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

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Communicated by Thomas M. Jessell, Columbia University College of Physicians and Surgeons, New York, NY, July 12, 2005 (received for review April 27, 2005)

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/R asymmetry (Fig. 1*A*). 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. 1*A*, 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 autoregulate 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-6^{prom}::gfp, rol-6 (d)*], *syIs63* [*cog-1::gfp*; *dpy-20* (+)], *syIs73* [*cog-1^{prom}::gfp*; *dpy-20* (+)], *otEx1749* [*mir-273^{prom2}::gfp*; *unc-122^{prom}:gfp*], *otEx1759* [*ceh-36^{prom}::gfp::die-1$^{3'UTR}$*; *rol-6* (*d*)], *ntIs1* [*gcy-5^{prom}::gfp*; *lin-15* (+)], *otIs3* [gcy-7^{prom}::*gfp*; *lin-15* (+)], *otIs162* [*gcy-6^{prom}::gfp*; *lin-15* (+)], *otIs160* [*lsy-6^{prom}::gfp*; *unc-122^{prom}:gfp*], *otIs151* [*ceh-36^{prom}::rfp*; *rol-6* (*d*)], *otEx1382* [*ceh-36^{prom}::lsy-6*; *rol-6* (*d*)], *otEx959* [*die-1::gfp*; *rol-6* (*d*)], *otEx1192* [*gcy-5^{prom}::die-1*; *unc-122^{prom}:gfp*], *ynIs54* [*flp-20^{prom}::gfp*], *ynIs* [*flp-4^{prom}::gfp*]. The *otEx2302* [*gcy-22^{prom}::gfp*; *unc-122^{prom}:gfp*] transgene contains 2 kb of the *gcy-22* promoter. *hen-1^{ASER}::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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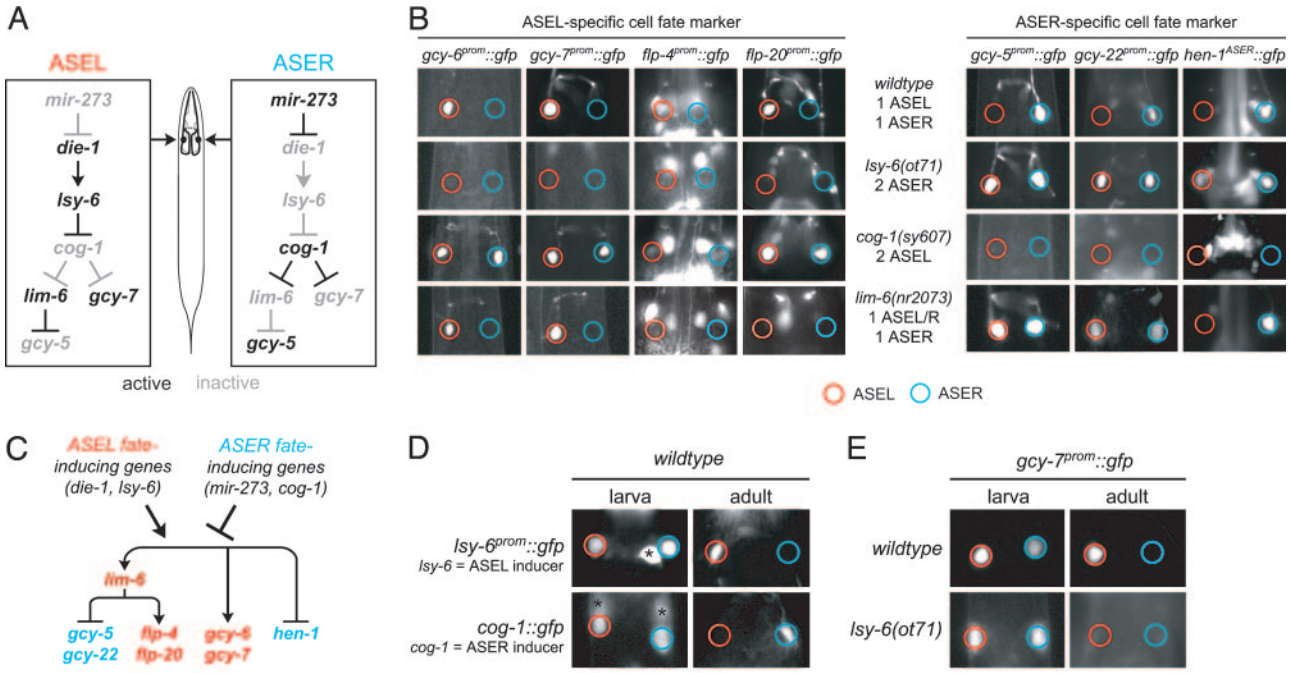

