MicroRNAs acting in a double-negative feedback loop to control a neuronal cell fate decision

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The elucidation of the architecture of gene regulatory networks that control cell-type specific gene expression programs represents a major challenge in developmental biology. We describe here a cell fate decision between two alternative neuronal fates and the architecture of a gene regulatory network that controls this cell fate decision. The two Caenorhabditis elegans taste receptor neurons “ASE left” (ASEL) and “ASE right” (ASER) share many bilaterally symmetric features, but each cell expresses a distinct set of chemoreceptors that endow the gustatory system with the capacity to sense and discriminate specific environmental inputs. We show that these left/right asymmetric fates develop from a precursor state in which both ASE neurons express equivalent features. This hybrid precursor state is unstable and transitions into the stable ASEL or ASER terminal end state. Although this transition is spatially stereotyped in wild-type animals, mutant analysis reveals that each cell has the potential to transition into either the ASEL or ASER stable end state. The stability and irreversibility of the terminal differentiated state is ensured by the interactions of two microRNAs (miRNAs) and their transcription factor targets in a double-negative feedback loop. Simple feedback loops are found as common motifs in many gene regulatory networks, but the loop described here is unusually complex and involves miRNAs. The interaction of miRNAs in double-negative feedback loops may not only be a means for miRNAs to regulate their own expression but may also represent a general paradigm for how terminal cell fates are selected and stabilized.

left/right asymmetry | bistable | network motif | regulatory RNA | cellular diversification

Nervous systems are characterized by a striking degree of cellular diversity. The molecular correlates to morphological and functional diversity of nervous systems are neuron-type specific gene expression programs. The experimental accessibility of the nematode Caenorhabditis elegans offers the opportunity to (i) determine the nature of neuron-type specific gene expression programs on a single-cell level and (ii) to genetically dissect the mechanisms that establish and maintain these single-cell specific programs. The two main gustatory neurons of C. elegans, ASE left (ASEL) and ASE right (ASER), display a particularly intricate level of neuronal diversity. Although bilaterally symmetric in many different regards (cell position, axodendritic morphology, synaptic connectivity, and molecular features), each neuron expresses a distinct spectrum of putative chemoreceptors, a feature that the worm requires to navigate through complex sensory environments (1, 2). The ASE neurons therefore not only provide a model to study sensory neuron fate diversification but also to study neuronal laterality, a common but poorly understood feature of many nervous systems.

To elucidate the nature of the gene regulatory program that diversifies ASEL and ASER, we have isolated mutants in which ASE asymmetry is disrupted (3–6). In “class I mutants,” both ASE neurons adopt the ASEL fate; in contrast, in “class II mutants,” both ASE neurons adopt the ASER fate. These phenotypic categories indicate that both ASE cells are endowed with the capacity to express either the ASEL or ASER fate and that once the two ASE neurons are generated, specific gene products ensure that each neuron expresses either the ASEL or ASER terminal cell fate. We extend these initial observations here by demonstrating that after their birth, the ASE neurons rapidly transition from an equipotent, hybrid precursor state to their terminal and stable ASEL and ASER end states.

Our previous molecular analyses of class I and class II mutants identified several gene regulatory factors including transcription factors and microRNAs (miRNAs) that control ASEL/ASER asymmetry (Fig. 1A). However, a key question left unanswered was how left/right asymmetric expression of the miRNA mir-273, the most upstream regulatory factor in the cascade shown in Fig. 1A, is controlled. We show here that these previously described gene regulatory factors interact with one another in a double-negative feedback loop that provides a simple explanation for the stability of ASEL and ASER cell fates.

Although feedback loops have previously been found as regulatory motifs that regulate cell fate decisions, the loop that we describe here is unique in its involvement of multiple miRNAs. A substantial, but still largely unknown, number of genes in metazoan genomes codes for miRNAs. Despite their abundance, the cellular and molecular contexts in which miRNAs exert their function in vivo are only beginning to be defined (7). Our analysis provides previously uncharacterized insights into the integration of miRNAs into gene regulatory networks. Moreover, the regulatory interactions that we describe here demonstrate that miRNAs can auto-regulate their expression through double-negative feedback regulation. Our findings corroborate the role of miRNAs as important developmental switches that control terminally differentiated cellular states.

