New Roles for the Heterochronic Transcription Factor LIN-29 in Cuticle Maintenance and Lipid Metabolism at the Larval-to-Adult Transition in *Caenorhabditis elegans*

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ABSTRACT Temporal regulation of gene expression is a crucial aspect of metazoan development. In the roundworm *Caenorhabditis elegans*, the heterochronic pathway controls multiple developmental events in a time-specific manner. The most downstream effector of this pathway, the zinc-finger transcription factor LIN-29, acts in the last larval stage (L4) to regulate elements of the larval-to-adult switch. Here, we explore new LIN-29 targets and their implications for this developmental transition. We used RNA-sequencing to identify genes differentially expressed between animals misexpressing LIN-29 at an early time point and control animals. Among 230 LIN-29-activated genes, we found that genes encoding cuticle collagens were overrepresented. Interestingly, expression of *lin-29* and some of these collagens was increased in adults with cuticle damage, suggesting a previously unknown function for LIN-29 in adult cuticle maintenance. On the other hand, genes involved in fat metabolism were enriched among 350 LIN-29-downregulated targets. We used mass spectrometry to assay lipid content in animals overexpressing LIN-29 and observed reduced fatty acid levels. Many LIN-29-repressed genes are normally expressed in the intestine, suggesting cell-nonautonomous regulation. We identified several LIN-29 upregulated genes encoding signaling molecules that may act as mediators in the regulation of intestinally expressed genes encoding fat metabolic enzymes and vitellogenins. Overall, our results support the model of LIN-29 as a major regulator of adult cuticle synthesis and integrity, and as the trigger for metabolic changes that take place at the important transition from rapid growth during larval life to slower growth and offspring production during adulthood.

KEYWORDS *Caenorhabditis elegans*; heterochronic; gene expression; collagen; metabolism

FOR successful animal development to occur, a large number of cellular events, including the proper regulation of gene expression, must occur in the right place but also at the right time. While much is known about regulation of metazoan development in the spatial dimension, less is known about the equally important temporal coordination of such events. Here we examine the temporal control of gene expression during the last phase of development in the nematode *Caenorhabditis elegans*.

After embryogenesis is completed inside an eggshell, this ecdysozoan nematode worm goes through four larval stages (L1–L4), molting its outer, collagen-rich cuticle between stages, before becoming an adult that is capable of laying eggs (Altun and Hall 2009). Genetic and molecular analyses have uncovered the heterochronic pathway as the main regulator of developmental timing in *C. elegans*. This pathway consists of a network of proteins and microRNAs (miRNAs) that interact to control the expression and stability of key transcription factors that regulate developmental events in a stage-specific manner (Nimmo and Slack 2009; Rougvie and Moss 2013; Moss and Romer-Seibert 2014). Mutations in components of this pathway lead to either the precocious

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or retarded occurrence of stage-specific events, particularly events involved in the development of the single-layer skin of the worm, the hypodermis. Several members of the heterochronic pathway are conserved in vertebrates and control developmental timing and stem cell fate in those organisms as well (Houbaviy *et al.* 2003; Moss 2007; Su *et al.* 2012; Ecsedi and Großhans 2013; Worringer *et al.* 2014; Tsialikas and Romer-Seibert 2015).

The most downstream heterochronic pathway regulator is LIN-29, a Kruppel-family zinc finger transcription factor (Rougvie and Ambros 1995) with homology to mammalian early growth response (EGR) proteins (Harris and Horvitz 2011). *lin-29* function is required for a number of developmental events that take place in the L4 stage in coordination with the worm's transition from larval to adult life. Some of these LIN-29-regulated events include the formation of the adult cuticle, the terminal differentiation and fusion of the lateral hypodermal cells (also called seam cells), the cessation of the molting cycle, the migration of the developing gonad, and the formation of various somatic reproductive structures in both hermaphrodites and males (Rougvie and Ambros 1995; Bettinger *et al.* 1996, 1997; Euling *et al.* 1999; Newman *et al.* 2000; Inoue *et al.* 2005; Sternberg 2005; Hayes *et al.* 2006; Abraham *et al.* 2007; Fielenbach *et al.* 2007; Ririe *et al.* 2008; Harris and Horvitz 2011; Blum *et al.* 2012; Gupta *et al.* 2012). According to whole-body RNA-sequencing (RNA-seq) data, *lin-29* transcript levels peak in the L3 stage, while immuno-staining and reporter fusions show that a major accumulation of LIN-29 protein takes place in hypodermal cells starting in the L4 (Bettinger *et al.* 1996; Gerstein *et al.* 2010; Harris and Horvitz 2011; Aeschimann *et al.* 2017). In these cells, LIN-29 expression is negatively regulated before the L4 stage by two upstream heterochronic proteins: HBL-1/Hunchback, which presumably acts by repressing *lin-29* transcription in the L2; and LIN-41/Trim, which represses by binding the 5′ end of the *lin-29a* transcript and blocking its translation in the L3 stage (Slack *et al.* 2000; Lin *et al.* 2003; Aeschimann *et al.* 2017). Negative regulation of HBL-1 and LIN-41 by members of the *let-7* miRNA family subsequently allows LIN-29 accumulation to occur at the correct time (Slack *et al.* 2000; Abrahante *et al.* 2003; Abbott *et al.* 2005; Aeschimann *et al.* 2017).

A number of target genes regulated by LIN-29 that may function in stage-specific developmental events have been identified. In the hypodermal seam cells, LIN-29 regulates expression of genes involved in cell division (Hong *et al.* 1998; Rausch *et al.* 2015), cell fusion (Friedlander-Shani and Podbilewicz 2011), molting (Harris and Horvitz 2011), and the adult-specific cuticle collagen (*col*) genes *col-7* and *col-19* (Liu *et al.* 1995; Rougvie and Ambros 1995). In recent work, we found that LIN-29 also regulates the L4-expressed *col* genes *col-38*, *col-49*, *col-63*, and *col-138*, and showed that mutation of specific LIN-29 binding sites abolished expression of a *col-38* reporter transgene *in vivo* in the L4 hypodermis (Abete-Luzi and Eisenmann 2018). In the anchor cell of the somatic gonad, LIN-29 activates expression of *lag-2*, a Notch ligand that promotes uterine cell differentiation and the formation of the uterine-seam cell connection (Newman *et al.* 2000). Finally, LIN-29 was also shown to act nonautonomously to regulate expression of vitellogenin genes *vit-1*, *vit-2*, *vit-3*, and *vit-6* in the intestine, promoting an adult-specific event required for fertility (Dowen *et al.* 2016).

The transition to adulthood is a fundamental life history event for all animals and it involves at least three major changes: the conclusion of a period of rapid somatic growth and differentiation, the acquisition of reproductive capabilities (*e.g.*, sexual organogenesis), and the associated metabolic adjustment underlying a switch in energy investment from somatic to germinal functions. To further explore the network of events coordinated by LIN-29, and to uncover potential new roles for this heterochronic protein, we temporally misexpressed LIN-29 and examined changes in development and gene expression. Using RNA-seq analysis, we identified several hundred genes for which expression was up- or downregulated upon temporal misexpression of LIN-29. These include 33 upregulated genes encoding cuticle collagens, suggesting a rather preponderant cell-autonomous role for LIN-29 in cuticle production at the last molt. Interestingly, our data suggests that LIN-29 and most likely its upstream regulators are also used to upregulate collagen gene expression in the adult in response to defects in cuticle integrity. Among target genes with decreased expression upon LIN-29 overexpression, we identified genes encoding enzymes involved in lipid metabolism, many of which are normally expressed in the intestine and are downregulated in the L4 stage. We found that several signaling molecules encoded by LIN-29-upregulated targets are required for both the positive expression of intestinal vitellogenin genes, and for the repression of some intestinal metabolic enzyme genes. Together, these results indicate that in addition to its roles in hypodermal developmental events, including cuticle collagen gene expression, LIN-29 may play a broader, cell-nonautonomous role in the regulation of fat metabolism perhaps contributing to a metabolic restructuring at the larval-to-adult transition.

Materials and Methods

C. elegans growth and strains used

C. elegans animals were cultured using standard methods (Brenner 1974). Worms were grown on NGM plates and fed with *Escherichia coli* OP50, or HT115 in the case of RNA interference (RNAi) experiments. Experiments were performed at 20° unless indicated otherwise. Bristol strain N2 of *C. elegans* was the wild-type strain. The following strains and alleles were used in this work:

NL2099: *rrf-3*(*pk1426*) *II*
NR222: *rde-1*(*ne219*) *V*; *kzIs9*[pKK1260(*lin-26p*::*nls*::*gfp*), pKK1253(*lin-26p*::*rde-1*), pRF4(*rol-6*(*su1006*)] (Qadota *et al.* 2007)
EG669: *ttTi5605 II*; *unc-119(ed3) III*
CB6147: *bus-8(e2882) X* (Partridge *et al.* 2008)

SV1009: *heIs63* [*wrt-2p*::*gfp*::*ph* + *wrt-2p*::*gfp*::*H2B* + *lin-48p*::*mCherry*] *V*
HW1692: *lin-29(xe37)* II (Aeschimann *et al.* 2019)
HW1695: *lin-29(xe40)* II (Aeschimann *et al.* 2019)
CB769: *bli-1*(*e769*) *II*
hs::*lin-29*: *deSi5*[*pPA5* = *hsp-16.2p*::*lin-29a*::*unc-54-3′UTR*; *unc-119(+)*] II; *unc-119(ed3) III* (Abete-Luzi and Eisenmann 2018)
hs::*control*: *deSi6*[*pPA4* = *hsp-16.2p*::*unc-54-3′UTR*; *unc-119(+)*] II; *unc-119(ed3) III* (Abete-Luzi and Eisenmann 2018)

Ectopic induction of LIN-29 via heat shock

Embryos obtained from bleaching *hs*::*lin-29* and *hs*::*control* strains were hatched overnight in liquid in the absence of food, the resulting synchronized early L1 stage animals were grown for a given amount of time with food at 20° (or at 25° when indicated), induced by heat-shock exposure for 30 min at 37°, then returned to growing temperature until scoring, imaging, or collection for RNA preparations. Specific developmental stages were determined by time in hours postfeeding (hpf) and verified by the extent of gonad migration and/or vulval cell division/morphology.

Induction protocol for analyses of body morphology and vulva phenotypes: Animals were grown at 20°. Heat shocks corresponding to the late L2, late L3, and mid L4 stages were done at 23, 33, and 43 hpf, respectively.

Induction protocol for analysis of precocious seam cell fusion: Strains also carried the *heIs63* array. For late L2 induction, animals were grown at 20°, heat-shocked at 23 hpf, and scored at 28 hpf. For single L3 induction animals were grown at 25°, heat-shocked at 22 hpf (early L3), and scored at 25 hpf. For double L3 induction, animals were grown at 25°, heat-shocked at 22 and 25 hpf (mid L3), and scored at 27 hpf.

Induction protocols for analyses of precocious alae and gonad migration defects: Worms were grown at 25°, heat-shocked at 22 and 25 hpf, and then scored at the early-to-mid L4 stage (29–32 hpf).

Induction protocols for assessment of LIN-29 target gene expression: Animals were grown at 20°. Induction for RNA-seq analysis was done by heat shock in the early L3 at 28 hpf. Adult induction for quantitative RT-PCR (RT-qPCR) assessment of intestinal targets was carried out in gravid adults (66 hpf).

"Young adult animals" indicates pregravid adult animals that have yet to accumulate or lay eggs; "day 1 adult animals" indicates animals in the first day of egg laying.

Imaging

Animals were mounted on 2.5% agarose pads and suspended in anesthetic solution (5 mM levamisole in M9). Nomarski (DIC) and epifluorescence microscopy was performed on a Zeiss Axioplan 2 and recorded with a Lumenera Infinity 3 camera and Infinity Analyze software.

RNA-seq and target gene identification

hs::*lin-29* and *hs*::*control* worms grown at 20° were induced in the early L3 (28 hpf), given a 1-hr recovery at 20°, collected, and frozen at −80° for a minimum of 15 min. Pellets (50–100 μl) were washed three times, resuspended in DEPC water (600 μl), and homogenized with a gentleMAC dissociator (Miltenyi Biotec). RNA preparations were performed with Quick-RNA MiniPrep kit (Zymo Research). A total of six samples (three biological replicates for *hs*::*lin-29* and *hs*::*control* each) were sequenced with single-end 50 base reads on an Illumina HiSeq 2500. Bioinformatics quality controls were done using FastQC, version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). The ce10 reference genome was aligned using STAR, version 2.5.1b. The number of reads mapped to genes were counted using htseq, version 0.6.1p1. Differentially expressed genes were determined using DESeq2, version 1.12.3 with the cut-off of 0.05 on false discovery rate. Transcriptomic data from this work has been deposited in the Gene Expression Omnibus archive under accession number GSE118433.

RNAi treatments

In most cases, synchronized L1 stage animals were incubated at 20° [except for *lin-29*(*RNAi*) and *bli-1*(*RNAi*) experiments, in which worms were grown at 25°] and fed with HT115 *E. coli* previously transformed with specific RNAi clones (Kamath *et al.* 2000). The RNAi control was empty "feeding" vector L4440 (gift from Andrew Fire; plasmid 1654; Addgene). RNAi clones used in this work were from the Ahringer RNAi library (Kamath and Ahringer 2003) (*lin-29*, *wrt-6*, *grd-11*), the Vidal RNAi library (Rual *et al.* 2004) (*ins-37*, *grl-14*), or previous work (Jackson *et al.* 2014) (*bli-1*).

RNA isolation and RT- qPCR

For each experiment, relative transcript levels were assessed by two-step RT-qPCR with three-to-four independent biological replicates. RNAi-treated, heat-shocked, and control animals were collected and stored at −80° for a minimum of 15 min. Worm pellets (50–100 μl) were washed three times, resuspended in DEPC water (∼600 μl), homogenized with a gentleMAC dissociator (Miltenyi Biotec) and used for RNA preparations via Quick-RNA MiniPrep kit (Zymo Research). Total RNA was reverse transcribed with a mix of oligo(dT) and random primers using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCRs were performed with exon-exon spanning primers (Supplemental Material, Table S5) and the iTaq Universal SYBR Green Supermix system (Bio-Rad). All Ct values were normalized to housekeeping gene *gpd-2* and data were analyzed by the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Protein category (Gene Ontology term) and tissue enrichment analyses

Analysis of target gene lists for protein function was performed using UniProt Knowledgebase (www.uniprot.orgl);

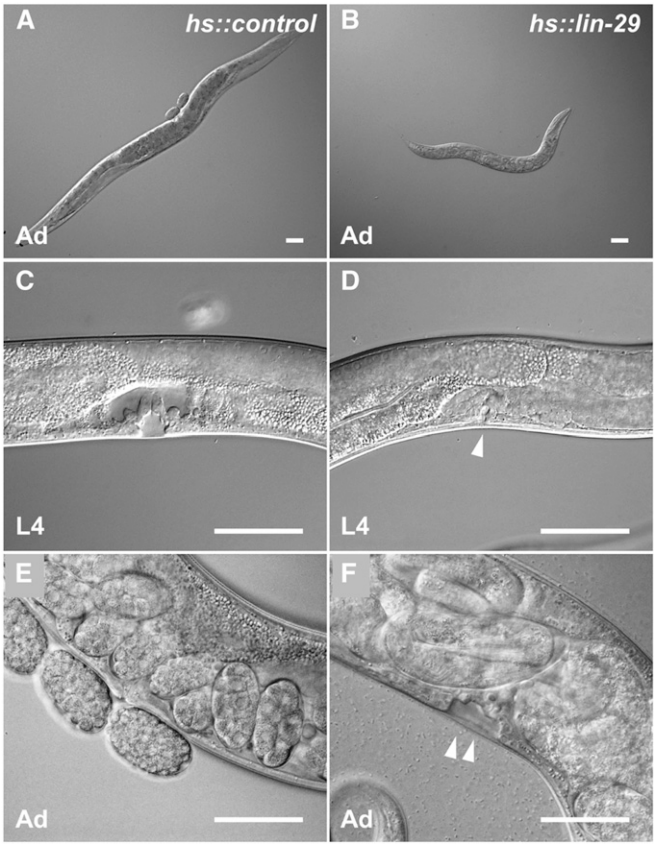

Figure 1 Early expression of LIN-29 results in body morphology and vulval defects. Nomarski images of *hs*::*control* (A, C, and E) or *hs*::*lin-29* (B, D, and F) animals that were subjected to heat shock early in development. (A and B) Adult animals that were given a single heat shock in both the L2 and L4 stages. (C–F) Animals were given a single heat shock in both the L2 and L3 stages, and scored in the L4 (C and D) or the adult (E and F) stage. Single arrowhead in D indicates an underinduced vulva in the L4 stage. Double arrowhead in F indicates an L4 stage vulva (compare to *hs*::*control* L4, C) in an adult *hs*::*lin-29* animal (note the presence of unlaid, late-stage embryos in the uterus). Bar, 50 μm.