Fig. 1. Mutations in *lsy* genes cause a state transition between the ASEL and ASER fates. (*A*) Gene regulatory factors controlling ASE laterality, as deduced by our previous genetic analysis (3–6). The permissively acting, ASEL/R-expressed genes *unc-37*/*Groucho*, *lin-49*, and *ceh-36* (4) are left 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 from the *hen-1* promoter (*hen-1*^{ASER}*::gfp*). 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 ASEL 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 guanyl 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. 1*B*). Moreover, reexamining the expression of a number of genes previously reported to be expressed in amphid sensory neurons (8), we find that two genes, *flp-4* and *flp-20*, which code for FMRFamide-type neuropeptides, are expressed in ASEL but not ASER (Fig. 1*B*). Last, a transcriptional regulatory region from the *hen-1* gene, which codes for a secreted low-density lipoprotein-receptor motif protein (10), exclusively monitors the ASER cell fate (Fig. 1*B*).

To address whether the ASEL-specific *flp-4*, *flp-20*, and previously unexamined *gcy-6* reporters and the ASER-specific *gcy-22* and *hen-1*^{ASER} 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-1*^{ASER} is lost (Fig. 1*B*). In contrast, in *lsy-6* null mutant animals (class II mutant), the expression of the ASEL markers *flp-4*, *flp-20*, and *gcy-6* is lost in ASEL, with a concomitant gain of the ASER markers *gcy-22* and *hen-1*^{ASER} (Fig. 1*B*). *flp-4*, *flp-20*, *gcy-6*, *gcy-7*, *gcy-5*, *gcy-22* and *hen-1*^{ASER} are therefore terminal markers of two alternative states, regulated by class I and class II genes (Fig. 1*C*).

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. 1*B*). 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 ASER fate markers *gcy-5* and *gcy-22*, the *lim-6* gene has no role in repressing the other known ASER terminal marker *hen-1*^{ASER} (Fig. 1*B*). 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. 1*C*). 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 ASEL and ASER Neurons Progress Through an Equipotent Precursor State. So far, all of our analyses of the ASE fate decision have exclusively focused on adult animals that express the terminal end state of the system, characterized by the 100% stereotyped expression of terminal effector genes in ASEL or ASER throughout adulthood. These end states could, in theory, develop in two distinct manners: (*i*) After their birth, each neuron could immediately adopt either the ASEL or ASER fate, which is then maintained throughout the life of the neurons; or (*ii*) as a reflection of their equipotency, each neuron may initially pass through a mixed, hybrid state, in which it expresses both ASEL and ASER fate markers, and subsequently selects only one of the two states. To address this issue, we characterized the expression of regulatory factors and terminal effector genes at embryonic (before and after the birth of the ASE cells) and early larval stages. Expression of effector genes and regulatory loop components can first be observed in the 3-fold stage (500 min after fertilization), long after the ASE neurons are born (350 min after fertilization) (Fig. 6). This 2.5-h lag in onset of reporter gene expression can only in part be explained by the estimated <30-min-long maturation time of the GFP chromophore, indicating that the ASEL/R fate determinants act postmitotically. This notion is supported by experiments 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 (*gcy-6*, *gcy-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. 1*D* 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 *gcy-5* and a complete lack of expression of the ASEL-specific markers *gcy-7* and *lim-6* in the adult stage (6). We find that in the complete absence of *lsy-6*, these ASEL-specific markers are expressed normally in both ASEL and ASER in embryonic and early larval stages (Fig. 1*E*). Therefore, even in a situation where both ASE neurons are fated to eventually express the ASER fate throughout their adult life, they will nevertheless pass through the hybrid ASEL/R stage.

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. 1*A*). 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. 1*A*, is regulated. Differential expression of *mir-273* in ASER vs. ASEL can be examined with a reporter gene construct in which transcriptional regulatory regions of the *mir-273* locus are fused to *gfp* (5). Surprisingly, we find that left/right asymmetry of *mir-273* expression is lost in animals that lack the *lsy-6* miRNA, such that *mir-273* expression is derepressed in ASEL (Fig. 2*A*). Together with our previous finding that *mir-273* can regulate *lsy-6* expression through negative regulation of *die-1* (5), this finding demonstrates that left/right asymmetric expression of either miRNA depends on the left/right asymmetric expression of the other miRNA.