Materials and Methods

All mutant strains were described in refs. 4–6. The following transgenes were used (4–6, 8, 9): otIs114 [lim-6prom;::gfp, rol-6 (d)], syIs63 [cog-1prom;::gfp, dpy-20 (+)], syIs73 [ceh-36prom;::gfp, unc-122prom;::gfp], otEx1749 [mir-273prom2;::gfp, unc-122prom;::gfp], otEx1759 [ceh-36prom;::gfp, die-1prom;::gfp, rol-6 (d)], nts1 [gcy-5prom;::gfp, lin-15 (+)], otIs3 [gcy-7prom;::gfp, lin-15 (+)], otIs162 [gcy-6prom;::gfp, lin-15 (+)], otIs160 [lsy-6prom;::gfp, unc-122prom;::gfp], otIs151 [ceh-36prom;::gfp, rol-6 (d)], otEx1382 [ceh-36prom;::gfp, rol-6 (d)], otEx959 [die-1prom;::gfp, rol-6 (d)], otEx1192 [gcy-7prom;::gfp, die-1prom;::gfp, unc-122prom;::gfp], yns54 [flp-20prom;::gfp, yns54 [flp-4prom;::gfp]. The otEx2302 [gcy-22prom;::gfp, unc-122prom;::gfp] transgene contains 2 kb of the gcy-22 promoter. lin-1prom;::gfp will be described elsewhere. Each array was crossed into the respective genetic backgrounds. In all cases where gfp expression was observed in cells other than ASE, the ASE neurons were unambiguously identified through the use of a transgene that expresses rfp bilaterally in ASEL and ASER (otIs151). In some cases, the subjective assessment of relative expression levels was confirmed.

Abbreviations: ASEL, ASE left; ASER, ASE right; miRNA, microRNA.

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ASER cell fate (Fig. 1 not ASER (Fig. 1 FMRFamide-type neuropeptides, are expressed in ASEL but previously reported to be expressed in amphid sensory neurons.

mutant animals, they initially express both ASEL and ASER markers. See Fig. 6 for quantification of effects.

and gcy-5 genes cause a state transition between the ASEL and ASER fates. (A) Gene regulatory factors controlling ASE laterality, as deduced by our previous genetic analysis (3–6). The permissively acting, ASEL/R-expressed genes unc-37/Groucho, lin-49, and ceh-36 (4) are left out for clarity. mir-273 likely acts together with other mir-273-related miRNAs (D. Didiano and O.H., unpublished data), yet throughout this paper, we only show mir-273 for clarity. (B) ASEL- and ASER-specific cell fate markers and their regulation by lsy genes. ASER-specific expression can be observed with a subfragment of the hen-1 promoter (hen-1asgfp). In all cases, reporter gene expression in ASE was unambiguously determined by using a chromosomally integrated rfp transgene in the genetic background, which is expressed in ASEL/R. Fig. 5, which is published as supporting information on the PNAS web site, shows the quantification of data. (C) Summary of the genetic interactions deduced from B. (D) Early bilateral expression of the ASE inducer lsy-6 and of the ASER inducer cog-1. Early bilateral expression can also be observed for gcy-6, gcy-7, and lim-6 (Fig. 6, which is published as supporting information on the PNAS web site, shows the quantification of all observations). * gfp-expressing cells other than ASE, which are out of focus in Right. (E) Even if both ASE neurons are fated to become ASER in class II lsy-6 mutant animals, they initially express both ASEL and ASER markers. See Fig. 6 for quantification of effects.

by double-blind acquisition of images and subsequent automated quantification of fluorescence intensity with Improvision’s (Lexington, MA) OPENLAB software.

Results

Regulation of Terminal Differentiation Features of ASEL and ASER Cell Fate. Aside from a multitude of bilaterally expressed terminal differentiation features, the only known terminal differentiation genes that distinguished the ASEL and ASER neurons were three guanylyl cyclase (gcy) receptors (1). In adult animals, two of these genes, gcy-6 and gcy-7, are stereotypically expressed in ASEL only, whereas gcy-5 is stereotypically expressed in ASER only. Expression of these genes is stable and maintained throughout adulthood. We find that the ASEL and ASER fates are defined by a number of additional genes. By extending the previous expression pattern analysis of gcy genes (1), we find that the gcy-22 gene is exclusively expressed in the ASER neuron (Fig. 1B).