Gene Ontology term enrichment was performed using DAVID (https://david.ncifcrf.gov; Huang *et al.* 2009) and AmiGO 2/PANTHER (http://amigo.geneontology.org; Carbon *et al.* 2009; Munoz-Torres and Carbon 2017); and tissue enrichment analysis (TEA) was performed using the WormBase Enrichment Analysis tool (www.wormbase.org; Angeles-Albores *et al.* 2016). Enrichment analyses were done using default parameters. Published data on target genes (IDs; RNAi phenotypes, sites of expression, times of expression, *etc.*) from Table S2 was retrieved using the WormBase Simplemine tool (www.wormbase.org; Lee *et al.* 2018).

Fatty acid gas chromatography–mass spectrometry analysis

For animals overexpressing LIN-29, synchronized L1 stage *hs*::*lin-29* and *hs*::*control* worms were grown at 20°, induced by heat shock (30 min 37°) twice in the L3 stage (28 and 33 hpf) and once in the L4 stage (43 hpf), and returned to 20° until 65 hpf. For animals with reduction of *lin-29* function only in the hypodermis, synchronized L1 larvae of strain NR222 were grown at 20° and fed HT115 bacteria containing either *lin-29*(*RNAi*) construct or empty-vector control. In all cases, adults (65 hpf) were washed from plates with water (four to six 60-mm plates per biological replicate) and transferred to preweighed glass vials. Worm samples were processed for fatty acid methyl ester analysis as described (Watts and Browse 2002) with the modification that naphthalene d8 (1 ng/μl final in injection mix) was added as an internal loading standard. Samples (1 μl) of the organic phase were analyzed by gas chromatography using a PerkinElmer Clarus 680 Gas Chromatograph equipped with a PerkinElmer (Norwalk, CT) Elite 5-MS column and helium as the carrier gas at 1.5 ml/min. Samples were injected without splitting at 250° and the following temperature program was used: 100° hold 2 min, 4°/min to 150° hold 4 min, 6°/min to 320° hold 4 min. Fatty acid methyl esters were identified by EI+ using a PerkinElmer Clarus SQ 8C Mass Spectrometer and TurboMass Ver6.0.0 software in the range 50.00–200.0 m/z. All biological replicates were processed and analyzed on the same day. For the *hs*::*lin-29* *vs.* *hs*::*control* study, a total of four biological trials of each strain were analyzed on two separate dates [two trials per gas chromatography–mass spectrometry (GC-MS) run for each strain]. Trials performed on different dates were not averaged. For the *lin-29*(*RNAi*) *vs.* control RNAi analysis, four biological replicates of each treatment were assessed together in a single GC-MS run (all trials averaged). For each fatty acid, the quantities determined by GC-MS were successively normalized to the naphthalene internal standard and to the weight of the sample.

Table 1 Adult phenotypes following *lin-29* misexpression in larval life

Time of heat shock	Strain	% WT	% Dpy	% Egl	% Small	% Small-Egl	*N*
No heat shock	*hs*::*lin-29*	98	0	0	2	0	130
L2	*hs*::*control*	99	0	0	1	0	73
	hs::*lin-29*	55	7	1	16*	21*	73
L3	*hs*::*control*	94	0	2	2	0	48
	hs::*lin-29*	22	8	38*	8	23*	86
L4	*hs*::*control*	100	0	0	0	0	72
	hs::*lin-29*	100	0	0	0	0	56
L2 + L3	*hs*::*control*	98	0	0	2	0	111
	hs::*lin-29*	12	5	55*	8	20*	145
L2 + L4	*hs*::*control*	99	0	0	1	0	224
	hs::*lin-29*	17	11*	7	28*	43*	161

Strains carrying *hs*::*lin-29* or *hs*::*control* were submitted to different protocols of heat shock (column 1) to test the consequences of LIN-29 induction at different times during development. Day 1 adults were assessed for body morphology phenotypes by direct observation. * $P < 0.001$ (Fisher's exact test) compared to the corresponding *hs*::*control*. WT, wild type; Dpy, Dumpy, Egl, Egg-laying defective; Small, substantial decrease in body size.

Survival analysis

L1-synchronized *hs*::*lin-29* and *hs*::*control* worms were grown at 20° until the first eggs were laid, then transferred

Table 2 Vulva defects induced by early expression of *lin-29*

		Vulva phenotypes				
Stage	Strain	% Wild type	% Abnormal	% Underinduced	% L4-like lumen	*N*
L4	*hs::control*	100	0	0	n.d.	73
L4	*hs::lin-29*	66	18	15	n.d.	110
Adult	*hs::control*	100	0	n.d.	0	30
Adult	*hs::lin-29*	21	46	n.d.	33	67

Vulva developmental defects were assessed in the indicated strains after early induction of LIN-29 by a single heat shock in both the L2 and L3 stages. Phenotypes were scored first in the L4 stage, then in adults of the same cohorts. In all cases $P < 0.001$ (Fisher's exact test) compared to the corresponding *hs::control*.

to 5-fluoro-2′-deoxyuridine (FUdR) solid media (to induce sterility) at 25°. It was previously shown that life span tends to be intrinsically shorter when animals are fed proliferating bacteria (most likely due to an age-related susceptibility to infection) and one recommended alternative is to use UV-killed bacteria as source of food for survival analysis (Garigan *et al.* 2002; Sutphin and Kaeberlein 2009). In this study, we tested both conditions and animals were fed with either live bacteria (*E. coli* OP50) or dead bacteria (UV-killed *E. coli* OP50 on 50 μg/ml carbenicillin NGM plates). Heat-shock inductions of LIN-29 started in day 1 adults and were repeated either daily or every other day. All cohorts were followed until 100% mortality, and survival curves were determined with OASIS 2 using the Kaplan–Meier method and statistically analyzed with the log rank test (https://sbi.postech.ac.kr/oasis2; Oncotarget 11269; Han *et al.* 2016).

Data availability statement

Original gene expression data underlying this work are fully available without restriction from the Gene Expression Omnibus archive (www.ncbi.nlm.nih.gov/geo/) under accession number GSE118433. After publication, some of the data from this paper will be available in the publicly accessible, curated database WormBase (wormbase.org). Any reagents and strains utilized in this work will either be available from a publicly accessible strain repository (the *Caenorhabditis* Genetic Center) or freely available upon request from the corresponding author. Supplemental material available at figshare: https://doi.org/10.25386/genetics.11663883.

Results

hs::lin-29 induction before the L4 stage leads to defects in body morphology, vulval development, and gonad migration

Loss-of-function mutations in upstream heterochronic pathway regulators precociously express LIN-29 earlier in development (Slack *et al.* 2000; Aeschimann *et al.* 2017); however, the consequences of direct misexpression of LIN-29 have not previously been assayed. To that end we used a strain containing a single-copy, integrated transgene containing a full-length *lin-29a* complementary DNA downstream of a heat-shock promoter (referred to as *hs::lin-29*) and a control strain with the identical heat-shock promoter and no insert (*hs::control*) (Abete-Luzi and Eisenmann 2018). We previously showed that this reagent was sufficient to drive ectopic expression of a reporter for the LIN-29 target *col-38* (which is normally expressed in the L4 stage) in either the L2/L3 stage or in the adult, when induced at those respective times of development (Abete-Luzi and Eisenmann 2018). In these cases, the *col-38* reporter expression was only observed in the hypodermal cells that normally express *col-38*, indicating that temporal but not spatial expression was affected. Here, we used this reagent to test whether temporal misexpression of LIN-29 during development was sufficient to cause phenotypes in processes associated with *lin-29*.

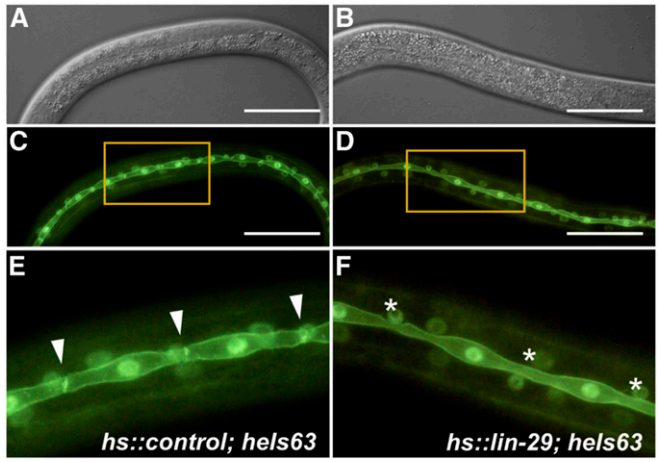

Figure 2 Early expression of LIN-29 is sufficient to cause precocious seam cell fusion. Shown here are synchronized L3 stage animals expressing nuclear and plasma membrane localized GFP in the hypodermal seam cells (from array *hels63*) and carrying either *hs::control* (A, C, and E) or *hs::lin-29* (B, D, and F). Populations of animals were given a heat shock in the late L2 (see Table 3; *Materials and Methods*). Nomarski (A and B) and epifluorescence (C and D) microscopy of larvae 5 hr after heat shock. Precocious seam cell fusion is observed, as seen in the magnified view (E and F; from insets shown in C and D): cell junctions between seam cells are still present in the *hs::control* strain (E; arrowheads) but are absent in animals carrying *hs::lin-29* (F; asterisks). Bar, 50 μm.

We exposed *hs::lin-29* animals to a heat-shock pulse either once in the L2, L3, or L4 stage, or twice (in the L2 and L3 stages, or in the L2 and L4 stages), and looked for morphological defects in these same animals as young adults. Adults that were subject to early temporal overexpression of LIN-29 displayed three morphological phenotypes: whereas a few animals displayed a Dumpy phenotype (Dpy), many more were egg-laying defective (Egl) or showed a substantial decrease in body size (Small), or both (Figure 1B, Figure S1, and Table 1). We noted that in many of the Small animals the pharynx was bent inside the head of the animal, as if the pharynx was too large to fit inside a smaller body (penetrance = 38% of Small adults, $n = 35$; Figure S2). No morphological phenotypes were observed when LIN-29 expression was induced only in the L4 stage when LIN-29 is normally present. We did note that the penetrances of both the Egl and Small

Table 3 LIN-29-induced seam cell fusion in earlier developmental stages

Time of heat shock	Time observed	Strain	% Multiple or all seam cells fused	% Only one fusion between two cells	*N*
Late L2	Early L3	*hs*::*control*; *heIs63*	0	7	42
		hs::*lin-29*; *heIs63*	45	0	42
Early L3	Mid L3	*hs*::*control*; *heIs63*	0	0	30
		hs::*lin-29*; *heIs63*	82	3	60
Early L3 + mid L3	Late L3	*hs*::*control*; *heIs63*	0	0	18
		hs::*lin-29*; *heIs63*	100	0	30

Animals expressing GFP in the nucleus and at the plasma membrane of the seam cells (from *heIs63*; see *Materials and Methods*) and carrying either *hs*::*lin-29* or *hs*::*control* were heat-shocked as indicated and observed for precocious seam cell fusion at the time shown. Multiple and single fusion events were scored with epifluorescence microscopy. In all cases $P \leq 0.0001$ (Fisher's exact test) compared to the corresponding *hs*::*control*.

phenotypes in animals subjected to a heat shock in the L2 were increased with an additional L4 induction, suggesting that excess LIN-29 in the L4 stage can contribute to these phenotypes. The morphological phenotypes we observed in *lin-29* gain-of-function conditions are similar to those seen in *lin-41* loss-of-function mutants, in which there is early accumulation of LIN-29 in the L3 stage: these animals also show Dpy, Small, and slightly Egl phenotypes (Slack *et al.* 2000; Tocchini *et al.* 2014).

To investigate the basis for the Egl phenotype observed upon misexpression of LIN-29, we assessed the L2 + L3 heat-shocked animals for vulval defects at the L4 and young adult stages; we saw vulval abnormalities with significant penetrance at both times (see Figure 1D, Figure S1, and Table 2). In the L4 stage, heat-shock-treated animals showed too few cells adopting vulval fates ("underinduced") or vulval inductions that were abnormal in morphology ("abnormal"). *lin-29* is known to be required for development of the egg-laying apparatus: *lin-29* mutants were first identified based on their loss-of-function Egl and protruding vulva (Pvl) phenotypes, and *lin-29* was later shown to be required for the formation of the connection between the uterus and vulva and for expression of certain genes in vulval cells (Ambros and Horvitz 1984; Bettinger *et al.* 1996, 1997; Newman *et al.* 2000; Inoue *et al.* 2005). However, one vulval phenotype we observed deserves comment: when examined as gravid adults, one-third of heat-shocked *hs*::*lin-29* animals showed a vulval morphology that resembled that found in mid-to-late L4 stage animals (Figure 1F, Figure S1, and Table 2). To our knowledge, this type of "arrested L4 vulva in an adult" phenotype has not been observed before in other heterochronic or vulval mutants. A possible explanation for both the "arrested vulva" phenotype and the "bent pharynx" phenotype in Small animals is that in animals experiencing an earlier than normal pulse of LIN-29, the hypodermis may have delayed or arrested development instead of progressing to adulthood, resulting in a hypodermis that is temporally out of sync with other body tissues. Thus, in some animals we observed an L4 vulva in an adult animal with embryos, while in other animals we found an adult-sized pharynx in an L4-sized body.

Finally, we also observed that overexpression of LIN-29 in the L3 stage was sufficient to cause a mild gonad migration phenotype. Although reduction of *lin-29* function does not cause a gonad migration defect on its own, genetic and molecular analyses indicate that *lin-29* acts on the migrating distal tip cell to control the timing of its turning event (Fielenbach *et al.* 2007; Huang *et al.* 2014). We found that 13% of *hs*::*lin-29* animals given two heat-shock pulses in the L3 stage had defects in gonad migration ($n = 48$, compared to 0% for *hs*::*control* animals, $n = 32$). In these animals the elongating gonad arms turned dorsalward correctly, but then one arm migrated in the incorrect direction along the anterior-posterior axis.

Early hs::lin-29 induction is sufficient to promote precocious seam cell fusion, but not precocious alae formation

LIN-29 is also known to regulate the fusion of the hypodermal seam cells with each other at the end of the L4 stage via expression of the fusogen *aff-1* (Friedlander-Shani and Podbilewicz 2011). Therefore, we looked at seam cell behavior when precociously inducing LIN-29 in the late L2 and in the L3 stage. We used the *heIs63* transgene, which expresses nuclear- and membrane-localized GFP from a seam-cell-specific promoter (Wildwater *et al.* 2011), to examine seam cell morphology in *hs*::*lin-29* and *hs*::*control* animals. We found that a single heat shock is enough to induce precocious seam cell fusion at high penetrance (Figure 2 and Table 3). We noted that the timing of the heat shock relative to the timing of the seam cell division affected the penetrance of the phenotype: cells that were newly divided and had not yet restored cell-cell contact upon heat shock did not display a precocious fusion phenotype, while single seam cells in contact with neighboring cells usually did show precocious fusion when LIN-29 was induced. Our results indicate that LIN-29 is not only required but also sufficient for seam cell fusion, at least in the L3 stage.