We corroborated this notion by examining the functional output of *mir-273* expression. *mir-273* can affect expression of the *die-1* gene by binding to two complementary sites in the *die-1* 3′ UTR, an event that can be assayed with a "sensor gene" in 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-36*^{prom}::*gfp*::*die-1*$^{3'UTR}$), 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-36*^{prom}::*gfp*::*die-1*$^{3'UTR}$ 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. 2*B*). *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. 2*C*). 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. 2*B*).

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-1*$^{3'UTR}$ sensor construct in ASER (Fig. 2*B*). Last, as expected from an ectopic activation of *die-1* expression in *cog-1*

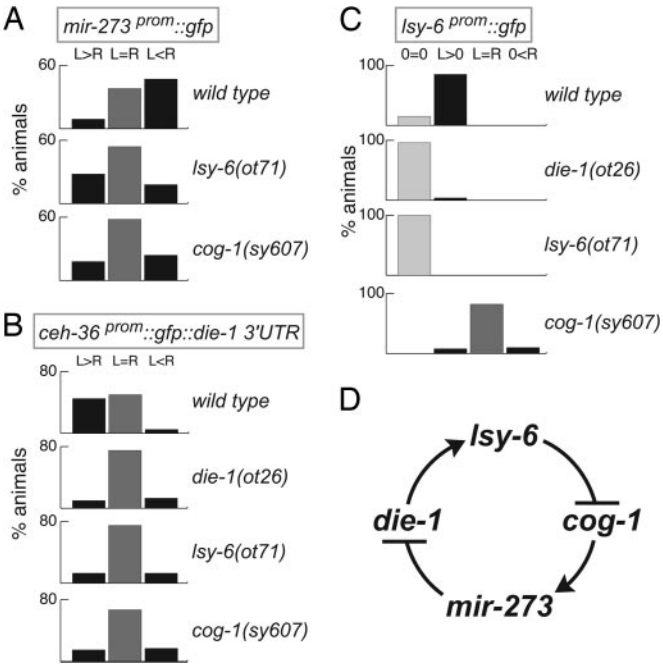

Fig. 2. The miRNAs *lsy-6* and *mir-273* act in a bistable feedback loop. "L > R" and "L < R" refer to relative *gfp* expression levels in ASEL vs. ASER, "L = R" to equal *gfp* levels (weak or strong), and "0 = 0" to no expression (used only in *C* because of potential mosaicism of the extrachromosomal lines in *A* and *B*). Sample size, $n = 34$ to >100 adult animals. (*A*) *lsy-6* and *cog-1* are required for the asymmetric expression of *mir-273*^{prom}::*gfp*. (*B*) *die-1*, *lsy-6*, and *cog-1* are required for asymmetric expression of the *die-1* sensor gene. (*C*) *die-1*, *lsy-6*, and *cog-1* are required for asymmetric expression of *lsy-6*^{prom}::*gfp*. (*D*) Model that summarizes genetic regulatory interactions.

mutants, we observe a gain of *lsy-6* expression in the ASER neuron of *cog-1* mutants (Fig. 2*C*).

The genetic data presented here demonstrate that *lsy-6*, *cog-1*, *mir-273*, and *die-1* act in a regulatory double-negative feedback loop (Fig. 2*D*). In this way, expression of the ASEL-inducing genes, *lsy-6* and *die-1*, defines and determines the stable ASEL state. In *lsy-6* and *die-1* mutants, which display a "2 ASER" phenotype, there is a complete loss of expression of not only the downstream ASEL effector genes but also ASEL-inducing loop components. Consequently, all known ASER-specific genes are derepressed in ASEL in these mutants. Similarly, expression of the ASER-inducing genes, *cog-1* and *mir-273* (and, likely other, *mir-273*-related miRNAs, D. Didiano and O.H., unpublished data), defines and determines the alternate stable ASER state. In *cog-1* mutants, expression of ASER inducer and effector genes is affected, with a concomitant derepression of ASEL-specific gene expression in ASER. In summary, the ASEL/R cell fate choice is a system in which ASEL- or ASER-inducing genes activate other inducer and effector genes to determine one of two alternate states. What triggers the left/right differential activity of these factors is not known.

***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. 3*A*). *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. 2*B*), 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. 3*A*). This effect does not require the *lsy-6*-mediated repression of *cog-1* expression, because the effect of ectopic *die-1* expression persists in *lsy-6* null mutants, excluding the possibility that *lsy-6* is the output regulator of the loop (Fig. 3*A*). 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 *lsy-6*-mediated repression of *cog-1* (Fig. 3*B*).

