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Upregulation of the *let-7* microRNA with precocious development in *lin-12/Notch* hypermorphic *C. elegans* mutants

Aharon Solomon[a], **Yousaf Mian**[a], **Cesar Ortega-Cava**[a], **Victor Liu**[a,c,#], **Channabasavaiah Basavaraju Gurumurthy**[b], **Mayumi Naramura**[a], **Vimla Band**[b,c], and **Hamid Band**[b,c,*]

a *Divisions of Molecular Oncology, Weinberg College of Arts and Sciences, Northwestern University, Evanston IL, 60201, USA*

b *Division of Cancer Biology, Evanston Northwestern Healthcare Research Institute and Department of Medicine, Feinberg School of Medicine, Weinberg College of Arts and Sciences, Northwestern University, Evanston IL, 60201, USA*

c *Department of Biochemistry, Molecular Biology and Cell Biology, Weinberg College of Arts and Sciences, Northwestern University, Evanston IL, 60201, USA*

Abstract

The *lin-12*/Notch signaling pathway is conserved from worms to humans and is a master regulator of metazoan development. Here, we demonstrate that *lin-12*/Notch gain-of-function (*gf*) animals display precocious alae at the L4 larval stage with a significant increase in *let-7* expression levels. Furthermore, *lin-12*(*gf*) animals display a precocious and higher level of *let-7 gfp* transgene expression in seam cells at L3 stage. Interestingly, *lin-12*(*gf*) mutant rescued the lethal phenotype of *let-7* mutants similar to other known heterochronic mutants. We propose that *lin-12*/Notch signaling pathway functions in late developmental timing, upstream of or in parallel to the *let-7* heterochronic pathway. Importantly, the human microRNA *let-7a* was also up-regulated in various human cell lines in response to Notch1 activation, suggesting an evolutionarily-conserved cross-talk between *let-7* and the canonical *lin-12*/Notch signaling pathway.

Keywords

Heterochronic genes; developmental timing; *lin-12*/Notch; *let-7*; *lin-41*; microRNA molting; cell fate; seam cells

Introduction

MicroRNAs are single strand non-coding RNAs of ~22 nucleotides that function as regulators of gene expression and are found in viruses, fungi, plants and animals [1–3]. In animals,

*Corresponding author: E-mail: hband@unmc.edu, Tel: 402-559-8572, Fax: 402-559-8270, University of Nebraska Medical Center, Eppley Science Hall 9027, 986805 Nebraska Medical Center, Omaha, NE 68198-6805.
#Current Addresses: A. Solomon: Chicago Biomedical Consortium (CBC), Northwestern University, Hogan 2-100, 2205 Tech Drive, Evanston, Il 60208
Y. Mian, B. Gurumurthy and V. Band: Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE 68198-5805
C. Ortega-Cava, M. Naramura and H. Band: Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-6805
V Liu: Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts.

microRNAs bind to complementary sequences within the 3′ untranslated regions (UTRs) of their target mRNAs leading to translational repression and mRNA destabilization [4]. Importantly, these new class of regulators have been implicated in stem cell differentiation, organ development and oncogenesis [5]. The molecular mechanisms regulating microRNA expression and function are therefore of great interest in understanding both normal and abnormal biological pathways.

The evolutionarily-conserved microRNA genes *lin-4* and *let-7* play key roles in the temporal control of larval developmental in *C. elegans* [6]. *lin-4* functions in early (L1 to L2) larval developmental decisions, while *let-7* functions as a core regulator of the late (L4-to-Adult) larval developmental pathway [7,8]. In wild-type animals, *let-7* is temporarily expressed in seam cells (hypodermal skin cells) at the L4 larval stage (L3 molt) and negatively regulates *lin-41*, which encodes a RING-finger B-box Coiled-coil-containing protein [9,10]. The down-regulation of the LIN-41 protein levels releases the repression on the adult-specific transcription factor LIN-29. Upon LIN-29 activation, seam cells exit the cell cycle, fuse and secrete a cuticular structure known as alae [11]. Furthermore, LIN-29 promotes the synthesis of adult cuticle and cessation of molting [12,13]. Loss-of-function mutations in *lin-41* result in a precocious alae phenotype. In this situation, seam cells prematurely adopt the adult fate and secrete alae during the L3 molt. Conversely, mutations in *let-7* or *lin-29* [9,13] cause a retarded phenotype where seam cells continue to proliferate and fail to adopt the adult cell fate at the adult stage (L4 molt). Although the role of *let-7* has been well established in *C. elegans*, its regulation in both *C. elegans* and mammals is poorly understood.

The canonical *lin-12*/Notch signaling pathway controls a broad spectrum of cell fate decisions and developmental processes in diverse metazoans [14]. In *C. elegans*, LIN-12 is involved in cell fate specification of the vulval precursor cells, sex myoblasts and *II* cells during the hermaphrodite gonad development [15]. The LIN-12/Notch receptor is activated upon binding to the DSL (Delta/Seratte/Lag-2) family of ligands on the surface of adjacent signal-sending cells. Ligand binding leads to cleavage of the extra-cellular domain of the receptor by the ADAM family proteases generating the membrane tethered intracellular domain (ICN). The ICN is subsequently cleaved from the membrane by the γ-secretase complex and translocated to the nucleus to interact with CSL (CBF1, Human/Su(H) Drosophila/Lag-1 *C. elegans*), the major downstream effector of Notch signaling, to regulate transcription of specific target genes [14]. In this report, we demonstrate that *lin-12*/Notch gain-of-function (*gf*) animals display precocious alae at the L4 larval stage with a significant increase in *let-7* expression levels. Our genetic and molecular data suggests that *lin-12*/Notch can positively regulate the *let-7* in the heterochronic pathway *in C. elegans.* Furthermore, we provide evidence that activated human Notch1 also increases the levels of *let-7a* microRNA in human cell lines, suggesting an evolutionarily conserved cross-talk between *let-7* and Notch receptors in metazoans.

