**RESEARCH ARTICLE**

**daf-16/FOXO blocks adult cell fate in \textit{Caenorhabditis elegans} dauer larvae via lin-41/TRIM71**

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Abstract

Many tissue-specific stem cells maintain the ability to produce multiple cell types during long periods of non-division, or quiescence. FOXO transcription factors promote quiescence and stem cell maintenance, but the mechanisms by which FOXO proteins promote multipotency during quiescence are still emerging. The single FOXO ortholog in \textit{C. elegans}, daf-16, promotes entry into a quiescent and stress-resistant larval stage called dauer in response to adverse environmental cues. During dauer, stem and progenitor cells maintain or re-establish multipotency to allow normal development to resume after dauer. We find that during dauer, daf-16/FOXO prevents epidermal stem cells (seam cells) from prematurely adopting adult characteristics. In particular, dauer larvae that lack daf-16 misexpress collagens that are normally adult-enriched. Using \texttt{col-19p::gfp} as an adult cell fate marker, we find that all major daf-16 isoforms contribute to opposing \texttt{col-19p::gfp} expression during dauer. By contrast, \texttt{daf-16(0)} larvae that undergo non-dauer development do not misexpress \texttt{col-19p::gfp}. Adult cell fate and the timing of \texttt{col-19p::gfp} expression are regulated by the heterochronic gene network, including \texttt{lin-41} and \texttt{lin-29}. \texttt{lin-41} encodes an RNA-binding protein orthologous to LIN41/TRIM71 in mammals, and \texttt{lin-29} encodes a conserved zinc finger transcription factor. In non-dauer development, \texttt{lin-41} opposes adult cell fate by inhibiting the translation of \texttt{lin-29}, which directly activates \texttt{col-19} transcription and promotes adult cell fate. We find that during dauer, \texttt{lin-41} blocks \texttt{col-19p::gfp} expression, but surprisingly, \texttt{lin-29} is not required in this context. Additionally, \texttt{daf-16} promotes the expression of \texttt{lin-41} in dauer larvae. The \texttt{col-19p::gfp} misexpression phenotype observed in dauer larvae with reduced daf-16 requires the downregulation of \texttt{lin-41}, but does not require \texttt{lin-29}. Taken together, this work demonstrates a novel role for daf-16/FOXO as a heterochronic gene that...
promotes expression of *lin-41/TRIM71* to contribute to multipotent cell fate in a quiescent stem cell model.

**Author summary**

In adults and juveniles, tissue-specific stem cells divide as needed to replace cells that are lost due to injury or normal wear and tear. Many stem cells spend long periods of time in cellular quiescence, or non-division. During quiescence, stem cells remain multipotent, where they retain the ability to produce all cell types within their tissue. In this study, we define a new role for the FOXO protein *daf-16* in promoting multipotency during the quiescent *C. elegans* dauer larva stage. *C. elegans* larvae enter dauer midway through development in response to adverse environmental conditions. Epidermal stem cells are multipotent in *C. elegans* larvae but differentiate at adulthood, a process controlled by the "heterochronic" genes. We found that *daf-16* blocks the expression of adult cell fate specifically in dauer larvae by promoting the expression of the heterochronic gene *lin-41*. *lin-41* normally blocks adult fate by repressing the expression of another heterochronic gene, *lin-29*, but surprisingly, *lin-29* is not needed for the expression of adult cell fate in this context. These findings may be relevant to mammals where the orthologs of *daf-16* and *lin-41* are important in stem cell maintenance and opposing differentiation.

**Introduction**

Tissue-specific stem cells divide as needed to replenish cells lost due to injury or normal wear and tear. Many stem cell types retain the capacity to produce multiple cell types during lengthy periods of quiescence, or non-division, and increased cell division can lead to compromised multipotency and stem cell maintenance [1]. However, the connections between quiescence and multipotency are incompletely understood. *Caenorhabditis elegans* (*C. elegans*) development can be interrupted by a quiescent and stress-resistant stage called dauer in response to adverse environmental conditions [2]. Stem cell-like progenitor cells maintain or re-establish multipotency during dauer, a situation analogous to mammalian stem cells [3–5].

Dauer formation is regulated by three major signaling pathways [6,7]. One of these pathways is insulin/IGF signaling, where favorable environmental cues lead to the production of multiple insulin-like peptides in sensory neurons. [8–13]. These signals are released and then received in multiple tissues where insulin signaling blocks the activity of the downstream DAF-16/FOXO transcription factor [14–16]. In adverse environments, DAF-16 is active and regulates the expression of genes that promote dauer formation [6,10].

The role of the DAF-16/FOXO transcription factor in promoting dauer formation is analogous to the role of the FOXO proteins in promoting quiescence in mammalian stem cells [17]. In addition, DAF-16/FOXO is required for stem cell maintenance and the ability of stem/progenitor cells to differentiate into the correct cell types in both systems [5,10,18–22]. For example, in *C. elegans* dauer larvae, *daf-16* is required in a set of multipotent vulval precursor cells to block EGFR/Ras and LIN-12/Notch signaling from prematurely specifying vulval cell fates [5].

Another group of progenitor cells that must remain quiescent and multipotent during dauer is the lateral hypodermal seam cells that make up part of the worm skin. Seam cells undergo self-renewing divisions at each larval stage and then terminally differentiate at
adulthood [23]. Larval vs. adult seam cell fate is regulated by a network of heterochronic genes [24]. In general, heterochronic transcription factors and RNA-binding proteins that specify early cell fates are expressed early in development. These early cell fate-promoting factors are then downregulated by microRNAs in order to allow progression to later cell fates [25]. This pathway culminates with the expression of the LIN-29 transcription factor. LIN-29 is the most downstream regulator of adult cell fate and directly activates the expression of adult-specific collagens such as col-19 [26–29]. LIN-29 protein is not expressed in the hypodermis until late in larval development due to the combined action of the early-promoting heterochronic genes lin-41 and hbl-1 [27,30,31]. lin-41 encodes an RNA-binding protein that binds to the lin-29 mRNA and blocks translation of the lin-29a isoform [30,32]. lin-29 is also required for adult cell fate in post-dauer animals. However, many genes that act earlier in the heterochronic pathway are dispensable for post-dauer development, suggesting that the regulation of adult cell fate differs in continuous and dauer life histories [3].

Here, we examine the role of daf-16/FOXO in maintaining multipotent seam cell fate during dauer. We find that daf-16 is required to block the expression of multiple adult-enriched collagens during dauer, including col-19, defining a novel role for daf-16 as a dauer-specific heterochronic gene. Using the adult cell fate marker col-19p::gfp as a readout, we find that daf-16 acts via lin-41 to regulate adult cell fate. Surprisingly, lin-29 plays at most a minor role in the regulation of col-19p::gfp during dauer. mRNA-seq experiments identified 3603 genes that are regulated by daf-16 during dauer, including 112 transcription factors that may mediate repression of adult-enriched collagens during dauer downstream of daf-16.

Results

daf-16 blocks adult-specific collagen expression in dauer larvae

Progenitor cells remain multipotent throughout dauer diapause, and daf-16 is required to maintain or re-establish multipotency in the vulval precursor cells (VPCs) of dauer larvae [5]. We asked whether daf-16/FOXO might also promote multipotency in lateral hypodermal seam cells, another multipotent progenitor cell type. The well-characterized adult cell-fate marker col-19p::gfp, where GFP expression is driven by the adult-specific col-19 promoter, is expressed in differentiated seam cells and the hyp7 syncytium in adult worms but is not expressed in multipotent seam cells or hyp7 in larvae [28,33]. To determine if daf-16/FOXO promotes multipotent, larval seam cell fate during dauer, we assayed larvae with reduced or absent daf-16, termed “daf-16(-)”, for precocious expression of col-19p::gfp. Specifically, we tested two daf-16 null alleles, daf-16(mu86) and daf-16(mgDf50), as well as daf-16(RNAi) dauer larvae. As daf-16(-) larvae are normally unable to enter dauer even in dauer-inducing environmental conditions (i.e., dauer defective), we took advantage of the temperature-sensitive dauer-constitutive allele daf-7(e1372) to enable dauer formation in daf-16(-) larvae. When grown at restrictive temperatures, both daf-16(-); daf-7(-) and control daf-7(-) larvae enter dauer even in favorable environments [34,35]. For simplicity, daf-7(e1372) is not always mentioned, but this allele was present in all daf-16(-) and control dauer larvae (see S1 Table for a complete list of strains used in this study). We found that daf-16(-) dauer larvae displayed penetrant col-19p::gfp expression in seam cells and hyp7 (Fig 1A). col-19p::gfp was expressed throughout the hypodermis, but was consistently brighter in the posterior third of the worm, gradually dimming toward the anterior. These findings indicate that daf-16 is necessary to block col-19p::gfp expression during dauer. For the remainder of the paper, “daf-16(0)” is used to indicate the daf-16(mgDf50) null allele, a large deletion that removes most of the daf-16 coding sequence, including the DNA-binding domain [15].
**Fig 1. daf-16 is required to prevent precocious onset of adult cell fate during dauer.** (A) Merged DIC and fluorescence images taken with a 63x objective displaying a portion of the lateral hypodermis in dauer larvae. Unless otherwise specified, the background for all strains in this and subsequent figures was daf-16(0); col-19p::gfp. (See S1 Table for full genotypes). Compromised daf-16 activity due to either of two null alleles (left) or RNAi (right) resulted in col-19p::gfp expression. Numbers indicate larvae expressing col-19p::gfp over the total number of dauer larvae scored. (B) Percent of daf-7 (control) and daf-16(0); daf-7 larvae that expressed col-19p::gfp before dauer formation and over time in dauer. GFP expression began during the molt, peaked soon after entry into dauer, and declined thereafter. (C) Percent of wild-type (control) and daf-16(0) animals that expressed col-19p::gfp at different stages. These worms were wild-type for daf-7 and therefore did not enter L2d or dauer. Larvae at L1 arrest were hatched in the absence of food and were monitored for GFP expression over seven days. (D) Percent of dauer larvae lacking some or all daf-16 isoforms that expressed any hypodermal col-19p::gfp (see S1 Table for full genotypes). Note that daf-16(a,f) displayed decreased expressivity compared to daf-16(0); the dauer larvae expressing col-19p::gfp were noticeably dimmer than in the null allele (S2 Fig). NS: p = 0.55, Fisher Exact Test. (E) Heat maps depicting relative expression of adult-enriched collagens (left) or dauer-enriched collagens (right) in daf-16(0); daf-7 dauer larvae or daf-7 (control) dauer larvae from mRNA-seq data. The row-normalized reads for each of two biological replicates per strain are depicted. These collagens were expressed at significantly different levels (FDR $\leq 0.05$) in daf-16(0) vs control dauer larvae in all cases except those collagens indicated by †. Adult and dauer-enriched collagens were identified using modENCODE data (S3 Table). Adult-enriched collagens were upregulated in daf-16(0) dauer larvae whereas dauer-enriched collagens were downregulated.

