated four times in our set of 17 candidate genomes and we estimate that the probability of this happening by chance is ~2x10^-6. The rate at which these mutations were found (1/3500 genomes screened) suggest that they represent loss of function alleles and this will be supported below. K01G5.9 is annotated in WormBase (WS261) as an L-asparaginase but we suggest that it is better described as the C. elegans homolog of Taspase; K01G5.9 has now been designated C. elegans tsp-1. Taspase is a conserved threonine-aspartic acid protease that cleaves mammalian and Drosophila MLL1/trithorax, as well as other nuclear proteins (e.g., TFIIA) associated with chromatin and transcription; mutations in human Taspasel have been associated with certain forms of cancer, particularly those of the head and neck (Hsieh et al. 2003a; Hsieh et al. 2003b; Khan et al. 2005; Takeda et al. 2006; Zhou et al. 2006; Oyama et al. 2013; Niizuma et al. 2015; Takeda et al. 2015; Stauber et al. 2016; Gribko et al. 2017). The four independent mutations are placed on the tsp-1 genomic locus as shown in Figure 5A. Sequence alignments of tspases from C. elegans, humans and Drosophila are shown in Figure 5B; residues highlighted in green are part of the human tspase active site and distinguish tspases from asparaginases (Khan et al. 2005). Highlighted in red is the conserved threonine residue that becomes, following autoproteolysis, the N-terminal nucleophile that cleaves after the adjacent aspartate residue (Khan et al. 2005). Individual mutations in the four candidate strains are denoted in blue; consistent with loss of function alleles, one mutation produces a stop codon and two further mutations occur either in or adjacent to conserved residues implicated in catalysis, tsp-1 expression appears wide-spread throughout the embryo and not obviously restricted to the developing endoderm. Levels of tsp-1 transcripts are detectable in all life stages but appear to be highest in the early embryo (probably the oocyte) (Gerstein et al. 2010; Hashimshony et al. 2015; Cao et al. 2017).

As an initial validation of tsp-1 as a candidate modifier, we performed feeding RNAi on the parent strain JM246 (single copy end-1p::elt-2 rescued by the multicopy array). A low level of rescue was observed; 4% of animals that had lost the rescuing array survived to adulthood compared to 0% for the ges-1 RNAi control. Furthermore, the average ELT-2 protein levels in 2E cell stage (array negative) embryos were increased ~threefold (3.1 +/- SD = 3.0; P < 10^-5) (Figure 3A, row 3). We then used CRISPR/Cas9 to produce a 29 bp deletion (allele ca18) in the endogenous tsp-1 gene (Figure 5A; see Methods), which causes a frame shift mutation leading to a premature translational stop and likely represents a null. The tsp-1(ca18) allele was introduced into the single copy MosSCI caSi2 [end-1p::elt-2]; end-1 end-3 background to produce strain JM274. This strain shows 12%

Whole genome sequencing identifies candidate modifier genes

We sequenced the full genomes of each of the 17 independent candidate strains, with the rationale that candidate modifiers could be identified if the same gene was mutated independently in more than one strain. Sequencing was performed at a median coverage of 35-fold (range = 25-58-fold); coding sequence variants are collected in Supplementary Material Table S1. Table 2 collects the identities of all genes that sustained two or more coding variant mutations. Table 2 also contains the calculated probability that each of the candidates was identified by chance, based on estimates of the intrinsic mutability of the identified genes from the Million Mutation Project (MMP) (Thompson et al. 2013). None of the strains included promoter or coding region mutations in the endogenous elt-2 locus nor, as expected because the starting alleles are deletions, revertants of end-1/3. We chose two candidates, K01G5.9 (renamed tsp-1 below) and pqn-82, to follow up in more detail. For the moment, we have not pursued other two-hit genes, e.g., Y18H1A.8, because of lack of transcripts in the early embryo, lack of promising annotation, because several of these candidate strains were also mutated for tsp-1 or pqn-82, and because of increasing probability that these genes were mutated by chance alone.
rescue and ~twofold (1.8 +/- SD = 1.4; P < 10^{-3}) increase in ELT-2 protein levels measured at the 2E cell stage (Figure 3A, row 4); although these protein levels fall within the range produced by the multicopy transgene (shown in Figure 3A, row 2), survival rates are much lower. Nonetheless, we conclude that loss of function in the tasp-1 gene does indeed allow a single copy of the \( \text{elt-2} \) cDNA transgene to rescue the \( \text{elt-2} \) lethal allele, at least partially.