Unlike seam cell fusion, we found that early overexpression of LIN-29 was not sufficient to induce adult alae formation. It has long been known that *lin-29* mutants lack adult alae, indicating *lin-29* is necessary for production of these adult cuticular structures in the L4 stage (Ambros and Horvitz 1984). We gave *hs*::*lin-29* animals two heat-shock treatments in the L3 stage and observed them from 2 to 5 hr after the second heat-shock period. Although we observed short,

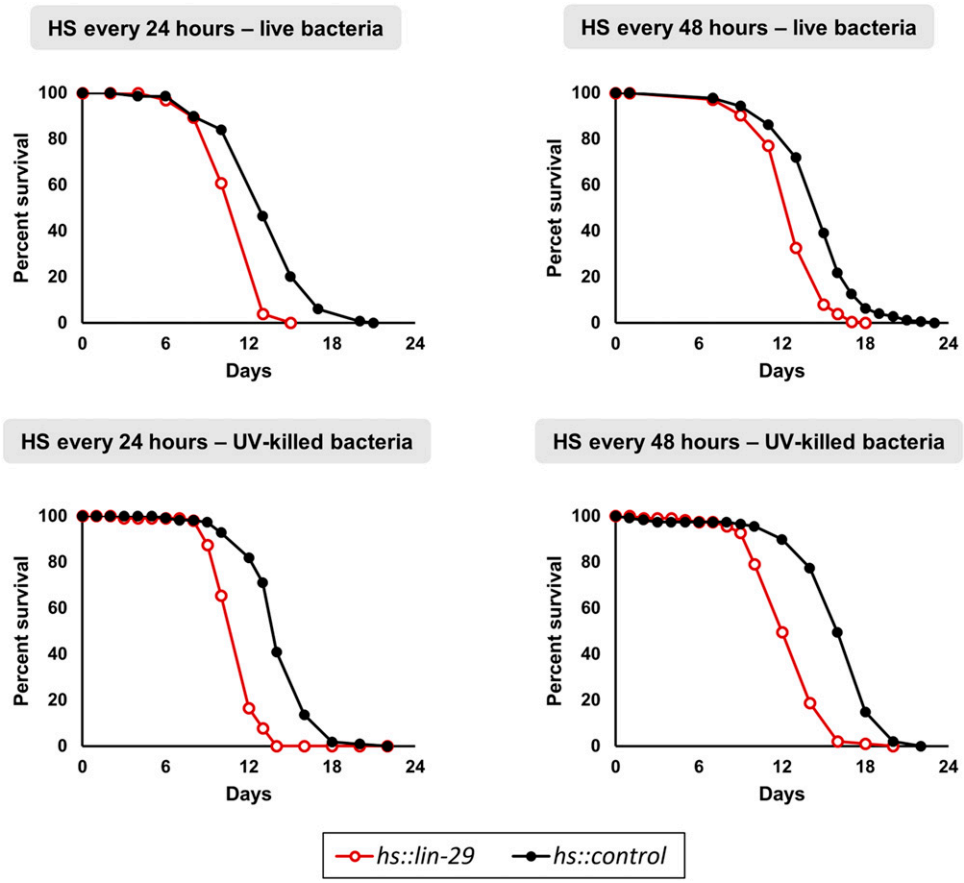

Figure 3 Periodic adult overexpression of LIN-29 shortens life span. Synchronized adult animals carrying either *hs*::*lin-29* or *hs*::*control* were periodically exposed to heat shock either every 24 or every 48 hr, and fed with either live or dead bacteria. Cohorts were FUdR-sterilized and followed until the last individual died. Survival curves were computed using the Kaplan–Meier estimator and statistical differences between *hs*::*lin-29* and *hs*::*control* groups were calculated with the log-rank test (in all cases $P < 0.0001$). In all four conditions both mean and maximum life span were shorter in *hs*::*lin-29* animals (see Table S1).

disorganized striations in rare animals, in no case did we observed the presence of unambiguous adult alae, even in small amounts. Precocious adult alae have been observed at the L3 molt in lin-41*(lf)* mutants and in *lin-41* or *hbl-1* RNAi-treated animals (Slack *et al.* 2000; Lin *et al.* 2003; Fielenbach *et al.* 2007) in which LIN-29 accumulated early. These results indicate that overexpression of the LIN-29a isoform under the conditions we assayed is not sufficient for production of adult alae in the L3 stage, suggesting that perhaps another LIN-29 isoform, or the repression of other upstream heterochronic regulators is necessary for this phenotype.

Misexpression of LIN-29 in the adult shortens life span

Previous work showed that two heterochronic genes that function early in the larval life, *lin-14* and *lin-4*, can affect life span even when their expression is manipulated solely in the adult (Boehm and Slack 2005). This was surprising because both *lin-14* and *lin-4* were known as key regulators of the L1 to L2 transition and any effects in the adult were unknown. We know that the expression of *lin-29* in the L4 promotes the developmental transition to the adult in some tissues and were curious whether this LIN-29 "maturing" instruction could have any beneficial or adverse effect on the aging of the animal if overexpressed during adulthood. We tested multiple pulses of LIN-29 only in the adult stage and assessed survival rates in four different conditions: a single heat shock every 24 hr or every 48 hr, and feeding with either dead bacteria or live bacteria. Worms subjected to these protocols showed no change in foraging behavior, no altered pharyngeal pumping, and no other visible phenotype or sickness, yet all four experiments showed significantly shorter mean life span and maximum life span (see Figure 3 and Table S1). Although we do not know the cause of this effect on life span, this result indicates that the disruption of normal temporal gene expression patterns by the misexpression of LIN-29 later during adulthood is detrimental to the animal. The observation that LIN-29 regulates cuticle collagen genes and genes involved in energy metabolism (this work, see below; Liu *et al.* 1995; Rougvie and Ambros 1995; Dowen *et al.* 2016; Abete-Luzi and Eisenmann 2018), and that both of these types of genes have effects on life span (Ewald *et al.* 2015; Duffy *et al.* 2016; Bustos and Partridge 2017), may be relevant to this observation.

Identification of genes regulated after LIN-29 temporal misexpression

To identify target genes regulated by LIN-29 we used a gain-of-function approach in which we examine global changes in gene expression following overexpression of wild-type LIN-29 using the heat-shock promoter, as we did for the transcription factor BAR-1 (Jackson *et al.* 2014; Gorrepati *et al.* 2015).

Table 4 Main categories of gene products among LIN-29-regulated genes

	Genes with significant differential expression		1.7-Fold upregulated genes		1.7-Fold downregulated genes	
Category	Count	%	Count	%	Count	%
Unknown	502	45.6	104	45.2	180	51.4
Lipid metabolism (Zhang)	65	5.9	2	0.9	25	7.1
Cuticle collagen	46	4.2	33	14.3	3	0.9
F-box protein	26	2.4	3	1.3	18	5.1
Nuclear hormone receptor	25	2.3	4	1.7	11	3.1
Other transcription factor	21	1.9	8	3.5	6	1.7
C-lectin	14	1.3	9	3.9	1	0.3
7TM receptor	13	1.2	12	5.2	1	0.3
Cytochrome P450	11	1.0	3	1.3	6	1.7
Transthyretin-domain	11	1.0	0	0.0	6	1.7
UDP-glucuronosyltransferase	10	0.9	3	1.3	3	0.9
Prion-like-(Q/N-rich)-domain	10	0.9	3	1.3	2	0.6
Noncoding RNA	8	0.7	2	0.9	0	0.0
O-acyltransferase	8	0.7	2	0.9	5	1.4
Nematode specific protein B	6	0.5	5	2.2	0	0.0
Zinc metalloprotease	6	0.5	10	4.3	2	0.6
Protein phosphatase	4	0.4	4	1.7	2	0.6
Extracellular signaling protein	4	0.4	4	1.7	0	0.0
Neuropeptide like protein	4	0.4	0	0.0	2	0.6
Peroxisomal assembly factor	3	0.3	0	0.0	3	0.9
Solute carrier protein	3	0.3	0	0.0	3	0.9

"Genes with significant differential expression" are those with a differential change between *hs*::*lin-29* and *hs*::*control* strains; $P < 0.05$ ($n = 1101$). The other gene sets are those genes showing ≥1.7-fold upregulation ($n = 230$) or downregulation ($n = 350$) in *hs*::*lin-29* compared to *hs*::*control* and $P < 0.05$.

To our knowledge, most work previously done on LIN-29 has been done using *lin-29* reduction-of-function mutation or RNAi-treated strains. For example, while investigating targets regulated by the miRNA *let-7*, Hunter *et al.* conducted microarray analyses of L4 stage *lin-29*(*n333*) mutant animals *vs.* wild type (Hunter *et al.* 2013). However, a caveat of looking for target genes with a reduction-of-function approach is that observed changes in gene expression or phenotype may be an indirect, downstream consequence of changes in cell fate or other defects caused by the loss of a regulatory factor during development. Although there are caveats to the gain-of-function approach as well (see *Discussion*), we believe the approach of expressing LIN-29 at a discrete time in otherwise normally developed animals and then examining changes in gene expression a short time later, may be more likely to avoid such secondary downstream effects. We believe that genes showing altered regulation shortly after LIN-29 overexpression at an earlier time in development are likely to represent targets of LIN-29 during its normal role in the L4 stage.

Strains carrying either *hs*::*lin-29* or *hs*::*control* were given a single heat shock in the early L3 stage and RNA-seq analysis was performed on triplicate samples collected 1 hr after the end of the heat-shock period. We chose this time since it is close to but earlier than the normal peak of LIN-29 gene expression, so other aspects necessary for LIN-29 function such as the presence of other transcription factors or a permissible chromatin state, may be present. We found 1101 genes that were differently expressed ($P < 0.05$) between the two heat-shocked strains.

Using an arbitrary cut-off of ≥1.7-fold, we narrowed our target list to 230 and 350 genes that were upregulated and downregulated respectively, upon early overexpression of LIN-29 (Table S2). To gain insight into the role of these differentially regulated genes we characterized them based on their molecular function (Table 4 and Table S2). The major categories of upregulated genes included those encoding proteins of unknown function ($n = 104$), cuticle collagens ($n = 33$), seven transmembrane receptors ($n = 12$), transcription factors ($n = 12$), zinc metalloproteases ($n = 10$), and C-type lectins ($n = 9$). For the downregulated genes, the major categories were proteins of unknown function ($n = 180$), enzymes functioning in fatty acid metabolism ($n = 25$), F-box proteins ($n = 18$), and transcription factors ($n = 17$). In addition to these gene classes, other types of genes that were found in large numbers in the set of all 1101 LIN-29-regulated genes were those encoding nuclear hormone receptor transcription factors (25 genes), cytochrome P450 enzymes (11 genes), UDP glycosyl transferase genes (11 genes), and proteins with transthyretin domains (11 genes) or prion-like (polyQ/N) domains (10 genes).

Genes encoding cuticle collagens are major upregulated targets of LIN-29 and are activated in response to cuticle defects in the adult

We performed Gene Ontology analyses to search for over-represented categories of genes in three data sets: all significant regulated genes (1101 genes), genes upregulated ≥1.7-fold (230 genes), and genes downregulated ≥1.7-fold (350 genes) (Table 5). The sole significantly enriched

Table 5 Enrichment analysis of LIN-29 target genes

All LIN-29-regulated targets (n = 1101)		Gene count				
Category/GO term		In genome	Found	Expected	Fold	P value
GO biological process	Peroxisome organization	18	7	0.97	7.23	1.89E−04
	Fatty acid metabolic process	105	21	5.65	3.72	1.56E−06
	Defense response to Gram-positive bacterium	60	12	3.23	3.72	2.65E−04
	Innate immune response	345	57	18.56	3.07	2.55E−12
	Oxidation-reduction process	618	60	33.25	1.8	3.76E−05
	Cellular response to chemical stimulus	612	57	32.92	1.73	1.57E−04
GO molecular function	Structural constituent of cuticle	168	48	9.04	5.31	2.90E−18
	Iron ion binding	118	20	6.35	3.15	2.38E−05
	Oxidoreductase activity	539	56	29	1.93	1.08E−05
GO cellular component	Peroxisomal membrane	18	8	0.97	8.26	3.08E−05
	Membrane raft	80	14	4.3	3.25	2.87E−04
	Extracellular space	294	37	15.82	2.34	7.31E−06
Lipid metabolism[a]	N/A	471	65	25.33	2.56	1.72E−12
1.7-fold upregulated targets (n = 230)		**Gene count**				
Category/GO term		In genome	Found	Expected	Fold	P value
GO molecular function	Structural constituent of cuticle	168	33	1.9	17.36	5.46E−29
Lipid metabolism[a]	N/A	471	2	5.29	0.94	6.84E−02
1.7-fold downregulated targets (n = 350)		**Gene count**				
Category/GO term		In genome	Found	Expected	Fold	P value
GO biological process	Fatty acid metabolic process	105	11	1.79	6.16	3.99E−06
	Innate immune response	345	18	5.87	3.07	4.40E−05
GO cellular component	Peroxisomal membrane	18	5	0.31	16.34	3.33E−05
Lipid metabolism[a]	N/A	471	25	8.05	3.11	4.61E−07

Enrichment analyses were done for all LIN-29 significant targets and for both 1.7-fold LIN-29 up- and downregulated subsets using Gene Ontology (GO) Consortium (see *Materials and Methods*).

[a] Genes were also compared to the list of 471 *C. elegans* metabolic genes from Y. Zhang *et al.* (2013).

category among the upregulated genes was "*structural constituent of cuticle*," which consists of 33 cuticle collagen genes (Table 6). This group includes *col-38*, *col-49*, *col-63*, and *col-138*, which we previously showed by qPCR were upregulated in *hs*::*lin-29* animals and downregulated in *lin-29*(*RNAi*) animals, and *col-19*, which was previously shown to be regulated by LIN-29 (Liu *et al.* 1995; Abrahante *et al.* 1998; Abete-Luzi and Eisenmann 2018). The fact that 33 of the 187 *col* genes in *C. elegans* were found to be upregulated in our analysis suggests that *col* genes are a major target of regulation by LIN-29.

As an independent assessment of this result, we used the SPELL search engine, which analyzes 400 data sets covering 6524 *C. elegans* microarray and RNA-seq experiments, to identify genes with a similar pattern of expression to query genes (Hibbs *et al.* 2007). We queried SPELL using the three *col* genes we previously showed were regulated by LIN-29 (*col-38*, *col-49*, and *col-63*; Abete-Luzi and Eisenmann 2018). Among the top 100 genes identified, 48 were cuticle *col* genes and 24 of these 48 genes were also identified as upregulated targets of LIN-29 in our analysis (Table S3). This result corroborates that a large number of cuticle *col* genes are coregulated under a variety of normal and experimental conditions. The fact that most of these *col* genes show a peak of expression in the L4 stage during normal development (see below) suggests they are likely to represent a battery of *col* gene targets of LIN-29 at the L4 to adult transition for use in synthesis of the adult cuticle.

Recent work showed that one of the LIN-29 *col* gene targets, *col-19*, is upregulated in adult animals in which cuticle integrity has been damaged via RNAi against the major cuticle collagen gene *bli-1* (Zhao *et al.* 2019). BLI-1 collagen localizes to the medial strut layer of the cuticle, and when *bli-1* function is compromised by mutation or RNAi, large fluid-filled blisters cover the surface of the worm (Lints and Hall 2009) (Figure 4A). A mechanism exists within the hypodermis to sense cuticle damage such as that caused by *bli-1*(*RNAi*) or physical damage, and alter gene expression to induce an innate immune response (Zhang *et al.* 2015). Interestingly, *bli-1*(*RNAi*) animals also show upregulation of the heterochronic miRNA gene *let-7* and downregulation of the heterochronic genes *hbl-1* and *lin-41* (Zhao *et al.* 2019). Since LIN-41 is a direct regulator of *lin-29* expression (Slack *et al.* 2000; Aeschimann *et al.* 2017), we reasoned that *lin-29* may be upregulated in response to adult cuticle damage, perhaps to induce expression of *col* gene targets that were

Table 6 33 cuticle collagen genes upregulated ≥1.7-fold upon *lin-29* overexpression

Gene name	Fold change
*col-49**	48.1
*col-38**	21.1
col-124	16.5
col-140	15.8
col-178	15.4
col-139	11.5
col-71	10.4
col-120	9.8
col-20	8.9
col-129	8.4
bli-6	7.3
rol-1	6.8
col-81	6.0
col-79	5.9
col-88	5.7
*col-138**	5.0
col-19§	4.6
*bli-1**	4.4
col-77	4.1
col-60	3.7
col-176	3.3
col-101	3.1
*col-63**	2.7
lon-3	2.1
col-150	2.1
col-182	2.0
col-109	2.0
col-91	2.0
col-73	1.8
col-8	1.8
col-179	1.8
col-142	1.8
col-48	1.7

Cuticle collagen genes which were previously shown to be regulated by LIN-29 are indicated as * (Abete-Luzi and Eisenmann 2018) and § (Liu *et al.* 1995; Rougvie and Ambros 1995).

used to synthesize the adult cuticle initially. Consistent with this hypothesis, we found that in day 1 adult *bli-1* RNAi-treated animals, *lin-29* expression was increased twofold, and the expression of four LIN-29 *col* gene targets (*col-38*, *col-49*, *col-63*, and *col-138*), which are normally expressed in the L4 stage, was increased in these *bli-1*(*RNAi*)-treated adult animals (Figure 4B). To corroborate this result, we examined the expression of *lin-29* and its L4 *col* gene targets in adult *bus-8* mutant animals. *bus-8* encodes a hypodermally expressed glycosyltransferase, and reduction-of-function mutants are hyperpermeable to drugs and other reagents due to defects in epidermal and cuticle integrity (Partridge *et al.* 2008). We found that in *bus-8*(*e2882*) mutant adults, *lin-29* and the L4 *col* gene targets were also upregulated (Figure 4C). Furthermore, for three of four *col* genes tested, the upregulation in the *bus-8* background was dependent on *lin-29* function (Figure 4D). We were unable to test the *lin-29* dependence in the *bli-1* background because the Bli phenotype is completely dependent on *lin-29* function (Table S4). The result that heterochronic proteins participate in a hypodermal response to cuticle damage suggests that this pathway not only contributes to normal cuticle synthesis before the adult stage, but also functions in cuticle maintenance in adults in response to damage or breaches in integrity.