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Wild type daf-16/FOXO promotes dauer formation largely in parallel to the DAF-3/SMAD–DAF-5/Ski complex [6,7]. To test whether this complex is also required to block col-19p::gfp expression during dauer, we examined daf-5(0) dauer larvae. Similar to daf-16(-) larvae, daf-5(0) larvae do not develop through dauer. To obtain daf-5(0) dauer larvae, we used the daf-2(e1370) allele to drive dauer formation in the daf-5(-) background. [34,35]. We found that daf-5(0); daf-2(e1370) dauer larvae never expressed col-19p::gfp (S1 Fig). Therefore, while daf-16 and daf-5 act in parallel to promote dauer formation, the regulation of adult cell fate is specific to daf-16.

We next asked whether daf-16/FOXO regulates col-19p::gfp expression during stages other than dauer. First, we examined the timing of col-19p::gfp expression in larvae before, during, and after their entry into dauer. In wild-type animals, col-19p::gfp expression begins during the L4 molt, peaks soon after molting to adulthood, and then declines over time. Similarly, in daf-16(0) mutants, col-19p::gfp expression begins during the L2d-to-dauer molt, peaks soon after larvae enter dauer, and then declines over time in dauer (Fig 1B). Next, we asked whether larvae that develop continuously express precocious col-19p::gfp. For this experiment, we used larvae that were wild-type for daf-7. col-19p::gfp expression was never observed in daf-16(0) larvae during continuous development, and col-19p::gfp was expressed normally in adults (Fig 1C). Finally, we asked whether daf-16(0) larvae in L1 arrest express col-19p::gfp. Dauer shares some similarities with L1 arrest, which is a developmentally arrested and quiescent stage occurring when L1 larvae hatch in the absence of food [36]. daf-16/FOXO is important for L1 arrest; arrested daf-16(0) mutant larvae precociously initiate post-embryonic development including V lineage seam cell divisions [21]. However, we saw no precocious col-19p::gfp expression in these larvae (Fig 1C).

We next wondered which daf-16 isoforms block col-19p::gfp expression during dauer. There are three major isoforms of daf-16: a, b, and f [15,16,37]. The a and f isoforms together are the major players with respect to the role of daf-16 in longevity, dauer formation, and stress resistance [38]. Isoform a plays a larger role than f, but the f isoform still contributes because mutants lacking isoforms a and f display a stronger phenotype than mutants lacking either single isoform. By contrast, the b isoform is dispensable because animals lacking a and f isoforms were indistinguishable from null mutants for the phenotypes tested [38]. In contrast, the b isoform regulates neuronal morphology and function [39,40]. To determine which daf-16 isoforms contribute to maintaining seam cell multipotency during dauer, we used the same isoform-specific alleles used by Chen et al. (2015) [38] to assess col-19p::gfp expression during dauer. Loss of only daf-16a produced a stronger phenotype than loss of only daf-16f, and loss of both daf-16a and f produced an even stronger phenotype than loss of either individual isoform examined, indicating that both isoforms contribute (Figs 1D and S2). Loss of a and f
together caused expression of col-19p::gfp at high penetrance (Fig 1D), however this expression was dimmer and in fewer cells than the expression observed with in the null background (S2 Fig). Therefore, since loss of daf-16a/f did not produce a phenotype as strong as that of the complete null, the b isoform must also play a role. Taken together, we conclude that all three isoforms of daf-16 contribute to blocking col-19p::gfp expression during dauer.

To extend these findings beyond col-19p::gfp reporter expression, we performed mRNA-seq and differential gene expression analysis on daf-16(0) vs. control dauer larvae. Consistent with the data from col-19p::gfp experiments, we found that endogenous col-19 was highly and significantly upregulated in daf-16 mutants. Furthermore, 19/22 other adult-enriched collagens were also upregulated (Fig 1E and S3 Table). Similar to col-19p::gfp, most of these adult-enriched collagens appear to be expressed in seam cells and in hyp7 (S4 Table). Consistent with the upregulation of adult-enriched collagens, 23/24 dauer-enriched collagens were downregulated in daf-16(0) dauer larvae (Fig 1E), indicating an overall shift to genes required for adult cuticle formation in daf-16(0) dauer larvae.

In wild-type animals, the onset of col-19p::gfp expression coincides with several other characteristics of adult seam cell fate, including the production of adult alae, cell-cycle exit, and seam-cell fusion [23,24,41]. Although daf-16(0) dauer larvae express adult collagens, they do not display these other adult characteristics. Consistent with previous reports, we observed that daf-16(0) dauer larvae display dauer alae, which are distinct from adult alae [15,34]. Using the wls78 transgenic strain that expresses GFP in both seam cell nuclei (scm::gfp) and apical junctions (ajm-1::gfp) [42], we found that seam cells in daf-16(0) dauer larvae remained unfused (S3 Fig). Additionally, seam cells in daf-16(0) dauer larvae failed to maintain quiescence and underwent cell divisions (S3 Fig). These cell divisions were similar to the asymmetric divisions that normally occur during the L1, L3, and L4 stages (S3 Fig). Seam cell division does not occur during either adult or dauer stages in wild-type animals, however daf-16/FOXO is required for cell-cycle arrest in other contexts [21,22]. Therefore, during dauer, daf-16/FOXO both promotes seam cell quiescence and blocks adult cell fate, where its role in regulating seam cell fate is predominantly to control stage-specific collagen expression.

**daf-16 promotes expression of lin-41 during dauer**

During continuous development, the timing of col-19p::gfp expression is regulated by the heterochronic gene network [24,28,33,43]. To determine how daf-16 interacts with heterochronic genes, we began by asking whether heterochronic genes that oppose adult cell fate during continuous development might also be required to block col-19p::gfp expression during dauer. We focused on the three most downstream of these heterochronic genes, lin-14, hbl-1, and lin-41 (Fig 2A). We found that knockdown of lin-14 and hbl-1 resulted in little to no col-19p::gfp expression in hypodermal cells whereas lin-41 RNAi resulted in penetrant col-19p::gfp expression in lateral hypodermal cells during dauer (Fig 2B). Similar to daf-16(RNAi), col-19p::gfp expression in lin-41(RNAi) dauer larvae was brightest in the posterior of the worm, and dim or absent in the anterior. However, overall col-19p::gfp expression was dimmer in lin-41(RNAi) dauer larvae compared to daf-16(RNAi) (S4 Fig). In addition, some col-19p::gfp expression was observed in non-hypodermal cell types in hbl-1(RNAi) and lin-41(RNAi) dauer larvae (S5 Fig).

Since both daf-16 and lin-41 block col-19p::gfp expression in dauer larvae, we asked whether daf-16 regulates lin-41 expression. We examined our mRNA-seq data and also performed qPCR on daf-16(0) and control dauer larvae. In these experiments, lin-41 was downregulated approximately 2-fold in daf-16(0) dauers compared to control dauer larvae (Fig 3A). In contrast, there was no significant change in mRNA levels of the other core heterochronic genes (S6 Fig). Together, these data suggest that during dauer, daf-16 specifically promotes lin-41
expression to block col-19p::gfp and adult cell fate, and that in daf-16(−) dauers, lower levels of lin-41 lead to precocious col-19p::gfp expression.

To test whether reduced lin-41 levels are required for precocious col-19p::gfp expression in daf-16(0) dauers, we performed daf-16 RNAi on a strain in which lin-41 is misexpressed. To do this, we took advantage of the lin-41(xe8[Δ3’UTR]) allele which prevents the normal

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**Fig 2.** lin-41 is required to prevent precocious adoption of adult cell fate during dauer. (A) A simplified diagram of the network of heterochronic genes that regulates stage-specific seam cell fate during continuous development. “3 let-7s” indicates the let-7 family members, mir-48, mir-84, and mir-241. Genes in red are the most downstream positive regulators of larval cell fates [25]. (B) Precocious hypodermal expression of col-19p::gfp in dauer larvae in response to RNAi of heterochronic genes compared to the lacZ and daf-16 RNAi controls. Images were taken with a 63x objective. Numbers indicate the number of larvae expressing any hypodermal col-19p::gfp over the total number of dauer larvae scored. lin-14 RNAi induced only dim expression of col-19p::gfp, at low penetrance. hbl-1 RNAi did not induce hypodermal expression of col-19p::gfp. lin-41 RNAi caused highly penetrant expression of col-19p::gfp during dauer.

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downregulation of lin-41 in late larval stages and causes a strong gain-of-function phenotype [44,45]. We found the col-19p::gfp phenotype produced by daf-16(RNAi) was markedly suppressed in lin-41(xe8) dauer larvae, consistent with the hypothesis that daf-16 works through lin-41 to block col-19p::gfp expression during dauer (Fig 3B). To ensure that the observed suppression was not due to the lin-41(xe8) strain being less sensitive to RNAi, we tested the response of lin-41(xe8) and control strains to unc-22 RNAi and found a similar response in both strains (S8 Fig).

**lin-29 is not required for col-19p::gfp expression in lin-41(-) dauer larvae**

During continuous development, lin-41 opposes col-19p::gfp and adult cell fate by directly repressing the translation of the LIN-29 transcription factor [30,32]. LIN-29 is in turn a direct activator of col-19 transcription and is thought to be the most downstream regulator of other aspects of adult cell fate [26,27,31]. If the col-19p::gfp expression observed in lin-41(RNAi) dauer larvae is due to misexpression of LIN-29, then loss of lin-29 should prevent col-19p::gfp expression. To ask this question, we used the lin-29(xe37) deletion allele that removes all but 27 amino acids of LIN-29 [45]. Surprisingly, we saw no effect of the loss of lin-29 on the expression of col-19p::gfp in lin-41(RNAi) dauer larvae (Fig 4A). As a control, we established that our strain produced the expected lin-29(0) phenotypes in adults: a drastic reduction of col-19p::gfp and a complete lack of adult alae formation (S9 Fig).