Materials and Methods

C. elegans culture and strains

C. elegans strains were maintained on NGM plates with OP50 as described [30]. The following strains were used in this study: N2 (wild-type), *lin-12*(*n137gf*), *lin-12*(*n137n460gf*), *lin-12* (*n472gf*), *lin-12*(*n302gf*), *lin-12*(*n676n930lf*), *lin-12*(*n676gf*), *let-7*(*n2853lf*), *let-7*(*mn112*); *unc-3*(*e151*), *lin-41*(*ma104lf*), *lin-29*(*n333lf*), *lag-1(om13lf), wls78* and *ZaEx5.* The double mutants: *let-7*(*n2853lf); lin-12*(*n137n460gf*), *let-7*(*mn112null*); *lin-12*(*n137n460gf*), *lin-41* (*ma104lf*); *lin-12*(*n137n460gf*), *lin-29*(*n333lf*); *lin-12*(*n137n460gf*) and *lin-12(427gf); lag-1 (om13lf).* Details on double mutant crosses are upon request.

Phenotype analysis

The precocious and retarded alae phenotypes were observed in L4 stages using Nomarski optics. The different developmental stages were determined by stage-specific gonad morphology [11] and by specific stage developmental markers: *col-19*, *col-17* and *col-3* [31]. To visualize seam cell junctions and nuclei, we used an integrated array (*wIs*78) containing both *ajm-1::gfp* and *scm-1::gfp*.

Northern analysis

Total RNA was extracted from synchronized cultures of worms or sub-confluent cultures of human cell lines using the Trizol reagent (Invitrogen). The small RNAs were resolved on pre-cast 15% Urea-TBE gels (Bio-Rad) for 1h and transferred to Zeta-probe blotting membrane (Bio-Rad) overnight using a Bio-Rad Criterion blotter. After transfer, the membranes were air-dried, UV cross-linked (1200J) and baked for 1h at 80°C. Blots were pre-hybridized in 50 ml hybridization solution (5X SCC, 20mM NaH_2PO_4, 7% SDS, 1X Denhardt's Solution) for 1 hour and subsequently probed overnight with $\propto$-^{32}P labeled *let-7* probe (enhanced with LNA, Exiqon) with a probe concentration of 10^6 cpm/ml. The *C. elegans let-7* and human *let-7a* detection utilized the same probe sequence 5′-aactatacaacctactacctca-3′ and for loading control we used the U6 probe 5′ cacgaatttgcgtgtcatcctt-3′. Prior to film exposure, the blots were washed twice for 30 min each in 6X SCC, 20X Denhardt's Solution, 10% SDS, 50 mM NaH_2PO_4 and twice for 10 minutes each in 1X SCC, 1% SDS.

qRT-PCR and RT-PCR

The quantitative real-time PCR (qRT-PCR) experiments were performed by the TaqMan method and the Stratagene MX3000P QPCR detection system. The fold-change in *lin-41* mRNA levels was calculated using the ΔΔCt method and normalized to *eft-2* loading control as described [4]. Standard RT-PCR experiments were performed using the MMLV Reverse Transcriptase kit (Stratagene) followed by PCR with primers that span the 3′-UTR of the target gene as described [4].

Cell Culture

The human embryonic kidney cell Hek293T and the mammary epithelial cell (MEC) lines 16A5 and 76NTert were used in our study. 16A5 and 76NTert cell lines are HPV-E6/E7 and hTert-immortalized, respectively, derivatives of 76N normal MEC line established from a reduction mammoplasty [32]. Mammary cells were cultured in the DFCI-1 medium supplemented with 12.5 ng/ml EGF and 293T cells were cultured in Dulbecco's Modified Essential Medium supplemented with 5 % fetal bovine serum.

Ectopic Notch expression

Intracellular region of human Notch-1 (ICN1) cloned in a retroviral vector (MIGR1) was kindly provided by Dr. Warren Pear (University of Pennsylvania). Retroviral supernatants were generated by transfecting 10ug of the MIGR1-ICN1 or control vector along with the pIK packaging vector into tsa54 packaging cells (CellGenesys) [33] using the calcium phosphate method. Viral supernatants were collected, cleared through a 45um cellulose filter and freshly applied to proliferating target cells during two serial incubations as previously described [34]. Aliquots of cells were processed for RNA extraction (see Northern blotting) or for protein extraction and Western blot.

Western blot analysis

Cells were lysed in 3% SDS, 50mmol/L Tris-HCl, pH 7.4. Aliquots of 100 ug protein were measured using the bicinchonic acid protein assay kit (Pierce), separated on SDS-PAGE gels,

transferred to a PVDF membrane (PerkinElmer) and blotted with a rabbit anti-human Notch 1 cleaved N terminal polyclonal antibody (100-401-407; Rockland) or anti-beta-actin mouse monoclonal antibody (Sigma- Aldrich) as loading control. Blots were incubated with the primary antibody for 1h at room temperature and washed 3 times in Tween20-TBS buffer. Appropriate HRP-labeled secondary antibodies were applied for 1h and washed again in Tween20-TBS buffer. Signals were developed using the Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer). The specificity of anti-Notch antibody was verified by competition with the specific control peptide (Rockland, data not shown).

Results and discussion

lin-12/Notch genetically interacts with the heterochronic gene lin-41

During an RNAi screen for potential regulators of *lin-12*/Notch in *C. elegans* (Liu, Solomon & Band, unpublished), we observed a molting defect (Mlt) phenotype associated with increased *lin-12*/Notch activity (Fig 1A). We found that the gain-of-function (*gf*) temperature sensitive *lin-12*(*n137n460gf*) animals display an intriguing Mlt phenotype at all developmental stages at 15°C (total 18%, n=248) (Fig 1A). Temperature shift experiments with *lin-12* (*n137n460gf*) animals confirmed that Mlt is associated with increased *lin-12/*Notch activity same as the multi-vulva (Muv) phenotype described by Greenwald and colleagues [16]. A significant suppression of both Mlt and Muv phenotypes was observed in these worms when shifted to 25°C: ~2% Mlt, (n=675) and 6.3% Muv (n=64) vs. 18% Mlt and 100% Muv at 15° C (n>100) ($P<0.05$). We could not detect any Mlt defects in a loss-of-function and null mutant strain of *lin-12* (Table 1 and data not shown).