Substrates and preferred cleavage sites of the taspase enzyme in \( C. \text{elegans} \) are not yet known. However, there are 239 proteins in WormBase (WS250) that contain the cleavage site (Q[F/I/L/M]D(Y/G) (Figure 3C). ELT-2 does not contain an obvious candidate cleavage site, suggesting that increased levels of ELT-2 in the early embryo do not result from abolishing direct taspase cleavage of ELT-2. Similarly, candidate taspase cleavage sites could not be detected in other factors acting in the core endoderm specification pathway (SKN-1, POP-1, WRM-1, SYS-1, MED-1/2, END-1/3 and ELT-7).

We note an intriguing feature of all the \( \text{caSi2} \); \( \text{tasp-1} \); \( \text{end-1} \) strains: they appear “mortal” and eventually die out after multiple (~10) generations. This ultimate mortality was seen with all four of the original candidate strains, with the strain reconstituted from the CRISPR/Cas9 \( \text{ca18} \) deletion, as well as with array-negative segregants from JM246 produced and propagated by continued exposure to \( \text{tasp-1} \) RNAi. (Similar mortality was not obvious with \( \text{tasp-1} \)(ca18) animals with an otherwise wildtype background; however, an eventual but impenetrant mortality cannot be ruled out). Could \( \text{caSi2} \); \( \text{tasp-1} \); \( \text{end-1} \) mortality possibly be associated with more general phenomena such as mortal germlines and “transgenerational epigenetic inheritance”? Work from several laboratories has shown that mutations in three genes, \( \text{hrdc-1} \), \( \text{met-2} \) and \( \text{prg-1} \), are associated with both mortal germlines and transgenerational RNAi (reviewed in (Brown and Montgomery 2017)). Both HRDE-1 and MET-2 proteins contain sequence motifs that match the Q[I/F/I/L/M]D(G) consensus cleavage site for Drosophila taspase (QMDG and QFDG respectively). PRG-1 protein contains a QVDG sequence and we note that the second position V seems improbable that three proteins chosen for an independent property all could be (potential) taspase targets.

\( \text{pqn-82} \): The second validated candidate was \( \text{pqn-82} \) (Y39A3CR.7), annotated in Wormbase as a “prion-like glutamine/asparagine rich domain bearing protein”. We estimate the probability that \( \text{pqn-82} \) was mutated twice by chance in our screen as 5x10^{-4}. \( \text{pqn-82} \) transcripts are high in oocytes and early embryos, apparently in most cell lineages, but decline sharply by mid-embryogenesis; transcripts can be detected again when adults become gravid (Gerstein et al. 2010; Hashimshony et al. 2015; Cao et al. 2017).

There are no obvious \( \text{pqn-82} \) homologs outside of nematodes; even within nematodes, conservation is modest (Blast P scores >10^{-7}). Nonetheless, other members of the PQN family have been associated with transcription and chromatin: for example, PQN-49/LET-19 is related to the conserved transcriptional coactivator subunit TRAP240, PQN-50/SEA-2 is a zinc finger protein involved in interpreting the X:A chromosomal ratio for sex determination and dosage compensation, PQN-81 has been redesignated \( \text{ssl-1} \) (Sw2/Snf2 like) and PQN-85 is homologous to Drosophila NIPPED-B involved in DNA repair and chromosome mechanics. PQN-51 is the \( C. \text{elegans} \) homolog of the general transcription factor TFIIA, noted above to be a candidate target of Taspase (Figure 5C).

As shown on Figure 6A, both \( \text{pqn-82} \) mutations isolated in the screen are nonsense alleles with premature termination toward the 3’-end of the gene and presumably cause a loss-of-function. The two candidate strains show 40–50% rescue and ELT-2 protein levels are increased two-to-three fold at the 2E cell stage (Figure 3A; 3.3 +/- SD = 2.5 for candidate strain 14 (row 5) and 2.2 +/- SD = 1.8 for candidate strain 24 (row 6); P < 10^{-8} and p<10^{-6}, respectively). RNAi performed against \( \text{pqn-82} \) on the parent strain JM246 does indeed lead to rescue, i.e., production of \( \text{caSi2} \); \( \text{end-1} \) offspring that can now survive without the multicopy extrachromosomal transgenic array...
Do tasp-1 and pqn-82 function in parallel pathways?

