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Caenorhabditis elegans *aristaless*/Arx gene *alr-1* restricts variable gene expression

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Variable expressivity of mutant phenotypes in genetically identical individuals is a phenomenon widely reported but poorly understood. For example, mutations in the gene encoding the transcription factor ALR-1 in *Caenorhabditis elegans* result in variable touch receptor neuron (TRN) function. Using single-molecule in situ hybridization, we demonstrate that this phenotypic variability reflects enhanced variability in the expression of the selector gene *mec-3*, which is needed, together with *unc-86*, for the differentiation of the TRNs. In a yeast expression system, ALR-1 enhances MEC-3/UNC-86-dependent transcription from the *mec-3* promoter, showing that ALR-1 can enhance bulk *mec-3* expression. We show that, due to stochastic fluctuations, autoregulation of *mec-3* is not sufficient for TRN differentiation; ALR-1 provides a second positive feedback loop that increases *mec-3* expression, by restricting variability, and thus ensures TRN differentiation. Our results link fluctuations in gene expression to phenotypic variability, which is seen in many mutant strains, and provide an explicit demonstration of how variable gene expression can be curtailed in developing cells to ensure their differentiation. Because ALR-1 and similar proteins (*Drosophila* *Aristaless* and human *ARX*) are needed for the expression of other transcription factors, we propose that proteins in this family may act to ensure differentiation more generally.

Aristaless family | LIM homeodomain proteins | stochastic gene expression | terminal differentiation

Phenotypic variability in genetically identical mutant organisms is widely reported (1–3) but poorly understood. Gene expression studies in unicellular organisms have shown that stochastic events can lead to molecular and phenotypic variability (4). In multicellular organisms, where cells should acquire particular cell fates, such noise in gene expression could have dramatic developmental consequences, implying that tight regulatory control is needed for robust development. Little is currently known about the mechanisms by which this noise is prevented in differentiating cells.

In general, cells are thought to acquire specific fates during development because regulatory transcription factors (encoded by selector or terminal selector genes) activate the expression of cell-type-specific realizer or effector genes that define cell identity (5, 6). Hobert (7) and Colman and Dreesen (8) have proposed that the stability and maintenance of a newly acquired state is mediated by autoregulatory loops whereby selector genes initiate and maintain the cell-specific gene program by autoregulating their own expression throughout the life of the cell.

The differentiation of the six touch receptor neurons (TRNs) in the nematode *Caenorhabditis elegans* uses such an autoregulatory loop. These cells sense gentle touch to the body. Screens for touch-insensitive mutants identified two genes, *unc-86* and *mec-3*, that are needed for the generation and specification of the TRNs (9, 10). *unc-86* encodes a homeodomain protein (UNC-86), which is expressed in the TRNs and their immediate precursors (11), whereas *mec-3* encodes a LIM-type homeodomain protein that is expressed only in the terminal cells (12). Mutants lacking *unc-86* activity do not generate the cells that become TRNs. In contrast,

these cells are produced in *mec-3* mutants, but they do not differentiate as TRNs (13). Therefore, *mec-3* acts as a typical terminal selector gene. UNC-86 is needed not only to produce touch cell lineages but also to initiate transcription from the *mec-3* gene (12). The maintained expression of *mec-3* and the subsequent expression of TRN characteristics require the combinatorial action of MEC-3 and UNC-86; MEC-3 acts (as a coactivator with UNC-86) in an autoregulatory loop to maintain its own expression and to induce the expression of TRN-specific genes (13–18).

In this study, we investigate the function of the transcription factor ALR-1 in the regulation of TRN cell fate. Tucker et al. (19) found that *alr-1*, the *C. elegans* homolog of the *Drosophila* paired-type homeobox gene *aristaless* (*al*), is expressed in these cells. *Aristaless*-like proteins have important developmental roles in several organisms. *alr-1* regulates the development of sensory neurons in the head and GABAergic motor neurons (19, 20). *Drosophila aristaless* is needed for the development of several organs, including the aristae and wings (21, 22). The mouse ortholog *Arx* controls neuroblast proliferation and migration of GABAergic neurons (23, 24). Finally, defects in human *Arx* cause mental retardation (23, 25–28).

