

REPORT



## *acn-1*, a *C. elegans* homologue of ACE, genetically interacts with the *let-7* microRNA and other heterochronic genes

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### ABSTRACT

The heterochronic pathway in *C. elegans* controls the relative timing of cell fate decisions during post-embryonic development. It includes a network of microRNAs (miRNAs), such as *let-7*, and protein-coding genes, such as the stemness factors, LIN-28 and LIN-41. Here we identified the *acn-1* gene, a homologue of mammalian angiotensin-converting enzyme (ACE), as a new suppressor of the stem cell developmental defects of *let-7* mutants. Since *acn-1* null mutants die during early larval development, we used RNAi to characterize the role of *acn-1* in *C. elegans* seam cell development, and determined its interaction with heterochronic factors, including *let-7* and its downstream interactors – *lin-41*, *hbl-1*, and *apl-1*. We demonstrate that although RNAi knockdown of *acn-1* is insufficient to cause heterochronic defects on its own, loss of *acn-1* suppresses the retarded phenotypes of *let-7* mutants and enhances the precocious phenotypes of *hbl-1*, though not *lin-41*, mutants. Conversely, the pattern of *acn-1* expression, which oscillates during larval development, is disrupted by *lin-41* mutants but not by *hbl-1* mutants. Finally, we show that *acn-1(RNAi)* enhances the *let-7*-suppressing phenotypes caused by loss of *apl-1*, a homologue of the Alzheimer's disease-causing amyloid precursor protein (APP), while significantly disrupting the expression of *apl-1* during the L4 larval stage. In conclusion, *acn-1* interacts with heterochronic genes and appears to function downstream of *let-7* and its target genes, including *lin-41* and *apl-1*.

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### Introduction

A fundamental feature of development is the relative timing of biological processes – whether proliferation, migration, or cell fate decisions. *Caenorhabditis elegans* (*C. elegans*) has proved immensely powerful in identifying genes and pathways involved in developmental timing due to the organism's discrete developmental stages and invariant cell lineages.<sup>1</sup> Many studies in *C. elegans* have focused on a pathway involved in developmental timing named the heterochronic pathway.<sup>2–4</sup> Heterochronic genes control the *relative* timing of development – the timing of developmental events relative to the developmental stage of the organism – and operate independent of spatial controls.<sup>2,4</sup> Mutations in these genes lead to bypass (“precocious” phenotype) or repeat (“retarded” phenotype) of stage-specific cell fate decisions.<sup>5</sup> Analyses of these mutations, both alone and in combinations, have both revealed numerous genes that promote correct cell fate choices and elucidated how they genetically interact in the heterochronic pathway.<sup>3,4,6</sup>

The key heterochronic genes responsible for controlling the timing pattern of stem-cell-like hypodermal seam cells in the later larval stages of *C. elegans* (L3 and L4) include the *let-7* microRNA (miRNA), its family members (*mir-48*, *mir-84*, and *mir-241*), and its downstream interactor genes (*hbl-1*, *lin-41*, *lin-42*, and *lin-29*).<sup>7–12</sup> Here, we focus on *let-7* and its two direct

targets: *lineage-abnormal 41* (*lin-41*), a Ring finger-B box-Coiled coil (RBCC) protein whose family members include several E3 ubiquitin ligases, and *hunchback-like 1* (*hbl-1*), a Cys<sub>2</sub>-His<sub>2</sub> zinc-finger transcription factor homologous to *Drosophila melanogaster's* *hunchback* (*hb*) segmentation gene.<sup>9–16</sup> During the L4 stage, *let-7* suppresses *hbl-1* and *lin-41* via miRNA-mediated post-transcriptional suppression mechanisms to promote the L4-to-adult transition.<sup>9,11,12</sup> Loss-of-function mutations in *let-7* result in reiteration of L4 cell fates, leading to an extra round of seam cell division, a delay in seam cell terminal differentiation and adult alae production, and a transient increase in the apparent number of seam cells.<sup>8</sup> In contrast, mutations in the *lin-41* or *hbl-1* genes cause seam cells to skip the L4 cell divisions and terminally differentiate precociously, albeit with less than 100% penetrance.<sup>9,11,12</sup> Simultaneous knock-down of both genes enhances these precocious phenotypes, suggesting that LIN-41 and HBL-1 function in parallel.<sup>9,12</sup> HBL-1 in turn inhibits transcription of *let-7*, forming a negative feedback loop between the adult- and larval-fate promoters.<sup>17</sup> *hbl-1* is also suppressed by the *let-7*-family-member miRNAs *mir-48*, *mir-84*, and *mir-241* in the seam cells during the L3 stage.<sup>7</sup>

While many key regulators of the heterochronic pathway are known, questions remain as to how the tightly timed expression

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patterns of these regulators promote stage-specific cell fate decisions. To answer these questions, several studies, including our own, have examined the roles of the downstream factors through which these regulators act in developmental timing. Of the several downstream genes of *let-7*, the *amyloid precursor protein-like 1* (*apl-1*), is of particular interest.<sup>18</sup> *apl-1* is a homologue of the human amyloid precursor protein (APP), a gene known to be associated with the progression of Alzheimer's disease.<sup>19,20</sup> In *C. elegans*, *apl-1* has been shown to be involved in neuronal plasticity, pharyngeal pumping, and molting.<sup>21-23</sup> Notably, Niwa et al. showed that *apl-1* suppresses the *let-7* retarded phenotypes and genetically interacts with *hbl-1*, *lin-41*, and *lin-42*.<sup>18</sup> In this study, we aimed to further elucidate the role of *apl-1* in developmental timing in order to both gain insights into the mechanisms of the heterochronic pathway and potentially elucidate the function of an Alzheimer's disease-associated gene.