In the RNAi screen (Liu, Solomon & Band, unpublished), we identified the heterochronic gene *lin-41*, which encodes a RING-finger, B-box and Coiled-Coil (RBCC) protein. We found that reduction of *lin-41* mRNA levels in *lin-12*(*n137n460gf*) animals by RNAi feeding enhanced the Mlt phenotype (from 18% to 44% Mlt at 15°C, n=760), without affecting the Muv phenotype (100% Muv). Notably, the Mlt defect occurred predominantly at the L4 to adult transition and most of the Mlt animals were trapped in their old cuticle. A similar but weaker defect was observed in *lin-41*(*ma104*) (13% Mlt, n=54) (Fig 1B) and in wild-type animals fed with *lin-41* RNAi construct (5% Mlt, n>100). The enhanced Mlt phenotype observed in *lin-12* (*n137n460gf*); *lin-41*(RNAi) animals suggested that *lin-12* and *lin-41* may cooperate to regulate molting.

Activated lin-12/Notch mutants display heterochronic defects

Molting defects were previously reported in retarded and precocious heterochronic pathway mutants [11], and recently *let-7* was shown to be involved in the regulation of molting [17]. Therefore, based on the genetic interaction observed between *lin-12* and *lin-41*, and the fact that *lin-41* is a target of *let-7*, we hypothesized that Mlt and heterochronic defects may be linked. To determine whether *lin-12*/Notch *gf* animals display heterochronic defects, we analyzed several *lin-12*/Notch (*gf*) mutants and found that they indeed displayed a phenotype of precocious alae and fused seam cells at the L3 molt phase (Fig 1 and Table 1). To verify the identity of the different developmental stages analyzed, we performed RT-PCR for developmental stage-specific markers and examined the gonad morphology, and found that they indeed correspond to the appropriate developmental stages (Fig 1K and data not shown). We also analyzed a partial loss-of-function strain and null mutant strain of *lin-12* and could not detect any retarded developmental phenotypes in these animals (Table 1 and data not shown). To address the possibility that our negative results with loss-of-function and null animals reflect redundant roles of the two Notch receptors, *lin-12* and *glp-1*, we depleted the *glp-1* mRNA from *lin-12*(*null*) and *lin-12*(*lf*) animals. Unfortunately, we could not analyze the F1 progeny because of larval arrest and/or embryonic lethality. Alternatively, our results could

be explained by a redundant role of *lin-12* in development timing in *C. elegans*. However, it is possible that the observed effects represent an aberrant effect of gain of function *lin-12* alleles and that normal *lin-12*does not play a physiological role in developmental timing.

Next, we examined if *lin-12* and *let-7* pathways interact genetically. For this analysis, we used the *lin-12*(*gf*) strain *lin-12*(*n137n460gf*) since it showed the most penetrant Mlt and heterochronic defects (Table 1). Our results demonstrate that the precocious alae in the L4 stage observed in *lin-12*(*gf*) animals were fully suppressed in null and loss-of-function mutant backgrounds of *let-7* and *lin-29*, respectively (Table 1). In addition, the precocious alae phenotype in double mutant *lin-12*(*n137n460gf*); *lin-41*(*ma104*) was comparable to that in the single mutant *lin-12*(*n137n460gf*) (Table 1). Our result indicates that *lin-41* may act downstream of *lin-12* in regulating alae formation. Taken together, we propose a genetic model wherein *lin-12* functions upstream of, or in parallel to, the *let-7* late larval development pathway in *C. elegans* (Fig. 5). Furthermore, our data suggest that *lin-12*/Notch might regulate *let-7* expression in *C. elegans*

lin-12/Notch positively regulates the expression of let-7

To determine whether *lin-12*/Notch regulates *let-7* expression, we generated the *lin-12* (*n137n460gf); zaEx5* strain carrying a reporter construct for *let-7* expression (*plet-7*::GFP) and analyzed GFP expression levels at different developmental stages. This strain exhibited precocious GFP expression in seam cells as well as enhanced GFP expression in the intestine at the L3 stage (90%, n=67) (Fig. 2). On the other hand, wild-type animals never showed GFP expression in seam cells before the L3 to L4 transition, as reported previously [18]. A brighter GFP signal was also detected in *lin-12*(*n137n460gf); zaEx5* L4 and adult animals in hypodermal and intestinal cells (80–90%, n>100, and data not shown). In addition to the *in vivo* observations, Northern blot analysis demonstrated a 2-fold increase in mature *let-7* microRNA levels in *lin-12*(*gf*) compared to wild-type worms (Fig 2E). Because of the negative regulatory role of *let-7* on *lin-41* mRNA levels [4], we expected lower levels of *lin-41* mRNA in *lin-12*(*gf*) worms. Indeed we found a substantial reduction in *lin-41* mRNA levels in L2, L4 and young adults stages in *lin-lin-12(gf)* worms, and this phenotype was reversed in the *let-7* null background (Fig 2G). The lower *lin-41* mRNA level in *lin-12*(*gf*) animals is expected to lead to a higher level of LIN-29 activity to specify premature larva to adult transition. Taken together, our results provide evidence that *lin-12*/Notch may negatively regulates *lin-41* via the up-regulation of the *let-7* microRNA.

To determine if *let-7* regulation by *lin-12*/Notch is cell-autonomous, we analyzed LIN-12 expression in seam cell using a strain carrying a functional *lin-12*::LacZ fusion construct [19]. However, we were unable to detect LacZ staining in seam cells although we observed the typical staining of vulva precursor cells and sheath cells around the tip of the gonad (data not shown). Our inability to detect the expression of LIN-12 in seam cells is likely to be due to either a very low level of expression and/or short temporal expression of LIN-12 in these cells, as is the case in certain other tissues types and cells known to require LIN-12 activity such as rectum, anal depressor muscle cells, rectal sphincter cells and some L1 intestinal cells [20].