Here we report that, in contrast to *mec-3*, *alr-1* does not act as a selector gene to determine the fate of the TRNs. Instead, ALR-1 ensures TRN differentiation by providing a second positive feedback circuit to maintain *mec-3* expression. We show that loss of *alr-1* produces variable touch sensation that correlates with increased variability in *mec-3* expression. These results demonstrate that *mec-3* autoregulation cannot maintain *mec-3* mRNA levels. These levels are maintained in wild-type animals because ALR-1 restricts *mec-3* expression to the high end of its expression range, a process that we call refinement. Our results demonstrate that stochastic fluctuations in gene expression can account for phenotypic variability and provide a mechanistic explanation for these genetic phenomena. In addition, we provide an example of how transcription factors like ALR-1 may function to reduce the variability of gene expression and ensure differentiation. Given that *alr-1* and its homologs (*aristaless* and *Arx*) are required for the expression of other transcription factors (20, 23, 29, 30), our data support the hypothesis that proteins in the *Aristaless*/ARX family may function as stabilizers of gene expression that ensure differentiation.

Results

***alr-1* Expression in the TRNs Depends on MEC-3.** Because MEC-3 controls TRN differentiation, we tested whether *alr-1* expression required *mec-3*. Using a transcriptional fusion in which the *alr-1* upstream sequences regulate *gfp* expression (*P_{alr-1} gfp*) (19), we confirmed that *alr-1* is expressed in the ALM, PLM, and AVM

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TRNs, but not in the TRN PVM (Fig. S1A). This expression, but not that in other cells, was completely abolished in *mec-3(e1338)* animals (Fig. S1B). This *mec-3*-dependent expression is consistent with the finding of the heptanucleotide AATGCAT (the consensus core for MEC-3 targets) (31) in the *alr-1* promoter. An ALR-1::GFP protein fusion was localized exclusively to the nucleus of all of the expressing TRNs throughout all larval development (Fig. S1C and Fig. S2), a result consistent with a role for ALR-1 in transcriptional control.

***alr-1* Mutants Are Variably Touch-Insensitive.** The TRNs sense gentle touch to the body in *C. elegans* (9). As expected from a role in the differentiation or function of the cells, mutations of *alr-1* (including the null mutation *oy42*) (20) cause touch insensitivity (Fig. 1A). The touch insensitivity of *alr-1(oy42)* mutants could be rescued with a genomic fragment of *alr-1* using a *mec-3* promoter (Fig. 1A), indicating that the touch-insensitive phenotype was a consequence of loss of *alr-1* function cell-autonomously in the TRNs.

The *alr-1* touch-insensitive phenotype was variably expressed, ranging from animals that were indistinguishable from wild type (responding to 7–8 of 10 touches) to those that were completely insensitive to touch (responding to 1–2 touches) (Fig. 1A). This great variability is not a characteristic of all strains with partial touch insensitivity because the loss of *mec-18* results in partial, but more restricted, touch insensitivity (Fig. 1A). Such in-

complete penetrance and variable expressivity have also been observed for phenotypes associated with the AWA and ASG cells in *alr-1* mutants (20). Individuals with a particular touch response showed similar touch responses during the course of development, suggesting that this phenotypic variability was set early during their differentiation and remained stable (Fig. S3).

The variability of the *alr-1* phenotype appeared to be cell-specific. We observed that individual *alr-1* mutants respond to anterior and posterior touch to different degrees, implying a cell-specific requirement for ALR-1 in ALM/AVM (sensing anterior touch) and PLM (sensing posterior touch) neurons, respectively. We also found that the ability of *alr-1* mutants to be attracted to diacetyl, which shows a similar variability in *alr-1* mutants (20), was independent of their ability to respond to touch, a result that is consistent with cell-autonomous activity.

***alr-1* Is Required Throughout Development for TRN Function.** *alr-1* is expressed in the TRNs at all larval stages (Fig. S2). To determine whether *alr-1* was needed for touch sensitivity throughout this time, we tested the touch sensitivity of adults that expressed *alr-1* for different amounts of times in larvae. We generated a strain that expressed *alr-1* in a temperature-dependent manner by inserting a *mec-8*-dependent intron before the coding region of *alr-1* and using a temperature-sensitive allele of *mec-8* (32). The extensive temperature-sensitive period (region of crossover between the downshift and upshift curves) (Fig. 1B) seen for touch sensitivity in this strain suggests that *alr-1* activity is needed throughout larval development, which is consistent with the ALR-1::GFP expression pattern.

ALR-1 Functions in TRN Differentiation as a Transcriptional Activator. Aristaless-like proteins are thought to act as either transcriptional activators or transcriptional repressors (29, 30, 33–37). In *C. elegans*, Melkman and Sengupta (20) found that *alr-1* blocked expression of the DD motor neuron marker *flp-13* in VD motor neurons and both down-regulated and up-regulated *lin-11* expression in the AWA and ASG neurons, respectively. These changes, however, could be indirect.