Genes encoding enzymes involved in fatty acid metabolism are enriched among LIN-29 downregulated targets

The most highly enriched category for the genes downregulated upon LIN-29 induction was "*peroxisomal membrane*": this category contained three genes encoding peroxisomal assembly factors (*prx-1*, *prx-5*, *prx-11*), and two genes encoding enzymes acting within the peroxisome (*ndx-8*, *maoc-1*) (AbdelRaheim and McLennan 2002; Zhang *et al.* 2010). Surprisingly, the second highest overenriched category among the downregulated genes was "*fatty acid metabolism*." To further explore the potential relevance of this result, we compared our gene target sets to a compendium of 471 *C. elegans* genes known to be involved in lipid metabolism (Y. Zhang *et al.* 2013). We found 25 of our 350 downregulated genes on this list, a number significantly higher than that expected by random sampling (hypergeometric $P = 4.61E{-}7$; Table 5 and Table S2). Likewise, when we searched the list of all 1101 LIN-29-regulated genes against the lipid metabolism gene list, we found 65 genes in total, 57 of which were downregulated upon LIN-29 misexpression ($P = 1.72E{-}12$; Table 5 and Table S2). These genes encode enzymes involved in a range of processes; however, many of them function in the synthesis of fatty acids, their storage, mobilization, and beta-oxidation (Figure 5). A potential link between the two enriched downregulated gene categories is the fact that many of the downregulated metabolic genes act in fatty acid beta-oxidation, a process which occurs in the peroxisome (and mitochondria) (Figure 5). Indeed, three lipid metabolic genes that are downregulated 30–50% by overexpression of LIN-29 function in peroxisomal beta-oxidation (*maoc-1*, *dhs-28*, and *daf-22*) (Figure S3), and reduction of their function by RNAi has been shown to cause an increase in lipid droplet size (Zhang *et al.* 2010). Similarly, reduction of function of the three peroxisomal assembly factor genes we identified (*prx-1*, *prx-5*, *prx-11*; Figure S3) also leads to an increase in lipid droplet size (Zhu *et al.* 2018), suggesting that both of these enriched categories of downregulated genes may impinge on lipid storage and utilization.

Also of note is the identification of genes encoding three transcription factors that regulate metabolic enzyme gene expression (Figure S4). The nuclear hormone receptor gene *nhr-80* was the gene most downregulated upon *lin-29* induction (more than fivefold decreased expression): NHR-80 physically interacts with NHR-49 to regulate genes involved in fatty acid metabolism, including *fat-5* that was downregulated almost twofold upon LIN-29 overexpression (Van Gilst *et al.* 2005; Brock *et al.* 2006; Pathare *et al.* 2012). Likewise, *sbp-1* encodes an SREBP (sterol regulatory element binding protein) homolog that is a major regulator of lipid metabolism genes (Lemieux and Ashrafi 2015). SBP-1 also regulates expression of *fat-5* (Watts 2009) as well as several other genes that showed decreased expression upon LIN-29

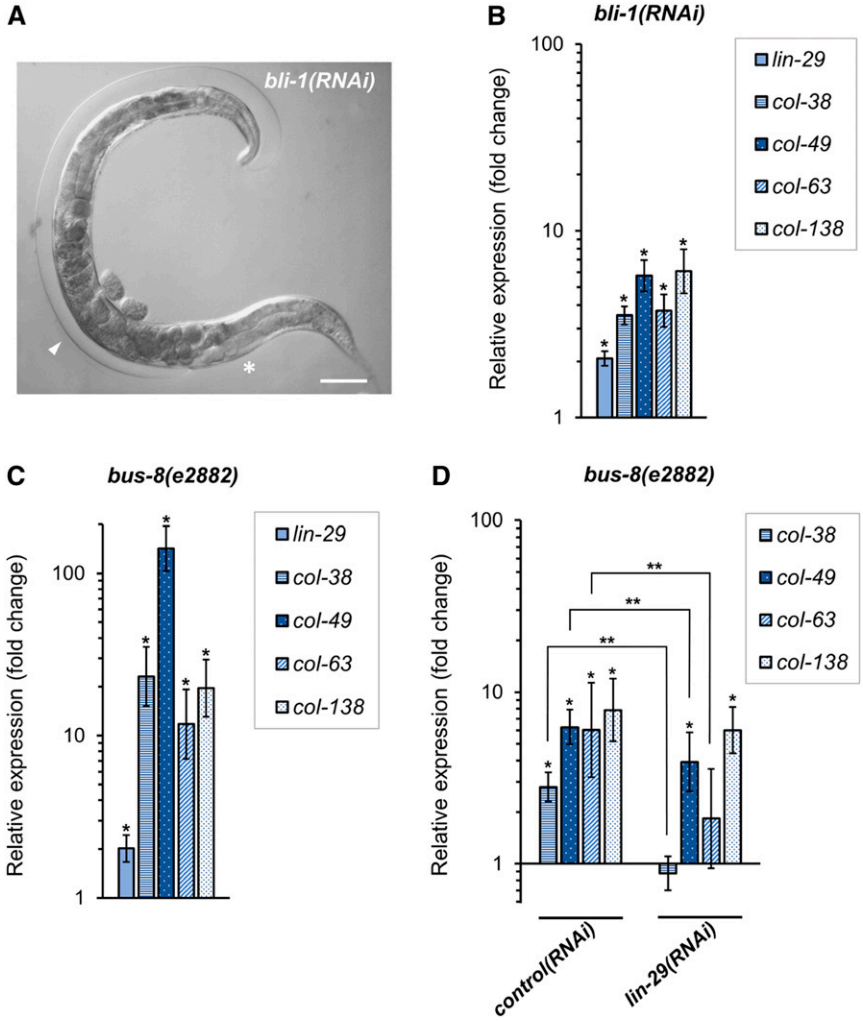

Figure 4 Upregulation of *lin-29* and *col* gene targets of LIN-29 in adults in response to defects in cuticle integrity. (A) Adult *bli-1*(*RNAi*) hermaphrodite showing Blister phenotype. Asterisk indicates normal cuticle, arrowhead indicates fluid-filled, Blistered cuticle. Bar, 50 μm. (B and C) Endogenous expression of *lin-29* and known *lin-29*-regulated cuticle collagen genes *col-38*, *col-49*, *col-63*, and *col-138* assessed by RT-qPCR in synchronized (B) young adults after *bli-1*(*RNAi*) feeding treatment (quantification was relative to expression in animals fed HT115 bacteria carrying empty RNAi vector control; see *Materials and Methods*); and (C) day 1 adults with *bus-8*(*e2882*) loss-of-function background (quantification was relative to expression in wild-type animals; both groups were fed standard OP50 bacteria). (D) RT-qPCR was used to measure endogenous *col* gene expression in day 1 adult *bus-8*(*e2882*) animals fed with HT115 bacteria containing either *lin-29*(*RNAi*) vector or empty-vector control. In both groups, quantification was relative to wild type animals fed HT115 with empty-vector control. Error bars represent SEM. Unpaired *t*-test analyses were performed comparing to respective controls (* $P < 0.05$) or between the indicated groups (D; ** $P < 0.05$).

induction: *elo-5*, *elo-6*, *fil-2* (Kniazeva *et al.* 2004), and the nuclear hormone receptor gene *nhr-64*, which itself regulates lipid metabolism (Liang *et al.* 2010) (Figure S4).

The discovery of fatty acid metabolic enzyme genes among LIN-29-regulated target genes suggests the possibility that LIN-29 may act to regulate developmentally linked changes in metabolism that are part of the larval-to-adult transition. Indeed, LIN-29 activity from the hypodermis was shown to be required for intestinal expression of the *vit* genes, which encode lipid transport proteins necessary to move lipids from the intestine into the developing oocytes (Dowen *et al.* 2016). In this same work, the authors showed that *lin-29*(*n333*) adult animals had slightly reduced overall fat levels based on Oil Red O staining, although the cause for this decrease was not clear.

To test the idea that LIN-29 plays a broader role in regulating fatty acid metabolism in the larval-to-adult transition, we used GC-MS analysis to look for differences in the levels of various fatty acid species in young adult *hs*::*lin-29* and *hs*::*control* animals that were subjected to heat-shock inductions in the L3 and L4 stages (see *Materials and Methods*). Consistent with the hypothesis, we found that levels of most individual fatty acid species, as well as total fatty acid levels, were decreased in adult animals subjected to early overexpression of LIN-29 (Table 7). Although the opposite of what may have been expected from the *lin-29*(*n333*) Oil Red O experiment (Dowen *et al.* 2016), this result supports the hypothesis that LIN-29 may normally regulate fatty acid metabolic gene expression in the L4 stage that affects fat content in the adult.

As showed above, repeated expression of LIN-29 in the adult life results in shorter mean life span and maximum life span. While we do not know the cause of this shortened life span, we consider a model in which repeated adult expression of *lin-29* over time results in persistent downregulation of genes encoding metabolic enzymes that are required to keep metabolic homeostasis. To corroborate whether LIN-29 is capable of repressing metabolic targets in the adult context, we chose four downregulated genes encoding enzymes that function in lipid metabolism (*acs-7*, *dhs-18*, *hacd-1*, *fat-5*) and the peroxisome assembly factor gene *prx-11*, and examined their expression after LIN-29 induction in gravid adults. We found that three of these genes (*dhs-18*, *hacd-1*, and *prx-11*) were downregulated (Figure S5), suggesting that perhaps metabolic functions may be perturbed in

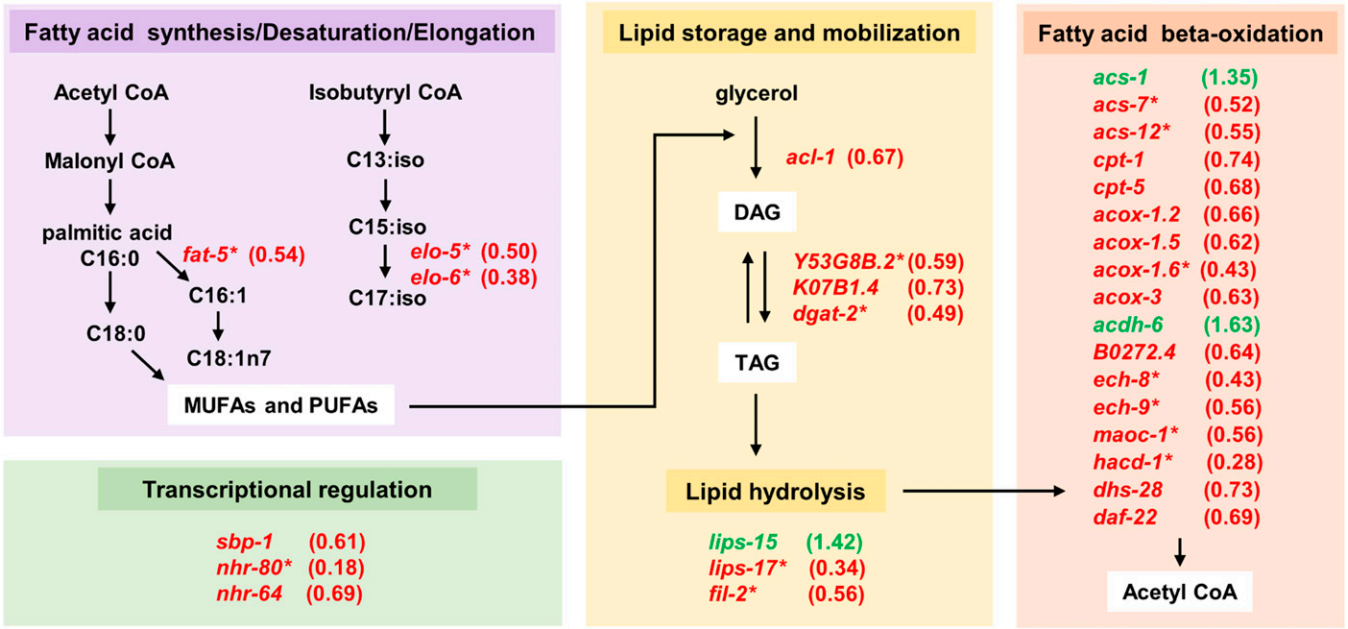

Figure 5 LIN-29-regulated genes involved in lipid metabolism. Genes that were upregulated (green font) or downregulated (red font) upon misexpression of LIN-29 in the L3 stage are shown, with their respective fold change in parenthesis. Genes are grouped into broad categories (colored boxes) based on their gene product function in lipid metabolism. Note that there are more enzymes involved in these processes; however, only genes with a significant change ($P < 0.05$) upon LIN-29 overexpression are shown here. *LIN-29 target genes that were regulated ≥1.7-fold.

these adult *hs*::*lin-29* animals, contributing to their shortened life span.

The intersection of gain-of-function and loss-of-function transcriptomic data identifies a set of high-confidence LIN-29-regulated genes

We compared our list of genes differentially regulated by overexpression of wild-type LIN-29 in the L3 stage to data from Hunter *et al.* that examined gene expression in *lin-29*(*n333*) mutants *vs.* wild type in the L4 stage (Hunter *et al.* 2013). Although this mutation causes reduction-of-function phenotypes, the size and levels of *lin-29* transcripts are not altered in *n333* mutant animals (Rougvie and Ambros 1995). The *n333* mutation causes a G > A mutation in the 3′ splice junction upstream of exon 5 of *lin-29a* (Blum *et al.* 2012), and mutations like this have been reported to retain some level of wild-type splicing (Blumenthal and Steward 1997), suggesting this is unlikely to be a true null allele. The intersection of these gene expression data sets gives a list of 21 strong candidates for LIN-29-activated genes: genes with increased expression in our gain-of-function/temporal misexpression approach and decreased expression in the Hunter *et al.* reduction-of-function data (hypergeometric $P = 5.40E{-}06$), and a list of 35 genes likely to be directly or indirectly repressed by LIN-29 (genes with decreased expression in our gain-of-function approach and increased expression in the Hunter *et al.* reduction-of-function data; hypergeometric $P = 2.34E{-}20$). We refer to the genes in common between these two transcriptomic data sets as genes for which LIN-29 is "necessary and sufficient" (N/S genes) and consider that these represent some of the best candidates for LIN-29 target genes (Table S2).

We were surprised by the small number of genes in this overlap, in particular the low number of cuticle collagen genes among the activated N/S genes (four genes). We previously showed that RNAi targeting *lin-29* significantly reduced expression of the L4 cuticle *col* genes *col-38*, *col-49*, *col-63*, and *col-138* in the L4 stage (Abete-Luzi and Eisenmann 2018), and *col-49* was the most highly upregulated gene upon LIN-29 overexpression (48-fold; Table S2). Yet of these four genes, only *col-38* was found as an activated N/S gene (Table S2). Examination of the *lin-29*(*n333*) data shows that *col-49*, *col-63* and *col-138* were downregulated 3- to 10-fold in the *lin-29*(*n333*) mutant; however, the data for these genes was slightly above the $P < 0.05$ cut-off. This suggests one (statistical) reason for the small overlap between the data sets.