Heterochronic phenotypes in lin-12/Notch gain-of-function mutants are lag-1 dependent

To determine if the canonical *lin-12* signaling pathway is involved in late larval developmental timing in *C. elegans*, we tested whether the precocious alae in *lin-12*(*gf*) worms are dependent on *lag-1*. LAG-1 is a conserved transcription cofactor that serves as a major effector of *lin-12* signaling [23]. We generated the *lin-12*(*n427gf*);*lag-1*(*om13*) and *lin-12* (*n137n460gf*);*lag-1*(*om13*) double mutants and found that both show a very slow growth rate with embryonic and larval arrest. Since the *n137n460* allele showed a higher penetrance of

embryonic lethality, we analyzed the *lin-12*(*n427gf*);*lag-1*(*om13*) mutant and found a complete suppression of the Muv and the precocious alae phenotypes but not of the Egl phenotype (Table 1). These results suggest that *lin-12*/Notch act in a canonical *lag-1* dependent manner to control alae formation in *C. elegans*.

The lethality phenotype of let-7 mutants is suppressed by lin-12/Notch gf mutant

The *let-7* microRNA is essential in *C. elegans* and the *let-7*(*mn112)* null homozygous animals die by a characteristic bursting of the vulva [8]. A similar phenotype was also described for the temperature sensitive *let-7*(*n2853)* mutant [8]. Previous studies have shown that the lethality of *let-7* mutants can be rescued by crossing with specific *let-7* target gene mutants, such as *lin-41, hbl-1, let-60/Ras* and *daf-12* [8,10]. To our surprise, we found that *lin-12* (*n137n460gf*) mutant rescued the lethality phenotypes of *let-7*(*n2853*) and *let-7*(*mn112*) mutants (Fig 3 and Table 2). The previous studies [8,10] suggest that dysregulated expression of *let-7* target genes contributes to the lethal and sterile phenotypes observed in *let-7* mutants. Rescue of lethality in *let-7* mutants by activated *lin-12* suggests the possibility that *lin-12* may also regulate *let-7* targets independent of *let-7* gene product (Table 2). This hypothesis can explain the rescue of the *let-7* mutants and it is currently under investigation.

Activated Notch1 regulates the let-7a microRNA in human cells

The fact that *lin-12*/Notch regulates seam cell fate via a conserved transcription cofactor (*lag-1*) and a phylogentically conserved microRNA (*let-7*), raises the possibility that a crosstalk between Notch receptors and the *let-7* microRNA might be conserved in higher metazoans. To test if *let-7* regulation by Notch receptor is conserved in humans, we introduced the intracellular domain (ICN) of mammalian Notch1 into human cell lines and analyzed the effect on human *let-7a* expression. Over-expression of Notch1 ICN resulted in a ~2 fold increase in *let-7a* RNA levels in the human embryonic kidney cell line 293T, and the immortalized human mammary epithelial cell lines 16A5 and 76NTert (Fig 4). These results suggest a conserved regulation of *let-7* by *lin-12*/Notch receptors across evolution. Notch signaling regulates important cell fate decisions in mammals, including stem cell maintenance, cell differentiation and proliferation, and dysregulation of Notch pathway is strongly associated with cancer. For example, activating mutations of Notch1 are found in more than 50 % of human T cell acute lymphoblastic leukemia [24], and activated ICN is found at high levels in breast cancer cell lines and tissues [25]. On the other hand, in an animal model, Nicolas *et al.* [26] demonstrated that Notch1 functions as a tumor suppressor in mammalian skin cells. Specifically, Notch1 deficiency in mouse epidermis and primary keratinocytes resulted in basal-cell carcinomas. It was also suggested that *let-7* may function as a tumor suppressor in human lung cancer by down-regulating the expression of Ras [27]. In addition, *let-7* was shown to inhibit cell growth of human lung adenocarcinoma and colon cancer cell lines [28,29]. Thus, it is conceivable that the novel Notch-*let-7* axis defined here could play an important role in cell fate determination in higher organisms.

Conclusions

The role of the canonical *lin-12*/Notch signaling pathway in the regulation of cell fates, cell proliferation and cell death during development has been well established. Our study revealed that activated *lin-12*/Notch alleles can affect late larval development timing in *C. elegans* by upregulating the *let-7* microRNA expression. We were not able to answer the question whether *lin-12*/Notch has a normal role in developmental timing because of lethality issues, and potentially redundant roles of the two Notch receptors (*lin-12* and *glp-1*). However, the observations that activated *lin-12* mutants display precocious alae at L4 larval stage with a precocious and elevated expression of *let-7*, and the ability to rescue the lethal phenotype of *let-7* mutations with *lin-12 gf* mutants collectively suggest that *lin-12*/Notch has a redundant

role in developmental timing in *C. elegans.* Our finding that activated human Notch1 can regulate *let-7a* expression in human cell lines suggests an evolutionarily-conserved cross-talk between *let-7* and the canonical *lin-12*/Notch signaling pathway. Future studies aimed at understanding the molecular mechanisms by which *lin-12*/Notch signaling regulates *let-7* could provide mechanistic insights relevant to developmental biology and may help understand the mechanisms of cellular transformation and tumor progression in higher organisms.