To this end, we identified a list of genes that could potentially interact with both *apl-1* and other components of the heterochronic pathway. Human APP is intensely studied due to its connection with Alzheimer's disease, so there is a vast body of literature, including several genome-wide association studies (GWAS), on APP. We hypothesized that the *C. elegans* homologues of human genes that have been shown to interact with APP may in turn interact with the nematode *apl-1* gene. Thus, we screened the *C. elegans* homologues of genes implicated in Alzheimer's disease for interactions with heterochronic genes.<sup>24-37</sup> *Angiotensin-converting enzyme-like non-peptidase* (*acn-1*), whose homologue ACE has been shown to interact with APP in GWAS, was one such gene. Here we identify *acn-1* as a novel component of the heterochronic pathway. Specifically, we demonstrate that *acn-1* suppresses *let-7* phenotypes and enhances the phenotypes of *apl-1* and *hbl-1*, though not *lin-41*. Conversely, loss of *lin-41* but not *hbl-1* alters the oscillatory pattern of *acn-1* expression. This study suggests that *acn-1* is a new factor involved in the developmental timing of seam cells in *C. elegans*. Moreover, as several studies have identified an association between mammalian ACE and Alzheimer's disease, our characterization of ACE's homologue in a model organism might prove useful in elucidating the nature of this association.

## Results

### Knockdown of *acn-1* suppresses *let-7(n2853)* lethality

GWAS of Alzheimer's disease yielded 22 human genes with single nucleotide polymorphisms (SNPs) exhibiting statistically significant correlation with the disease phenotype.<sup>24-26,28-34,37</sup> An additional 23<sup>rd</sup> gene on our list was beta-secretase (BACE), which is known to cleave APP.<sup>35,36</sup> Of these 23 human genes, 14 of them have *C. elegans* homologues as identified by the Basic Local Alignment Search Tool (BLAST) (Fig. 1). Due to constraints in RNAi construct availability and previously reported lethal effect of some RNAi constructs, we ultimately examined 24 *C. elegans* genes corresponding to 10 of these 14 human genes (Fig. 1). Fig. 1 shows all genes that were examined here.

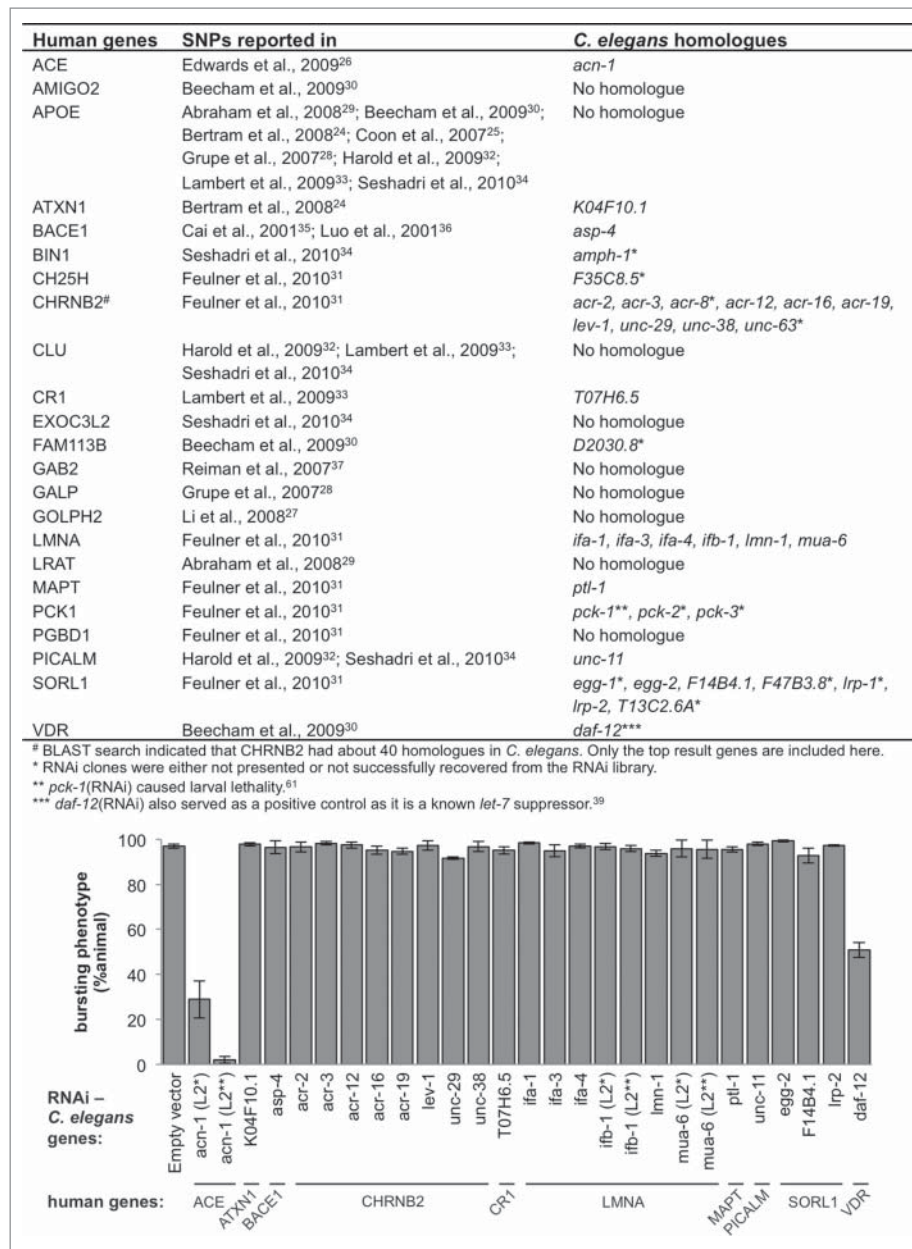
We hypothesized that some of these 24 *C. elegans* genes would interact with *apl-1* and by extension the heterochronic

genes with which *apl-1* interacts. *let-7* has one of the strongest mutant phenotypes of any heterochronic gene: loss of *let-7* causes both retarded development of the seam cells and loss of vulval structure, resulting in lethality by bursting through the vulva soon after the L4/adult transition.<sup>8</sup> Identifying suppressors of *let-7* mutant phenotypes is thus one of the most amenable ways to screen for novel heterochronic genes. For instance, loss of most of *let-7*'s several downstream target genes suppresses both the vulval-related lethality and abnormal seam cell development phenotypes of *let-7* mutants.<sup>8,9,11,12,38,39</sup> In order to identify suppressors of the *let-7* mutant phenotypes, we first examined whether RNAi knockdown of our candidate genes diminished the lethality caused by loss of *let-7*. For most RNAi constructs, *let-7* mutant animals were fed *E. coli* carrying RNAi plasmids starting at the L1 larval stage; however, in order to bypass the early lethal effects of *ifb-1* and *mua-6* RNAi knockdowns, *let-7(n2853)* animals were fed the same RNAi construct-carrying bacteria starting from the L2 stage instead of L1. The majority of the RNAi constructs we screened resulted in more than 90% of animals bursting through the vulva, indicating no suppression of *let-7* mutant phenotypes (Fig. 1).