We next confirmed the lin-41 RNAi results using the null allele lin-41(n2914). Since lin-41(0) hermaphrodites are sterile [30], we used an available transgene that is integrated close to...
the lin-41 locus as a balancer: nIs408[lin-29::mCherry, ttx-3p::gfp] [46]. This transgene had the added advantage of rescuing some of the lin-29(0) defects, making the strain easier to maintain (see Methods). When we induced dauer formation in the progeny of lin-41(0)/nIs408 and lin-41(0); lin-29(0) dauers expressed col-19p::gfp. nIs408 is a transgene used to balance lin-41(0) (see text). lin-41(0) larvae are dauer-defective [47]. Numbers indicate the number of dauers expressing col-19p::gfp over total dauers scored. (C, D) lin-29::gfp expression imaged on a compound microscope (C) or a confocal microscope (D). Images were taken with a 63x objective. Arrowheads indicate hypodermal nuclei with visible lin-29::gfp expression. Numbers indicate the number of larvae expressing any detectable lin-29::gfp over total number of larvae. lin-41(RNAi) produced substantial precocious expression of lin-29::gfp in continuously developing L3 staged larvae, easily discernable under the compound microscope. However, lin-41(RNAi) produced only very dim expression in dauer larvae. Dauer larvae produce substantial autofluorescence at the microscope settings required to visualize lin-29::gfp.

Fig 4. lin-41 regulates col-19p::gfp independently of lin-29 in dauer larvae. (A) lin-29 is not required for col-19p::gfp expression in lin-41(RNAi) dauers. Fluorescence levels were determined as described in S7 Fig. ns = not significant (p = 0.4856, two-tailed Mann-Whitney Test). n = 33–41; see S7 Table for complete underlying numerical data. (B) lin-41(0); lin-29(0) dauer larvae expressed col-19p::gfp. nIs408 is a transgene used to balance lin-41(0) (see text). lin-41(0) larvae are dauer-defective [47]. Numbers indicate the number of dauers expressing col-19p::gfp over total dauers scored. (C, D) lin-29::gfp expression imaged on a compound microscope (C) or a confocal microscope (D). Images were taken with a 63x objective. Arrowheads indicate hypodermal nuclei with visible lin-29::gfp expression. Numbers indicate the number of larvae expressing any detectable lin-29::gfp over total number of larvae. lin-41(RNAi) produced substantial precocious expression of lin-29::gfp in continuously developing L3 staged larvae, easily discernable under the compound microscope. However, lin-41(RNAi) produced only very dim expression in dauer larvae. Dauer larvae produce substantial autofluorescence at the microscope settings required to visualize lin-29::gfp.

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41(0)/nIs408; lin-29(0) mothers, we found that lin-41(0); lin-29(+) homozygous larvae, recognized by the lack of ttx-3p::gfp expression, were largely dauer defective (see [47]). Of the two lin-41(0) dauer larvae we recovered, one expressed col-19p::gfp (Fig 4B). Interestingly, 62% of larvae expressing ttx-3p::gfp displayed col-19p::gfp expression. This percentage is close to the 2/3 of transgene-containing larvae segregating from the heterozygous parent that would be predicted to be lin-41(0)/nIs408 heterozygotes, suggesting that lin-41 is haploinsufficient in its role in blocking col-19p::gfp during dauer. To bolster this supposition, we confirmed that dauer larval homozygous for nIs408 do not express col-19p::gfp (Fig 4B).

Although lin-41(0) homozygous larvae display a dauer-defective phenotype, lin-41(0); lin-29(0) larvae do not [47]. We were therefore able to test lin-41(0); lin-29(0) dauer larvae for col-19p::gfp expression. Approximately half of these larvae expressed col-19p::gfp (Fig 4B). Although the dauer-defective phenotype of lin-41(0) single mutants prevented us from determining the extent to which loss of lin-29 suppresses the col-19p::gfp phenotype in lin-41(0) homozygous mutant dauer larvae, these results do confirm that col-19p::gfp can be misexpressed in lin-41(0) dauer larvae even in the absence of lin-29.

To better understand the relationship between lin-41 and lin-29 during dauer, we asked whether lin-41 is required to block LIN-29 expression in dauer larvae. During continuous development, lin-41 mutants display precocious hypodermal expression of LIN-29 [30,32]. We used lin-29(xe61[lin-29::gfp]), which tags both isoforms of lin-29 [32] to assess the effect of lin-41 RNAi during dauer. Using settings that allow unambiguous visualization of hypodermal lin-29::gfp during L4 and adult stages, we saw only extremely dim lin-29::gfp expression in lin-41(RNAi) dauer larvae, just at the edge of detection (Fig 4C). We used confocal microscopy as a more sensitive assay to confirm that lin-29::gfp is expressed, albeit at very low levels in lin-41 (RNAi) but not control dauer larvae (Fig 4D). In control RNAi experiments run in parallel, lin-41(RNAi) was able to cause strong col-19p::gfp expression in dauer larvae (24/27 larvae examined), demonstrating that there were no technical problems in the RNAi experiment that would lead to such low expression. Finally, we confirmed that lin-41(RNAi) produces the expected precocious lin-29::gfp expression in L3 staged larvae that developed continuously (Fig 4C). Taken together, contrary to the role of lin-29 during continuous development, our data indicate that lin-41 regulates col-19p::gfp expression in dauer larvae and that lin-29 does not play a significant role in this regulation.

**daf-16 acts at least partially independently of lin-29 to regulate col-19p::gfp during dauer**

As described above, daf-16 promotes lin-41 expression during dauer, and misexpression of lin-41 suppresses the precocious col-19p::gfp expression observed in daf-16(0) dauer larvae. Since lin-29 was not required for misexpression of col-19p::gfp in lin-41(-) dauer larvae, we hypothesized that lin-29 would also be dispensable for col-19p::gfp expression in daf-16(-) dauer larvae. We found that neither of two lin-29 null alleles affected the penetrance of col-19p::gfp expression in daf-16(0) mutant dauers, demonstrating that the misexpression of col-19p::gfp in daf-16(0) dauer larvae does not depend on lin-29 (Fig 5A). However, when we compared levels of expression between the strains, we found a small but statistically significant decrease in expression in dauer larvae that lack lin-29, indicating that the presence of lin-29 bolsters col-19p::gfp expression slightly (Fig 5B). We next asked whether loss of daf-16 affects expression of lin-29. Examining our mRNA-seq data, no significant difference in lin-29 mRNA levels were observed in daf-16(0) vs. control dauer larvae (S6 Fig). However, during continuous development, regulation of lin-29a by lin-41 occurs translationally and may not be evident from mRNA levels [32,48]. We next examined lin-29::gfp expression in daf-16(0) dauer larvae. Unlike the dim lin-
expression we observed in lin-41(RNAi) dauer larvae, lin-29::gfp expression in the hypodermis was completely undetectable in daf-16(0) dauer larvae. Using confocal microscopy, 0/24 daf-16(0); lin-29(n546) dauer larvae displayed detectable lin-29::gfp. Taken together, these experiments demonstrate that daf-16 regulates col-19p::gfp expression largely independently of lin-29.

Since the key downstream regulator of col-19 expression is not required for the misexpression of col-19p::gfp in daf-16(0) dauer larvae, one or more different regulators must activate or derepress col-19p::gfp expression in this context. One possibility is that DAF-16 itself is a direct activator. Two pieces of indirect evidence initially argued against this possibility. First, the promoter sequence used in the col-19p::gfp transgene does not contain a canonical DAF-16/FOXO binding element (TGTTTAC) [49]. Furthermore, ChIP-seq experiments performed as part of modENCODE and displayed on WormBase did not show binding of DAF-16 to the col-19 promoter [50]. However, this evidence is not conclusive. The consensus sequence for DAF-16/FOXO binding elements is very broad, leaving open the possibility that DAF-16 could recognize a non-canonical site [51]. In addition, the ChIP-seq experiments were carried out during the L4/young adult stage, whereas the only time that we see aberrant col-19p::gfp expression in daf-16(0) mutant larvae is during dauer. To more definitively test DAF-16 binding at the col-19 promoter during the dauer stage, we used an endogenously tagged daf-16::zf1::wrmScarlet::3xFLAG strain to perform ChIP-qPCR during dauer. As expected, the promoter for a confirmed DAF-16 target, mtl-1 [52,53] showed 18–30 fold

**daf-16/FOXO regulates the expression of many genes during dauer**

Fig 5. daf-16 acts at least partially independently of lin-29 to block adult cell fate during dauer. (A) daf-16(0); lin-29(0) dauers expressed col-19p::gfp at a similar penetrance to daf-16(0) dauers. n546 is a nonsense mutation and xe37 is a deletion of all but 27 amino acids. Images were taken with a 63x objective. Numbers indicate the number of dauers expressing hypodermal col-19p::gfp over the total number of dauers. (B) Levels of col-19p::gfp are slightly reduced in daf-16(0); lin-29(xe37) dauer larvae compared to daf-16(0) dauers. Fluorescence levels were determined as described in S7 Fig. *** p<0.0001 (Two-tailed Mann-Whitney Test). n = 34–50; see S8 Table for complete underlying numerical data.

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enrichment in the daf-16::3xflag ChIP sample compared to wild type. By contrast, the promoter for col-19 showed only 2-4-fold enrichment in the daf-16::3xflag ChIP sample compared to wild type (Figs 6A and S11). This lower level of DAF-16 enrichment at the col-19 promoter was comparable to the DAF-16 enrichment at the coding region of the heterochromatinized gene bath-45, suggesting that the DAF-16 ChIP-qPCR experiments were inherently noisy. These data indicate that daf-16 blocks col-19p::gfp expression indirectly.