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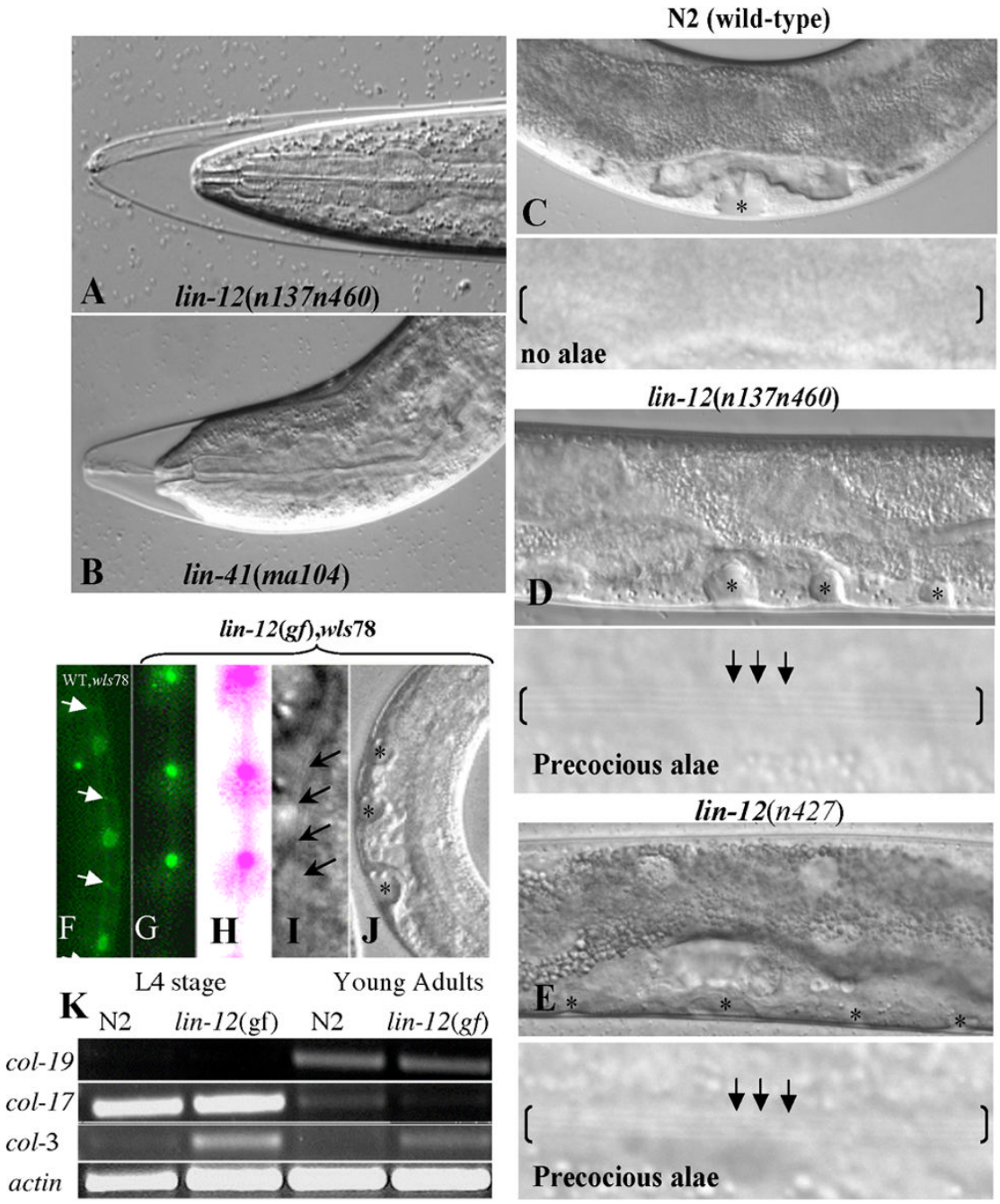

Fig 1.

Activated *lin-12*/Notch mutants display precocious alae and seam cell phenotypes. A and B. The *lin-12*(*n137n460gf*) gain-of-function (A) and *lin-41*(*ma104lf*) loss-of-function (B) animals displaying molt (Mlt) phenotype at the transition from L4 to adult stage. C-E. A wild-type animal (N2 strain) (C) at the mid-late L4 stage with no alae (i.e., normal development) in contrast to *lin-12*(*n137n460gf*) (D) or *lin-12*(*n427gf*) mutants (E) at the L4 stage with vulva invagination (black asterisks) and precocious alae (black arrows and parenthesis). F. An N2, *wIs*78 animal expressing AJM-1::GFP (adherens junction marker; white arrows) and SCM::GFP (in seam cell nuclei) depicting un-fused seam cells at the L4 stage. G. A *lin-12* (*n137n460gf*), *wIs78* animal expressing AJM-1::GFP and SCM::GFP demonstrating a fused

seam cell phenotype at mid L4 stage with no obvious cell-cell junctions as indicated by the lack of junctional GFP signal. The fused seam cells visualized with GFP are shown in green (G) or after pseudo-coloring (pink) (H). I-J. *lin-12*(*n137n460gf*) L4 stage larvae displaying multiple invaginations of the forming multi-vulva and precocious alae (arrows). K. RT-PCR of developmental stage-specific collagen transcripts at L4 vs. adult stage in N2 (wild-type) and *lin-12*(*n137n460gf*) worms. The adult-specific *col-19* marker [31] is highly expressed at the adult stage in both N2 and *lin-12*(*gf*) worms but is nearly undetectable at L4 stage. In contrast, L4-specific *col-17* marker shows a reciprocal expression pattern. *col-3* is expressed at all developmental stages but is higher at L4 and lower in adults [31]. All experiments were done at 15°C.