*acn-1* was the only gene whose knockdown appeared to suppress the *let-7* bursting vulva phenotype. Animals fed with *acn-1(RNAi)* from the L1 stage mostly arrested at the L2-L3 stages and therefore never survived to the L4/adult transition during which bursting occurs. The early larval arrest phenotype observed with L1 RNAi feeding was phenocopied by the *acn-1* (null) mutant.<sup>40</sup> In order to overcome the problem of premature developmental arrest, we began feeding *let-7(n2853)* animals with *acn-1(RNAi)* starting at the L2 stage; as a result, more than 90% of animals survived to the L4/adult molt when they could be scored for bursting. Among these, less than 30% died by bursting (Fig. 1). The animals that survived appeared smaller than N2 wild-type animals but were able to lay eggs, confirming that they had reached the adult stage.

### Knockdown of *acn-1* suppresses *let-7(n2853)* retarded seam cell defects

Since bursting is a vulval phenotype, we next investigated whether knockdown of *acn-1* affects development of the seam cells. While vulval cells undergo post-embryonic cell divisions, they do not divide during every larval stage as seam cells do; observing seam cells can therefore reveal more details of stage-by-stage development and heterochrony. *let-7(n2853)* mutants have retarded seam cell phenotypes: the seam cells do not stop dividing at the larval/adult transition and undergo terminal differentiation. This results in the transient detection of more than the normal 16 seam cells per side right after the L4/adult molt due to the presence of dividing daughter cells that should have already terminally differentiated;<sup>18</sup> additionally, adult alae are not completely produced at the young adult stage as normally observed in wild-type animals.<sup>8</sup> To visualize the seam cells in our knockout animals, we used a *let-7(n2853);wIs79* strain (the *wIs79* construct contains *ajm-1::gfp;scm-1::gfp* seam cell markers). Knocking down *acn-1* eliminated the supernumerary seam cell divisions (scored as the normal number of 16 seam cells), suggesting that a few seam cells that may still undergo cell division due to *let-7* mutation have stopped



**Figure 1.** Knockdown of *acn-1* partially suppresses *let-7* lethality. This graph shows the result of an RNAi screen for *let-7* suppressors. RNAi constructs targeting *C. elegans* homologues of the genes listed in the table in this figure were screened for suppression of the bursting-vulva phenotype of *let-7(n2853)* at the L4/adult transition. Except when noted, *let-7(n2853)* animals were placed on RNAi bacteria at L1. Animals marked with L2\* were fed with empty vector RNAi clones for the first 16 hours before being moved to plates with the indicated RNAi constructs. Animals marked with L2\*\* were fed with the OP50 strain of *E. coli* for 16 hours before being moved. Empty L4440 vector<sup>60</sup> was used as a negative control; *daf-12* RNAi was used as a positive control.<sup>39</sup>

dividing when *acn-1* was reduced (Table 1). Moreover, silencing *acn-1* by RNAi also suppressed the retarded alae production phenotype caused by loss of *let-7* (Table 1). Even though knocking down *acn-1* restored some adult alae formation ('any alae' column), it disrupted the morphology of alae such that alae were not complete ('>50% alae' column) (Table 1) – an observation consistent with previous studies.<sup>40</sup>

#### Loss of *acn-1* enhances the loss-of-*apl-1* suppressor phenotype and affects *apl-1* expression

The *let-7(n2853)*-suppressing phenotypes of *acn-1* knockdowns are similar to those of *apl-1* mutants;<sup>18</sup> thus, we tested whether subjecting the animals to both RNAi simultaneously would

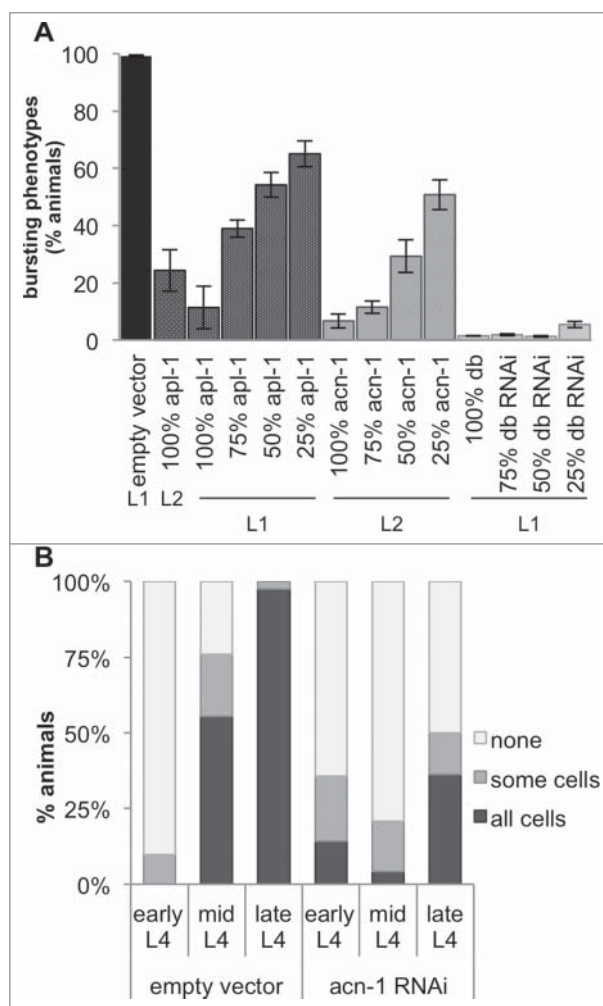
enhance the heterochronic defects of the individual knockdowns. Since both *apl-1*(RNAi) and *acn-1*(RNAi) already suppressed the bursting vulva phenotype very effectively, any enhancement of this suppression could not be easily distinguished (Fig. 2A those labelled with '100%'); thus, we lowered the concentration of RNAi construct-carrying *E. coli* strain HT115 by mixing in *E. coli* containing only empty vector (L4440). We found that knocking down *apl-1* and *acn-1* together, at concentrations where knocking down either alone had little effect, suppressed the bursting vulva phenotype at a significantly higher rate (Fig. 2A). Thus, *apl-1* and *acn-1* synergistically suppress the *let-7* phenotype.