Likewise, we were surprised that very few of our "metabolic" downregulated targets were in common with the *lin-29*(*n333*) data set for significantly upregulated genes. We first validated the RNA-seq result that some fatty acid metabolic genes are downregulated upon temporal misexpression of LIN-29 by performing qPCR on five of them (*acs-7*, *dhs-18*, *hacd-1*, *fat-5*, and *prx-11*) in a new set of biological replicates comparing *hs*::*lin-29* and *hs*::*control* animals after early L3 induction: all five genes were downregulated (Figure 6A). We then determined whether reduction of *lin-29* function by RNAi (which should unambiguously affect all *lin-29* isoforms, unlike the *n333* allele) caused increased expression of these metabolic genes in the L4 stage when LIN-29 protein normally accumulates. Consistent with our *hs*::*lin-29* results,

all five genes showed increased transcript levels at the L4 stage in *lin-29* RNAi-treated animals (Figure 6B), suggesting that these genes are indeed repressed by LIN-29 activity at the L4 stage, yet for unknown reasons, the *lin-29*(*n333*) allele failed to significantly derepress them.

Temporal expression patterns of LIN-29-regulated genes

If the upregulated genes we identified upon early misexpression of LIN-29 are actual targets of LIN-29 regulation during the L4 stage, we would predict that these genes may show an increase in expression in the L4 stage during normal development; conversely, genes in our downregulated gene set would be predicted to decrease in expression at that time. To determine the pattern of temporal expression for our set of LIN-29 targets, we examined modENCODE developmental expression data for these genes (Gerstein *et al.* 2010), and categorized them based on whether they show a peak of expression in any particular stage of the worm life cycle. We then compared the pattern of temporal expression of our LIN-29 target genes to the pattern for all 16,183 *C. elegans* genes in the modENCODE data sets (Figure 7).

Notably, the proportion of our upregulated genes that show a peak of expression in the L4 stage during normal development is significantly larger than the percentage of L4-peak genes in the genome as a whole (27% *vs.* 7%; chi-square with Yates correction $P < 0.0001$; Figure 7). For the cuticle *col* genes specifically, we found that 85% (28 of 33) of the cuticle collagen genes upregulated upon LIN-29 misexpression show a single peak of expression in either the L4 or young adult stage during normal development, while 36% of all *col* genes show peak expression in those developmental times (Figure S6). We also looked at the distribution of temporal expression patterns for the smaller set of LIN-29-upregulated N/S genes and observed that genes that peak in the L4 stage are even more overrepresented (43% *vs.* 7%; chi-square with Yates correction $P < 0.0001$).

On the other hand, in the case of our LIN-29 downregulated targets, the proportion of genes that peak in the L4 stage during normal development was significantly smaller than that expected based on the known genomic distribution (3% *vs.* 7%; chi-square with Yates correction $P = 0.0064$; Figure 7). This was also true for genes with peak expression in the adult (2% *vs.* 7%; chi-square with Yates correction $P = 0.0003$; Figure 7). When we examined the temporal expression patterns of the N/S subset of LIN-29-downregulated genes, we noted that the proportion of genes with a peak of expression in stages before the L4 (63%) was much higher than that observed in the total genomic set (31%). Additionally, we examined postembryonic expression profiles generated from the modENCODE data for the 25 lipid metabolism genes downregulated ≥1.7-fold by early LIN-29 expression and found that 20 of 25 showed either a permanent (*e.g.*, *dhs-18*; Figure S7) or temporary (*e.g.*, *elo-5*, Figure S7) downregulation in the L4 stage during normal development.

Table 7 Relative percent of fatty acid content in animals overexpressing *lin-29 vs.* control

		% FA in *hs*::*lin-29* relative to *hs*::*control*	
Fatty acid (FA)		Run 1	Run 2
C14:0	*Myristic acid*	72	143
C15:1		58	103
C15:0	*Pentadecanoic acid*	112	123
C16:1	*Palmitoleic acid*	102	155
C16:0	*Palmitic acid*	82	87
C17:2		50	60
C17:1	*Heptadecanoic acid*	69	81
C17:0	*Margaric acid*	80	66
C18:3	*Linolenic acid*	72	92
C18:2	*Linoleic acid*	90	85
C18:1	*Oleic acid*	81	88
C18:0	*Stearic acid*	79	76
C19:1		147	108
C19:0	*Nonadecanoic acid*	58	60
C20:5	*Eicosapentaenoic acid*	106	81
C20:4	*Arachidonic acid*	91	65
C20:3		77	51
C20:2		61	73
C20:0	*Eicosanoic acid*	67	65
Total		84	82

Amounts of individual fatty acid in young adults were assessed by their esterification to fatty acid methyl esters (FAMEs) and quantification via gas chromatography–mass spectrometry in *hs*::*lin-29* and *hs*::*control* animals after heat-shock treatment in the larva (see *Materials and Methods*). Each run included two independent biological trials. Shown is the percentage of each FAME in *hs*::*lin-29* animals relative to the amount in *hs*::*control* animals. "Total" indicates the sum of all FAME species in *hs*::*lin-29* animals relative to *hs*::*control* animals.

Together, these trends are consistent with the hypothesis that many of the genes we identified as upregulated upon misexpression of LIN-29 in the early L3 are normally upregulated by the peak of LIN-29 protein in the L4 stage during development, and that many of the genes we identified as downregulated upon LIN-29 temporal misexpression may be expressed earlier in larval life and are normally downregulated in the L4 stage, when LIN-29 levels peak.

Spatial expression patterns of LIN-29-regulated genes are consistent with both cell-autonomous and -nonautonomous regulation

A major site of LIN-29 expression based on reporter gene expression and antibody staining is in hypodermal cells, with accumulation beginning in the early L4 in the seam cells, followed by expression in other hypodermal cells and the hypodermal syncytium, and remaining through adulthood (Bettinger *et al.* 1996; Harris and Horvitz 2011). However *lin-29* expression is also seen earlier in the L3 stage in the hypodermal vulval cells, and the anchor cell and distal tip cells of the hermaphrodite gonad (Bettinger *et al.* 1996; Harris and Horvitz 2011). In males, LIN-29 is expressed in the linker cell—the cell that controls gonad migration in this sex—during the L3 stage and disappears in the late L4 stage, when this cell dies facilitating the connection of the gonad with the cloaca (Euling *et al.* 1999). Finally, LIN-29 shows steady expression in the pharynx of both males and

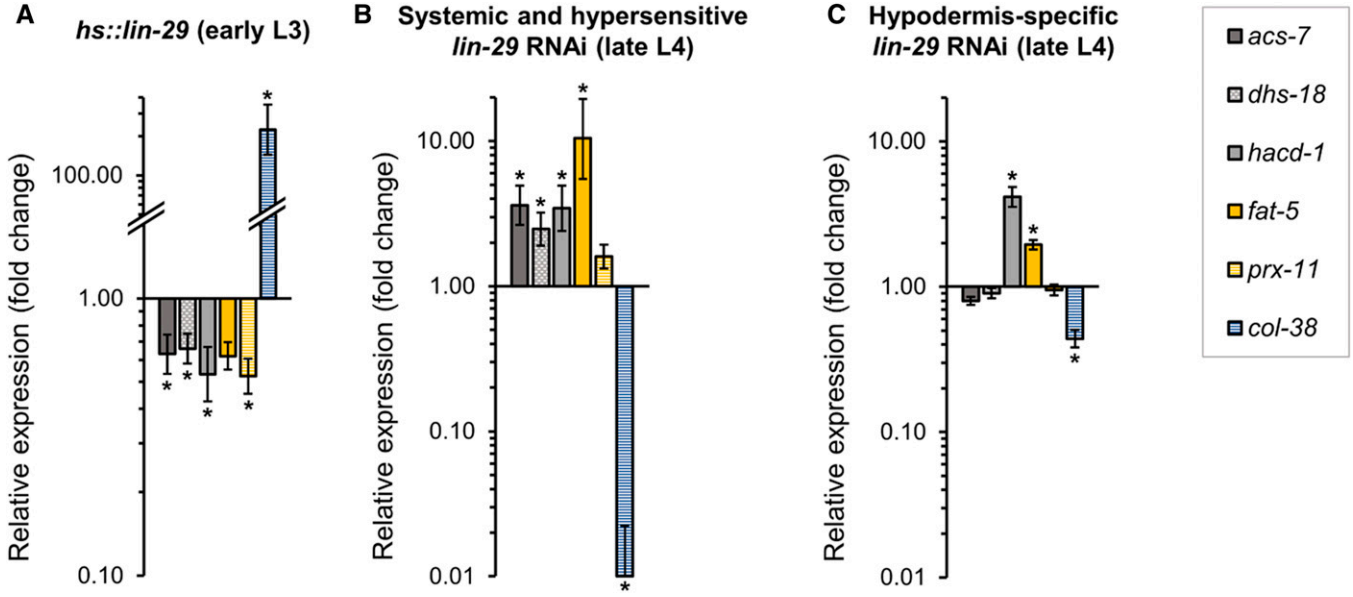

Figure 6 LIN-29 represses intestinal genes involved in fatty acid metabolism and beta-oxidation in the L4 stage. Endogenous expression of intestinally expressed genes *acs-7*, *dhs-18*, *hacd-1*, *fat-5*, as well as peroxisome factor *prx-11*, was assessed by RT-qPCR in (A) *hs*::*lin-29* animals 1 hr after induction in the early L3 stage (quantification relative to *hs*::*control* strain), and (B and C) L4 stage larvae after *lin-29*(*RNAi*) treatment (quantification relative to empty-vector control) in two different backgrounds: (B) a strain containing the RNAi-hypersensitive mutation *rrf-3*(*pk1426*), in which RNAi is stronger and effective in all tissues; and (C) a nonhypersensitive strain, where RNAi is only effective in the hypodermis (NR222; see *Materials and Methods*). In all cases, known LIN-29 upregulated gene *col-38* (Abete-Luzi and Eisenmann 2018) was analyzed as a control for efficacy of *lin-29* heat-shock induction and *lin-29* RNAi. Error bars represent SEM. * $P < 0.05$ (unpaired *t*-test).

hermaphrodites, beginning in the L1 stage and persisting through adulthood (Bettinger *et al.* 1996; Euling *et al.* 1999; Harris and Horvitz 2011); however, to our knowledge, a role of LIN-29 in pharyngeal cells remains to be determined.

We examined the known spatial expression patterns of our LIN-29 differentially regulated genes in the *C. elegans* database (Table S2; see *Materials and Methods*). There is published gene expression data for 193 of the 230 upregulated genes, and based on this data, almost 75% of the upregulated genes show expression in at least one tissue known to express LIN-29 protein (Figure 8; Bettinger *et al.* 1996; Harris and Horvitz 2011). When the pattern of spatial expression for the upregulated genes is compared to the genome as a whole by tissue enrichment analysis (TEA) (Angeles-Albores *et al.* 2016), the most overrepresented expression site is the "epithelial system" with 74 of 193 of the upregulated genes ($P =$ 1.0E−08; including all of the cuticle *col* genes with known expression). For the downregulated genes, there is spatial expression information for 316 of 350; 78% of the downregulated genes show expression in at least one tissue known to express LIN-29 (Figure 8). The fact that ≥75% of our up- and downregulated genes express in sites where LIN-29 is present is consistent with these genes being targets of LIN-29 during normal development.

Nevertheless, for both the up- and downregulated gene sets, >20% of the genes show expression in tissues not known to express LIN-29. This site is most often the intestine, although this trend is much more prevalent for the downregulated genes: 213 out of 316 genes have intestinal expression, with 49 of these apparently expressing solely in this tissue (Figure 8 and Table S2). When we performed TEA for the downregulated gene set, the two overrepresented expression sites with the greatest numbers of genes are "intestine" (210 genes; TEA $P =$ 1.5E−28) and "epithelial system" (100 genes; TEA $P =$ 3.20E−06). Finally, among the 65 genes involved in lipid metabolism that we identified as possible LIN-29-regulated genes (from the full set of 1101 significant *hs*::*lin-29*-responsive genes), 75% of them show expression in the intestine, a major site of metabolic activity in the worm that is not known to express LIN-29.

One explanation for the identification of large numbers of intestinally expressed genes as potential LIN-29 targets is that expression from the heat-shock promoter led to the presence of LIN-29 in the intestine where it is not normally found, which bound to these genes and regulated their expression. Alternatively, LIN-29 expression in another tissue could have caused indirect (cell nonautonomous) regulation of these genes in the intestine. There is precedent for the idea of a signal from the hypodermis regulating intestinal gene expression. First, MacNeil *et al.* showed that hypodermis-specific transcription factors (*e.g.*, LIN-26) can regulate the expression of reporters for intestinal genes (*e.g.*, *acdh-1*) and proposed the existence of a signal that propagates regulatory information from one tissue to another (MacNeil *et al.* 2015). Second, Dowen *et al.* hypothesized that LIN-29 in the hypodermis activates expression of a secreted signal that mediates LIN-29-dependent regulation of vitellogenin gene

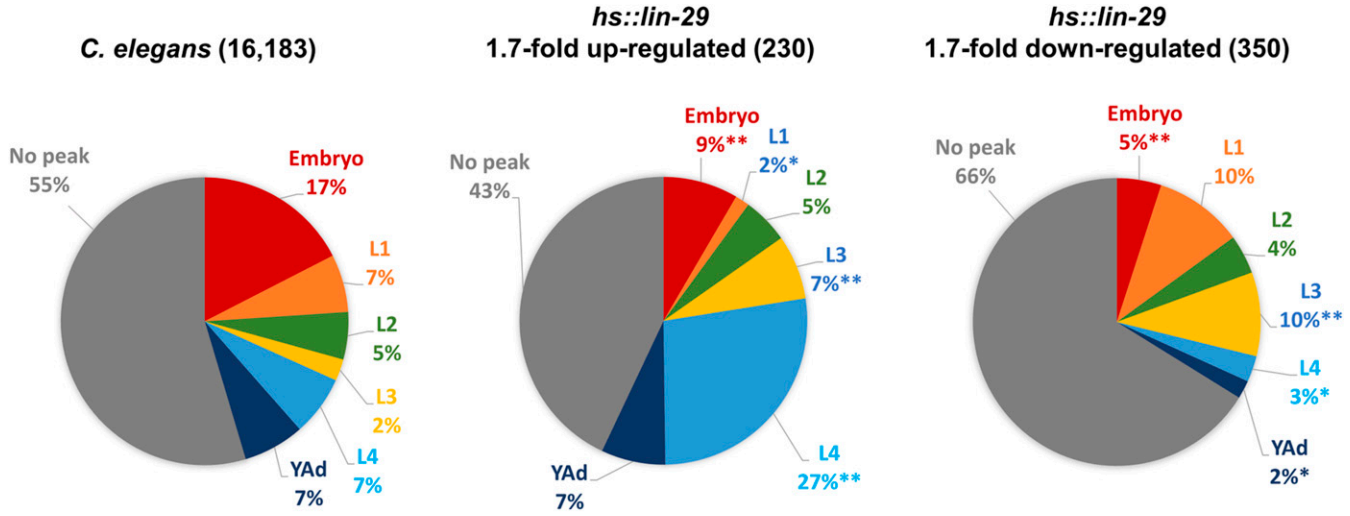

Figure 7 Genes that normally peak in the L4 stage are overrepresented among LIN-29 upregulated targets. Temporal expression peaks were assessed for the indicated gene sets based on modENCODE RNA-seq data (Gerstein *et al.* 2010) using criteria from Jackson *et al.* (2014): genes showing ≥35% of their total developmental expression in one stage were identified as having a *peak* in that stage (color coded), the remainder are indicated as "no peak" (gray). Distributions for all genes in each set were calculated, displayed as percentages and compared to the genomic distribution (left). ** $P <$ 0.0001 and * $P < 0.01$ (chi-square with Yates correction).

expression in the intestine, acting through both insulin and mTORC2 signaling pathways (Dowen *et al.* 2016). Finally, Clark *et al.* suggest that activation of a BMP signaling pathway in the hypodermis leads to changes in fat accumulation in the intestine via insulin signaling (Clark *et al.* 2018). Supporting the idea that the downregulation of intestinal gene expression by ectopic LIN-29 may be indirect is the fact that at least some of these metabolic genes are expressed almost exclusively in the intestine (*i.e.*, *acs-7*, *dhs-18*, and *hacd-1*; Table S2) and yet are derepressed by *lin-29* RNAi treatment, a situation in which no ectopic/intestinal LIN-29 is involved (see above; Figure 6B). To bolster this result, we repeated the *lin-29* RNAi treatment in a strain in which RNAi is only effective in the hypodermis (Figure 6C). Since this strain does not contain the *rrf-3* mutation that renders animals hypersensitive to RNAi (Simmer *et al.* 2002), the *lin-29* RNAi treatment was less effective (based on the smaller fold change observed for the hypodermal gene *col-38*; compare Figure 6B and Figure 6C). However, two of the lipid metabolic genes tested, *hacd-1* and *fat-5*, which are expressed in the intestine but not the hypodermis, showed an increase in expression in L4 animals in which *lin-29* function was compromised only in the hypodermis. We also performed fatty acid quantitation on these animals in which *lin-29* function was reduced only in the hypodermis and found that levels of most fatty acid species, as well as total fatty acid content, were increased relative to control RNAi animals (Table 8), which is the opposite of the result obtained when we overexpressed *lin-29* (Table 7). Together, these observations suggest that in addition to acting cell-autonomously to regulate gene expression in the hypodermis in the L4 stage, LIN-29 may also act cell-nonautonomously from the hypodermis to regulate expression of genes in the intestine, including many genes involved in lipid metabolism.