To address whether the ASEL-specific flp-4, flp-20, and previously unexamined gcy-6 reporters and the ASER-specific gcy-22 and hen-1ASER reporters are regulated by the same set of regulatory factors that control the expression of the gcy-5 and gcy-7 genes, we crossed the respective reporter transgenes into class I (“2 ASEL cells”) and class II (“2 ASER cells”) mutant backgrounds. We find that in animals that lack the cog-1 homeobox gene (class I mutant), the normally ASEL-specific flp-4, flp-20, and gcy-6 reporters are ectopically activated in ASER, whereas expression of the ASER markers gcy-22 and hen-1ASER is lost (Fig. 1B). In contrast, in lsy-6 null mutant animals (class II mutant), the expression of the ASE markers flp-4, flp-20, and gcy-6 is lost in ASER, with a concomitant gain of the ASER markers gcy-22 and hen-1ASER (Fig. 1B). flp-4, flp-20, gcy-6, gcy-7, gcy-5, gcy-22 and hen-1ASER are therefore terminal markers of two alternative states, regulated by class I and class II genes (Fig. 1C).

We gained further insights into the regulatory architecture of ASEL-expressed genes by examining gene expression profiles in animals that lack the lim-6 LIM homeobox gene, an ASEL-specific transcription factor (3). lim-6 is a negative regulator of gcy-5 expression in ASEL but has no impact on ASEL-specific expression of gcy-6 and gcy-7 (3). We find that lim-6 also represses the new ASER marker gcy-22 in ASEL. In contrast, lim-6 is a positive regulator of flp-4 and flp-20 expression in ASEL (Fig. 1B). In genetic terms, lim-6 therefore behaves as either an activator or repressor, depending on the target gene. In contrast to its repressive effect on expression of the ASE fate markers gcy-5 and gcy-22, the lim-6 gene has no role in repressing the other known ASER terminal marker hen-1ASER (Fig. 1B). These observations suggest that lim-6 plays a role in the control of a branch in the network of terminal effector genes, whereas the upstream regulatory genes lsy-6 and cog-1 control all aspects of lateral cell fate specification (Fig. 1C). Consistent with this conclusion, lim-6 is, in contrast to lsy-6, not sufficient to drive ASEL fate upon ectopic misexpression in ASER (4).
The miRNAs lsy-6 and mir-273 Act in a Double-Negative Feedback Loop. How do ASEL and ASER lock into their terminal fates? Our previous genetic analyses identified a set of gene regulatory factors, including transcription factors and miRNAs, that are required for expression of either of the two alternate ASE fates (Fig. 1A). A key question, left unanswered by our previous studies, was how left/right asymmetric expression of mir-273, the most upstream miRNA in the cascade shown in Fig. 1A, is regulated. Differential expression of mir-273 in ASER vs. ASEL can be examined with a reporter gene construct in which the expression of regulatory loop components under the control of heterologous, postmitotically active promoters was shown to rescue the respective mutant phenotypes (4–6).

Surprisingly, the expression of genes that are exclusively expressed in adult ASEL neurons is initially observed in both ASEL and ASER throughout late embryonic and early larval stages (gcv-6, gcv-7, lsy-6, and lim-6 were tested). Moreover, the adult-ASER-specific regulatory factor cog-1 is also expressed bilaterally in embryonic and early larval stages (Figs. 1D and 6). These data indicate the existence of a hybrid precursor state adopted by both neurons after their birth. To corroborate this notion, we examined temporal expression profiles in lsy-6 null mutants that are characterized by bilateral expression of the ASER marker gcv-5 (5). When fused to the ASEL and ASER (5). When fused to the ASER-promoter construct, but also likely extends to endogenous expression, because the activation of the cog-1 promoter in both ASEL and ASER (5).