*apl-1* is expressed from the L4 stage into adulthood,<sup>18</sup> while *acn-1* is expressed in all larval stages and disappears during

**Table 1.** Knockdown of *acn-1* partially suppressed the retarded phenotypes in seam cells of *let-7* mutants. *let-7* mutants have retarded seam cell phenotypes – displaying supernumerary divisions and transiently producing more than the normal 16 seam cells and delaying adult alae formation.<sup>8</sup> *let-7(n2853);wls79* animals were scored right after the L4/adult transition but before bursting. Knocking down *acn-1* partially suppressed both phenotypes.

RNAi treatment of <i>let-7(n2853);wls79</i>	Number of seam cells per side	
empty vector control RNAi (n = 58)	18.3 ± 0.95	
<i>acn-1</i> RNAi (n = 81)	16.3 ± 0.87 *	
*p-value < 0.001 as judged by Mann-Whitney U test		
RNAi treatment of <i>let-7(n2853);wls79</i>	Any alae (including both ≤50% and >50% alae)	>50% alae
empty vector control RNAi (n = 52)	42.31%	11.54%
<i>acn-1</i> RNAi (n = 53)	67.92% **	13.21% <sup>ns</sup>

\*\*p-value < 0.01 and <sup>ns</sup> not statistically significant as judged by chi-square test



**Figure 2.** Loss of *acn-1* enhanced the *apl-1(RNAi)* phenotype and affected *apl-1* expression. (A) RNAi-mediated knockdown of *acn-1* and *apl-1* enhanced the suppression of the *let-7* bursting-vulva phenotype. This effect was dose-dependent. A gradient of the quantity of RNAi was achieved by mixing the indicated percentage of tested RNAi construct with the corresponding amount of empty vector RNAi (experimental details in Materials and Methods). We observed a significant enhancement of the suppression phenotype in animals fed with a double RNAi construct (dbRNAi) targeting both *acn-1* and *apl-1* sequences. 10–19% of animals fed with dbRNAi stalled at the L1 or L2 stage and were excluded from the calculation of the percentage of animals exhibiting the bursting-vulva phenotype. The L1 and L2 labels designate the developmental stage at which animals were placed on RNAi bacteria. (B) Expression of the *apl-1::gfp::unc-54* 3'UTR construct in hypodermal seam cells at different developmental stages in nematodes fed empty vector (L4440) or *acn-1* RNAi. Shown is the percentage of animals with seam cell GFP expression.

adulthood.<sup>40</sup> We thus tested whether *acn-1* regulates *apl-1* expression, employing wild-type animals with *apl-1 promoter::gfp::unc-54* 3'UTR constructs to measure *apl-1* transcription. Only ~10% of these animals expressed GFP at the early L4 stage, and when they did it was found in only a few seam cells (Fig. 2B); expression of *apl-1::gfp* then increased during the mid-L4 stage to almost 100% of animals expressing it in all seam cells (Fig. 2B). Knockdown of *acn-1* disrupted this expression pattern (Fig. 2B). Whereas about 75% and 100% of control animals expressed *apl-1* at the mid- and late-L4 stages respectively, only about 20% and 50% of *acn-1* knockdowns did the same (Fig. 2B). This effect of *acn-1* knockdown on *apl-1* transcription parallels that of *lin-41* silencing.<sup>18</sup>

### *acn-1(lf)* is not sufficient to cause heterochronic phenotypes

Since *acn-1* is able to suppress *let-7(n2853)* retarded phenotypes, we hypothesized that *acn-1(lf)* might be sufficient to cause precocious phenotypes in hypodermal seam cells. However, this was not the case. *acn-1* knockdown animals consistently failed to exhibit precocious hypodermal seam cell fusion or adult alae (data not shown). Therefore, *acn-1* does not behave as a canonical heterochronic gene by itself.

### Knockdown of *acn-1* enhances the precocious phenotypes of *hbl-1* mutants but not of *lin-41* mutants

In wild-type animals, lateral hypodermal seam cells divide during the L1, L2, L3, and L4 stages before terminally differentiating and fusing together at the L4-adult molt. After fusion, these cells secrete a cuticular structure called the adult alae. Knockdown or mutation of *hbl-1* results in precocious seam cell terminal differentiation and adult alae formation, albeit with less than 100% penetrance.<sup>9,12</sup>

Consistent with previous studies,<sup>9,12</sup> we observed that 60% of *hbl-1(mg285);wls79(ajm-1::gfp;scm-1::gfp)* animals exhibited at least one seam cell fusion event in L4; while 7%–12% did so during the early and late L3 stage respectively (Table 2). On the other hand, in this background, 100% of *acn-1(RNAi)* knockdowns exhibited at least one precocious seam cell fusion event at the L4 stage (Table 2). More notably, 58% and 47% did the same at the late L3 and early L3 stages respectively (Table 2) – the first time that precocious seam cell fusion has been reported in *hbl-1(mg285)* animals at a stage earlier than the L3 to L4 molt.