To test how ALR-1 acts in the TRNs, we determined whether we could restore ALR-1 function with fusions of the ALR-1 homeodomain (ALR-1HD) with either the activation domain of VP16 (VP16AD::ALR-1HD) or the repressor domain of Engrailed (EnRD::ALR-1HD). The VP16AD::ALR-1HD fusion partially rescued the touch-insensitive phenotype of *alr-1(oy42)* mutants (three of five stable lines), but the EnRD::ALR-1HD fusion did not (six stable lines) (Fig. S4). These results suggest that ALR-1 affects TRN fate by acting mainly as a transcriptional activator.

These observations are consistent with the lack of the octapeptide domain and polyaniline tract 4 domain in ALR-1 (28), which McKenzie et al. (38) showed were needed for ARX repression. We cannot, however, exclude the possibility that ALR-1 can act as a repressor because our VP16 fusion only partially rescued the *alr-1* mutant phenotype in the TRNs and did not rescue the chemosensory phenotype of *alr-1* mutants (although the Engrailed fusion also failed to rescue this phenotype).

***alr-1* Is Required Selectively for TRN Gene Expression.** Consistent with the loss of touch sensitivity and ALR-1 being a homeodomain transcription factor, we found that the expression of genes required for TRN function was reduced in *alr-1* mutants. All three available mutant alleles of *alr-1*, including the null allele *oy42*, greatly reduced MEC-18 protein (Fig. S5A) and mRNA (Fig. S5B). The decreased expression was more dramatic at the early larval stages of the animals although it was still obvious at later stages (Table S1).

The expression of *mec-2*, *mec-7*, and *mec-17*, detected using antibodies or GFP fusions, was similarly reduced by the *oy42*

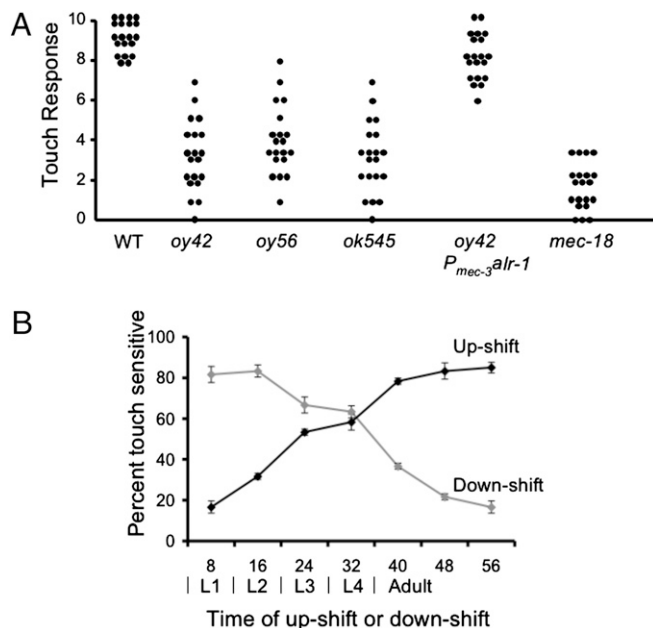


Fig. 1. ALR-1 is necessary for touch sensation, and it is needed in all stages of larval development for adult touch sensation. (A) Touch insensitivity varies in *alr-1* mutants. Wild-type, *alr-1(oy42)* mutants rescued in the TRNs with *P_{mec-3} alr-1*, and *mec-18* mutants do not show this extreme variability. Each dot corresponds to an individual animal ($n = 20$). The touch response was measured as the number of times that animals moved away from 10 touches delivered alternately near the head and the tail (Materials and Methods). (B) ALR-1 is needed throughout TRN development for adult touch sensation. Temperature sensitivity of *mec-8ts*; *alr-1* mutants, expressing MEC-8-regulated *alr-1* (*P_{alr-1} intron 9::alr-1*). Temperature shifts were conducted as described previously (10): Animals were grown at the appropriate temperature for two generations and then synchronized at hatching and shifted from 15 °C to 25 °C (Up-shift, black line; at 25 °C, ALR-1 protein cannot be made) or from 25 °C to 15 °C (Down-shift, gray line; at 15 °C, ALR-1 protein can be made) at the indicated times and tested for touch sensitivity as adults. Values are the mean \pm SEM of three replicates ($n = 20$).

mutation in all of the TRNs except PVM (Fig. 2A and Fig. S5C and D), the only TRN that does not express *alr-1* (Fig. S1). The reduction, as scored by antibody staining, seemed to vary from animal to animal, mimicking the variability in the loss of touch sensitivity (Table S1). We could not see variability in GFP-expressing animals, probably because of the stability of GFP.

Importantly, mutations of *alr-1* did not reduce all gene expression in the TRNs. TRN expression of the pan-neuronal genes *unc-119* (39) and *sng-1* (40) was unaffected by the *alr-1* (*oy42*) mutation at all developmental stages (Fig. 2B; Fig. S6A; Table S1). These results argue against ALR-1 acting as a general enhancer of transcription in the TRNs.