LIN-29-regulated extracellular signaling proteins are required for the regulation of LIN-29 intestinal targets

Our results on LIN-29-dependent downregulation of intestinal gene expression, combined with the fatty acid analysis in LIN-29 overexpressing and reduction-of-function animals, suggest a possible role for LIN-29 in coordinating intestinal metabolic activity at the larval to adult transition from the hypodermis.

We identified four genes encoding signaling molecules among our LIN-29 upregulated genes: three encoding *C. elegans* proteins related to the Hedgehog family of signals (*grd-11*, *grl-14*, *wrt-6*) and one encoding an insulin-like peptide (*ins-37*). When examining the wild-type postembryonic expression data (modENCODE) for these genes, we observed that *grd-11* and *grl-14* show low expression during larval life, but both *wrt-6* and *ins-37* show a marked peak of expression in the L4 stage, when hypodermal LIN-29 protein is active (Figure S7). Expression of three of these signal genes goes down in the *lin-29*(*n333*) L4 data set (Hunter *et al.* 2013) [*grl-14* (0.57, $P = 0.065$); *wrt-6* (0.17, $P = 0.046$); *ins-37* (0.51, $P = 0.058$)]. Moreover, a *wrt-6* reporter is expressed only in the hypodermis and in the socket cells of the amphids (Aspöck *et al.* 1999).

We individually tested the requirement for each of these four signaling genes both for repression of LIN-29-downregulated intestinal metabolic gene targets and for activation of *vit* genes in the late L4 stage using RNAi and qPCR. For the metabolic genes tested (*acs-7*, *dhs-18*, *hacd-1*, *fat-5*, and *prx-11*), at least one of the genes showed significantly increased expression in *wrt-6*, *grd-11*, or *grl-14* RNAi-treated

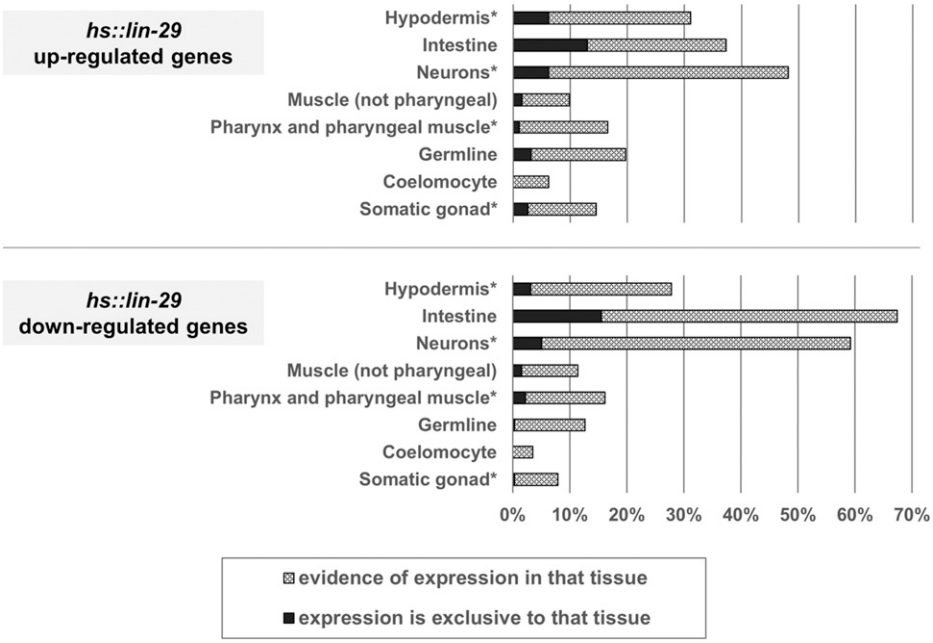

Figure 8 Spatial expression patterns of LIN-29 target genes. Spatial expression data available for 193 of 230 upregulated genes (top) and for 316 of 350 downregulated genes (bottom) was obtained (see *Materials and Methods*) and plotted as percentages of genes with expression in the indicated tissues. Tissues where LIN-29 is known to be expressed are denoted with an asterisk. Percentages sum to >100% because many genes are expressed in multiple tissues.

animals (Figure 9A). In the case of *grd-11*(*RNAi*), four of the metabolic genes showed significant increases, and for *grl-14* (*RNAi*), three of five genes did so. The gene *hacd-1* showed an increase in expression in all three treatments of reduction-of-function for a Hedgehog-related factor. In the case of *ins-37*(*RNAi*) animals, four of five genes showed an almost twofold increase in expression, but the results were not significant at $P < 0.05$, so it is unclear if *ins-37* functions to regulate these genes in the L4 stage.

The results for the *vit* genes were more variable (Figure 9B), with a significant decrease in expression for *vit-3* in *ins-37*(*RNAi*) animals, and for *vit-1* and *vit-3* in *wrt-6*(*RNAi*) animals. Unlike the case for repression of the metabolic genes, the gene *grd-11* appears to not be required for the activation of the *vit* genes in the intestine at the L4 stage, and while the results with *grl-14* RNAi were consistent with a role, they were not significant. Nonetheless, it is interesting to note that the L4 expression of these *vit* genes is affected when the function is reduced for the two signaling genes that show a normal peak of expression at the L4 stage.

Together, these data suggest that several genes encoding signaling molecules that we identified as upregulated targets of LIN-29 may function in the repression of intestinal metabolic gene expression in the L4 stage when LIN-29 levels peak. These results support the hypothesis that LIN-29 may play a role in mediating the cell-nonautonomous regulation of at least some of the intestinal targets of LIN-29 through activation of signaling molecules (this work; Dowen *et al.* 2016).

Discussion

The LIN-29 transcription factor is the terminal effector of the heterochronic pathway in *C. elegans* and is necessary for the execution of a number of developmental processes at the larval-to-adult transition; however, the sufficiency of *lin-29* for these processes has not be assessed. Here, we utilize a gain-of-function reagent to examine the phenotypic and gene regulatory effects caused by direct expression of *lin-29* at earlier (L3) or later (adult) time points. We show that early expression of LIN-29 causes Dumpy, Egg-laying defective, and Small body size phenotypes, as well as precocious fusion of seam cells, all phenotypes displayed by *lin-41*(*lf*) mutant and *lin-41*(*RNAi*) animals (Slack *et al.* 2000; Tocchini *et al.* 2014). This result, together with our previous demonstration that early and late misexpression of LIN-29 can induce expression of endogenous *col* genes and a *col-38* reporter (Abete-Luzi and Eisenmann 2018), indicates that LIN-29 alone is sufficient to initiate a number of processes occurring at the larval-to-adult transition. This is consistent with recent work indicating that there are only four relevant targets downstream of the heterochronic miRNA *let-7*, two of which are *lin-29* and *mab-10*, which encodes a LIN-29-interacting protein (Aeschimann *et al.* 2019). We also found that early expression of *lin-29* caused a curious "L4 vulva in an adult body" phenotype, which we hypothesize may be the result of the *lin-29* causing precocious differentiation of the vulval cells; interestingly, vertebrate homologs of LIN-29 also have known prodifferentiation activities (Pagel and Deindl 2011). Finally, overexpression of LIN-29 in the adult was found to shorten life span. Taken together, these phenotypic effects suggest the importance of keeping *lin-29* levels properly restrained until the correct time for the important transition to adulthood has been attained.

To further characterize the role of this transcription factor, we identified target genes regulated by LIN-29 using a gain-of-function approach involving temporal misexpression of LIN-29 at an earlier than normal time in development,

Table 8 Relative percent of fatty acid content in animals treated with hypodermis-specific *lin-29(RNAi)* *vs.* control RNAi

Fatty acid (FA)		% of FA in hypodermis-specific *lin-29*(*RNAi*) animals relative to control
C14:0	*Myristic acid*	134
C15:1		110
C15:0	*Pentadecanoic acid*	118
C16:1	*Palmitoleic acid*	145
C16:0	*Palmitic acid*	127
C17:2		98
C17:1	*Heptadecanoic acid*	109
C17:0	*Margaric acid*	117
C18:3	*Linolenic acid*	135
C18:2	*Linoleic acid*	119
C18:1	*Oleic acid*	143
C18:0	*Stearic acid*	126
C19:1		81
C19:0	*Nonadecanoic acid*	116
C20:5	*Eicosapentaenoic acid*	182
C20:4	*Arachidonic acid*	159
C20:3		180
C20:2		185
C20:0	*Eicosanoic acid*	147
Total		132

Amounts of individual fatty acid in young adults were assessed by their esterification to fatty acid methyl esters (FAMEs) and quantification via gas chromatography–mass spectrometry in NR222 animals fed with *lin-29*(*RNAi*) and empty RNAi vector control (see *Materials and Methods*). A single run was performed on three independent biological trials per treatment.

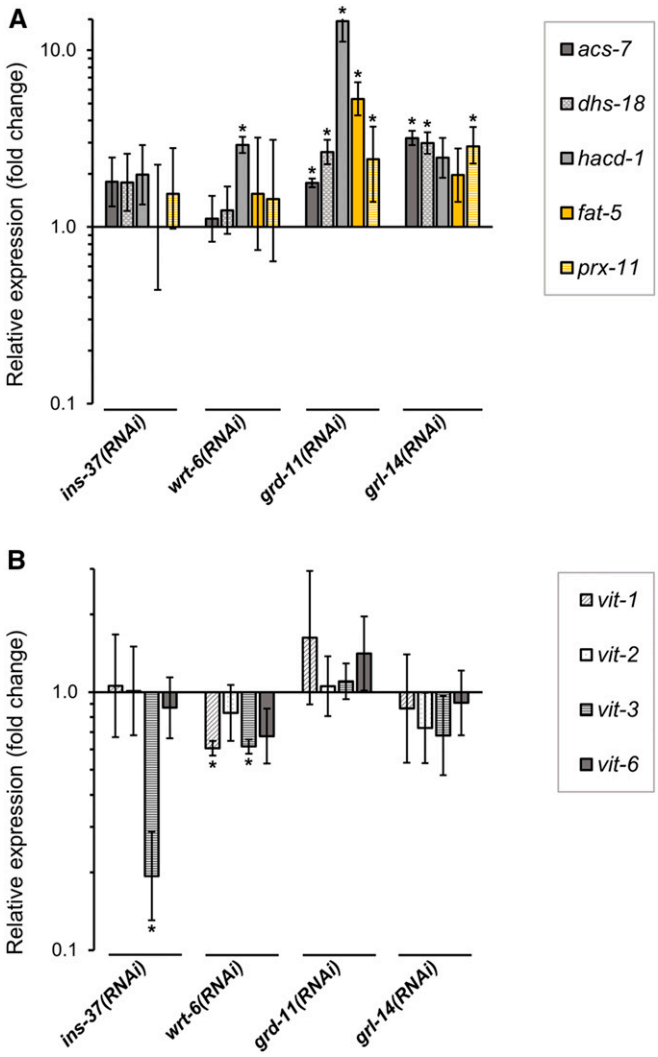

Figure 9 Four *lin-29* target genes that encode signaling molecules regulate expression of LIN-29 intestinal targets in the L4 stage. Endogenous expression of five metabolic genes downregulated upon LIN-29 expression (*acs-7*, *dhs-18*, *hacd-1*, *fat-5*, and *prx-11*; A), and four vitellogenin genes previously shown to require *lin-29* for their expression (*vit-1*, *vit-2*, *vit-3* and *vit-6*; Dowen *et al.* 2016; B) was evaluated by RT-qPCR in late L4 stage *ins-37*(RNAi), *wrt-6*(RNAi), *grd-11*(RNAi)l or *grl-14*(RNAi) animals. Quantifications were relative to expression in animals treated with empty-vector RNAi control. Error bars represent SEM. * $P < 0.05$ (unpaired *t*-test).

followed by analysis of transcriptional changes an hour later. We believe this approach may more closely reflect the biological situation—a rapid increase in LIN-29 levels from a low starting point—and does not suffer from the caveat that observed changes in gene expression could be due to changes in cell fate earlier in development caused by a mutant background. One caveat of the gain-of-function approach is that overexpression of LIN-29 in tissues where it is not normally expressed may lead to the identification of spurious targets. However, it is worth pointing out that temporal misexpression of LIN-29 both earlier and later in development leads to *col* gene reporter expression only in the tissues in which it is normally detected, simply at an earlier or later time (Abete-Luzi and Eisenmann 2018). This suggests that lack of necessary cofactors, or a nonpermissive chromatin state, may prevent LIN-29 from activating targets genes in inappropriate tissues. This is consistent with work showing that after heat-shock expression of a neuronal transcription factor, targets genes only showed ectopic spatial expression when activity of a chromatin factor was compromised (Tursun *et al.* 2011). Although we cannot rule out that ectopically expressed LIN-29 may be directly affecting gene expression due to its presence in tissues where it is not normally expressed or due to higher levels of expression than normal, the fact that many of the putative LIN-29 target genes we identified in our gain-of-function approach were regulated in the opposite manner when *lin-29* function was reduced by RNAi, and that many of them naturally show changes of expression in the L4 stage, strongly suggests that LIN-29 is likely to participate in the regulation of expression of these genes during normal development.

The major category of known genes upregulated by LIN-29 are those encoding cuticle collagen (*col*) genes (14% of known 1.7-fold upregulated genes). We identified 33 *col* genes, and the majority of them have a known peak of expression in the L4 or adult stage, after LIN-29 levels peak in the hypodermis. Although LIN-29 was previously shown to regulate several *col* genes (Liu *et al.* 1995; Rougvie and Ambros 1995; Abete-Luzi and Eisenmann 2018), this result suggests a much more pervasive role for LIN-29 in regulating

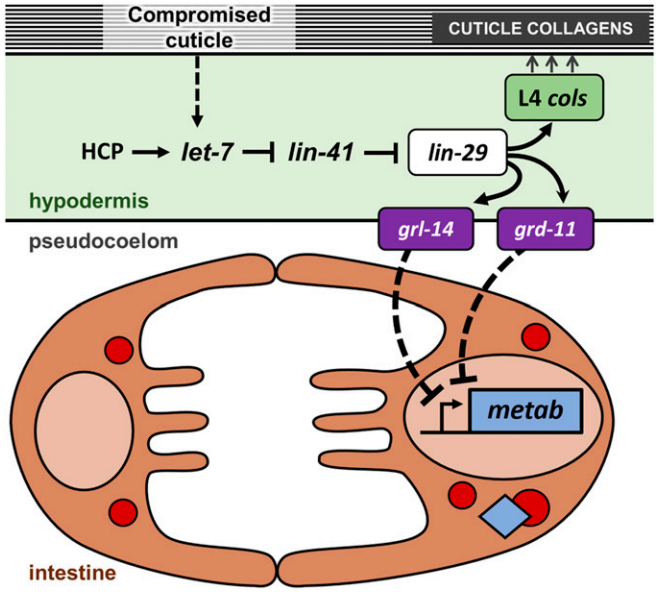

Figure 10 Model of the roles of LIN-29 and its target genes in the hypodermis and intestine. In the hypodermis, *lin-29* expression is regulated by the heterochronic pathway (HPC) acting through *let-7* and *lin-41*. LIN-29 activates expression of many L4 and adult specific cuticle collagen (*col*) genes which contribute to the adult cuticle. Cuticle damage in the adult [*e.g.*, in *bli-1*(*RNAi*) animals] signals through *let-7* to increase expression of *lin-29* and *col* genes. Dashed lines indicate that a mechanism is not yet known. Hypodermal LIN-29 also activates expression of signaling genes (*grd-11* and *grl-14* are shown as examples), which act to reduce expression of genes involved in fat metabolism in the intestine.

the formation of the adult cuticle. The large number of L4 and adult *col* genes regulated by LIN-29 may reflect the fact that the adult cuticle is the most structurally complex of the cuticles synthesized by the worm (Page and Johnstone 2007), and unlike other cuticles, which need to last for less than a day, the adult lifelong cuticle may require more robustness.