**Table 2.** Knockdown of *acn-1* enhanced the precocious phenotypes of *hbl-1(mg285)* but did not affect those of *lin-41(ma104)*. *hbl-1(mg285)* led to precocious hypodermal seam cell fusion and adult alae production though not with complete penetrance.<sup>9,12</sup> RNAi knockdown of *acn-1* enhanced these phenotypes further, promoting seam cell fusion and alae production as early as the L3 stage. *lin-41(ma104)* animals also exhibited precocious seam cell fusion and adult alae production during the L4 stage,<sup>11</sup> but knocking down *acn-1* did not enhance these phenotypes.

Strains and RNAi constructs fed	Percentage of animals with any:				
	Fused seam cells (n)	Fused seam cells (n)	Fused seam cells (n)	Alae (n)	Alae (n)
<i>hbl-1(mg285);wIs79</i> , empty vector(RNAi)	Early L3 12.0 (25)	Late L3 6.7 (45)	L4 60.0 (45)	Early L3 4.0 (25)	Late L3 10.0 (20)
<i>hbl-1(mg285);wIs79, acn-1</i> (RNAi)	46.7 (45)**	57.8 (45)***	100.0 (20)***	28.9 (45)*	48.9 (45) <sup>ns</sup>

Strains and RNAi constructs fed	Percentage of animals with any:	
	Fused seam cells (n)	Alae (n)
<i>lin-41(ma104);wIs79</i> , empty vector(RNAi)	L4 26.0 (50)	L4 16.0 (50)
<i>lin-41(ma104);wIs79, acn-1</i> (RNAi)	28.0 (50) <sup>ns</sup>	18.0 (50) <sup>ns</sup>

As judged by chi-square test, \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001, and <sup>ns</sup> not statistically significant when the percentages of animals on *acn-1*(RNAi) were compared to those on empty vector(RNAi) at the same stage.

Similarly, we also examined whether *acn-1* knockdown enhanced the precocious adult alae production characteristic of *hbl-1(mg285)* mutants. Whereas 4% and 10% of *hbl-1(mg285);wIs79* animals produced at least some alae at the early and late L3 stages respectively, 29% and 49% of *acn-1* RNAi knockdowns did the same (Table 2) – a marked increase due to *acn-1* knockdown. Incidentally, the percentage of animals with any alae at late L3 was not significantly different from that at early L3.

These increases in the rates of both precocious seam cell fusion and alae production are statistically significant, as analyzed by Fisher's exact test (Table 2). These results clearly demonstrate that knockdown of *acn-1* enhances the precocious phenotypes caused by loss of *hbl-1*.

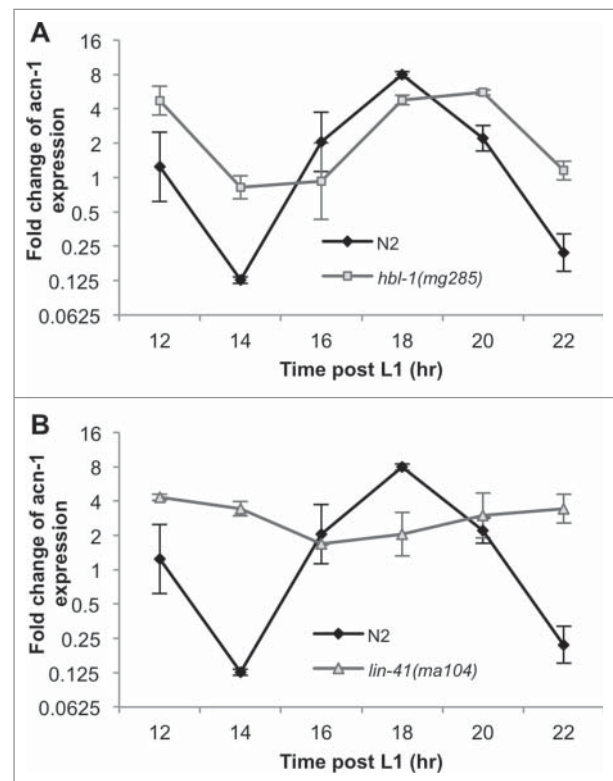
Since *hbl-1* and *lin-41* are both targets of the *let-7* miRNA, and since *hbl-1* mutants exhibit enhanced penetrance of the precocious phenotypes of *lin-41* mutants, it has been proposed that *hbl-1* and *lin-41* function in parallel.<sup>9,11,12</sup> Therefore, we investigated whether *acn-1* could enhance *lin-41* mutant phenotypes in the same manner that it did *hbl-1* mutant phenotypes. We observed that *lin-41(ma104);wIs79* animals exhibited precocious seam cell fusion and adult alae production, consistent with a previous report.<sup>11</sup> However, knockdown of *acn-1* produced no increase in the percentage of *lin-41* mutants exhibiting those phenotypes (Table 2). Thus, although knockdown of *acn-1* enhances the precocious phenotypes of *hbl-1* mutants, it does not have the same effect on *lin-41* mutants.

### The oscillatory expression pattern of *acn-1* is influenced by *lin-41* but not *hbl-1*

It has previously been reported that *acn-1::gfp* fusion reporter lines – which include the 5' UTR sequence, *acn-1* coding sequence, and GFP – exhibit GFP expression in hypodermal seam cells starting during embryogenesis and continue until the young adult stage.<sup>40</sup> However, Frand et al. (2005) allude to unpublished data demonstrating that *acn-1p::gfp-pest* (a shorter-lived form of GFP) displays cyclic expression during all four molts.<sup>41</sup> In addition, two recent global studies of mRNA expression during *C. elegans* larval development demonstrate

that *acn-1* exhibits an oscillatory expression pattern.<sup>42,43</sup> We confirmed this observation in wild-type animals by collecting RNA every 2 hours between the early L2 and early L3 stages, and measuring *acn-1* transcription levels using qRT-PCR (Fig. 3A and B).