To identify candidate regulators of col-19p::gfp and endogenous adult collagen expression during dauer, we looked more broadly at the changes in gene expression that occur downstream of daf-16/FOXO using our mRNA-seq data comparing daf-16(0) vs. control dauer larvae. We found that 2027 genes were downregulated ≥2-fold in daf-16(0) dauer larvae, and 1576 genes were upregulated ≥2-fold in daf-16(0) dauer larvae (FDR ≤ 0.05) (Fig 6B). Interestingly, when we performed functional annotation clustering on these differentially expressed genes using the Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov/), terms related to collagens and cuticle structure were highly enriched among both downregulated and upregulated genes (S12 and S13 Figs). Signaling-related terms were also highly enriched, including terms related to ion transport associated with the downregulated genes and terms related to protein kinases and phosphatases associated with the upregulated genes (S12 and S13 Figs). Of the genes whose expression was affected, 112 encode
transcription factors (91 downregulated and 21 upregulated) and are candidate genes to directly regulate col-19p::gfp expression and adult cell fate during dauer (S14 Fig).

Discussion

Heterochronic genes specify stage-specific seam cell fate at each larval stage and at adulthood [24]. During continuous development, heterochronic genes function as a cascade where successive microRNAs act as molecular switches to downregulate early cell fate-promoting transcription factors and RNA-binding proteins, allowing progression to the next cell fate [25,54]. The decision to enter dauer drastically alters the timing of developmental progression [2]. Perhaps for this reason, extensive modulation of the heterochronic pathway has been identified in pre- and post-dauer stages [3,55–57]. For example, many heterochronic genes that are essential for stage-specific cell fate specification during continuous development are dispensable after dauer [3,42,43,55]. However, the mechanisms that act to prevent precocious specification within the dauer stage itself have not been addressed. Here, we find a novel role for daf-16/FOXO as a dauer-specific heterochronic gene. daf-16 opposes adult cell fate during dauer via a modified heterochronic pathway involving lin-41 but not lin-29.

The heterochronic gene lin-41 encodes an RNA-binding protein that promotes larval cell fate and opposes adult cell fate during continuous development [30,58]. We found that lin-41 acts downstream of daf-16 to block adult cell fate during dauer. Specifically, both daf-16 and lin-41 are required to prevent precocious expression of the col-19p::gfp adult cell fate marker during dauer, and lin-41 expression is downregulated in daf-16(0) dauer larvae (Figs 1A, 2B and 3A). In contrast, the other early-promoting heterochronic genes tested, lin-14 and hbl-1, do not appear to play a role in regulating col-19p::gfp hypodermal expression during dauer, and their expression was not significantly affected in daf-16(0) dauer larvae (Figs 2B and S6). Finally, a lin-41 gain-of-function allele partially blocked the misexpression of col-19p::gfp caused by daf-16 RNAi (Fig 3B). All together, these data are consistent with a linear pathway involving lin-41 but not lin-29.

The mechanism by which daf-16 promotes lin-41 expression is currently unknown. One possibility is that daf-16 regulates expression of lin-41 via the let-7 microRNA. During continuous development, lin-41 is expressed in early stages but is downregulated by the let-7 microRNA during the L4 stage to allow progression to adult cell fate. let-7 opposes lin-41 expression by binding to the lin-41 3'UTR and mediating silencing [30,58]. The ability of the lin-41(xe8 [Δ3'UTR]) allele to interfere with the daf-16(-) phenotype suggests the possibility that let-7 is involved in this regulatory pathway.

During continuous development lin-41 blocks adult cell fate by directly repressing the translation of the most downstream component in the heterochronic pathway, the LIN-29 transcription factor that promotes all aspects of adult cell fate [24,26,27,31,32]. LIN-29 expression remains low in the lateral hypodermis during early larval development due to the combined action of lin-41 and hbl-1 which regulate distinct lin-29 isoforms [30–32]. Surprisingly, we found that daf-16 and lin-41 regulate adult cell fate mostly independently of lin-29 (Fig 6D). During continuous development, the precocious phenotypes observed in lin-41(-) larvae are completely suppressed by compromising lin-29 activity [30]. Indeed, the phenotypes of every precocious heterochromic mutant tested during continuous development are suppressed by lin-29(-) alleles [26,30,42,43,59]. In contrast, loss of lin-29 had little to no effect on the precocious col-19p::gfp phenotype observed in daf-16(-) or lin-41(-) dauer larvae (Figs 4A, 4B, 5A and 5B). Therefore, whereas lin-29 is a direct transcriptional activator of col-19 and other
adult-enriched collagens in the context of adults [27–29], our work demonstrates that during dauer, daf-16 blocks adult collagen expression through factor(s) other than lin-29. We found over 3600 genes whose expression changed in daf-16(0) dauer larvae, including 112 transcription factors. One or more of these factors may control the expression of the col-19p::gfp transcriptional reporter.

In addition to regulating col-19p::gfp, we found that daf-16 is required during dauer to block expression of nearly all adult-enriched collagens. The expression of adult-enriched collagens in daf-16(0) dauer larvae is accompanied by a concomitant decrease in the expression of dauer-enriched collagens (Fig 1E). The effect of daf-16 mutations on the dauer cuticle may explain some of the previously observed defects in daf-16(0) dauer larvae. Two defining features of dauer larvae are the presence of dauer alae on the cuticle and resistance to treatment with detergents such as SDS. SDS-resistance depends in part on the specialized dauer cuticle [2]. daf-16(0) dauer larvae possess dauer alae that are slightly less defined than wild-type, and daf-16(0) dauers are only partially SDS-resistant [15,34,60,61]. It is possible that the shift from dauer-enriched collagens to adult-enriched collagens in daf-16(0) dauer larvae is responsible for these phenotypes. Notably, our functional annotation clustering analysis found that collagen-related terms were highly enriched among both upregulated and downregulated genes. This finding suggests that regulation of stage-specific collagen expression is a key role of daf-16/FOXO during dauer.

Dauer interrupts development midway through the larval stages [2]. During dauer, progenitor cells that have not yet completed development must maintain or re-establish multipotent fate, neither differentiating prematurely nor losing their previously acquired tissue identity. The role we identified for daf-16 in blocking adult cell fate in lateral hypodermal cells during dauer appears to contribute to the maintenance of multipotency in lateral hypodermal seam cells. We have previously described an analogous role for daf-16 in promoting multipotent VPC fate [5]. In both contexts, daf-16 activity is important to prevent precocious adoption of cell fates that normally occur later in development, after recovery from dauer. However, the mechanisms by which these cell fates are adopted differ in each cell type. Adult seam cell fate is regulated by the heterochronic genes, whereas VPC fate specification is regulated primarily by EGFR/Ras and LIN-12/Notch signaling [24,25,62]. The ability of daf-16/FOXO to influence these distinct developmental pathways suggests that daf-16/FOXO has a broad role in promoting multipotent cell fate during dauer. As one of the major regulators of dauer formation, daf-16 is well-positioned to coordinate the decision to enter dauer with the necessary alterations to developmental pathways that are paused during dauer.

daf-16 is the sole C. elegans ortholog of the genes encoding the FOXO proteins [15,16]. FOXO transcription factors regulate both stem cell quiescence and stem cell plasticity across species, from Hydra to mammals [20,63]. However, the mechanisms by which FOXO promotes multipotency in stem cells are still emerging. The protein encoded by the lin-41 ortholog, LIN41/TRIM71 also promotes cell fate plasticity in mammalian stem cells [64]. Our work provides the first connection between daf-16/FOXO and lin-41/TRIM71 and may be relevant to mammalian stem cells.

Methods

Strains and maintenance

A full list of strains and their genotypes used in this study is located in S1 Table. Balanced strains are described in more detail here. Homozygous lin-41(0) hermaphrodites are sterile, therefore for experiments involving the null allele lin-41(n2914), lin-41 was balanced with transgene nls408[ttx-3p::gfp, lin-29::mCherry] [46], which we found to be closely linked to lin-41. At each generation, larvae that expressed ttx-3p::gfp were singled out and their progeny
were monitored for segregation of lin-41(0) homozygotes that were Dpy, Ste, and lacked ttx-3p::gfp. The rescuing lin-29::mCherry enabled more robust growth in the lin-41(0)/nIs408; lin-29(0) strain than typical strains homozygous for lin-29(0). Although lin-29(0) mutants are homozygous viable, they are Egl, Pvl, and have reduced brood size [24]. For experiments, lin-41(n2914) homozygous larvae were identified based on the lack of ttx-3p::gfp expression. For more details about experiments involving these strains see Cale & Karp (2020) [47].

Animals homozygous for the lin-41(xe8[Δ3'UTR]) allele burst at young adulthood [44]. This gain-of-function allele was balanced over the lin-41(bch28 xe70) allele, where bch28 is a complex insertion of eft-3p::gfp that disrupts the lin-41 locus, and xe70 is a deletion of the lin-41 3'UTR [45,65]. For experiments, lin-41(xe8) larvae were identified based on the lack of eft-3p::gfp expression.

Dauer induction
All strains used for experiments involving dauer larvae contained daf-7(e1372), which is a temperature-sensitive, hypomorphic allele that induces dauer entry at 24˚C or 25˚C [34,67]. Unless otherwise specified, dauer larvae were obtained by allowing 10–20 gravid adult hermaphrodites to lay embryos for 2–8 hours at 24˚C, then removing the parents and allowing the embryos to incubate at 24˚C. For all experiments except those in Fig 1B, dauer larvae were scored approximately 48–52 hours after egg-laying, a time soon after dauer formation (“early dauer” or 0-day dauer larvae). In Fig 1B when different stages were examined, L2d larvae were scored at 24 hours after egg-laying; L2d-dauer molt larvae were scored at 39 hours after egg-laying; 1-day dauer larvae were scored at 72 hours after egg-laying. Dauer formation was verified by looking for crisp, defined dauer alae and radial constriction [67].

For experiments involving homozygous egg-laying-defective strains, including those with lin-29(0) or lin-29(xe61), embryos were obtained by sodium hypochlorite treatment consisting of two 2-minute incubations in 1M NaOH, 10% Clorox bleach. For experiments with XV254 daf-16(mgDf50); lin-29(xe37); daf-7(e1372); maIs105[co1-19p::gfp] and controls, embryos were obtained by dissecting gravid adult hermaphrodites.

Nondauer stages
Continuous development (Fig 1C). Strains XV33 mals105[co1-19p::gfp] and VT1750 daf-16(mgDf50); mals105 were synchronized by allowing gravid adult hermaphrodites to lay embryos at 24˚C or 25˚C and then incubating the progeny until the desired stage was reached. Developmental stage was evaluated by the extent of gonad and/or vulval development and scored for co1-19p::gfp or lin-29::gfp expression.