Sample size, n = 34 to >100 adult animals. (A) lsy-6 and cog-1 are required for the asymmetric expression of mir-273prom:gfp. (B) die-1, lsy-6, and cog-1 are required for asymmetric expression of the die-1 sensor gene. (C) cog-1, lsy-6, and cog-1 are required for asymmetric expression of lsy-6prom:gfp. (D) Model that summarizes genetic regulatory interactions.

which gfp mRNA is equally expressed under the control of the ceh-36 promoter in both ASEL and ASER (5). When fused to the 3’ UTR of die-1 (ceh-36prom:gfp::die-13’UTR), expression of the reporter construct is down-regulated in ASER, but not ASEL; this down-regulation depends on mir-273 target sites (5). We find that the asymmetry of ceh-36prom:gfp::die-13’UTR expression is lost and that gfp expression levels are low in ASEL and ASER in lsy-6 null mutants. This observation suggests that the transcriptional derepression of mir-273 in ASEL seen in lsy-6 mutants results in subsequent translational repression of die-1 through its 3’ UTR (Fig. 2B). lsy-6-mediated repression of die-1 expression cannot only be inferred with the die-1 3’ UTR reporter construct, but also likely extends to endogenous die-1 expression, because the activation of the lsy-6 promoter, which is controlled by die-1 (5), is lost in lsy-6 mutants (Fig. 2C). The lsy-6 miRNA therefore indirectly regulates the activity of its own promoter. Last, the effects of lsy-6 on the functional output of mir-273 (= die-1 repression) can also be observed in die-1 mutants. die-1 therefore regulates the activity of its own 3’ UTR (Fig. 2B).

How does lsy-6 repress mir-273 expression to positively regulate its own expression? The most obvious candidate to mediate this effect is the cog-1 homeobox gene, a direct target of the lsy-6 miRNA (6). If derepression of mir-273 activity in ASEL, observed in lsy-6 mutants, is indeed due to an increase in cog-1 function, one would expect that a loss of cog-1 would cause the opposite effect, that is, a loss of mir-273 activity in ASER. In cog-1 null mutants, we indeed observe a disruption of asymmetric mir-273 expression as well as a loss of mir-273 function as demonstrated by a derepression of the die-13’UTR sensor construct in ASER (Fig. 2B). Last, as expected from an ectopic activation of die-1 expression in cog-1
die-1 Is the Output Regulator of Downstream Asymmetrically Expressed Genes. Regulatory loops must contain output regulator(s) of downstream effectors. In our system, we define effector genes as those genes that either are not an integral part of the loop and/or define the terminal fate of the neuron. The output regulator of the loop that we describe here is not immediately obvious because disruption of the activity of any loop member will disrupt expression of the effector genes, likely due to the deregulation of the unknown output regulator.

Through genetic epistasis analysis, we infer that die-1 is the likely output regulator of the loop. In cog-1 null mutants, lim-6 is ectopically expressed in ASER. Genetic removal of die-1 in cog-1 null mutants causes a complete loss of lim-6 expression in both ASEL and ASER (Fig. 3A). die-1 is therefore required for cog-1 to exert its effect, consistent with the notion that die-1 is the output regulator of the effector genes. Because mir-273 function is lost in cog-1 mutant animals (Fig. 2B), the suppression of cog-1 by die-1 therefore also demonstrates that mir-273 cannot be the output regulator of the loop. The epistatic nature of die-1 was corroborated with additional sets of experiments. Overexpression of die-1 in ASER activates lim-6 expression in ASER (Fig. 3A). This effect does not require the lys-6-mediated repression of cog-1 expression, because the effect of ectopic die-1 expression persists in lys-6 null mutants, excluding the possibility that lys-6 is the output regulator of the loop (Fig. 3A). Last, we extended our marker analysis from the ASEL marker lim-6 to the ASER marker gcy-5. We find that die-1 shows the same epistatic relationship in terms of its effect on gcy-5. die-1 regulates gcy-5 independent of the lys-6-mediated repression of cog-1 (Fig. 3B).