We then repeated this temporal *acn-1* transcriptional analysis in *hbl-1(mg285)* animals and found that *hbl-1* mutant animals also exhibited an oscillatory *acn-1* expression pattern, albeit with some small differences in the



**Figure 3.** *lin-41* influences *acn-1*'s oscillating expression pattern. (A and B) qRT-PCR of *acn-1* mRNA from wild-type (N2) nematodes shows oscillating expression. These time points cover the period between early L2 and early L3 (the L2/L3 transition takes place between 18 and 20 hrs post-L1.) (A) *hbl-1(mg285)* mutation led to a slight rightward phase shift in the oscillation of *acn-1* expression but no significant differences in any oscillation parameters, while (B) *lin-41(ma104)* mutation significantly dampened the amplitude of *acn-1*'s oscillations.

oscillatory function parameters. Loss of *hbl-1* did not affect the period of *acn-1*'s oscillations and the oscillations were only slightly phase-shifted to the right ( $p$ -value = 0.81) relative to those of wild-type animals (Fig. 3A). Additionally, their amplitude was somewhat dampened ( $-0.87 \pm 0.86$  cycles, corresponding to a 0.65-fold change ( $p$ -value = 0.31) from that in wild-type animals) (Fig. 3A). These changes observed were too small and variable to prove any functionally significant influence by *hbl-1* on the expression profile of *acn-1*.

On the other hand, *lin-41*(*ma104*) mutant animals exhibited a significantly disrupted *acn-1* expression pattern (Fig. 3B). Though the mean *acn-1* expression level remained relatively unchanged from that of wild-type animals, the amplitude of *acn-1*'s oscillations appeared to be greatly dampened ( $-1.73 \pm 0.71$  cycles, corresponding to a 0.29 fold change ( $p$ -value = 0.01) from that in wild-type animals) (Fig. 3B), indicating that a functional copy of the *lin-41* gene is necessary to maintain the normal oscillatory pattern of *acn-1* expression. For other oscillation parameters, loss of *lin-41* does not significantly affect the phase (slightly right-shifted) or the period of *acn-1*'s oscillations (Fig. 3B).

In order to test whether *acn-1* regulates expression of *hbl-1* or *lin-41* in a feedback loop, we treated wild-type animals with empty vector (L4440) and *acn-1* RNAi constructs, collected them at 12 (L2), 21 (L3), and 29 (L4) hours post-treatment, and measured levels of *hbl-1* and *lin-41* mRNA using qRT-PCR. We found that the temporal expression levels of *hbl-1* and *lin-41* mRNAs remained unchanged in *acn-1* knockdowns relative to those in control animals (data not shown). Therefore, it is unlikely that a feedback loop exists between *acn-1* and either *hbl-1* or *lin-41*.

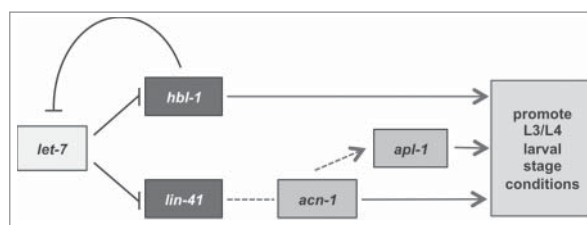
## Discussion

### *acn-1* genetically interacts with genes in the heterochronic pathway

In this study, we reveal several genetic interactions between *acn-1* and certain heterochronic genes. Although knockdown of *acn-1* does not on its own produce heterochronic phenotypes in the hypodermal seam cells, it partially suppresses the retarded phenotypes of *let-7* mutants and enhances the precocious phenotypes of *hbl-1* and *apl-1* mutants. These results suggest that *acn-1* promotes the larval identity of hypodermal seam cells, presumably in cooperation with other genes.

Since the *acn-1* 3' UTR contains no sequence with partial complementarity to *let-7* or *let-7* family member miRNAs (*mir-48*, *mir-84*, and *mir-241*), it is unlikely that *acn-1* is a direct target of *let-7* or its family members. Rather, *acn-1* is likely an indirect target of *let-7* that acts further downstream in the heterochronic pathway (Fig. 4).

It has been proposed that *hbl-1* and *lin-41* function in parallel for three reasons: both genes are targets of *let-7* and its family members, the majority of *hbl-1* and *lin-41* mutant phenotypes are identical, and knockdown of either gene by RNAi enhances the penetrance of the other's precocious phenotype.<sup>9,11,12</sup> In this study, we identify distinct features between *hbl-1*'s and *lin-41*'s interactions with *acn-1*. Knockdown of



**Figure 4.** A proposed model of the relationships between *acn-1* and the heterochronic genes *let-7*, *hbl-1*, and *lin-41*.

*acn-1* enhances the precocious phenotypes of *hbl-1* mutants but does not affect those of *lin-41* mutants. Furthermore, whereas loss-of-function *lin-41*(*ma104*) mutations affect the oscillatory pattern of *acn-1* expression, *hbl-1*(*mg285*) mutations do not. These data suggest that *acn-1* may lie in the *lin-41* arm of the pathway (Fig. 4) and that *lin-41* but not *hbl-1* regulates *acn-1* transcription in some way.

While mean *acn-1* expression is not significantly diminished in *lin-41*(*ma104*) mutant animals, the amplitude of *acn-1*'s oscillations is considerably reduced. It is therefore unlikely that *lin-41* functions in a straightforward way to promote or suppress *acn-1* transcription. In a study of global mRNA expression, *lin-41* was also shown to exhibit an oscillatory expression pattern with the same 8-hour period as that of *acn-1*, albeit with a much smaller amplitude (1.48 units) than that of *acn-1* (3.94 units).<sup>42</sup> In wild-type nematodes during the L2-L3 sampling period, we observed some fluctuation in *lin-41* expression, though not large enough to verify a pattern of oscillation (data not shown). On the other hand, *hbl-1* is expressed at a steady level until it is down-regulated during the L3 stage.<sup>9,12,42,43</sup> These findings indicate that *lin-41* may play a role in regulating *C. elegans*'s cyclic progression through the larval stages,<sup>42</sup> and our results suggest that this regulation may at least partially occur through its interaction with *acn-1*. Such a model would explain why loss of *lin-41* disrupts *acn-1*'s oscillatory expression pattern. However, it may also be the case that loss of *lin-41* causes animals to develop less synchronously, thus making it difficult to fit a single sinusoidal curve to an asynchronous population.