We asked if tasp-1 and pqn-82 act in parallel pathways. If so, phenotypes of null alleles should show synergistic interactions. Table 3 collects brood sizes and survival rates of progeny produced by the strains that have lost the function of tasp-1 and/or pqn-82. Both single mutants show lower brood sizes and a low degree of lethality (4–6%) compared to wildtype; both effects appear at most “additive” in the pqn-82 tasp-1 double mutant rather than synergistic. For strain JM280 caSi2 pqn-82 (gk3768); end-1 end-3, roughly 50% of the laid embryos will reach adulthood. For strain JM274 caSi2 tasp-1(ca18); end-1 end-3, 19% of array-negative animals reach adulthood. When both pqn-82 and tasp-1 are removed, an intermediate number (30%) of array-negative animals reach adulthood. Considering the large inter-brood variability and the possibility that tasp-1 RNAi might be less effective than the tasp-1(ca18) knockout, we suggest that the data are most consistent with a model in which tasp-1 and pqn-82 act in the same pathway with respect to brood size and viability.

We next tested whether tasp-1 and pqn-82 act in parallel pathways with respect to endoderm specification/differentiation. The maternal provided SKN-1 transcription factor specifies the fate of the EMS cell, the progenitor of the entire endoderm and of a fraction of pharynx and bodywall muscle (Bowerman et al. 1992; Bowerman et al. 1993; Raj et al. 2010). Removal of SKN-1, either by RNAi or by mutation, causes 100% embryonic lethality. However, birefringent gut granules, the standard marker for endoderm specification/differentiation, can still be detected in roughly one-third of these embryos, reflecting mostly the parallel POP-1 dependent pathway of endoderm specification (Maduro et al. 2005; Shetty et al. 2005). As shown in Table 4, the fraction of arrested embryos that show gut granules increases from 33% in skn-1 (RNAi) to 63% in skn-1(RNAi); tasp-1(ca18). In contrast, deletion of pqn-82 does not lead to enhanced expression of endoderm markers following skn-1 RNAi (Table 4), consistent with tasp-1 and pqn-82 functioning in part in different pathways. We also note that the increased expression of gut granules in the tasp-1(ca18) skn-1 RNAi embryos provides evidence that TASP-1 has functions on endoderm specification/differentiation in C. elegans beyond the transgenes on which the present screen is based.

The rescuing ability of tasp-1 and pqn-82 is not solely due to increases in ELT-2 protein levels

Both tasp-1 and pqn-82 loss-of-function lead to an increase in the level of ELT-2 protein detectable at the 2E cell stage of the embryo (Figures 3A and B). Although ELT-2 levels in single copy end-1p:elt-2 embryos were comparable for tasp-1 and pqn-82 mutants, rescue was considerably higher for pqn-82, suggesting that ELT-2 levels may not be the sole factor determining rescue. To test whether increased ELT-2 by itself is sufficient to cause rescue, we constructed strain JM279 carrying three separate copies, each on a different chromosome, of an end-1p:elt-2 cDNA insert. Figure 3A, row 7, shows that the ELT-2 protein levels at the 2E cell stage are increased roughly threefold (3.2 +/− SD = 4; P < 10−4) relative to the single insertion used for the mutagenized parental strain. However, when these three copies were introduced into the end-1 end-3; caEx10 background and after counter-selection against the rescuing array, we were unable to detect rescued progeny, suggesting that ~threefold increase in ELT-2 levels at the 2E cell stage is insufficient, by itself, to rescue end-1 end-3 lethality. The average level of ELT-2 protein at the 2E cell stage of these triple MosSCI embryos is roughly half of the average ELT-2 level detected in the same stage embryos of the multicopy strain JM229 (compare rows 7 and 3 of Figure 3A), suggesting there could be a threshold level of ELT-2 needed to survive. However, the distributions of ELT-2 levels in individual embryos strongly overlap in the two strains and the upper range of the non-rescuing triple MosSCI strain is above that of the rescuing pqn-82 strain (Figure 3A). We suggest that tasp-1 and pqn-82 may have an additional function(s) beyond simply increasing ELT-2 protein levels in the early embryo. Perhaps a time window, rather than a concentration threshold,
Figure 5 A. Four independent mutations identified in tasp-1 are shown above the gene. The CRISPR mutation (ca18) introduced into the gene is also shown (below). B. Alignment (Clustal Omega performed at www.ebi.ac.uk with default settings) of the protein sequences from C. elegans tasp-1, human Taspase1 and Drosophila taspase 1. The Threonine residue highlighted in red depicts the active site nucleophile, which lies immediately downstream of the aspartic acid residue in the autocleavage site. Residues highlighted in green on the sequence of human Taspase1 have been identified as part of the active site and are not found in asparaginases (Khan et al. 2005). Changes in the amino acid sequence resulting from the four candidate mutations identified in the current screen are shown in blue. C. Alignment (Clustal Omega) of the C-terminal portions of TFIIA, showing that the taspase cleavage site known in humans and Drosophila is also conserved in C. elegans (highlighted).
could be critical and the tasp-1 or pqn-82 strains (as well as the original multicopy strain) could produce more ELT-2 earlier in the 2E cell cycle than the simple three-copy MosSCI insertions. We have previously commented that ELT-2 protein cannot be detected at the 1E cell stage in the JM229 multicopy strain (Gilleard et al. 1999; Gilleard and McGhee 2001), even though the endoderm ultimately ends up being specified (Wiesenfahrt et al. 2016). Perhaps even more of a delay in the appearance time of ELT-2 is sufficient to abolish rescue.