***alr-1* Maintains *mec-3* Expression.** Although *alr-1* is a target of *mec-3*, we found that *alr-1* was also needed for appropriate *mec-3* expression. Both the fluorescence of an unstable GFP expressed from the *mec-3* promoter (*P_{mec-3} praja::gfp*) (Fig. 3A and Table S1) and the amount of *mec-3* mRNA (Fig. S6B) was reduced in the TRNs (except PVM) (Fig. S6C) of young larvae and adult *alr-1*(*oy42*) animals. The reduction of *mec-3* expression observed with *P_{mec-3} praja::gfp* displayed a similar variability to that observed with MEC-18 immunostaining (Table S1). [We tried to test whether addition of *mec-3*(+), which rescued *mec-3* mutants, could rescue *alr-1* mutants (it did not) (Table S2). However, this experiment is difficult to interpret because we do not know whether sufficient *mec-3* can be generated in *alr-1* mutants.]

The initial expression of *mec-3* requires the POU-type homeo-domain transcription factor UNC-86 (13, 41) whereas its maintained expression requires both MEC-3 and UNC-86 (13). For example, *mec-3* mutants expressed *P_{mec-3} gfp* less often (~60%) and less intensely than wild type did at hatching (Table S1). This expression disappeared as animals matured. This initial *mec-3*-independent *mec-3* expression was also *alr-1*-independent (Fig. 3B and Table S1). Moreover, expression from *unc-86* promoter-*gfp* fusions was no different with and without *alr-1* (Fig. S7).

The extended requirement for *alr-1* in touch sensation implicates it in the maintenance of *mec-3* expression. To test this hypothesis, we measured *P_{mec-3} praja::gfp* expression in adult *mec-8ts; alr-1* mutant animals, expressing MEC-8-regulated *alr-1* (*P_{alr-1} intron 9::alr-1*) either early or late in development (before or after

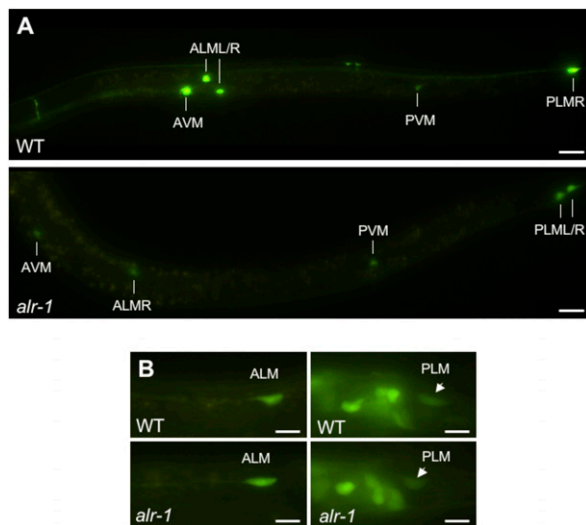


Fig. 2. ALR-1 is needed for TRN expression of *mec-17::gfp* but not for the expression of *P_{unc-119} gfp*. (A) *mec-17::gfp* expression in wild-type (Upper) and *oy42* mutant (Lower) animals. GFP levels remain unaffected at the PVM neuron where *alr-1* is not expressed. (Scale bars: 20 μ m.) (B) *P_{unc-119} gfp* is expressed equally in TRNs (ALM, Left; PLM, Right) of wild-type and *alr-1* (*oy42*) adults. (Scale bars: 5 μ m.)