A simple explanation of why knocking down *acn-1* enhances the penetrance of the *hbl-1*(*mg285*) mutant's precocious phenotypes is that *acn-1* functions in a pathway parallel to that of *hbl-1* (Fig. 4), most likely the pathway regulated by *lin-41*. Such an indirect relationship between *acn-1* and *hbl-1* is supported by our observation that *hbl-1*(*mg285*) does not significantly affect the expression of *acn-1*. The slight change in *acn-1* expression in *hbl-1*(*mg285*) mutants might have resulted from the negative feedback loop between *hbl-1* and *let-7*,<sup>17</sup> which would indirectly affect *lin-41* expression via *let-7*. Moreover, the hypothesis that *acn-1* operates downstream of *lin-41* is consistent with our findings that *acn-1* knockdown does not enhance *lin-41*(*ma104*)'s heterochronic phenotypes in hypodermal seam cells as *lin-41*(*ma104*)'s seam cell phenotypes would accordingly already encompass the extent of *acn-1* knockdown's phenotypes (Fig. 4). Thus, it appears that while both *lin-41* and *hbl-1* promote the same larval stage cell fate decisions, they may work through different genes to complement and achieve the same goal.

## Heterochronic genes and molting

Previous studies have connected the process of molting to several genes that were originally shown to control the timing of hypodermal seam cell development. It has been demonstrated that *mir-84* and *let-7* promote the cessation of the molting cycle, and that several heterochronic genes – namely, *lin-28*, *lin-14*, *lin-41*, *lin-42*, and *hbl-1* – are necessary to that end.<sup>44</sup> Two of the main signaling molecules that regulate molting (generating new cuticles and promoting ecdysis) are the nuclear hormone receptors, *nhr-23* and *nhr-25*.<sup>45–47</sup> *mir-84* and *let-7* have been shown to require functional copies of *nhr-23* and *nhr-25* to terminate molting.<sup>44</sup> Moreover, knockdown of *apl-1* was found to suppress the cuticle defect caused by the *mir-48* and *mir-84* mutations.<sup>18</sup> In addition, *nhr-25* was shown to regulate *apl-1* expression, and these two genes were found to act synergistically to cause a molting defect.<sup>48</sup>

On the other hand, *acn-1* was initially demonstrated to be necessary to promote the molting process.<sup>40,41</sup> Feeding *acn-1* (*RNAi*) at the L1 larval stage leads to unsuccessful molting (generally at the L2/L3 molt).<sup>40,41</sup> Furthermore, knocking down *nhr-23* and *nhr-25* reduces *acn-1::gfp* expression in the hypodermal seam cells.<sup>40</sup>

In this study, we reveal novel interactions between the canonical heterochronic genes and *acn-1*, a gene that was previously thought to be just a molting gene. Indeed, *acn-1* is involved not only in the molting cycle, which occurs at regular intervals during the progression of *C. elegans* through the larval stages, but also in cell fate decisions leading up to terminal differentiation of the hypodermal seam cells.

## The interaction between human ACE and APP suggests a potential interaction between the *C. elegans* homologues, *acn-1* and *apl-1*

It is well-established that mutations in the APP gene lead to early-onset Alzheimer's disease (AD).<sup>20</sup> However, the protein's normal function is less well understood. ACE has emerged as a gene linked to APP and AD. While the main function of the ACE protease is to cleave angiotensin I to produce the active form, angiotensin II, as part of the renin-angiotensin aldosterone system (RAAS) that regulates blood pressure,<sup>49</sup> several GWAS studies have associated ACE with APP and Alzheimer's Disease (for a review see ref. 50). Indeed, several point and insertion/deletion mutations in *ACE1* exhibit high correlation with expression of the disease phenotype.<sup>50,51</sup> Moreover, ACE has been shown to degrade APP-derived A $\beta$  peptide *in vitro*.<sup>52–54</sup> Finally, administration of an ACE inhibitor was found to lead to improved learning and memory in mice.<sup>55–57</sup> Although these findings suggest a link between ACE, Alzheimer's disease, and APP processing, the exact mechanism of ACE's contribution to Alzheimer's disease remains unclear.<sup>27,31,57</sup>

In our exploration of the connection between the *C. elegans* homologues of ACE and APP we found them to form a mutual relationship in the heterochronic pathway. We found that loss of *acn-1* enhanced *apl-1*(*RNAi*)'s suppression of the *let-7* mutant phenotype. In addition, knockdown of *acn-1* led to changes in the temporal expression pattern of *apl-1*. The *C. elegans acn-1* protein lacks the key amino acids that bind

zinc molecules in the metallopeptidase active site of ACE; consequently, *acn-1* lacks ACE's peptidase activity.<sup>40</sup> However, ACN-1's active sites may still bind peptides without cleaving them.<sup>40</sup> Here we show that *acn-1* genetically and functionally interacts with *apl-1* even without the metallopeptidase active site sequences. Given the complexity of the relationship between ACE1 and APP in humans, there might be several facets of their interactions that remain unknown. It would be interesting to further examine interactions between not only the human homologues, but between homologues in other model organisms as well.

## Materials & methods

### Nematode strains and culture

*C. elegans* were grown on standard nematode growth media (NGM) plates and fed with the OP50 strain of *E. coli*.<sup>58</sup> Nematodes were kept at either 20°C or 23°C. They were synchronized by bleaching (5% 5N NaOH, 10% bleach in M9 solution) and hatched in M9 without *E. coli* overnight before starvation-arrested L1 were plated for each experiment.

Strains used were: wild-type N2 Bristol, *let-7*(*n2853*),<sup>8</sup> *let-7*(*n2853*);*wIs79*(*ajm-1::gfp*;*scm-1::gfp*),<sup>18</sup> *apl-1::gfp::unc-54* 3'UTR,<sup>18</sup> *wIs79*(*ajm-1::gfp*;*scm-1::gfp*),<sup>18</sup> *hbl-1*(*mg285*),<sup>9</sup> and *lin-41*(*ma104*).<sup>11</sup> *hbl-1*(*mg285*);*wIs79*(*ajm-1::gfp*;*scm-1::gfp*) and *lin-41*(*ma104*); *wIs79*(*ajm-1::gfp*;*scm-1::gfp*) were created by R. Niwa. *acn-1::gfp* (*rol-6* marker)<sup>40</sup> was kindly given to us by R. Elwyn Isaac.