Taken together, these data indicate that *die-1* is the output regulator of the downstream effector genes (Fig. 4*D*). We cannot however exclude the possibility that *cog-1* also has a direct effect on effector gene expression ("?" in Fig. 4*D*). 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 *lsy-6* miRNA (Fig. 2*D*), *cog-1* also regulates its own expression on the transcriptional

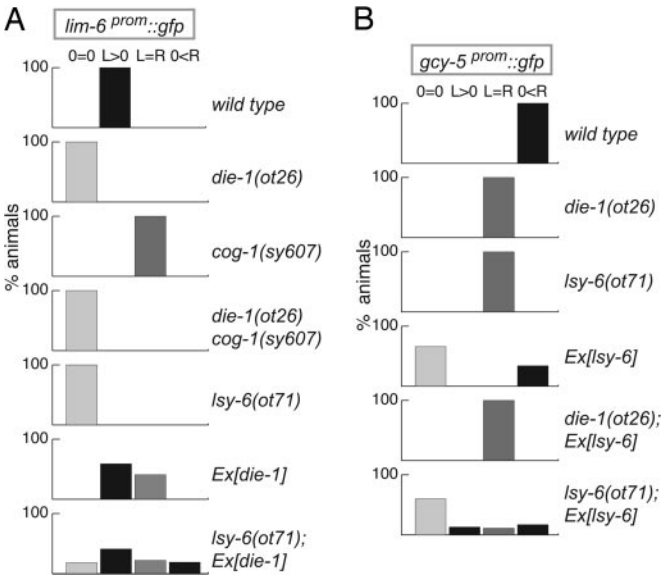

Fig. 3. *die-1* is the output regulator of effector genes. "0 = 0" refers to no *gfp* expression, "L > 0" or "0 < R" to exclusive *gfp* expression in ASEL or ASER, and "L = R" to equal expression. Sample size, $n = 32$ to >100 adult animals. (*A*) *cog-1* requires *die-1* and *die-1* does not require *lsy-6* to regulate *lim-6*. Ectopic expression of *die-1* in ASER is observed in transgenic animals, which carry extra copies of the *die-1* genomic locus (*Ex*[*die-1*]). These animals activate *lim-6*^{prom}::*gfp* independent of *lsy-6*. (*B*) *lsy-6* requires *die-1* to regulate *gcy-5* expression. *lsy-6* was ectopically expressed in both ASEL and ASER under control of the *ceh-36* promoter (*Ex*[*lsy-6*]).

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 *lsy-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-1*^{prom}::*gfp*, is left/right asymmetric and affected in *lsy-6* mutants. ASEL-specific *lsy-6* first affects translation of the COG-1 protein which, in turn, affects transcription of the *cog-1* locus in a *lsy-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 *hen-1*^{ASER} (Fig. 1*B*; 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. 4*D*).