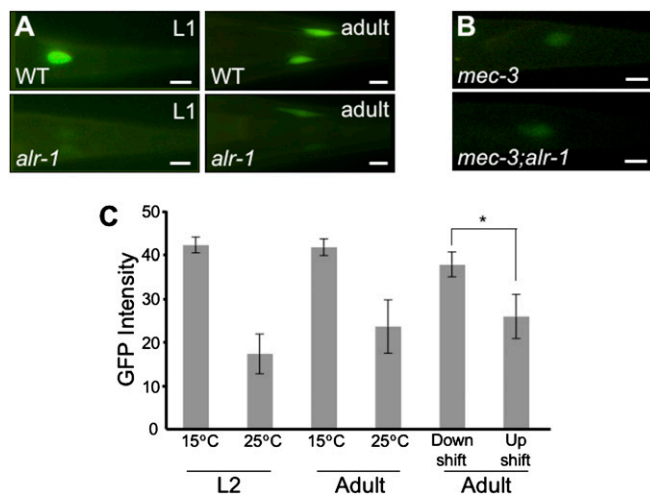


Fig. 3. ALR-1 is required to maintain *mec-3* expression. (A) Mutation of *alr-1* reduces expression of an unstable GFP in PLM neurons from *P_{mec-3} praja::gfp* in a late L1 larva (Left) and in an adult (Right). (Scale bars: 4 μ m.) (B) Newly hatched *mec-3* (Upper) and *mec-3; alr-1* (Lower) larvae express *P_{mec-3} gfp* to the same degree. (Scale bars are 4 μ m.) (C) Post-L2 expression of *alr-1* is sufficient for wild-type expression of unstable GFP from the *mec-3* promoter (*P_{mec-3} praja::gfp*) in PLM neurons of *mec-8ts; alr-1* mutants expressing MEC-8-regulated *alr-1* (*P_{alr-1} intron 9::alr-1*). Animals were grown at the permissive (15 °C) or nonpermissive temperature (25 °C) for two generations, and GFP intensity was measured at the L2 or the adult stage. Additionally, animals were grown at 15 °C or 25 °C until the L2 stage when they were shifted to the opposite temperature; GFP intensity was then measured at the adult stage. $P < 0.01$, Student's *t* test.

the L2 stage). Expression of *alr-1* only before the L2 stage was not sufficient for maximum levels of *P_{mec-3} praja::gfp* expression in adults, but expression of *alr-1* after the L2 stage was (Fig. 3C).

ALR-1 Enhances MEC-3/UNC-86-Induced Transcription from the *mec-3* Promoter. A likely hypothesis is that ALR-1 directly activates *mec-3* expression. To investigate this possibility, we examined its stimulation of β -galactosidase production from several promoters in yeast cells. We inserted *P_{mec-3}* or *P_{unc-119}* upstream of the yeast minimal promoter *P_{CYC-1}* to drive the expression of β -galactosidase in the presence of different combinations of MEC-3, UNC-86, and ALR-1. MEC-3 or UNC-86 alone did not stimulate β -galactosidase expression from the basal promoter (*P_{CYC-1}*), the *mec-3* promoter (*P_{mec-3} P_{CYC-1}*), or the pan-neuronal *unc-119* promoter (*P_{unc-119} P_{CYC-1}*). Together, MEC-3 and UNC-86 increased β -galactosidase activity 30-fold from *P_{mec-3} P_{CYC-1}*, but not from the other promoters (Fig. 4A). ALR-1 also increased expression from *P_{mec-3} P_{CYC-1}* and not the other promoters, but only slightly (1.4-fold) (Fig. 4A) and it did not have any effect on transcription in combination with either MEC-3 or UNC-86. ALR-1, however, had a greater effect in the presence of both MEC-3 and UNC-86, increasing expression to 60-fold (Fig. 4A). These results show that ALR-1 can enhance the bulk *mec-3* expression and echo the in vivo requirement for *alr-1* for TRN-specific gene expression.

Our results demonstrate that *mec-3* autoregulation without *alr-1* is not sufficient for maintenance of *mec-3* expression; *alr-1* provides a second positive feedback loop that enhances *mec-3* expression and maintains TRN differentiation (Fig. 4B).

TRN Expression of *mec-3* Is More Variable in *alr-1* Mutants. Our yeast expression studies demonstrate that ALR-1 can enhance *mec-3* expression, but these assays provide a bulk assessment of ALR-1 activity and do not indicate how ALR-1 increases *mec-3* expression. Using single-molecule fluorescence in situ hybridiza-

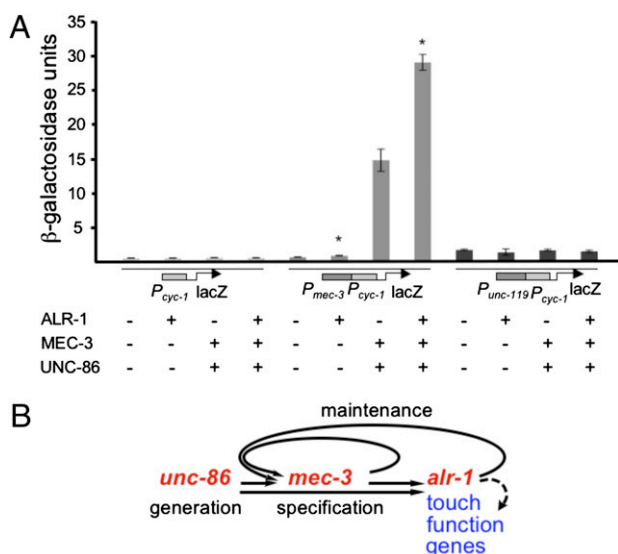


Fig. 4. ALR-1 enhances UNC-86/MEC-3-induced transcription in yeast cells. (A) ALR-1, MEC-3, and UNC-86 or combinations were expressed in yeast cells as described in *Materials and Methods*. Values are the mean \pm SEM of five replicates. $P < 0.006$, Student's *t* test. (B) A model for *alr-1* function in the TRNs. ALR-1 is needed to ensure maximum levels of *mec-3* expression and possibly to induce expression of TRN-specific genes (dashed line).