**Estimating the background rate of “false positives” or “bypass mutations”**

Before repeating the present screen on a scale that could approach saturation, we wished to assess whether identification of “false-positive” modifiers ever becomes a limitation. In other words, does the screen turn up large numbers of “bypass mutations” that do not depend on the behavior of the single copy end-1p:elt-2 MosSCI insert on which the current screen was based? We repeated the basic screen described above but replaced the caSi2 single copy MosSCI allele of end-1p:elt-2 with an integrated multicopy array of a construct in which the end-1 promoter now drives expression of a cDNA coding for the hypodermal GATA factor ELT-3 (Gilleard et al. 1999; Gilleard and McGhee 2001). Within the embryo, ELT-3 and ELT-2 clearly have distinct sets of transcriptional targets (Fukushima et al. 1998; Gilleard and McGhee 2001) and it would seem unlikely (but extremely interesting) to identify mutations that could “modify” normal ELT-3 hypodermal function such that it could now specify endoderm. Relevant to the current discussion, this screen would also produce end-1/3 bypass mutations. As for the major screen, end-1 end-3 lethality was rescued in the parent by an extrachromosomal array containing end-1p:elt-2, myo-3p::RFP and heatshock::

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**Table 3** Brood size and progeny survival to adulthood (number and percentage) associated with strains that have lost pqn-82 and/or tasp-1

| Strain         | Genotype / RNAi         | Average Brood Size ± SD | Average # | Average % | Broods Counted |
|----------------|------------------------|-------------------------|-----------|-----------|----------------|
| N2             |wildtype                | 287 ± 15                | 286 ± 15  | 99 ± 1    | 5              |
| VC3801         |pqn-82(gk3768)          | 226 ± 10                | 216 ± 14  | 96 ± 3    | 5              |
| JM281          |tasp-1(ca18)            | 173 ± 25                | 163 ± 34  | 94 ± 6    | 3              |
| JM282          |pqn-82(gk3768) tasp-1(ca18) |151 ± 20               |138 ± 18  | 92 ± 4    | 4              |
| JM280*         |caSi2; pqn-82(gk3768); end-1 end-3 | 163 ± 87              |81 ± 46   |50 ± 8    |5               |
| JM274*         |caSi2; tasp-1(ca18); end-1 end-3 |104 ± 61              |26 ± 22   |19 ± 15   |5               |
| JM280          |caSi2; pqn-82(gk3768); end-1 end-3; tasp-1 RNAi | 120 ± 46              |30 ± 8    |30 ± 15   |5               |

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*a JM274 and JM280 are maintained by the presence of the extrachromosomal array caEx10, which rescues the end-1 end-3 lethality. For these two strains, as well as for JM280 + tasp-1 RNAi, the progeny counted were only those that had lost the caEx10 array (i.e., that were NonRed).

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Figure 6 A. The two independent mutations identified in the structural gene of pqn-82 are shown above the gene. The genomic coordinates for the altered base pairs are 1,851,047 for Candidate # 24 and 1,852,218 for Candidate # 14. B. end-1 mRNA (green) in wild-type (N2) and pqn-82 embryos as imaged by single molecule fluorescence in situ hybridization (smFISH). Ubiquitously expressed set-3 mRNA transcripts (red) were co-hybridized as a control, and nuclei were stained with DAPI. Scale bar is 10 μm. C. Single molecules of end-1 and set-3 mRNA that were imaged by smFISH (B) were quantitated at three stages of embryonic development. Levels of set-3 transcripts stayed consistent across stages (mean of 444 molecules/embryo) but levels of end-1 transcripts increased (means of 47, 109, 214 molecules/embryo at 8-cell, 16-cell, and 32-cell stages). However, the relative amount of end-1 mRNA in N2 and pqn-82(-/-) embryos was not significantly different for each stage-specific, pairwise comparison as calculated by Student’s two-tailed t-test (20 embryos per stage and genotype). Upper boxplot whiskers represent the lesser of either the greatest value point or the upper quartile plus 1.5 times the interquartile range; lower whiskers represent the reverse. The data derive from one of two independent replicates, both of which reach the same conclusions.
Table 4 Influence of tasp-1 and pqn-82 mutations on the ability of skn-1 RNAi embryos to produce gut granules. RNAi was administered by feeding the parental screens and inspecting arrested embryos by birefringence.