### RNAi experiments

Target genes were knocked down by feeding RNAi to the animals. All RNAi constructs were taken from the Ahringer lab's RNAi library<sup>59</sup> except for the *apl-1*;*acn-1* RNAi construct, which was created by combining the two RNAi inserts in the library into a single construct via molecular cloning. All plasmids were expressed in the HT115 strain of *E. coli*. As a negative control, animals were fed *E. coli* containing the empty L4440 vector, the parental plasmid of other RNAi constructs.<sup>60</sup> These *E. coli* strains were selected on LB plates with ampicillin and tetracycline, grown in LB media with ampicillin, and induced with IPTG before being fed to the animals.<sup>60</sup> Except for *acn-1*(*RNAi*), synchronized nematodes were fed with RNAi bacteria starting from the L1 stage. Feeding animals with *acn-1* (*RNAi*) from the L1 stage resulted in arrested development at the L2/L3 molt (as also observed by Brooks et al.<sup>40</sup>); therefore animals were fed with *acn-1*(*RNAi*) starting from the L2 stage.

In the vulva bursting assay, 100–200 *let-7*(*n2853*) mutants were placed on each RNAi plate. Each data point shown is the average across 3 plates of the percentage of animals that died by bursting.

In the experiment using different dilutions of RNAi, we first adjusted the concentration of each RNAi construct with LB media to control for the concentration of bacteria, as determined by optical density at 600nm (OD<sub>600</sub>). We used that starting concentration of RNAi bacteria as '100% RNAi'. The RNAi dilutions labeled as '75%', '50%', and '25%' were diluted with *E. coli* containing the empty vector RNAi (L4440) at the same

OD600 at ratios of 3:1, 1:1, and 1:3 of specific RNA:L4440 respectively.

### Observation of animals by microscopy

Nematodes were anatomically staged based on the length of the gonad.<sup>9,11</sup> Early, mid, and late L4 animals were categorized by whether their gonadal distal arms were  $0 - \frac{1}{4}$ ,  $\frac{1}{4} - \frac{1}{2}$ , or  $> \frac{1}{2}$  the length of the proximal arms respectively. Early L3 animals were categorized both by the size of the animals to ensure that it has passed the L2/L3 molt and by a gonad length not significantly greater than that of the L2's gonad; late L3 animals were categorized by gonads approaching the gonadal turns. Animals were observed using an Axioplan II microscope (Carl Zeiss) equipped with an AxioCam MRm CCD camera (Carl Zeiss).

In Tables 1 and 2, animals were examined for alae production (either any at all or covering  $> 50\%$  of the length of the animal) and at least partial seam cell fusion.

The *apl-1::gfp::unc-54 3'UTR* was strongly expressed in the head region, so it was difficult to distinguish seam cells expression there.<sup>18</sup> In Fig. 2B, what we described as GFP expressed in “all cells” refers to all seam cells that could be distinguished, excluding those in the head region.

### RNA extraction, Reverse Transcription, and Quantitative RT-PCR

Animals were synchronized by bleaching (see ‘*nematode strains and culture*’). Starvation-arrested L1 animals were placed on NGM plates with OP50 and kept at 23°C for the amount of time specified before sample collection. RNA extraction was done using a mirVana miRNA Isolation Kit (ThermoFisher, <https://www.thermofisher.com/order/catalog/product/AM1560>). RNA samples were treated with Turbo DNase (ThermoFisher, <https://www.thermofisher.com/order/catalog/product/AM1907>). cDNA synthesis was performed according to the protocol for SuperScript III Reverse Transcriptase (ThermoFisher, <https://www.thermofisher.com/order/catalog/product/18080044>). We used SYBR Green-based real-time PCR for expression analysis (Roche, <https://shop.roche.com/shop/products/lightcycler14301-480-sybr-green-i-master> and the Light-Cycler 480 Instrument; <https://shop.roche.com/shop/products/lightcycler14301-480-instrument-ii>). 2.5 ng of cDNA was used per reaction. Primers used for qRT-PCR include: CTGCTGGA-CAGGAAGATTACG and CTCGGACATTCTCGAATGAAG for *cdc-42*; GTTCCCGTGTTCATCACTCAT and ACACCGT CGAGAAGCTGTAGA for *pmp-3*; GTCGCTTCAAATCAGT TCAGC and GTTCTTGTC AAGTGATCCGACA for *Y45F10 D.4*;<sup>61</sup> GACAAGTCCTCCCTTTCCATC and GACAAGTCCT CCCTTTCCATC for *acn-1*; GCAGTTCACAAATTTTCATC CGG and CTCGCCATCTTTCTCCATTGC for *hbl-1*; and CG GCTCCGTATTTCGTCTCT and GATCGTCCCACTGATTG GC for *lin-41*.

### Fit of the *acn-1* oscillatory pattern to a sinusoidal curve

Total RNA was collected every 2 hrs starting at  $t = 12$  hrs and ending at  $t = 22$  hrs post-hatching, and *acn-1* expression levels at each time point were measured using quantitative RT-PCR.

ddCt values were set to a sinusoidal function of time of the form  $y = A \sin(B \cdot t + C) + D$  in MatLab to determine the accuracy of the fit. Next, confidence intervals for each parameter were obtained using Bayesian statistical inference. A random walk was performed using the “Just Another Gibbs Sampling” (JAGS) package in R to obtain 100,000 sets of parameter values, from which statistical values could be inferred. All parameters were assumed to have uniform prior distributions, and the distribution of real data values about the fit was assumed to be normal.

### Abbreviations

<i>acn-1</i>	angiotensin-converting enzyme-like, non-peptidase-1
ACE	angiotensin-converting enzyme
APP	amyloid precursor protein
miRNA	microRNA

### Disclosure of potential conflicts of interest

The authors have no conflict of interest.

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