L1 arrest (Fig 1C). To induce L1 arrest, embryos from strains XV33 and VT1750 were obtained by sodium hypochlorite treatment and then incubated at 20˚C in M9 in a shaking incubator. L1 larvae were removed each day for 7 days and scored for co1-19p::gfp expression.

Adults (S9 Fig). Strains VT1777 daf-7(e1372); mals105 and XV253 lin-29(xe37); daf-7(e1372); mals105 were synchronized by sodium hypochlorite treatment. Embryos were placed on seeded NGM plates and grown at 20˚C for 72–75 hours. Adults were scored for the presence of stage-specific alae and co1-19p::gfp expression.

RNAi
RNAi bacteria were from the Ahringer library (Source Bioscience), except the lacZ RNAi clone was pXK10 [68]. RNAi plates were prepared by adding 300μL of an overnight culture of RNAi
bacteria to 60mm NGM plates containing 50μg/mL carbenicillin and 200μg/mL IPTG. Embryos obtained from sodium hypochlorite treatment were plated onto seeded RNAi plates and incubated at 24°C to allow daf-7(e1372) larvae to enter dauer or daf-7(+) larvae to develop continuously.

**Compound microscopy**

Animals were picked onto slides made with 2% agarose pads and paralyzed with 0.1 M levamisole. A Zeiss AxioImager D2 compound microscope with HPC 200 C fluorescent optics was used to image worms. DIC and fluorescence images were obtained using a AxioCam MRm Rev 3 camera and ZEN 3.2 software. GFP was visualized with a high efficiency GFP shift free filter.

**Confocal microscopy**

Animals were picked onto slides made with 2% agarose pads and paralyzed with 0.1 M levamisole. A Nikon A1R scanning laser confocal light microscope was used to image worms using an excitation laser set to at wavelength of 488 nm. To visualize lin-29::gfp, the laser power was set to 70%.

**Phenotypic data collection and analysis**

All phenotypic data presented are from at least two independent experiments, typically performed by independent researchers. Any experiments that involved subjective decisions were blinded by having a lab member not involved in the experiment code the strains and/or images before scoring. Statistical analyses were performed on Graphpad Prism (version 9.1) and specific tests are described in individual figure legends. P-values < 0.05 were deemed statistically significant.

**Sample collection for qPCR and RNA sequencing**

Synchronized populations of VT2317 daf-16(mgDf50); daf-7(e1372) and control CB1372 daf-7(e1372) dauer larvae were obtained by incubating embryos isolated by sodium-hypochlorite treatment at 24°C for 52 hours. Because some daf-16; daf-7 animals grown under these conditions fail to enter dauer [60], dauer larvae from both strains were handpicked into M9 solution, washed twice, and then pelleted to a volume of 100μl packed worms. TRIzol reagent (Invitrogen) was added to the samples at a 10:1 ratio of TRIzol to worm pellet. The samples were then frozen in dry-ice/ethanol. Two biological replicates were obtained for each strain for mRNA-seq. For qPCR, we obtained two biological replicates of the wild-type sample and three biological replicates of the daf-16(0) mutant sample.

**RNA isolation**

Total RNA isolation from dauer sample was conducted using TriReagent (Ambion) protocol with the following modifications: pelleted C. elegans in TRI-Reagent were subjected to three freeze/thaw/vortex cycles prior to BCP addition to improve extraction efficiency, isopropanol precipitation was conducted in the presence of glycogen for 1hr at -80°C, RNA was pelleted by centrifugation at 4°C for 30 minutes at 20,000 x g; the pellet was washed three times in 70% ethanol and resuspended in water. BioAnalyzer assay (Agilent Technologies) was used for quality control of the RNA sequencing samples prior to library creation, with a minimum RIN of 8.5. NanoDrop2000 (Thermo Scientific) was used to quantify and assess the RNA quality of samples analyzed by qPCR.
RT-qPCR

cDNA was synthesized from 250ng total RNA using SuperScript III Reverse Transcriptase (Invitrogen) and analyzed with a CFX96 Real-Time System (BioRad) using Absolute Blue SYBR Green PCR MasterMix (Life Technologies). Relative lin-41 mRNA levels were calculated based on the ΔΔ2Ct method [69] using eft-2 for normalization. Results presented are the average values of independent calculations from biological replicates.

RT-qPCR primers.

| Prime | Sequence |
|-------|----------|
| eft-2 F | ACGCTCGTGATGAGTTCA AG |
| eft-2 R | ATTTGGTCCAG TTCCGTCTG |
| lin-41 F | GGTTCaAATGCCACAAGAG |
| lin-41 R | AGGTCCAACTGCCAAATCAG |

mRNA-seq library preparation and sequencing

Libraries were constructed using the TruSeq RNA Library Prep Kit v2 (Illumina). The DNA concentration and fragment size of sequencing libraries were analyzed using the BioAnalyzer assay (Agilent Technologies). High-throughput sequencing was performed on the Illumina HiSeq 2500 platform to generate paired-end reads of 100 bp.

mRNA-seq analysis

Basecalling and base call quality were performed using Illumina’s Real-Time Analysis (RTA) software, and CIDSSeqSuite 7.1.0 was used to convert compressed bcl files into compressed fastq files. Using Trimomatic v. 0.39, Illumina adapters were clipped from raw mRNA-seq reads, followed by quality trimming (LEADING: 5; TRAILING: 5; SLIDINGWINDOW:4:15) [70]. Reads with a minimum length of 36 bases were retained (MINLEN: 36). Processed reads were aligned to C. elegans reference genome WBcel235 using STAR v. 2.4.2a with default parameters and—twopassMode Basic [71]. RSEM v. 2.1 was used to quantify gene abundance based on mapped reads [72]. Outlier samples were identified by assessing the quality of raw and processed reads with FastQC v. 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and by Principal Component Analysis (PCA) on log2-transformed normalized gene expression counts generated using DESeq2 v. 1.30.0 [73]. Differential gene expression analysis was performed using DESeq2 with a significance threshold of FDR \( \leq 0.05 \) and absolute value of log2-transformed fold changes of at least 2.

DAVID analysis

Differentially upregulated and downregulated genes with at least a log2-transformed fold change of 2 and FDR \( \leq 0.05 \) were individually analyzed using DAVID Functional Annotation Clustering (https://david.ncifcrf.gov/summary.jsp. Accessed 20210507). Terms from InterPro and Gene Ontology sub-ontologies cellular component (CC), biological process (BP), and molecular function (MF) were plotted by -log10(FDR) and ranked by fold enrichment.

ChIP-qPCR

GS8924 (daf-16(ar620[daf-16::zf1-wrmScarlet-3xFLAG])), a gift from Katherine Luo and Iva Greenwald (Columbia University) and control N2 embryos obtained after sodium-hypochlorite treatment were hatched overnight in M9 buffer. The L1 arrested worms were grown on NGM plates seeded with HB101 at 25˚C. After 5 days, the starved worm population was collected in M9 buffer and mutuated in 1% SDS solution for 30 minutes to isolate dauers. The worm pellet was washed 4x with autoclaved DI water and the worms were plated on an NGM
plate with no food. The live dauers crawled out and were collected as a ~500 μL packed pellet in M9 buffer.

The animals were nuted for 30 min at room temperature in 12 mL of 2.6% formaldehyde in autoclaved DI water for live crosslinking. The crosslinking was quenched with 600 μL of 2.5M glycine for 5 min at room temperature. The worms were washed 3x in water before flash freezing and storing at -80°C. Frozen pellets were ground twice for 1 min in the Retsch MM400 cryomill at a frequency of 30/s. The worm powder was lysed in 2 mL of 1x RIPA (1x PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) with 1x HALT Protease and phosphatase inhibitor (Thermo Scientific 78443) for 10 min at 4°C. The crosslinked chromatin was sonicated in the Diagenode Bioruptor Pico for 3 3min cycles, 30 s ON/OFF at 4°C. The chromatin was diluted to a concentration of 20-30 ng/μL. 10% of the volume was processed as the input sample. 10 μg of chromatin was incubated with 2 μg of Monoclonal Anti-Flag M2 antibody (Sigma-Aldrich F3165) overnight at 4°C and then for 2 hours with Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen 11201D). After 3 800 μL washes in LiCl buffer (100 mM Tris HCl pH 7.5, 500 mM LiCl, 1% NP40, 1% sodium deoxycholate), the samples were de-crosslinked by incubating with 80 μg of Proteinase K (Thermo Scientific 25530015) in Worm Lysis buffer (100 mM Tris HCl pH 7.5, 100 mM NaCl, 50 mM EDTA, 1% SDS) at 65°C for 4 hours. The samples were subjected to phenol-chloroform extraction and the DNA pellet was resuspended in TE buffer. RNase A (Thermo Scientific 12091021) treatment was performed for 1 hour at 37°C.

Quantitative PCR for promoter regions of interest was performed with Absolute Blue SYBR Green (Thermo Scientific AB4166B) on the CFX96 Real Time System Thermocyclers (Biorad) using custom primers. The cycle numbers in the ChIP samples were normalized to respective input values. The log 2 transformed fold change values in the daf-16::3xFLAG::wrmScarlet samples were normalized to the respective N2 samples. 2 biological replicates with 2 technical replicates each were done for the promoter regions of mtl-1 and col-19. Each biological replicate was analyzed separately.