tion (SM-FISH) (42) that renders each mRNA visible as a single fluorescent spot, we found that ALR-1 normally reduces the variability of the expression of *mec-3* (Fig. 5A and B). *mec-3* expression is much more variable in ALM and PLM neurons in *alr-1* first-stage larvae than in wild-type larvae (coefficient of variation: 13.7 and 27.8 for wild-type and *alr-1* PLM neurons and 13.3 and 26.3 for wild-type and *alr-1* ALM neurons). The maxi-

imum number of *mec-3* mRNAs is the same for both strains, but the range of values is greater in *alr-1* mutant TRNs. Moreover, consistent with cell-autonomous activity of *alr-1*, we found that the number of *mec-3* mRNAs in individual ALM and PLM neurons varied independently (Table S3 and S4). Thus, ALR-1 restricts the stochastic expression of *mec-3*.

The reduced *mec-3* expression and the greater variability of *mec-3* expression in *alr-1* mutants reflect the phenotypic variability seen in these mutants. Nonetheless, the maximum level of expression was the same for both strains. In other words, the presence of *alr-1*(+) restricts *mec-3* expression to the high end of its range, but does not increase the range. We call this process refinement to distinguish it from enhancement (Fig. 5C). In contrast, expression of *mec-3* in FLP neurons, which do not detectably express *alr-1*, was the same in wild-type and mutant animals (Fig. 5B). These results indicate that *alr-1* increases *mec-3* expression by restricting *mec-3* expression variability and suggest that the phenotypic variability observed in *alr-1* mutants correlates with variability in *mec-3* gene expression in the TRNs.

Discussion

Incomplete penetrance and variable expressivity are often characteristic features of mutant phenotypes (1–3). In this study, we provide evidence that links stochastic fluctuations in gene expression to phenotypic variability. In addition, we provide an explicit demonstration of how transcription factors can reduce variability in gene expression and ensure development.

Earlier studies have shown the cooperative role of the MEC-3 and UNC-86 transcription factors in defining the terminal fate of the TRNs and in maintaining the expression of MEC-3 (13–18). Here we demonstrate that, due to stochastic fluctuations, MEC-3 autoregulation is insufficient to maintain TRN differentiation. The second positive feedback loop provided by ALR-1 enhances *mec-3* expression by reducing the variability in *mec-3* expression. We propose that regulation of the variability in gene expression is important for controlling differentiation and that genes like