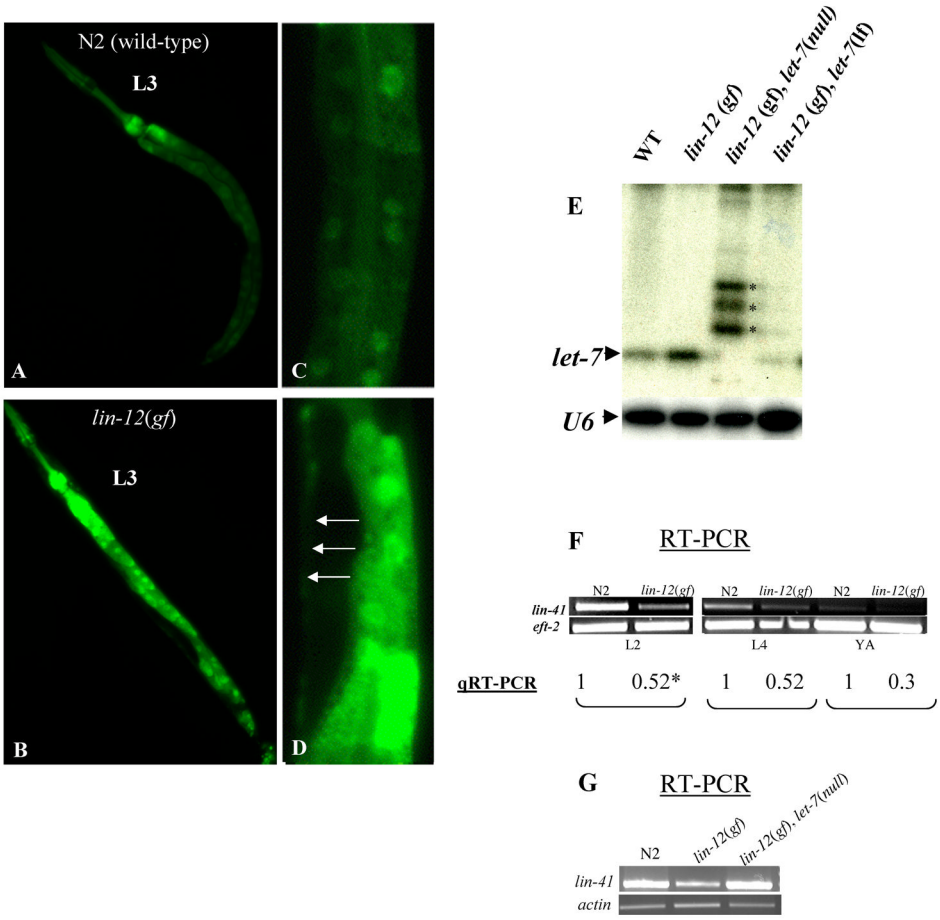

Fig. 2.

Up-regulation of *let-7* microRNA expression in *lin-12*/Notch gain-of-function mutant worms. A–D. N2, *plet-7*::GFP reporter animals at L3 stage display weak GFP expression in intestinal cells with undetectable GFP in seam cells (A and C). In contrast, *lin-12*(*n137n460gf*), *plet-7*::GFP worms display brighter GFP signals in intestinal cells and precocious GFP signals in seam cells (B, D) (white arrows). C-D. C and D are higher magnification of mid-body section of worms depicted in A and C respectively. E. Elevated *let-7* microRNA expression in *lin-12*/Notch *gf* worms. Total RNA was isolated form young adult animals of the N2 strain, or *lin-12*(*n137n460gf*), the doubles *lin-12*(*n137n460lf*);*let-7*(*mn112null*) and *lin-12* (*n137n460gf*);*let-7*(*n2853lf*) mutants, and subjected to Northern blot analysis for *let-7* microRNA and U6 (loading control). Note that *let-7* expression is 2–3-fold higher in *lin-12 (gf)* single mutants compared to wild-type animals but weak or undetectable in *let-7*(*n2853*) and *let-7*(*mn112*) null mutant background, respectively. The identity of the bands above the mature *let-7* microRNA (see asterisks) present in lanes 3 and 4 is unknown. F. RT-PCR (upper line) and real-time PCR (qRT-PCR) (lower line) quantification of the relative levels of *lin-41* mRNA in *lin-12*(*gf*) worms at L2, L4 and young adults (YA) stages in comparison to wild-type animals. The average fold-change in mRNA levels (see experimental procedure for qRT-PCR analyses) in each developmental stage is marked with an asterisk. G. RT-PCR analysis to demonstrate that the observed reduction of *lin-41* mRNA levels in the *lin-12* (*n137n460gf*) animals is reversed in a *let-7* null mutant background.