**ChIP-qPCR primers.**

mtl-1p F  TGAGCAGTCTTAATCATCCTTTGCAC
mtl-1p R  ACGTGAATGTTGCAAACCTCCTTTGCAC
col-19p F  TCCATCTCTCTTGGAACACAT
col-19p R  ACACCTTCAAACCTAACCA GTGT
bath-45 F  ATTTAAATGTGTTTGAGAAGGCAGC
bath-45 R  GTGCTTTCTGCATCAGAGCC

**Supporting information**

S1 Fig. daf-5 is not required to block col-19::gfp expression during dauer. The daf-7/TGFβ pathway regulates dauer formation in parallel to the insulin-like pathway [6,7]. daf-5 encodes a Sno/Ski protein that works in a complex with DAF-3/SMAD to regulate transcription downstream of DAF-7/TGFβ signaling [76]. daf-5(0) mutants are dauer-defective, but can be induced to enter dauer when combined with the daf-2(e1370) allele [34,35]. (A) daf-2(e1370) mutants develop slowly and acquire dauer characteristics approximately one day later than wild-type or daf-7(e1372) larvae [60,77]. Since daf-16; daf-7 dauer larvae express col-19p::gfp most penetrantly within one day of dauer entry, we used SDS-resistance to determine a time that might correspond to “early dauer” in this strain. SDS-resistance is acquired at the end of the L2d-to-dauer molt and continues throughout dauer [2]. Gravid adult hermaphrodites of the indicated strains were allowed to lay eggs for 3 hours and then removed. Embryos were incubated at 24°C for the indicated times and then incubated in 1% SDS for 10 minutes. The
percent of larvae alive after this treatment is shown; data were aggregated from four independent experiments. (B) Percent of dauer larvae expressing col-19p::gfp. This expression was never observed in either control or daf-5(0) dauer larvae at times when many early dauer larvae should have been present. Data shown were aggregated from 3–4 independent experiments.

S2 Fig. Dauer larvae lacking 1–2 isoforms of daf-16 display col-19p::gfp more dimly and in fewer cells than daf-16(0). (A) Representative micrographs of dauer larvae scored in Fig 1D. In Fig 1D, any col-19p::gfp expression in the lateral hypodermis was considered “on”, however there are qualitative differences in that expression in the different strains. Dashed lines in the control indicate the boundaries of the dauer larva, as determined by DIC optics. (B) Loss of 1–2 isoforms of daf-16 results in fewer cells expressing col-19p::gfp compared to daf-16(0) dauer larvae. Quantification of the number of cells expressing col-19p::gfp along one side of the lateral hypodermis between the pharynx and rectum. Each dot represents a dauer larva. \( p = 0.0460 \) (Two-tailed Mann-Whitney Test). (C) Loss of 1–2 isoforms of daf-16 results in dimmer expression of col-19p::gfp throughout the hypodermis compared to the daf-16(0) dauer larvae. Fluorescence levels were determined as described in S7 Fig. \( **p < 0.0001 \) (Two-tailed Mann-Whitney Test). Larvae shown in panels B-C were from an independent set of experiments from those in Fig 1D. \( n = 28–31 \); see S2 Table for complete underlying numerical data.

S3 Fig. Seam cells divide and remain unfused in daf-16(0) dauer larvae. (A) Micrographs showing apical junctions between seam cells in daf-7 (control) and daf-16(0); daf-7 (daf-16(0)) dauer larva, and seam cell divisions in daf-16(0) dauer larvae. The worms are oriented with anterior to the left and ventral down. Apical junctions were visualized by ajm-1::gfp as part of the wls78 transgene [42,78]. Junctions were present between all seam cells in both daf-7 control and daf-16(0); daf-7 dauer larvae. Several seam cells in the daf-16(0); daf-7 dauer shown have recently divided. An example of one dividing seam cell is bracketed. The region bounded by junctions is smaller and rounder in the anterior daughter that will fuse with hyp7 (h). The posterior daughter (s) will remain a seam cell. Seam cells that were not dividing are indicated with an arrow. These photos were taken soon after dauer formation ("early dauers"). (B) Percent of dauer larvae with one or more seam cells actively dividing at the time of scoring. Seam cell divisions were identified based on ajm-1::gfp expression. Cell divisions were only seen in daf-16(0) dauer larvae.

S4 Fig. daf-16(RNAi) dauer larvae express col-19p::gfp more strongly than lin-41(RNAi) dauer larvae. Larvae assessed for col-19p::gfp expression in Fig 2 were analyzed for fluorescence intensity using ImageJ. GFP-positive nuclei were outlined using the freehand tool and the mean pixel intensity for all of such nuclei were averaged together for each individual worm. These averages are underestimates for the expression of col-19p::gfp in daf-16(RNAi) dauer larvae because many nuclei had fully saturated pixels. \( ****p < 0.0001 \) (Two-tailed Mann-Whitney Test). \( n = 30 \).

S5 Fig. RNAi of lin-41 and hbl-1 induces col-19p::gfp expression in non-hypodermal tissues. Numbers indicate larvae expressing col-19p::gfp in at least one cell in the relevant tissue over the total number of dauer larvae scored. Dauer larvae are oriented with anterior to the left and ventral down. (Top) lin-41 RNAi induces col-19p::gfp expression in vulva precursor cells
(VPCs) during dauer at high penetrance. The larva shown expressed col-19p::gfp in P5.p and P7.p, but overall which VPCs expressed col-19p::gfp varied from worm to worm and did not correlate with the predicted cell fate they will adopt. The exposure time in the fluorescence channel was 200ms. (Bottom) hbl-1 RNAi induces col-19p::gfp expression in occasional ventral neurons during dauer at lower penetrance. As col-19p::gfp includes a nuclear localization sequence, expression is typically restricted to the nucleus. However, in the larva shown a neuronal process could be seen extending posteriorly to the cell body (arrow). The exposure time in the fluorescence channel was 50ms.

S6 Fig. Levels of most core heterochronic genes are essentially unaffected by daf-16 during dauer. (A) A diagram of the network of heterochronic genes that regulates stage-specific seam cell fate during continuous development. “3 let-7s” indicates the let-7 family members, mir-48, mir-84, and mir-241. mir-48 is also downregulated by lin-42 (not depicted) [79]. All protein-coding genes except lin-41 are indicated in blue. (B) Log2 fold change of the protein-coding heterochronic genes other than lin-41, comparing daf-16(0) to control dauer larvae. Log2 fold change was calculated by DESeq2 from mRNA-seq data. Dashed lines indicate 2x fold upregulation or downregulation. False discovery rates (FDR) are indicated for each gene.

S7 Fig. Fluorescence scale used to determine relative fluorescence intensity of col-19p::gfp. Fluorescence images of col-19p::gfp in dauer larvae were taken from a strain we found to produce a wide range of expression levels, XV160, daf-7(e1372); mals105; unk-1(xk6). These images were taken using compound microscopy and identical settings, including an exposure time of 125ms. The images were then exported as 8-bit tiffs for ImageJ analysis. We identified six representative images distributed across the range of ImageJ values (0–250). We assigned numbers (0.5–5) to these images that reflect their distribution. A value of 0.25 was used if expression was visible, but less than that in the 0.5 panel. The fluorescence scale values with ImageJ values in parentheses were as follows: 0.5 (22), 1 (45), 2 (104), 3 (149), 4 (200), and 5 (250). These six images were then used as a scorecard to compare to experimental images for Figs 3B, 4A, and 5B. For these experiments, a 63x objective was used to take 2–3 images along the length of the dauer larva, using identical settings for all strains being compared. Each image was then subjectively compared to the fluorescence scale and assigned a value 0–5, with 0.5 values being used if an image was between two whole-number images. The values for each worm were averaged together to create an overall score for the worm.

S8 Fig. The lin-41(xe8) strain is not defective for RNAi. Unc phenotypes produced by unc-22(RNAi) were assessed during forward locomotion and binned into categories Mild: occasional twitching that did not interrupt movement. Intermediate: occasional twitching that did interrupt movement. Severe: constant twitching but still capable of forward locomotion. Paralyzed: worms whose twitching was so severe that they were not able to move forward. unc-22 RNAi experiments were carried out in parallel to the experiments shown in Fig 3B. However, because dauer larvae did not display strong Unc phenotypes, dauer larvae were moved to new RNAi plates at 20°C and allowed to recover to the post-dauer L4 (PDL4) stage. Numbers indicate the total number of PDL4 larvae assessed.

S9 Fig. The lin-29(xe37) strain displays reiterative phenotypes in adults. (A-B) The background for all strains was daf-7(e1372); mals105[col-19p::gfp]. (A) lin-29(xe37) mutant adults do not have adult alae. Numbers indicate the number of worms with alae defects over the total
number of adults. (B) *lin-29(xc37)* mutant adults express very low levels of *col-19p::gfp*. The two images were taken with identical settings (10ms exposure). Representative images are shown (n = 37–38).

**S10 Fig. DAF-16 is unlikely to bind the *col-19* promoter to regulate transcription.** (A) Genetic region upstream of *mtl-1*, a confirmed transcriptional target of DAF-16 [52,53]. DNA sequence beginning immediately 3’ to W02F12.8, the gene upstream of *mtl-1* is shown. This sequence likely contains promoter elements that regulate *mtl-1* expression. A canonical DAF-16-Binding Element (DBE, red) is located 76bp upstream of the *mtl-1* ATG. (B) The 846bp upstream of the *col-19* ATG which comprises the regulatory sequence driving *col-19p::gfp* expression [28]. No canonical DBEs (GTAAACA or TGTTTAC) [49] were found in this sequence. Sequences were taken from WormBase (WS280).

**S11 Fig. DAF-16 is not enriched at the *col-19* promoter.** ChIP-qPCR experiments were performed on N2 or *daf-16(ar620[daf-16::zf1-wrmScarlet-3xFLAG])* dauer larvae. Binding of DAF-16-3xFLAG was first normalized to input, and then to the average of the respective wild-type value (mean +/- SD for two technical replicates) is shown. Binding to the *col-19* promoter was not observed, whereas there was substantial binding to the promoter of the known DAF-16 target *mtl-1* (Barysyt et al. 2001; Li et al. 2008). The coding region of the heterochromatinized gene *bath-45* was used as negative control. This figure shows the second of two biological replicates; the first replicate is shown in Fig 6A.

**S12 Fig. Functional annotation clustering for genes upregulated in *daf-16* mutants.** DAVID analysis showing gene ontology (GO) and InterPro terms that were significantly enriched (p ≤ 0.05, Bonferroni corrected) in genes whose expression was upregulated ≥2x (FDR ≤ 0.05) in *daf-16(0)*; *daf-7* dauer larvae vs. *daf-7* (control) dauer larvae. GO BP = GO term biological process; GO CC = GO term cellular compartment; GO MF = GO term molecular function, INTERPRO = InterPro protein classification.

**S13 Fig. Functional annotation clustering for genes downregulated in *daf-16* mutants.** DAVID analysis showing gene ontology (GO) and InterPro terms that were significantly enriched (p ≤ 0.05, Bonferroni corrected) in genes whose expression was downregulated ≥2x (FDR ≤ 0.05) in *daf-16(0)*; *daf-7* dauer larvae vs. *daf-7* (control) dauer larvae. GO BP = GO term biological process; GO CC = GO term cellular compartment; GO MF = GO term molecular function, INTERPRO = InterPro protein classification.