Interestingly, we found that several *col* gene targets of LIN-29 in the L4 stage also show increased expression in the adult when cuticle integrity is compromised in both *bli-1*(*RNAi*) and *bus-8*(*e2882*) animals. As it was previously shown that *bli-1*(*RNAi*) treatment alters transcript levels for the heterochronic genes *let-7*, *hbl-1*, and *lin-41*, this suggests that the regulation of *col* genes by LIN-29 may also occur in response to cuticle damage, via a signal that acts through upstream heterochronic regulators of LIN-29 expression (Zhang *et al.* 2015; Zhao *et al.* 2019). This suggests that a mechanism to repair the cuticle during adult life may exist, and that this mechanism acts via the same molecular pathway that was utilized to synthesize the adult cuticle initially (Figure 10). Additional work will be needed to establish the mechanism by which the presence of cuticle damage in the adult leads to regulation of heterochronic pathway components to impinge on LIN-29 activity.

Besides the *col* genes, several other genes previously shown to depend on *lin-29* activity for their expression were not identified in our list of *hs*::*lin-29* upregulated genes. Several explanations for this result are possible: (1) a target could be expressed in too few cells to be identified by our global approach, (2) a target could require a different *lin-29* isoform than *lin-29a*, (3) LIN-29 may be necessary but not sufficient for target gene regulation, or (4) a target gene was regulated in the expected direction but did not rise to statistical significance. However, two examples of putative LIN-29 targets bear commenting on. First, the *vit* genes (*vit-1*–*6*) have been shown to be positively regulated in the intestine by *lin-29* activity in the hypodermis (Dowen *et al.* 2016), yet only two *vit* genes show increased expression in our *hs*::*lin-29* animals (*vit-2* and *vit-6*). Recently it was shown that expression of the *vit* genes in the intestine is regulated by the conserved transcription factors CEH-60 and UNC-62 (Dowen 2019). Neither *ceh-60* nor *unc-62* showed a significant change in expression in our gain-of-function data (Table S2) or in the *lin-29*(*n333*) reduction-of-function data (Hunter *et al.* 2013), suggesting they may not function directly downstream of LIN-29. Therefore, the fact that we did not observe significant effects on most *vit* genes upon LIN-29 overexpression could be an example of genes for which *lin-29* is necessary but not sufficient for expression. Second, the transcription factor MAB-10 is known to physically interact with LIN-29 and participate in a number of *lin-29*-mediated processes (Harris and Horvitz 2011). In previous work, it was suggested that expression of *mab-10* was not regulated by *lin-29* (Harris and Horvitz 2011); however, we find *mab-10* transcripts levels increase 2.3-fold upon overexpression of LIN-29 (Table S2), and *mab-10* levels also decreased in both *lin-29*(*n333*) and *let-7(lf)* mutants (Hunter *et al.* 2013). This transcriptional regulation may be evolutionarily conserved, as the LIN-29 orthologs EGR1, EGR2, and EGR3 all regulate expression of the MAB-10 ortholog NAB2 through binding sites in the NAB2 gene promoter (Kumbrink *et al.* 2005, 2010). These results suggest that *mab-10* is likely to be a transcriptional target of LIN-29, and that *lin-29* and *mab-10* may have complicated effects on each other's expression and activity.

A major category of genes showing downregulation upon LIN-29 misexpression are genes involved in lipid metabolism (7% of 1.7× downregulated genes, $n = 25$). Many of the lipid metabolic genes we identified show temporary or permanent downregulation of expression in the L4 stage when LIN-29 levels peak. We also showed that *lin-29* reduction of function by RNAi causes an increase in expression of several of these genes at the L4 stage. LIN-29 overexpression also caused a decrease in expression of *nhr-80* and *sbp-1*, two transcription factors that regulate downstream metabolic genes (McKay *et al.* 2003; Kniazeva *et al.* 2004; Van Gilst *et al.* 2005; Brock *et al.* 2006; Nomura *et al.* 2010; Pathare *et al.* 2012; MacNeil *et al.* 2015), including *fat-5* (Watts and Browse 2000; Brock *et al.* 2006). Thus, our target gene identification suggests a broader role for LIN-29 in regulating fat metabolism at the adult transition than previously suggested. The demonstration that both increased expression of *lin-29* (*hs*::*lin-29* animals) and decreased expression of *lin-29* [*lin-29*(*RNAi*) animals] during larval life lead to opposite changes in fatty acid content in young adult animals is supportive

of the hypothesis that *lin-29* regulates genes involved in fat metabolism during normal development.

Many of the metabolic genes we identified have known expression in the intestine, a major site of metabolic activity that does not express *lin-29*, suggesting that *lin-29* may regulate metabolic gene expression cell-nonautonomously. We identified four LIN-29 upregulated target genes that encode extracellular signaling proteins belonging to the insulin-like peptide and hedgehog-related families and showed that they negatively regulate expression of several LIN-29 metabolic gene targets in the L4 stage. While RNAi of these individual genes in a sensitized background was sufficient to cause an effect on metabolic gene expression, we were unable to show that downregulation of those metabolic gene targets by *hs*::*lin-29* was significantly dependent on activity of the signaling genes (data not shown). Whether this is due to the increased level of signal gene transcript caused by the higher levels of LIN-29 induced by heat shock, or to other reasons, is currently unknown. Regardless, the identification of these signaling genes as activated targets of LIN-29 that can regulate fatty acid metabolic genes expressed in the intestine, combined with the demonstration that reduction of *lin-29* activity in only the hypodermal tissue can alter expression of these same fatty acid metabolic genes and increase fatty acid levels, supports a model that *lin-29* activity in the hypodermis cell-nonautonomously coordinates fat metabolic gene expression in the intestine during normal development (Figure 10).

Three of the consequences of the transition from larval life to adulthood are (1) the period of rapid growth in length and volume that occurs during larval life comes to an end (Knight *et al.* 2002); (2) consistent with this, the active metabolism seen in larval life slows down (Braeckman *et al.* 2009); and (3) worms become reproductive and mobilize fat from the intestine into developing oocytes via movement of yolk (the major protein component of which are vitellogenins) (Dowen *et al.* 2016; Watts and Ristow 2017). The identification of genes involved in fatty acid synthesis and beta-oxidation as downregulated targets of LIN-29 (this work), combined with the previous identification of *vit* genes as activated targets (Dowen *et al.* 2016), suggests that LIN-29 may play a major role in effecting these changes. For example, if the adult worm needs to slow its growth and mobilize fat stores from somatic tissues to germ cells, it must slow down the burning of fat, which is consistent with the decrease in expression of many genes involved in fatty acid beta-oxidation (Figure 5). Likewise, a decrease in the storage of fat in somatic tissues (to make it available for mobilization to oocytes) is consistent with a decrease in expression of *Y53G8B.2*, *K07B1.4*, and *dgat-2*, which encode three of four *C. elegans* DGAT enzymes that function in triacylglycerol synthesis (Figure 5) (Watts and Ristow 2017). Thus, the changes in fatty acid metabolic gene expression we observed on early overexpression of LIN-29 can be rationalized based on a normal role for LIN-29 in coordinating metabolic changes at the larval-to-adult transition. Intriguingly, one vertebrate homolog of LIN-29, EGR-1 (Harris and Horvitz 2011), is expressed in adipose tissue, and misexpression of *EGR-1* correlates with dietary-induced obesity and insulin resistance in both mice and humans (J. Zhang *et al.* 2013). Conversely, loss of *Egr-1* function protects mice from dietary-induced pathologies such as insulin resistance and hyperlipidemia, most likely due to an altered balance between energy expenditure and storage (J. Zhang *et al.* 2013). Therefore, it is possible that the regulation of energy metabolism by this type of zinc finger transcriptional regulator may have been conserved during evolution.

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Literature Cited

Abbott, A. L., E. Alvarez-Saavedra, E. A. Miska, N. C. Lau, D. P. Bartel *et al.*, 2005 The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev. Cell 9: 403–414. https://doi.org/10.1016/j.devcel.2005.07.009

AbdelRaheim, S. R., and A. G. McLennan, 2002 The Caenorhabditis elegans Y87G2A.14 Nudix hydrolase is a peroxisomal coenzyme A diphosphatase. BMC Biochem. 3: 5. https://doi.org/10.1186/1471-2091-3-5

Abete-Luzi, P., and D. M. Eisenmann, 2018 Regulation of C. elegans L4 cuticle collagen genes by the heterochronic protein LIN-29. Genesis 56: 1–8. https://doi.org/10.1002/dvg.23106

Abraham, M. C., Y. Lu, and S. Shaham, 2007 A morphologically conserved nonapoptotic program promotes linker cell death in Caenorhabditis elegans. Dev. Cell 12: 73–86. https://doi.org/10.1016/j.devcel.2006.11.012

Abrahante, J. E., E. A. Miller, and A. E. Rougvie, 1998 Identification of heterochronic mutants in Caenorhabditis elegans: temporal misexpression of a collagen:green fluorescent protein fusion gene. Genetics 149: 1335–1351.

Abrahante, J. E., A. L. Daul, M. Li, M. L. Volk, J. M. Tennessen *et al.*, 2003 The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Dev. Cell 4: 625–637. https://doi.org/10.1016/S1534-5807(03)00127-8

Aeschimann, F., P. Kumari, H. Bartake, D. Gaidatzis, L. Xu *et al.*, 2017 LIN41 post-transcriptionally silences mRNAs by two distinct and position-dependent mechanisms. Mol. Cell 65: 476–489.e4. https://doi.org/10.1016/j.molcel.2016.12.010

Aeschimann, F., A. Neagu, M. Rausch, and H. Großhans, 2019 let-7 coordinates the transition to adulthood through a single primary and four secondary targets. Life Sci. Alliance 2: e201900335. https://doi.org/10.26508/lsa.201900335

Altun, Z. F., and D. H. Hall, 2009 Introduction. In WormAtlas. https://doi.org/10.3908/wormatlas.1.1

Ambros, V., and H. Horvitz, 1984 Heterochronic mutants of the nematode Caenorhabditis elegans. Science 226: 409–416. https://doi.org/10.1126/science.6494891

Angeles-Albores, D., R. Y. N Lee, J. Chan, and P. W. Sternberg, 2016 Tissue enrichment analysis for C. elegans genomics. BMC Bioinformatics 17: 366. https://doi.org/10.1186/s12859-016-1229-9

Aspöck, G., H. Kagoshima, G. Niklaus, and T. R. Bürglin, 1999 Caenorhabditis elegans has scores of hedgehog-related genes: sequence and expression analysis. Genome Res. 9: 909–923. https://doi.org/10.1101/gr.9.10.909

Bettinger, J. C., K. Lee, and A. E. Rougvie, 1996 Stage-specific accumulation of the terminal differentiation factor LIN-29 during Caenorhabditis elegans development. Development 122: 2517–2527.

Bettinger, J. C., S. Euling, and A. E. Rougvie, 1997 The terminal differentiation factor LIN-29 is required for proper vulval morphogenesis and egg laying in Caenorhabditis elegans. Development 124: 4333–4342.

Blum, E. S., M. C. Abraham, S. Yoshimura, Y. Lu, and S. Shaham, 2012 Control of nonapoptotic developmental cell death in Caenorhabditis elegans by a polyglutamine-repeat protein. Science 335: 970–973. https://doi.org/10.1126/science.1215156

Blumenthal, T., and K. Steward, 1997 RNA processing and gene structure, pp. 117–145 in *C. elegans II*, edited by D. L. Riddle. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Boehm, M., and F. Slack, 2005 A developmental timing microRNA and its target regulate life span in C. elegans. Science 310: 1954–1957. https://doi.org/10.1126/science.1115596

Braeckman, B. P., K. Houthoofd, and J. R. Vanfleteren, 2009 Intermediary metabolism (February 16, 2009), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.146.1, http://www.wormbook.org. https://doi.org/10.1895/wormbook.1.146.1

Brenner, S., 1974 The genetics of Caenorhabditis elegans. Genetics 77: 71–94.

Brock, T. J., J. Browse, and J. L. Watts, 2006 Genetic regulation of unsaturated fatty acid composition in C. elegans. PLoS Genet 2: e108. https://doi.org/10.1371/journal.pgen.0020108

Bustos, V., and L. Partridge, 2017 Good Ol' fat: links between lipid signaling and longevity. Trends Biochem. Sci. 42: 812–823. https://doi.org/10.1016/j.tibs.2017.07.001

Carbon, S., A. Ireland, C. J. Mungall, S. Shu, B. Marshall *et al.*, 2009 AmiGO: online access to ontology and annotation data. Bioinformatics 25: 288–289. https://doi.org/10.1093/bioinformatics/btn615

Clark, J. F., M. Meade, G. Ranepura, D. H. Hall, and C. Savage-Dunn, 2018 Caenorhabditis elegans DBL-1/BMP regulates lipid accumulation via interaction with insulin signaling. G3 (Bethesda) 8: 343–351. https://doi.org/10.1534/g3.117.300416

Dowen, R. H., 2019 CEH-60/PBX and UNC-62/MEIS coordinate a metabolic switch that supports reproduction in C. elegans. Dev. Cell 49: 235–250.e7. https://doi.org/10.1016/j.devcel.2019.03.002

Dowen, R. H., P. C. Breen, T. Tullius, A. L. Conery, and G. Ruvkun, 2016 A microRNA program in the C. elegans hypodermis couples to intestinal mTORC2/PQM-1 signaling to modulate fat transport. Genes Dev. 30: 1515–1528. https://doi.org/10.1101/gad.283895.116

Duffy, J., A. S. Mutlu, and M. C. Wang, 2016 Lipid metabolism, lipid signalling and longevity, pp. 307–329 in *Ageing: Lessons from C. elegans*, Springer, Cham, Switzerland.

Ecsedi, M., and H. Großhans, 2013 Lin-41/Trim71: emancipation of a miRNA target. Genes Dev. 27: 581–589. https://doi.org/10.1101/gad.207266.112

Euling, S., J. C. Bettinger, and A. E. Rougvie, 1999 The LIN-29 transcription factor is required for proper morphogenesis of the Caenorhabditis elegans male tail. Dev. Biol. 206: 142–156. https://doi.org/10.1006/dbio.1998.9063

Ewald, C. Y., J. N. Landis, J. P. Abate, C. T. Murphy, and T. K. Blackwell, 2015 Dauer-independent insulin/IGF-1-signalling implicates collagen remodelling in longevity. Nature 519: 97–101. https://doi.org/10.1038/nature14021

Fielenbach, N., D. Guardavaccaro, K. Neubert, T. Chan, D. Li *et al.*, 2007 DRE-1: an evolutionarily conserved F Box protein that regulates C. elegans developmental age. Dev. Cell 12: 443–455. https://doi.org/10.1016/j.devcel.2007.01.018

Friedlander-Shani, L., and B. Podbilewicz, 2011 Heterochronic control of AFF-1-mediated cell-to-cell fusion in C. elegans, pp. 5–11 in Cell Fusion in Health and Disease, Vol. 713. Springer, Dordrecht, The Netherlands. https://doi.org/10.1007/978-94-007-0763-4_2

Garigan, D., A. L. Hsu, A. G. Fraser, R. S. Kamath, J. Abringet *et al.*, 2002 Genetic analysis of tissue aging in Caenorhabditis elegans: a role for heat-shock factor and bacterial proliferation. Genetics 161: 1101–1112.