Taken together, these data indicate that die-1 is the output regulator of the downstream effector genes (Fig. 4D). We cannot however exclude the possibility that cog-1 also has a direct effect on effector gene expression (“?” in Fig. 4D). ASEL-specific effector gene expression in ASEL may require both the presence of the activator die-1 and the absence of the repressor cog-1, whereas repression of ASEL-specific effector genes in ASER may require both the absence of the activator die-1 and the presence of the repressor cog-1.

Additional Feedback Interactions in the Regulatory Loop. Additional regulatory interactions of individual loop components provide further levels of complexities to the architecture of the regulatory loop. These regulatory interactions involve the cog-1 and lim-6 homeobox genes. Apart from translational autoregulation mediated by the feedback loop and the lys-6 miRNA (Fig. 2D), cog-1 also regulates its own expression on the transcriptional level. Specifically, we find that cog-1 reporter gene expression is lost in ASER in cog-1 null mutants and that this loss is not due to feedback regulation through the loop because expression is still lost when lys-6 is completely removed in a cog-1 mutant background (Fig. 7, which is published as supporting information on the PNAS web site). We infer that cog-1 positively regulates its own transcription. This conclusion also provides an explanation for the seemingly puzzling observations that transcription of the cog-1 locus, assayed with cog-1prom::gfp, is left/right asymmetric and affected in lys-6 mutants. ASEL-specific lys-6 first affects translation of the COG-1 protein which, in turn, affects transcription of the cog-1 locus in a lys-6-independent manner.

Another feedback interaction is exerted by the lim-6 gene. Complete loss of the activity of the ASEL-expressed lim-6 gene in lim-6(nr2073) null mutants causes a partial deregulation of several components of the loop, but it does not affect the expression of the ASEL effector genes gcy-6 or gcy-7 or the ASER effector gene hmn-1ASER (Fig. 1B; see also Fig. 8, which is published as supporting information on the PNAS web site). Two observations indicate that lim-6 may affect this regulatory network in a complex manner involving regulation of several genes in temporally independent ways. lim-6 null mutants display an almost complete derepression of gcy-5 in ASEL throughout development and adulthood, yet the feedback regulation of lim-6 on its own expression can only be observed in adult animals (Fig. 8 and data not shown). Taken together, lim-6 appears to augment the activity of the loop but does not affect its overall output (dashed line in Fig. 4D).

Discussion

A central question in biology concerns the transitions of reversible biochemical events into irreversible biological states, such as terminal cell fates in multicellular organisms. Using simple biochemical processes in bacteria as paradigms, Monod and Jacob (15) proposed that it is the interaction of gene regulatory
those shown here because genetic screens for mutants affecting ASEL bistable system. The feedback loop is likely to contain more components than (unpublished data).

outputs (11, 14, 18). (of the individual interaction, loops can either produce stable or oscillating lead into or out of the loop from either ''A'' or ''B.'' Depending on the signs Johnston et al. The ASEL

miRNAs can indirectly regulate their own expression through

stability of the system. Last, our findings firmly place miRNAs

negative feedback loop, that provides the molecular basis for

regulating transcription factors are also used in the determina-

bistable systems as defined in ref. 14. It is a system that

transitions from an equipotent precursor state to a stable end

state that is stabilized by a feedback loop (Fig. 4D). The system

is balanced, because neither state is favored (ASEL always

expresses ASEL features and ASER always expresses ASER

features). Once established, the feedback loop guarantees irre-

versibility of the system. The irreversibility of the system, an

extreme form of hysteresis (14), is not only observed in wild-type

animals but can also be inferred from the effect of removal of

lim-6, a terminal effector gene, whose activity generates a nested

feedback loop (Fig. 4D). lim-6 mutants display a partial disruption in the expression of regulatory loop components but still appropriately express the stable end states as measured by the correct expression of the ASEL markers gcy-7 and gcy-6 and the ASER marker hen-1. One explanation for this observation would be that once the system is established, it is difficult to flip to the alternate state.

Another feature of the few well characterized bistable systems are nonlinear response dynamics, or "ultrasensitivity" (14), defined as the ability of the system to amplify initial inputs into the system. As suggested in ref. 18, transcription factor-based bistable systems are ideally suited to provide such nonlinear response dynamics due to the often observed cooperativity of transcription factor action. The identification of more transcription factors whose activity is required for the regulatory loop (ref. 4 and R.J.J. and O.H., unpublished data) suggests the existence of such cooperative effects in the ASEX system as well.