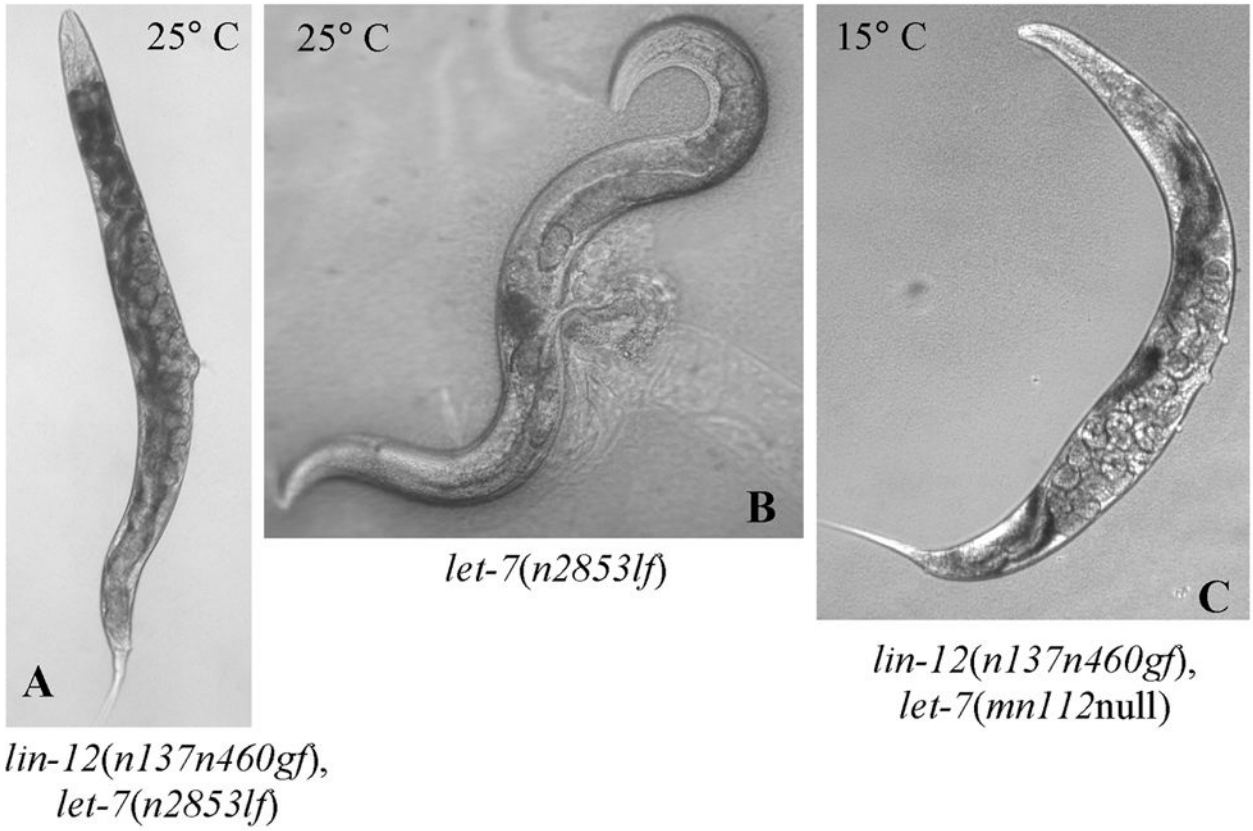

Fig. 3.

lin-12(*n137n460gf*) mutant suppresses the lethality and sterility phenotypes of *let-7* mutations. A–B. *lin-12*(*n137n460gf*); *let-7*(*n2853lf*) Egl double mutant (A) with fertilized eggs and progeny in contrast to *let-7*(*n2853lf*) animals which die as a result of characteristic bursting of the vulva at 25°C (B). C. A viable *lin-12*(*n137n460gf*), *let-7*(*nmn112*) null animal showing an Egl phenotype with progeny developing within the parental worm at 15°C, and also at 25°C (see table 2).

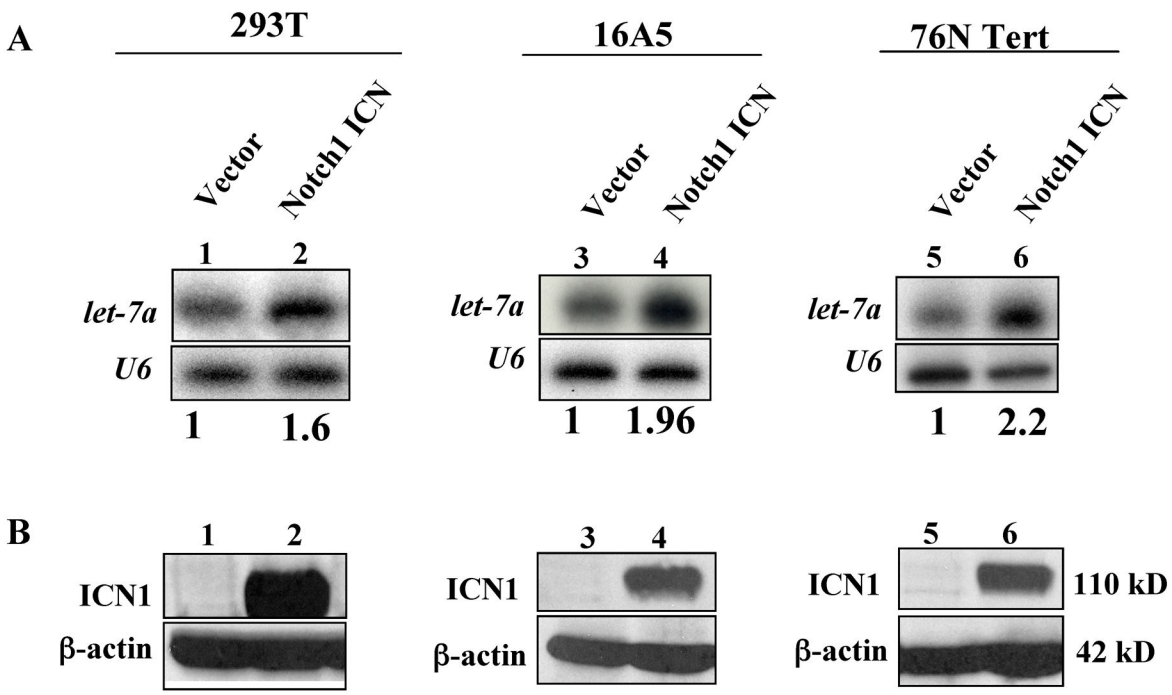

Fig 4.

Overexpression of activated Notch1 up-regulates the *let-7a* microRNA expression in human epithelial cell lines. A. The indicated cell lines were retrovirally transduced with intracellular domain of Notch1 (Notch1 ICN) or control vector and total RNA isolated from subconfluent cell monolayers followed by Northern analyses of the *let-7a* mature microRNA expression. The autoradiograms were scanned and the intensity of bands quantified using the LabWork™ imaging analysis software (UVP). The relative *let-7a* expression in ICN-over-expressing cells relative to their vector controls are shown below the lanes. A comparable RNA loading between control and ICN lanes is shown by U6. The results are representative of two independent experiments. B. Western blot analysis of Notch1 ICN and β-actin (loading control) proteins in aliquots of the cell samples used for Northern blotting in A.