**S14 Fig. Transcription factors differentially expressed in *daf-16(0)* dauer larvae compared to control dauer larvae.** Genes encoding transcription factors were defined based on their WormBase annotation (WS279). Although annotated as transcription factors, we noticed that the WormBase list also contained genes encoding RNA-binding proteins [80,81]. We therefore subtracted genes identified as encoding RNA-binding proteins from the transcription factor list. From among these genes, we searched our mRNA-seq data to find those whose expression changed ≥2x (FDR ≤ 0.05) by DESeq2 analysis. This analysis produced 112 genes that fit our criteria. Those genes are shown here as heat maps depicting relative expression of these genes in *daf-16(0)*; *daf-7* dauer larvae or *daf-7* (control) dauer larvae from mRNA-seq data. The row-
normalized reads for each of two biological replicates per strain are depicted.

S1 Table. List of strains used in this study.

S2 Table. Underlying numerical data for S2C Fig, fluorescence levels of col-19p::gfp in daf-16 isoform mutant dauers.

S3 Table. Collagen expression during development.

S4 Table. Adult-enriched collagen expression in seam cells and hyp7.

S5 Table. Raw lin-41 qPCR data for Fig 3A.

S6 Table. Underlying numerical data for Fig 3B, fluorescence levels of col-19p::gfp in lin-41(xe8) mutant dauers.

S7 Table. Underlying numerical data for Fig 4A, fluorescence levels of col-19p::gfp in lin-41(RNAi); lin-29(0) dauers.

S8 Table. Underlying numerical data for Fig 5B, fluorescence levels of col-19p::gfp in daf-16(0); lin-29(0) dauers.

S9 Table. Raw ChIP-qPCR data for Fig 6A.

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References

1. Orford KW, Scadden DT. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nature Reviews Genetics. 2008; 9: 115–128. https://doi.org/10.1038/nrg2269 PMID: 18202695

2. Cassada RC, Russell RL. The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental Biology. 1975; 46: 326–342. https://doi.org/10.1016/0012-1606(75)90109-8 PMID: 1183723

3. Liu Z, Ambros V. Alternative temporal control systems for hypodermal cell differentiation in Caenorhabditis elegans. Nature. 1991; 350: 162–165. https://doi.org/10.1038/350162a0 PMID: 2650247

4. Euling S, Ambros V. Reversal of cell fate determination in Caenorhabditis elegans vulval development. Development. 1996; 122: 2507–2515. PMID: 8756295

5. Karp X, Greenwald I. Control of cell-fate plasticity and maintenance of multipotency by DAF-16/FoxO in quiescent Caenorhabditis elegans. PNAS. 2013; 110: 2181–2186. https://doi.org/10.1073/pnas.1222377110 PMID: 23341833

6. Fielenbach N, Antebi A. C. elegans dauer formation and the molecular basis of plasticity. Genes & Development. 2008; 22: 2149–2165. https://doi.org/10.1101/gad.1701508 PMID: 18708575

7. Baugh LR, Hu PJ. Starvation responses throughout the Caenorhabditis elegans life cycle. Genetics. 2020; 216: 837–878. https://doi.org/10.1534/genetics.120.303565 PMID: 35268389

8. Fernandes-de-Abreu DA, Caballero A, Fardel P, Strousstrup N, Chen Z, Lee K, et al. An insulin-to-insulin regulatory network orchestrates phenotypic specificity in development and physiology. Plos Genet. 2014; 10: e1004225. https://doi.org/10.1371/journal.pgen.1004225 PMID: 24675767

9. Cornils A, Gloeck M, Chen Z, Zhang Y, Alcedo J. Specific insulin-like peptides encode sensory information to regulate distinct developmental processes. Development. 2011; 138: 1183–1193. https://doi.org/10.1242/dev.060905 PMID: 21343369

10. Murphy CT, Hu PJ. Insulin/insulin-like growth factor signaling in C. elegans. WormBook. 2013; 1–43. https://doi.org/10.1895/wormbook.1.164.1 PMID: 24395814

11. Zheng S, Chiu H, Boudreau J, Papanicolaou T, Bendena W, Chin-Sang I. A functional study of all 40 C. elegans insulin-like peptides. Journal of Biological Chemistry. 2018; jbc.RA118.004542. https://doi.org/10.1074/jbc.RA118.004542 PMID: 30206121

12. Ritter AD, Shen Y, Bass JF, Jeyaraj S, Deplancke B, Mukhopadhyay A, et al. Complex expression dynamics and robustness in C. elegans insulin networks. Genome Res. 2013; 23: 954–965. https://doi.org/10.1101/gr.150466.112 PMID: 23539137

13. Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, Buchman AR, et al. Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes & Development. 2001; 15: 672–686. https://doi.org/10.1101/gad.867301 PMID: 11274053

14. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science. 1997; 277: 942–946. https://doi.org/10.1126/science.277.5328.942 PMID: 9252323

15. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, et al. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature. 1997; 389: 994–999. https://doi.org/10.1038/40194 PMID: 9353126
16. Lin K, Dorman JB, Rodan A, Kenyon C. daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science. 1997; 278: 1319–1322. https://doi.org/10.1126/science.278.5341.1319 PMID: 9360933

17. Tothova Z, Gilliland DG. FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system. Cell Stem Cell. 2007; 1: 140–152. https://doi.org/10.1016/j.stem.2007.07.017 PMID: 18371346

18. Tothova Z, Kollipara R, Hunty BJ, Lee BH, Castrillon DH, Cullen DE, et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell. 2007; 128: 325–339. https://doi.org/10.1016/j.cell.2007.01.003 PMID: 17254970

19. Zhang X, Yalcin S, Lee D-F, Yeh T-YJ, Lee S-M, Su J, et al. FOXO1 is an essential regulator of pluripotency in human embryonic stem cells. Nature Cell Biology. 2011; 13: 1092–1099. https://doi.org/10.1038/ncb2293 PMID: 21804543

20. Liang R, Ghaffari S. Stem cells seen through the FOXO Lens: an evolving paradigm. Current topics in developmental biology. 2018; 127: 23–47. https://doi.org/10.1016/bs.ctdb.2017.11.006 PMID: 29433739

21. Baugh LR, Sternberg PW. DAF-16/FOXO Regulates Transcription of cki-1/Cip/Kip and Repression of lin-4 during C. elegans L1 Arrest. Current Biology. 2006; 16: 780–785. https://doi.org/10.1016/j.cub.2006.03.021 PMID: 16631585

22. Narbonne P, Roy R. Inhibition of germline proliferation during C. elegans dauer development requires PTEN, LKB1 and AMPK signalling. Development. 2006; 133: 611–619. https://doi.org/10.1242/dev.02232 PMID: 16407400

23. Sulston JE, Horvitz HR. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Developmental Biology. 1977; 56: 110–156. https://doi.org/10.1016/0078-6849(77)90158-0 PMID: 8381299

24. Ambros V, Horvitz HR. Heterochronic mutants of the nematode Caenorhabditis elegans. Science. 1984; 226: 409–416. https://doi.org/10.1126/science.6542614

25. Rougvie AE, Moss EG. Developmental transitions in C. elegans larval stages. Curr Top Dev Biol. 2013; 105: 153–180. https://doi.org/10.1016/B978-0-12-396968-2.00006-3 PMID: 23962842

26. Ambros V. A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell. 1989; 57: 49–57. https://doi.org/10.1016/0092-8674(89)90171-2 PMID: 2702689

27. Rougvie AE, Ambros V. The heterochronic gene lin-29 encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans. Development. 1995; 121: 2491–2500. PMID: 7671813

28. Liu Z, Kirch S, Ambros V. The Caenorhabditis elegans heterochronic gene pathway controls stage-specific transcription of collagen genes. Development. 1995; 121: 2471–2478. PMID: 7671811

29. Abete-Luzi P, Fukushige T, Yun S, Krause MW, Eisenmann DM. New roles for the heterochronic transcription factor LIN-29 in cuticle maintenance and lipid metabolism at the larval-to-adult transition in Caenorhabditis elegans. Genetics. 2020; 214: 669–690. https://doi.org/10.1534/genetics.119.302860 PMID: 31974205

30. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G. The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Molecular Cell. 2000; 5: 659–669. https://doi.org/10.1016/s1097-2765(00)80245-2 PMID: 10882102

31. Azzi C, Aeschimann F, Neagu A, Grohans H. A branched heterochronic pathway directs juvenile-to-adult transition through two LIN-29 isoforms. Elife. 2020; 9: e53387. https://doi.org/10.7554/eLife.53387 PMID: 32238899

32. Aeschimann F, Kumari P, Bartake H, Gaidatzis D, Xu L, Ciosk R, et al. LIN41 Post-transcriptionally silences mRNAs by two distinct and position-dependent mechanisms. Mol Cell. 2017; 65: 475–489.e4. https://doi.org/10.1016/j.molcel.2016.12.010 PMID: 28111013

33. Feinbaum R, Ambros V. The timing of lin-4 RNA accumulation controls the timing of postembryonic developmental events in Caenorhabditis elegans. Developmental Biology. 1999; 210: 87–95. https://doi.org/10.1006/dbio.1999.9272 PMID: 10364429

34. Vowels JJ, Thomas JH. Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. Genetics. 1992; 130: 105–123. https://doi.org/10.1093/genetics/130.1.105 PMID: 17321566

35. Larsen PL, Albert PS, Riddle DL. Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics. 1995; 139: 1567–1583. https://doi.org/10.1093/genetics/139.4.1567 PMID: 7789761