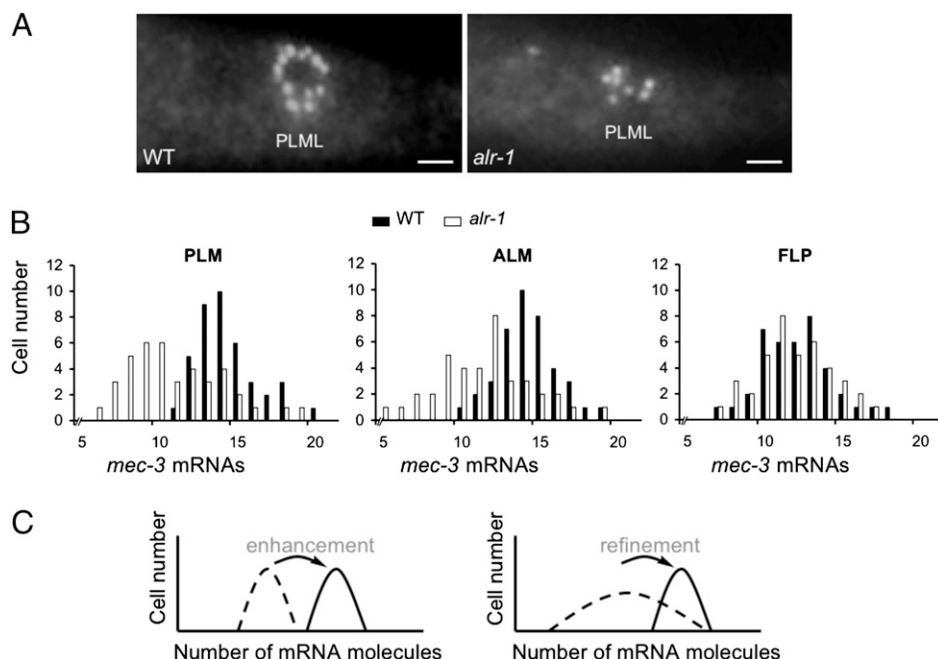


Fig. 5. ALR-1 restricts variability in *mec-3* expression (A) SM-FISH for *mec-3* in PLM neurons in wild-type (WT) and *alr-1*(oy42) L1 larvae. A single focal plane is shown. (Scale bars: 2 μ m.) (B) The number of *mec-3* mRNA molecules is restricted in wild-type L1 larvae (black bars) PLM and ALM, but not in FLP neurons compared with the same cells in *alr-1* L1 larvae (white bars). mRNAs in 40 cells were counted. (C) Two possible ways in which a similar increase in transcription can be achieved. For enhancement (Left), addition of a transcription factor shifts the range of expression to higher values. For refinement (Right), transcription is confined to the upper portion of a preexisting range. Dashed line: before activation; solid line: after activation.

alr-1 function to overcome stochastic fluctuations in gene expression and ensure differentiation.

ALR-1 Maintains Expression of MEC-3. Our results suggest that ALR-1 is a transcriptional activator that controls TRN activity by maintaining optimal *mec-3* expression throughout larval development. First, ALR-1 enhanced MEC-3/UNC-86-dependent transcription from the *mec-3* promoter in a yeast expression system. Second, the ALR-1HD with the VP16 activation domain can partially rescue the *alr-1* mutant phenotype. The hypothesis that ALR-1 enhances transcription from the *mec-3* promoter is further supported by ChIP-Seq experiments, demonstrating a strong association of ALR-1 with the *mec-3* proximal promoter (25th of 7,009 potential targets) (43).

The interactions of ALR-1 and MEC-3 provide a positive feedback loop: MEC-3 controls the expression of *alr-1*, and ALR-1 regulates the maintenance of *mec-3* expression. Even though it is expressed downstream of selector genes, ALR-1 appears to participate in the maintenance of *mec-3* expression by providing a second positive feedback loop parallel to the autoregulatory *mec-3* feedback loop (Fig. 4B). Regulatory interactions involving LIM-type homeodomain transcription factors and *aristaless* homodomain transcription factors have also been described in pretarsus development in *Drosophila* (AL and LIM1) (27, 29) chemosensory neuron development in *C. elegans* (ALR-1 and the LIM1 ortholog *lin-11*) (20) and in the neocortex and ganglionic eminence (ARX and Lhx6) and thalamic eminence (ARX and LHX9) in mice (23).

alr-1 does not function as a general transcription factor in the TRNs, but appears to activate the *mec-3*-dependent pathway. In addition to amplifying *mec-3* expression, which would indirectly increase the production from *mec-3* targets, *alr-1* may also directly affect the expression of those targets. The ChIP-Seq analysis of ALR-1 (43) shows a strong association of the protein with *mec-2*, *mec-7*, *mec-4*, *mec-9*, *mec-10*, *mec-14*, and *mec-17* and a low association with *mec-1*, *mec-6*, and *mec-12*, suggesting that ALR-1 may directly affect their transcription. These observations suggest that ALR-1 participates in *mec-3* expression and in the expression of at least some of the *mec-3* target genes (Fig. 4B).

ALR-1 Prevents Phenotypic Variability by Controlling Variable Gene Expression. Unlike MEC-3, ALR-1 does not act as a terminal selector gene (6); it ensures but does not determine the fate of the TRNs. TRNs in *alr-1* mutants still express *mec-3* target genes, albeit at lower levels. In addition, the PVM neurons, which do not express detectable *alr-1* in wild-type animals, nonetheless share differentiated features with the other TRNs. The PVM cells express *mec-3* target genes at lower amounts (e.g., Fig. 24), and they contain fewer of the TRN-specific microtubules than the other TRNs (44).

Our results link stochastic fluctuations in gene expression to incomplete penetrance and variable expressivity and provide a mechanism to explain these genetic phenomena. In our model, removal of a stabilizing component (e.g., ALR-1) results in stochastic expression that is so broad that some, but not all, cells express the selector gene (*mec-3*) at levels below the threshold level needed for cell function (touch sensitivity). Wild-type ALR-1 stabilizes gene expression and ensures that it is above the threshold needed for function.

These results also provide an explicit demonstration for how variability may be prevented during development when cell fate accuracy is essential. Because incomplete penetrance, phenotypic variability and variable expression of transcription factors have been reported for many mutants (1, 3, 45), we suggest that regulation of stochastic gene expression may be important for cell differentiation and function.