It is tempting to view a large number of cell fate decisions as bistable systems. For example, in analogy to the phage lambda system, double negative feedback loops that involve cross-regulating transcription factors are also used in the determination of several alternative neuronal fates in the vertebrate spinal cord that may also constitute bistable systems (19, 20). Other bistable systems that control alternative fate decisions involve both signaling proteins as regulatory loop components, such as the AC/VU cell fate decision in C. elegans vulval development (Fig. 4C). All these examples follow the initial prediction of Monod and Jacob that gene regulatory feedback loops are a key feature of cellular differentiation events in multicellular organisms (15).

We can infer from our mutant analysis that the double-
negative feedback loop operating in ASEL/R is not merely required to maintain asymmetry. In Isy-6 mutants, the ASE neurons are completely bilaterally symmetric at all stages. After the birth of the neuron, both neurons express ASEL and ASER fate markers (equipotent hybrid precursor state) and then both transition to the ASER state at the same rate. Regulatory components of the loop are therefore clearly required for the establishment of the asymmetry, but the key question concerning the trigger of differential activity of the regulatory feedback loop components still remains to be answered. This question also is poorly understood in other apparently bistable, cell fate-determining systems. For example, the differential activity of the lin-12 system in the AC/VU cells is triggered by unknown means through a birth-order bias of the AC/VU cells (21). We note that the ASE feedback loop is large, containing at least four, and likely many more, components (Fig. 4D). In theory, the multi-component nature of feedback loops allows for the introduction of multiple checkpoints through which the system could be regulated. Checkpoints could be regulated by specific external signals that bias the transition from the hybrid precursor state to a specific stable end state. Although such signals are not yet known in the context of ASEL/R development, two characteristics of such inputs are apparent: (i) the input only needs to be transient and can therefore be restricted to a specific developmental time window; (ii) the input must be spatially stereotyped because the left ASE neuron always takes on the ASEL cell fate and the right ASE neuron always takes on the ASER cell fate. This result differs from the stochastic, lin-12-controlled AC/VU bistable system in which either vulval precursor can take on the AC or VU fate (Fig. 4C). Another difference from the AC/VU system is that there is no communication between ASEL and ASER; each cell can adopt its fate in the absence of the other (R.J.J. and O.H., unpublished data). The multi-component nature of the regulatory loop also allows for multiple outputs from the loop. Although our analysis suggests that die-1 is the likely output regulator for some of the effector genes, it is conceivable that other as yet unknown effector genes may be regulated by other loop components.

miRNAs and Gene Regulatory Networks. Transcription factors have served as paradigms to understand how gene regulatory components are placed into defined regulatory networks. Intriguingly, transcription factor activities are linked to one another in a surprisingly limited number of network motifs, including feed-forward loops, bi-fans, single-input modules, multicomponent loops, and others (22–24). Many of these network motifs can be described and modeled in mathematical terms, thus revealing the underlying design principles of such motifs (16, 25). Feedback loops constitute a commonly observed network motif and are a defining feature of all of the above-mentioned bistable systems. We have described here that miRNAs can, similar to transcription factors, be components of such defined network motifs.

The placement of at least two miRNAs into a double-negative feedback loop also reveals a potential mechanism by which miRNAs can positively autoregulate their transcription, albeit indirectly. Positive autoregulation of transcription factors is a commonly observed phenomenon in cell fate specification (26) that has not been observed for miRNAs before. Negative autoregulation of miRNAs has been observed in plants in which the mir-159 miRNA represses its own activator, the transcription factor GAMYB (27), and also possibly exists in C. elegans (28). In contrast to the stable switch controlled by positive or double-negative feedback loops, such simple negative feedback loops are predicted to produce either transient or oscillating outputs (Fig. 4A).

In summary, our placement of miRNAs into a double-negative feedback loop provides a mechanism for how miRNAs can determine terminally stable end states. It remains to be seen whether miRNA-mediated negative feedback loops may represent a general paradigm for how neurons select amongst alternative fates and lock into stable states.

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