36. Johnson TE, Mitchell DH, Kline S, Kermal R, Foy J. Arresting development arrests aging in the nematode Caenorhabditis elegans. Mechanisms of Ageing and Development. 1984; 28: 23–40. https://doi.org/10.1016/0047-6374(84)90150-7 PMID: 6542614
37. Kwon E-S, Narasimhan SD, Yen K, Tissenbaum HA. A new DAF-16 isoform regulates longevity. Nature. 2010; 466: 498–502. https://doi.org/10.1038/nature09184 PMID: 20613724
38. Chen AT-Y, Guo C, Itani OA, Budaitis BG, Williams TW, Hopkins CE, et al. Longevity genes revealed by integrative analysis of isoform-specific daf-16/FoxO mutants of Caenorhabditis elegans. Genetics. 2015; 201: 613–629. https://doi.org/10.1534/genetics.115.177998 PMID: 26219299
39. Christensen R, Torre-Ubieta L de la, Bonni A, Colon-Ramos DA. A conserved PTEN/FOXO pathway regulates neuronal morphology during C. elegans development. 2011; 138: 5257–5267. https://doi.org/10.1242/dev.069062 PMID: 22069139
40. Sun S, Ohta A, Kuhara A, Nishikawa Y, Kage-Nakadai E. daf-16/FOXO isoform b in AIY neurons is involved in low preference for Bifidobacterium infantis in Caenorhabditis elegans. Neuroscience. 2019. https://doi.org/10.1016/j.neures.2019.01.011 PMID: 30731110
41. Podbielwicz B, White JG. Cell fusions in the developing epithelial of C. elegans. Developmental Biology. 1994; 161: 408–424. https://doi.org/10.1006/dbio.1994.1041 PMID: 8313992
42. Abrahante JE, Daul AL, Li M, Volk ML, Tennessen JM, Miller EA, et al. The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Developmental Cell. 2003; 4: 625–637. https://doi.org/10.1016/s1534-5807(03)00127-8 PMID: 12737799
43. Abrahante JE, Miller EA, Rougvie AE. Identification of heterochronous mutants in Caenorhabditis elegans. Temporal misexpression of a collagen::green fluorescent protein fusion gene. Genetics. 1998; 149: 1335–1351. https://doi.org/10.1093/genetics/149.3.1335 PMID: 9649524
44. Eccedi M, Rausch M, Großhans H. The let-7 microRNA directs vulval development through a single target. Developmental Cell. 2015; 32: 335–344. https://doi.org/10.1016/j.devcel.2014.12.018 PMID: 25669883
45. Aeschimann F, Neagu A, Rausch M, Großhans H. let-7 coordinates the transition to adulthood through a single primary and four secondary targets. Life Sci Alliance. 2019; 2: e201900335. https://doi.org/10.25669883
46. Harris DT, Horvitz HR. MAB-10/NAB acts with LIN-29/EGFR to regulate terminal differentiation and the transition from larva to adult in C. elegans. Development. 2011; 138: 4051–4062. https://doi.org/10.1242/dev.065417 PMID: 21862562
47. Cale AR, Karp X. lin-41 controls dauer formation and morphology via lin-29 in C. elegans. Micropublication. Biology. 2020; 2020: https://doi.org/10.17912/micropubbiology.000323 PMID: 33313484
48. Bettinger JC, Lee K, Rougvie AE. Stage-specific accumulation of the terminal differentiation factor LIN-29 during Caenorhabditis elegans development. Development. 1996; 122: 2517–2527. PMID: 8756296
49. Furuyama T, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J. 2000; 349: 629–634. https://doi.org/10.1042/0264-6021:3490629 PMID: 10880363
50. Gerstein MB, Lu ZJ, Nostrand ELV, Cheng C, Arshinoff BI, Liu T, et al. Integrative analysis of the Caenorhabditis elegans genome by the modENCODE project. Science. 2010; 330: 1775–1787. https://doi.org/10.1126/science.1196692
51. Tepper RG, Ashraf J, Kaletsky R, Kleemann G, Murphy CT, Bussemaeker HJ. PQM-1 complements DAF-16 as a key transcriptional regulator of DAF-2-mediated development and longevity. Cell. 2013; 154: 676–690. https://doi.org/10.1016/j.cell.2013.07.006 PMID: 23911329
52. Barsyte D, Lovejoy DA, Lithgow GJ. Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. Faseb J. 2001; 15: 627–634. https://doi.org/10.1096/fj.99-0966com PMID: 11259381
53. Li J, Ebata A, Dong Y, Rizki G, Iwata T, Lee SS. Caenorhabditis elegans HCF-1 Functions in Longevity Maintenance as a DAF-16 Regulator. Plos Biol. 2008; 6: e233. https://doi.org/10.1371/journal.pbio.0060233 PMID: 18828672
54. Rougvie AE. Control of developmental timing in animals. Nature reviews Genetics. 2001; 2: 690–701. https://doi.org/10.1038/35088566 PMID: 11533718
55. Karp X, Ambros V. Dauer larva quiescence alters the circuitry of microRNA pathways regulating cell fate progression in C. elegans. Development. 2012; 139: 2177–2186. https://doi.org/10.1242/dev.075986 PMID: 22619389
56. Ilbay O, Ambros V. Pheromones and nutritional signals regulate the developmental reliance on let-7 family microRNAs in C. elegans. Curr Biol. 2019; 29: 1735–1745.e4. https://doi.org/10.1016/j.cub.2019.04.034 PMID: 31104929
57. Hammell CM, Karp X, Ambros V. A feedback circuit involving let-7-family miRNAs and DAF-12 integrates environmental signals and developmental timing in Caenorhabditis elegans. PNAS. 2009; 106: 18668–18673. https://doi.org/10.1073/pnas.0908131106 PMID: 19828440
58. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000; 403: 901–906. https://doi.org/10.1038/3502607 PMID: 10706289

59. Lin SY, Johnson SM, Abraham M, Vella MC, Pasquinelli A, Gambheri C, et al. The C. elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Developmental Cell. 2003; 4: 639–650. https://doi.org/10.1016/s1534-5807(03)00124-2 PMID: 12737800

60. Nika L, Gibson T, Konkus R, Karp X. Fluorescent beads are a versatile tool for staging Caenorhabditis elegans in different life histories. G3. 2016; 6: 1923–1933. https://doi.org/10.1534/g3.116.030163 PMID: 27172224

61. Gottlieb S, Ruvkun G. daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in Caenorhabditis elegans. Genetics. 1994; 137: 107–20. PMID: 8056303

62. Sternberg PW. Vulval development. WormBook. 2005; 1–28. https://doi.org/10.1093/genetics/137.1.107 PMID: 18050418

63. Boehm A-M, Khalturin K, Anton-Enxleben F, Hemmrich G, Klostermeier UC, Lopez-Quintero JA, et al. FoxO is a critical regulator of stem cell maintenance in immortal Hydra. PNAS. 2012; 109: 19697–19702. https://doi.org/10.1073/pnas.1209714109 PMID: 23150562

64. Worringer KA, Rand TA, Hayashi Y, Sami S, Takahashi K, Tanabe K, et al. The let-7/LIN-41 pathway regulates reprogramming to human induced pluripotent stem cells by controlling expression of prodifferentiation genes. Cell Stem Cell. 2013; 14: 40–52. https://doi.org/10.1016/j.stem.2013.11.001 PMID: 24239824

65. Katic I, Xu C, Ciosk R. CRISPR/Cas9 genome editing in Caenorhabditis elegans: evaluation of templates for homology-mediated repair and knock-ins by homology-independent DNA repair. G3. 2015; 5: 1649–1656. https://doi.org/10.1534/g3.115.019273 PMID: 26044730

66. Brenner S. The genetics of Caenorhabditis elegans. Genetics. 1974; 77: 71–94. https://doi.org/10.1093 genetics/77.1.71 PMID: 4364676

67. Karp X. Working with dauer larvae. WormBook. 2018; 1–19. https://doi.org/10.1895/wormbook.1.180.1 PMID: 27417559

68. Karp X, Greenwald I. Post-transcriptional regulation of the E/Daughterless ortholog HLH-2, negative feedback, and birth order bias during the AC/VU decision in C. elegans. Genes & Development. 2003; 17: 3100–3111. https://doi.org/10.1101/gad.1160803 PMID: 14701877

69. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006; 1: 1559–1582. https://doi.org/10.1038/nprot.2006.236 PMID: 17406449

70. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

71. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013; 29: 15–21. https://doi.org/10.1093/bioinformatics/bts635 PMID: 23104866

72. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. Bmc Bioinformatics. 2011; 12: 323. https://doi.org/10.1186/1471-2105-12-323 PMID: 21816040

73. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15: 550. https://doi.org/10.1186/s13059-014-0550-8 PMID: 25516281

74. Oh SW, Mukhopadhyay A, Dixit BL, Raha T, Green MR, Tissenbaum HA. Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. Nat Genet. 2006; 38: 251–257. https://doi.org/10.1038/ng1723 PMID: 16380712

75. Honda Y, Honda S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. Faseb J. 1999; 13: 1385–1393. https://doi.org/10.1096/fasebj.13.11.1385 PMID: 10428762

76. da Graca LS, Zimmerman KK, Mitchell MC, Kozhan-Gorodetska M, Sekiewicz Z, Morales Y, et al. DAF-5 is a Ski oncprotein homolog that functions in a neuronal TGF beta pathway to regulate C. elegans dauer development. Development. 2004; 131: 435–446. https://doi.org/10.1242/dev.00922 PMID: 14681186

77. Ruaud AF, Katic I, Bessereau JL. Insulin/Insulin-like growth factor signaling controls non-dauer developmental speed in the nematode Caenorhabditis elegans. Genetics. 2011; 187: 337–343. https://doi.org/10.1534 genetics.110.123323 PMID: 20944013

78. Koh K, Rothman JH. ELT-5 and ELT-6 are required continuously to regulate epidermal seam cell differentiation and cell fusion in C. elegans. Development. 2001; 128: 2867–2880. PMID: 11532911
79. McCulloch KA, Rougvie AE. *Caenorhabditis elegans* period homolog lin-42 regulates the timing of heterochronic miRNA expression. Proc National Acad Sci. 2014; 111: 15450–15455. https://doi.org/10.1073/pnas.1414856111 PMID: 25319259

80. Matia-González AM, Laing EE, Gerber AP. Conserved mRNA-binding proteomes in eukaryotic organisms. Nature Structural & Molecular Biology. 2015; 22: 1027–1033. https://doi.org/10.1038/nsmb.3128 PMID: 26595419

81. Tamburino AM, Ryder SP, Walhout AJM. A compendium of *Caenorhabditis elegans* RNA binding proteins predicts extensive regulation at multiple levels. G3. 2013; 3: 297–304. https://doi.org/10.1534/g3.112.004390 PMID: 23390605