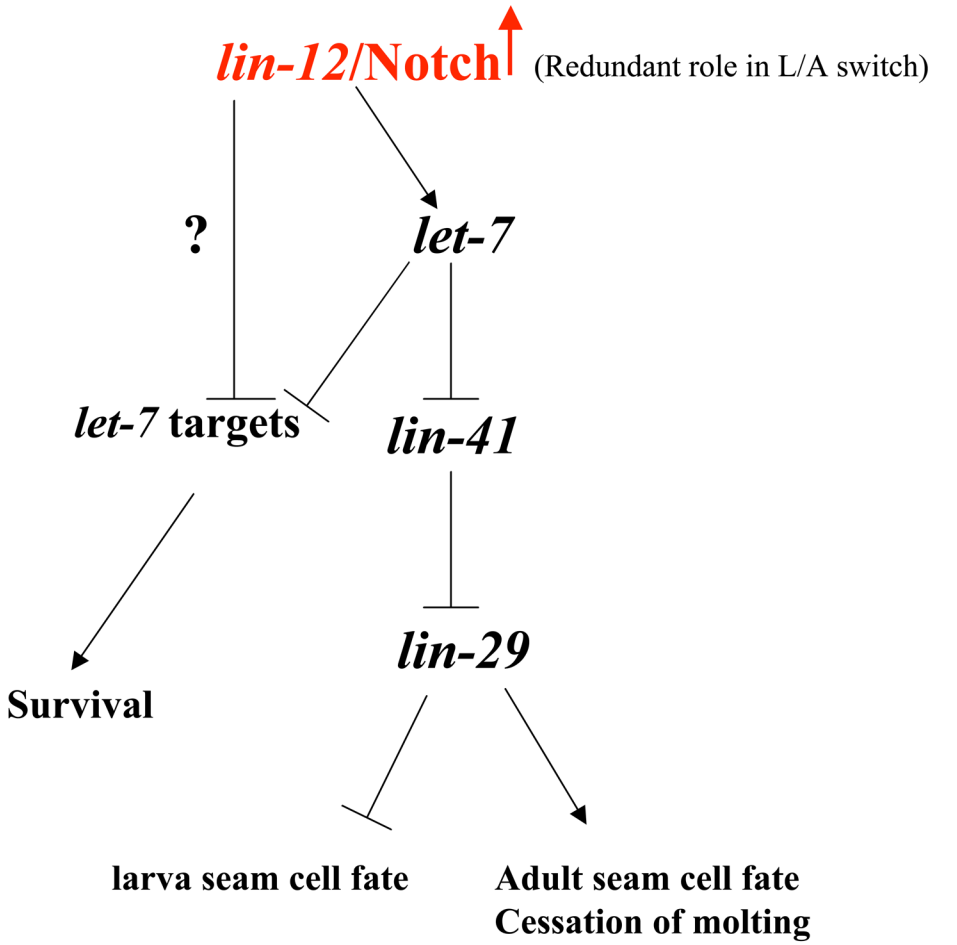

Fig 5.

The proposed genetic model for *lin-12*/Notch in regulating larva-to-adult switch (L/A) in *C. elegans*. We propose that *lin-12*/Notch positively regulates the *let-7* microRNA in a non-cell autonomous manner in the L4 stage to execute the L/A switch. Temporal up-regulation of *let-7* will ensure the proper down-regulation of *lin-41* mRNA and activation of the transcription factor *lin-29*. Upon LIN-29 activation, seam cells will exit the cell cycle and will differentiate into adult seam cells. In this final molt phase (L4), an adult cuticle and alae are formed, and molting cycle ceases. In addition, we propose that rescue of lethality in *let-7* mutants by activated *lin-12*/Notch suggests the possibility that *lin-12*/Notch may also regulate other *let-7* targets independent of the *let-7* microRNA.

Table 1

Precocious phenotype of activated *lin-12* gain-of-function (*gf*) mutant worms and interactions with the *let-7* heterochronic pathway components.

	% of animals with adult lateral alae			
Genotype	**L4 larva (L3 molt)**	**Adult (L4 molt)**	**Alae phenotype**	**Other phenotypes**
N2 (wild-type)	0% (48)*	100 % (56)	Normal	-
lin-12(*n137gf*)	35% (60)	100% (30)	Precocious	Muv, Egl[ab]
lin-12(*n137n460gf*)	54% (106)	100% (37)	Precocious	Mlt[c], Muv, Egl Dpy[d]
lin-12(*n427gf*)	51 % (80)	100% (29)	Precocious	Muv, Egl
Lin-12(427gf), lag-1 (om13lf)	0% (17)	100% (20)	Normal	Egl, Emb, Lva[ef]
lin-12(*n676gf*)	30% (40)	100 % (33)	Precocious	Egl
lin-12(*n302gf*)	29% (31)	100% (10)	Precocious	Egl
lin-12(*n676n930lf*)	0% (30)	100% (10)	Normal	Egl, Vul[g]
let-7(*n2853lf*)	0% (20)	0% (20)	Retarded	Mlt, Egl
let-7(*mn112null*); *unc-3* (*e151lf*)	0% (20)	0% (20)	Retarded	Mlt, Let
lin-41(*ma104lf*)	46% (57)	100% (25)	Precocious	Mlt, Egl, Dpy
lin-29(*n333lf*)	0% (15)	0% (31)	Retarded	Mlt, Egl,
let-7(*n2853lf*); *lin-12* (*n137n460gf*)	0% (20)	0% (20)	Retarded	Mlt, Muv, Egl
let-7(*mn112null*); *lin-12* (*n137n460gf*)	0% (16)	0% (25)	Retarded	Mlt, Muv, Egl
lin-41(*ma104lf*); *lin-12* (*n137n460gf*)	50% (32)	100% (16)	Precocious	Mlt, Muv, Egl
lin-29(*n333lf*); *lin-12* (*n137n460gf*)	0% (30)	0% (25)	Retarded	Mlt, Muv, Egl

*Number animals scored.

[a]Multi-vulva,

[b]Egg-laying defect,

[c]Molting defect,

[d]Dumpy,

[e]Embryonic lethal,

[f]Larval arrest,

[g]Vulvaless.

Table 2

Activated *lin-12*/Notch mutant suppresses the lethality and sterility phenotypes of *let-7* mutations

Genotype	No. of progeny [a]	Fertile worms[b]
N2 (wild-type)	280±12	100 %
lin-12(*n137n460gf*)	21±2	100 %
let-7(n2853lf)	0	2–5 %
lin-12(*n137n460gf*), *let-7(n2853lf)*	23±3	100 %
let-7(nm112null), unc-3(e151)	0	2–5 %
lin-12(*n137n460gf*), *let-7(nm112), unc-3(e151)*	32±3	100 %
lin-41(ma104lf)	30[c]	100 %[c]
lin-41(ma104lf), let-7(nm112), unc-3(e151)	19[c]	95 %[c]

[a] Average number of live progeny from ten adults worms at 25°C.

[b] Percent of worms (n >100) with fertile eggs scored at 25°C.

[c] From Slack *et al.* Molecular cell, 5 659–669, 2000.