| Strain          | gut granule + | gut granule - | n  | % gut granule + |
|-----------------|---------------|---------------|----|-----------------|
| N2              | 181           | 365           | 546| 33%             |
| tasp-1(ca18)    | 513           | 308           | 821| 63%             |
| pqn-82(gk3768) | 145           | 325           | 470| 31%             |

We screened ~30,000 mutagenized genomes, basically as described above, but found only one surviving strain that could be propagated without the multicopy rescuing array. However, follow-up PCR and RNAi experiments showed that the reason for this strain’s survival was the chance integration into the genome of one or more copies of the rescuing construct derived from the original extrachromosomal array.

Overall, even though we did not find mutations that allow ELT-3 to specify endoderm, we conclude that this type of screen is robust, with an acceptably low level of false positives or bypass mutations. Integration into the genome of copies of the rescuing construct occurs at an acceptably low frequency and moreover, is easily detected by PCR.

Future improvements

We have developed a general strategy to isolate mutations that rescue lethality of C. elegans mutants. tasp-1 and pqn-82 genes were identified by virtue of multiple alleles detected by whole genome sequencing without the need of prior out-crossing, mapping or complementation testing. Presumably, the remaining strains carry modifier mutations in genes that were hit only once in our screen; such modifiers therefore occur at the rate of ~1/14000 mutations/gene/gamete and are likely to include (rare) gain-of-function alleles or hypomorphs of essential genes. It is possible that such rare alleles could be detected by increasing the scale of the screen, to the point where these genes would also turn up as multiple independent hits. However, it is important to have an alternative strategy such as out-crossing followed by SNP mapping (Davis et al. 2005) or genomic sequencing (Joseph et al. 2017). In the present case, we found this was not practical. Outcrossing involved the simultaneous maintenance of the unlinked ca32 II MosSCI insertion and the end-1 end-3 V loci, as well as the candidate mutation, with the added complication that the candidate modifier could be acting maternally. Moreover, males from the candidate strains were either difficult to produce or, when produced, did not mate. Thus, any future repetitions of the screen would be best done with a new parental strain in which the end-1p::elt-2 cDNA single copy insertion is linked tightly to the end-1 end-3 locus. We suspect that future screens could be made even more powerful by using a wildtype end-1 or end-3 gene as the rescuing extrachromosomal transgene (rather than end-1p::elt-2 with its relatively inefficient rescue), coupled to a lower concentration or a tighter transcriptional control of the heatshock::peel-1 counterselection plasmid. These straightforward modifications would lead to a healthier parental strain, higher brood sizes following mutagenesis and hence greater numbers of mutagenized genomes that could be easily screened.

Summary and Conclusions

The screen described in this paper has allowed us to search for modifiers of the ability of a single copy end-1p::elt-2 cDNA transgene to rescue the lethality of the end-1 end-3 double mutant. Through whole genome sequencing, two modifier genes, tasp-1 and pqn-82, were identified and both were validated as loss-of-function modifiers by RNAi and by de novo introduction of knockout mutations using CRISPR/Cas9. More work will be required to understand how these two modifiers function at the molecular level but they both seem the type of genes that could influence the shape of a Waddington developmental landscape as depicted on Figure 1. Our plan for the future will be to repeat this screen (with optimizations) on a much larger scale, to better define the transcriptional landscape of the C. elegans endoderm and the reasons for its developmental robustness.

Finally, we suggest that the basic design of the present screen—rescuing the lethality of a hypomorph in a parent strain by an extrachromosomal array that can be counterselected following mutagenesis—could easily be applied to other interesting situations in C. elegans (see also Joseph et al. 2017)). Such screens could well be simpler and more efficient than more customary approaches using temperature sensitive alleles or classical balancers, or even GFP-marked balancers combined with a worm sorter.

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