Although touch insensitivity of *alr-1* mutants differs from animal to animal, individual animals maintain the same level of touch

insensitivity through the larval period. Thus, the stochastic processes that define *mec-3* expression levels in *alr-1* mutants appear to be set early and remain stable during the development of the TRNs. The temperature-shift experiments, however, demonstrate that ALR-1 can reset the expression level of *mec-3* at later times.

Our experiments indicate that MEC-3 (with UNC-86) sets the maximum levels of *mec-3* expression and ALR-1 ensures (by overcoming stochastic events) that *mec-3* expression will always be sufficient. In general, the effectiveness of any transcription factor will depend on its nuclear concentration, its capacity to bind DNA, and the efficiency with which it activates transcription. If the concentration of the transcription factor is not saturating, increasing the concentration should increase transcription and shift the range of expression to a higher position. Alternatively, if the concentration is already maximal, ensuring the efficiency of transcription (either by securing the association with the promoter or by adding to the intrinsic efficiency of activation) should shift expression to the top of the range (refinement in Fig. 5C). We feel that the alteration in *mec-3* expression caused by ALR-1 may be of this latter type.

Because variability similar to that seen in touch sensitivity was described for the *Dyf*, *Osm*, *Daf-d*, chemoattraction phenotypes, and *lin-11* expression in AWA and ASG neurons of *alr-1* mutants (20), the control of the variability of gene expression may be a general function for ALR-1. *alr-1* does not function as a classical selector gene, but it plays the critical role of fine-tuning the autoregulation of selector genes to ensure differentiation. This hypothesis is further supported by our preliminary results showing that ALR-1 reduces the variability of expression of another selector gene, *unc-30*, that defines the fate of the DD and VD GABAergic neurons (46) (Fig. S8). In addition, ChIP-Seq results (43) show that ALR-1 associates with *unc-30* and with some of the UNC-30 downstream targets (*unc-25*, *unc-46*, *unc-47*). These observations suggest that ALR-1 plays a more general role in ensuring neuronal terminal differentiation by securing the efficiency of the expression of selector genes and probably of their downstream targets.

Because *alr-1*, *Drosophila aristaless*, and mouse *Arx* regulate the LIM homeobox genes *lin-11* (20), *Lim1* (29, 30), and *Lhx6/Lhx9* (23), respectively, we hypothesize that *Aristaless* proteins may have a conserved role across species in regulating and restricting the variability of the expression of LIM-homeodomain transcription factors and, perhaps, other selector genes. Unfortunately, the null phenotypes of these genes are so severe that variability cannot be easily assessed (23, 47).

Materials and Methods

General Procedures. Unless otherwise indicated, strains were maintained and studied at 20° according to Brenner (48) on OP50 *Escherichia coli*. Detailed information on the strains used, reporter plasmids, plasmid constructions, microinjections, quantitative real-time PCR, and microscopy and immunofluorescence is given in *SI Materials and Methods*.

Touch Assays. We assayed gentle touch sensitivity in blind tests as described (9). We quantified the response by counting the number of responses to 10 touches delivered alternately near the head and tail in 20 animals (20 stable transformants for the rescuing experiments).

SM-FISH. We designed oligonucleotide probes and performed SM-FISH (42) on L1 stage animals as described at <http://www.singlemoleculefish.com>. Forty-three 20-nucleotide probes for *mec-3* mRNA were synthesized by BioSearch Technologies and coupled to Cy5 (GE Amersham). We imaged the animals using a Nikon TE 2000 microscope equipped with a Princeton Instruments Micromax 1024B camera and appropriate filters for Cy5. We collected stacks of 30–40 images spaced 0.3 μ m apart for each individual neuron and counted the number of fluorescent spots per neuron using ImageJ (<http://rsb.info.nih.gov/ij/>).

Yeast Transcription Assays. We constructed yeast strains expressing *P_{cyc-1} lacZ*, *P_{mec-3}P_{cyc-1} lacZ*, and *P_{unc-119}P_{cyc-1} lacZ* by digesting plasmids with these DNAs with *Apal* and integrating them in the URA locus of the yeast strain YPH499

(Stratagene). These plasmids (as well as plasmids encoding MEC-3, UNC-86, and ALR-1) were transformed using the Liac/SS carrier DNA/PEG method (49). Positive colonies were identified using single-colony PCR. Strains were grown at 30° in yeast synthetic drop-out media (Clontech #630426) in the presence of 2% glucose. Strains (at a concentration of OD₆₀₀ = 0.01) were induced with 0.2% galactose after growth overnight in synthetic drop-out media with 2% raffinose. Induction was achieved by growing the cultures overnight to OD₆₀₀ = 1. Liquid β -galactosidase assays were performed as described in Reynold and Lundblad (50).

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