Gerstein, M. B., Z. J. Lu, E. L. Van Nostrand, C. Cheng, B. I. Arshinoff, *et al.*, 2010 Integrative analysis of the Caenorhabditis elegans genome by the modENCODE project. Science 330: 1775–1787. https://doi.org/10.1126/science.1196914

Gorrepati, L., M. W. Krause, W. Chen, T. M. Brodigan, M. Correa-Mendez, *et al.*, 2015 Identification of Wnt pathway target genes regulating the division and differentiation of larval seam cells and vulval precursor cells in Caenorhabditis elegans. G3 (Bethesda) 5: 1551–1566. https://doi.org/10.1534/g3.115.017715

Gupta, B. P., W. Hanna-Rose, and P. W. Sternberg, 2012 Morphogenesis of the vulva and the vulval-uterine connection (November 30, 2012), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.152.1, http://www.wormbook.org.

Han, S. K., D. Lee, H. Lee, D. Kim, H. G. Son *et al.*, 2016 OASIS 2: online application for survival analysis 2 with features for the analysis of maximal lifespan and healthspan in aging research. Oncotarget 7: 56147–56152. https://doi.org/10.18632/oncotarget.11269

Harris, D. T., and H. R. Horvitz, 2011 MAB-10/NAB acts with LIN-29/EGR to regulate terminal differentiation and the transition from larva to adult in C. elegans. Development 138: 4051–4062. https://doi.org/10.1242/dev.065417

Hayes, G. D., A. R. Frand, and G. Ruvkun, 2006 The mir-84 and let-7 paralogous microRNA genes of Caenorhabditis elegans direct the cessation of molting via the conserved nuclear hormone receptors NHR-23 and NHR-25. Development 133: 4631–4641. https://doi.org/10.1242/dev.02655

Hibbs, M. A., D. C. Hess, C. L. Myers, C. Huttenhower, K. Li *et al.*, 2007 Exploring the functional landscape of gene expression: directed search of large microarray compendia. Bioinformatics 23: 2692–2699. https://doi.org/10.1093/bioinformatics/btm403

Hong, Y., R. Roy, and V. Ambros, 1998 Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in Caenorhabditis elegans. Development 125: 3585–3597.

Houbaviy, H. B., M. F. Murray, and P. A. Sharp, 2003 Embryonic stem cell-specific microRNAs. Dev. Cell 5: 351–358. https://doi.org/10.1016/S1534-5807(03)00227-2

Huang, D. W., B. T. Sherman, and R. A. Lempicki, 2009 Bioinformatics enrichment tools: paths toward the comprehensive functional

analysis of large gene lists. Nucleic Acids Res. 37: 1–13. https://doi.org/10.1093/nar/gkn923

Huang, T. F., C. Y. Cho, Y. T. Cheng, J. W. Huang, Y. Z. Wu *et al.*, 2014 BLMP-1/Blimp-1 regulates the spatiotemporal cell migration pattern in C. elegans. PLoS Genet. 10: e1004428. https://doi.org/10.1371/journal.pgen.1004428

Hunter, S. E., E. F. Finnegan, D. G. Zisoulis, M. T. Lovci, K. V. Melnik-Martinez *et al.*, 2013 Functional genomic analysis of the let-7 regulatory network in Caenorhabditis elegans. PLoS Genet. 9: e1003353. https://doi.org/10.1371/journal.pgen.1003353

Inoue, T., M. Wang, T. O. Ririe, J. S. Fernandes, and P. W. Sternberg, 2005 Transcriptional network underlying Caenorhabditis elegans vulval development. Proc. Natl. Acad. Sci. USA 102: 4972–4977. https://doi.org/10.1073/pnas.0408122102

Jackson, B. M., P. Abete-Luzi, M. W. Krause, and D. M. Eisenmann, 2014 Use of an activated beta-catenin to identify Wnt pathway target genes in Caenorhabditis elegans, including a subset of collagen genes expressed in late larval development. G3 (Bethesda) 4: 733–747. https://doi.org/10.1534/g3.113.009522

Kamath, R. S., and J. Ahringer, 2003 Genome-wide RNAi screening in Caenorhabditis elegans. Methods 30: 313–321. https://doi.org/10.1016/S1046-2023(03)00050-1

Kamath, R. S., M. Martinez-Campos, P. Zipperlen, A. G. Fraser, and J. Ahringer, 2000 Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. Genome Biol. 2: RESEARCH0002. https://doi.org/10.1186/gb-2000–2-1-research0002

Kniazeva, M., Q. T. Crawford, M. Seiber, C. Y. Wang, and M. Han, 2004 Monomethyl branched-chain fatty acids play an essential role in Caenorhabditis elegans development. *PLoS Biol.* 2: E257. https://doi.org/10.1371/journal.pbio.0020257

Knight, C. G., M. N. Patel, R. B. R. Azevedo, and A. M. Leroi, 2002 A novel mode of ecdysozoan growth in Caenorhabditis elegans. Evol. Dev. 4: 16–27. https://doi.org/10.1046/j.1525-142x.2002.01058.x

Kumbrink, J., M. Gerlinger, and J. P. Johnson, 2005 Egr-1 induces the expression of its corepressor Nab2 by activation of the Nab2 promoter thereby establishing a negative feedback loop. J. Biol. Chem. 280: 42785–42793. https://doi.org/10.1074/jbc.M511079200

Kumbrink, J., K. H. Kirsch, and J. P. Johnson, 2010 EGR1, EGR2, and EGR3 activate the expression of their coregulator NAB2 establishing a negative feedback loop in cells of neuroectodermal and epithelial origin. J. Cell. Biochem. 111: 207–217. https://doi.org/10.1002/jcb.22690

Lee, R. Y. N., K. L. Howe, T. W. Harris, V. Arnaboldi, S. Cain *et al.*, 2018 WormBase 2017: molting into a new stage. Nucleic Acids Res. 46: D869–D874. https://doi.org/10.1093/nar/gkx998

Lemieux, G. A., and K. Ashrafi, 2015 Insights and challenges in using C. elegans for investigation of fat metabolism. Crit. Rev. Biochem. Mol. Biol. 50: 69–84. https://doi.org/10.3109/10409238.2014.959890

Liang, B., K. Ferguson, L. Kadyk, and J. L. Watts, 2010 The role of nuclear receptor NHR-64 in fat storage regulation in caenorhabditis elegans. *PLoS One* 5: e9869. https://doi.org/10.1371/journal.pone.0009869

Lin, S.-Y., S. M. Johnson, M. Abraham, M. C. Vella, A. Pasquinelli *et al.*, 2003 The C. elegans hunchback homolog, hbl-1, controls temporal patterning and is a probable microRNA target. Dev. Cell 4: 639–650. https://doi.org/10.1016/S1534-5807(03)00124-2

Lints, R., and D. H. Hall, 2009 The cuticle, in *WormAtlas*. https://doi.org/10.3908/wormatlas.1.12

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

Livak, K. J., and T. D. Schmittgen, 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25: 402–408. https://doi.org/10.1006/meth.2001.1262

MacNeil, L. T., C. Pons, H. E. Arda, G. E. Giese, C. L. Myers *et al.*, 2015 Transcription factor activity mapping of a tissue-specific in vivo gene regulatory network. Cell Syst. 1: 152–162. https://doi.org/10.1016/j.cels.2015.08.003

McKay, R. M., J. P. McKay, L. Avery, and J. M. Graff, 2003 C elegans: a model for exploring the genetics of fat storage. Dev. Cell 4: 131–142. https://doi.org/10.1016/S1534-5807(02)00411-2

Moss, E. G., 2007 Heterochronic genes and the nature of developmental time. Curr. Biol. 17: R425–R434. https://doi.org/10.1016/j.cub.2007.03.043

Moss, E. G., and J. Romer-Seibert, 2014 Cell-intrinsic timing in animal development. Wiley Interdiscip. Rev. Dev. Biol. 3: 365–377. https://doi.org/10.1002/wdev.145

Munoz-Torres, M., and S. Carbon, 2017 Get GO! retrieving GO data using AmiGO, QuickGO, API, files, and tools, pp. 149–160 in *Methods in Molecular Biology*, Humana Press, New York.

Newman, A. P., T. Inoue, M. Wang, and P. W. Sternberg, 2000 The Caenorhabditis elegans heterochronic gene lin-29 coordinates the vulval-uterine-epidermal connections. Curr. Biol. 10: 1479–1488. https://doi.org/10.1016/S0960-9822(00)00827-7

Nimmo, R. A., and F. J. Slack, 2009 An elegant miRror: microRNAs in stem cells, developmental timing and cancer. Chromosoma 118: 405–418. https://doi.org/10.1007/s00412-009-0210-z

Nomura, T., M. Horikawa, S. Shimamura, T. Hashimoto, and K. Sakamoto, 2010 Fat accumulation in Caenorhabditis elegans is mediated by SREBP homolog SBP-1. Genes Nutr. 5: 17–27. https://doi.org/10.1007/s12263-009-0157-y

Page, A. P., and I. L. Johnstone, 2007 The cuticle, *WormBook*, ed. The *C. elegans* Research Community WormBook, http://www.wormbook.org, https://doi.org/doi/10.1895/wormbook.1.138.1

Pagel, J.-I., and E. Deindl, 2011 Early growth response 1–a transcription factor in the crossfire of signal transduction cascades. Indian J. Biochem. Biophys. 48: 226–235.

Partridge, F. A., A. W. Tearle, M. J. Gravato-Nobre, W. R. Schafer, and J. Hodgkin, 2008 The C. elegans glycosyltransferase BUS-8 has two distinct and essential roles in epidermal morphogenesis. Dev. Biol. 317: 549–559. https://doi.org/10.1016/j.ydbio.2008.02.060

Pathare, P. P., A. Lin, K. E. Bornfeldt, S. Taubert, and M. R. van Gilst, 2012 Coordinate regulation of lipid metabolism by novel nuclear receptor partnerships. *PLoS Genet.* 8: e1002645. https://doi.org/10.1371/journal.pgen.1002645

Qadota, H., M. Inoue, T. Hikita, M. Köppen, J. D. Hardin *et al.*, 2007 Establishment of a tissue-specific RNAi system in C. elegans. Gene 400: 166–173. https://doi.org/10.1016/j.gene.2007.06.020

Rausch, M., M. Ecsedi, H. Bartake, A. Müllner, and H. Großhans, 2015 A genetic interactome of the let-7 microRNA in C. elegans. Dev. Biol. 401: 276–286. https://doi.org/10.1016/j.ydbio.2015.02.013

Ririe, T. O., J. S. Fernandes, and P. W. Sternberg, 2008 The Caenorhabditis elegans vulva: a post-embryonic gene regulatory network controlling organogenesis. Proc. Natl. Acad. Sci. USA 105: 20095–20099. https://doi.org/10.1073/pnas.0806377105

Rougvie, A. E., and V. Ambros, 1995 The heterochronic gene lin-29 encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans. Development 121: 2491–2500.

Rougvie, A. E., and E. G. Moss, 2013 Developmental transitions in C. elegans larval stages. *Curr. Top Dev. Biol.* 105: 153–180. https://doi.org/10.1016/B978-0-12-396968-2.00006-3

Rual, J. F., J. Ceron, J. Koreth, T. Hao, A.-S. S. Nicot *et al.*, 2004 Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. Genome Res. 14: 2162–2168. https://doi.org/10.1101/gr.2505604

Simmer, F., M. Tijsterman, S. Parrish, S. P. Koushika, M. L. Nonet *et al.*, 2002 Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Curr. Biol. 12: 1317–1319. https://doi.org/10.1016/S0960-9822(02)01041-2

Slack, F. J., M. Basson, Z. Liu, V. Ambros, H. R. Horvitz *et al.*, 2000 The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol. Cell 5: 659–669. https://doi.org/10.1016/S1097-2765(00)80245-2

Sternberg, P. W., Vulval development 2005 WormBook, ed. The *C. elegans* Research Community, WormBook, https://doi.org/10.1895/wormbook.1.6.1, http://www.wormbook.org.

Su, J.-L., P.-S. Chen, G. Johansson, and M.-L. Kuo, 2012 Function and regulation of let-7 family microRNAs. MicroRNA 1: 34–39. https://doi.org/10.2174/2211536611201010034

Sutphin, G. L., and M. Kaeberlein, 2009 Measuring Caenorhabditis elegans life span on solid media. J. Vis. Exp. (27): 1152. https://doi.org/10.3791/1152

Tocchini, C., J. J. Keusch, S. B. Miller, S. Finger, H. Gut *et al.*, 2014 The TRIM-NHL protein LIN-41 controls the onset of developmental plasticity in Caenorhabditis elegans. *PLoS Genet.* 10: e1004533. https://doi.org/10.1371/journal.pgen.1004533

Tsialikas, J., and J. Romer-Seibert, 2015 LIN28: roles and regulation in development and beyond. Development 142: 2397–2404. https://doi.org/10.1242/dev.117580

Tursun B., T. Patel, P. Kratsios, and O. Hobert, 2011 Direct conversion of C. elegans germ cells into specific neuron types. Science 331: 304–308. https://doi.org/10.1126/science.1199082

Van Gilst, M. R., H. Hadjivassiliou, A. Jolly, and K. R. Yamamoto, 2005 Nuclear hormone receptor NHR-49 controls fat consumption and fatty acid composition in C. elegans. *PLoS Biol.* 3: e53. https://doi.org/10.1371/journal.pbio.0030053

Watts, J. L., 2009 Fat synthesis and adiposity regulation in Caenorhabditis elegans. Trends Endocrinol. Metab. 20: 58–65. https://doi.org/10.1016/j.tem.2008.11.002

Watts, J. L., and J. Browse, 2000 A palmitoyl-CoA-specific Δ9 fatty acid desaturase from Caenorhabditis elegans. Biochem. Biophys. Res. Commun. 272: 263–269. https://doi.org/10.1006/bbrc.2000.2772

Watts, J. L., and J. Browse, 2002 Genetic dissection of polyunsaturated fatty acid synthesis in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 99: 5854–5859. https://doi.org/10.1073/pnas.092064799

Watts, J. L., and M. Ristow, 2017 Lipid and carbohydrate metabolism in Caenorhabditis elegans. Genetics 207: 413–446. https://doi.org/10.1534/genetics.117.300106

Wildwater, M., N. Sander, G. de Vreede, and S. van den Heuvel, 2011 Cell shape and Wnt signaling redundantly control the division axis of C. elegans epithelial stem cells. Development 138: 4375–4385. https://doi.org/10.1242/dev.066431

Worringer, K. A., T. A. Rand, Y. Hayashi, S. Sami, K. Takahashi *et al.*, 2014 The let-7/LIN-41 pathway regulates reprogramming to human induced pluripotent stem cells by controlling expression of prodifferentiation genes. Cell Stem Cell 14: 40–52. https://doi.org/10.1016/j.stem.2013.11.001

Zhang, J., Y. Zhang, T. Sun, F. Guo, S. Huang *et al.*, 2013 Dietary obesity-induced Egr-1 in adipocytes facilitates energy storage via suppression of FOXC2. Sci. Rep. 3: 1–10. https://doi.org/10.1038/srep01476

Zhang, S. O., A. C. Box, N. Xu, J. Le Men, J. Yu *et al.*, 2010 Genetic and dietary regulation of lipid droplet expansion in Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 107: 4640–4645. https://doi.org/10.1073/pnas.0912308107

Zhang, Y., X. Zou, Y. Ding, H. Wang, X. Wu *et al.*, 2013 Comparative genomics and functional study of lipid metabolic genes in Caenorhabditis elegans. BMC Genomics 14: 164. https://doi.org/10.1186/1471-2164-14-164

Zhang, Y., W. Li, L. Li, Y. Li, R. Fu *et al.*, 2015 Structural damage in the C. elegans epidermis causes release of STA-2 and induction of an innate immune response. Immunity 42: 309–320. https://doi.org/10.1016/j.immuni.2015.01.014

Zhao, L., S. Dong, Y. Zhao, H. Shao, N. Krasteva *et al.*, 2019 Dysregulation of let-7 by PEG modified graphene oxide in nematodes with deficit in epidermal barrier. Ecotoxicol. Environ. Saf. 169: 1–7. https://doi.org/10.1016/j.ecoenv.2018.10.106

Zhu, X., Y. Liu, H. Zhang, and P. Liu, 2018 Whole-genome RNAi screen identifies methylation-related genes influencing lipid metabolism in Caenorhabditis elegans. J. Genet. Genomics 45: 259–272. https://doi.org/10.1016/j.jgg.2018.03.005

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