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

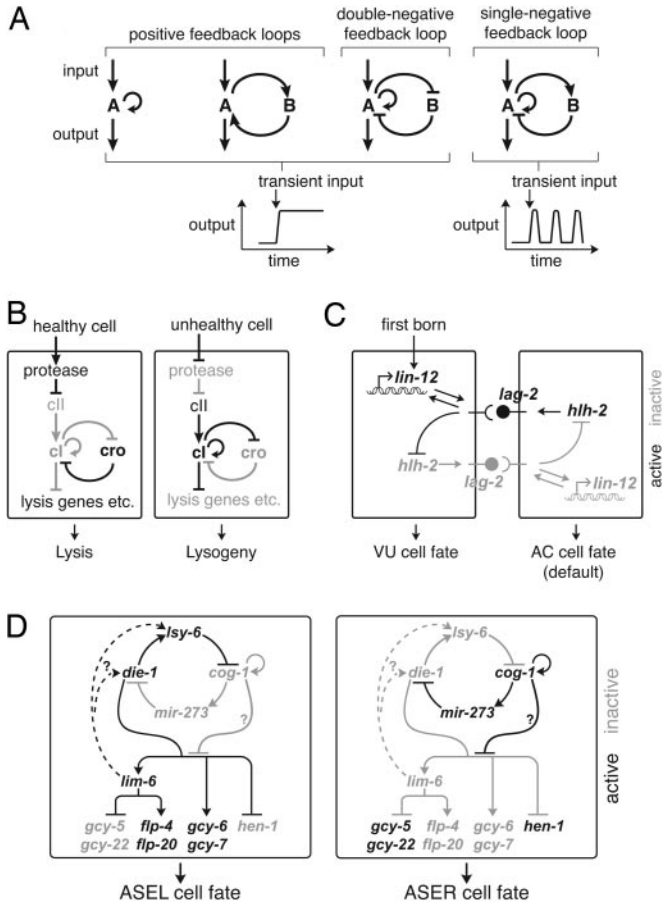

Fig. 4. Feedback loops and bistable systems. (*A*) Architecture of different types of feedback loops. In the multicomponent loops, inputs and outputs can lead into or out of the loop from either ''A'' or ''B.'' Depending on the signs of the individual interaction, loops can either produce stable or oscillating outputs (11, 14, 18). (*B*) The phage lambda system displays bistable behavior regulated by a simple double-negative feedback loop (12). (*C*) In the AC/VU cell fate decision in vulval development in *C. elegans*, two equipotent germline precursor cells interact through the Notch/*lin-12* receptor and its ligand Delta/*lag-2* to induce two distinct cell fates (13). (*D*) Summary of the ASE bistable system. The feedback loop is likely to contain more components than those shown here because genetic screens for mutants affecting ASEL/R asymmetry uncovered a number of additional, as yet uncharacterized genes (unpublished data).

factors in feedback loops that may be the mechanism responsible for creating terminally differentiated cellular states in multicellular organisms. Our findings not only confirm this hypothesis, but refine our understanding of feedback regulation in terminal cell fate determination. Specifically, we have defined the ASEL/R cell fate decision as a system that progresses from an equipotent, hybrid precursor state into one of two alternative end states. We have placed several previously known gene regulatory factors into a regulatory network motif, a double-negative feedback loop, that provides the molecular basis for the stability of the system. Last, our findings firmly place miRNAs into a defined gene regulatory network and demonstrate that miRNAs can indirectly regulate their own expression through double-negative feedback loops. We will discuss these points in more detail below.

The ASEL/R Cell Fate Decision May Constitute a Bistable System. The concept of bistability has been used to describe a select number of signal transduction and transcriptional regulatory events (14, 16, 17). Bistability denotes a system that exists almost exclusively in one of two possible states. These individual states are stabilized by feedback loops, which can either be positive or double-negative in nature (Fig. 4*A*). Depending on the strength of the feedback loop, the bistable system may be irreversible. From this loop, an output(s) affects downstream terminal targets. An upstream input into the feedback loop, dependent on signaling surpassing a given threshold, determines which of the two possible states is adopted. The stable nature of the feedback mechanism enables the system to respond to transient inputs provided, for example, in the form of a signal at a specific stage of development (Fig. 4*A*). The ability to respond to transient signals and translate them into stable end states illustrates that the concept of bistability may provide a useful formalism to describe a wide variety of cell fate decisions.

One prominent type of bistable system involves cellular differentiation from an equipotent precursor state into one of two discrete end states. Relatively simple systems of this type involve two transcription factors that repress each other's expression. A classic example is the decision of phage lambda to enter either the lytic or lysogenic state (Fig. 4*B*). This bistable system exhibits the criteria described above: the lytic or lysogenic states are balanced such that either state can be selected dependent on the health of the cell, state transitions are rapid, hysteresis is achieved, the *cI*/*cro* interactions constitute a stabilizing double-negative feedback loop, and *cI* controls the output of the loop.

The ASEL/R system appears to fulfill several key criteria of 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. 4*D*). 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 irreversibility 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. 4*D*). *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 ASE 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. 4*C*). 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 *lsy-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. 4*D*). In theory, the multicomponent 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. 4*C*). 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 multicomponent 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. 4*A*).

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.

We thank G. Miesenböck and U. Alon for stimulating discussions on network architecture and T. Jessell, V. Ambros, P. Sengupta, S. Pfaff, J. Briscoe, C. Desplan, J. Collins, and members of the O.H. laboratory for comments on the manuscript. This work was funded by National Institutes of Health Grant R01 NS050266-01 (to O.H.) and predoctoral fellowships from the National Science Foundation (to R.J.J.) and the National Institutes of Health (to S.C.).

1. Yu, S., Avery, L., Baude, E. & Garbers, D. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3384–3387.
2. Pierce-Shimomura, J. T., Faumont, S., Gaston, M. R., Pearson, B. J. & Lockery, S. R. (2001) *Nature* **410,** 694–698.
3. Hobert, O., Tessmar, K. & Ruvkun, G. (1999) *Development (Cambridge, U.K.)* **126,** 1547–1562.
4. Chang, S., Johnston, R. J., Jr., & Hobert, O. (2003) *Genes Dev.* **17,** 2123–2137.
5. Chang, S., Johnston, R. J., Frokjaer-Jensen, C., Lockery, S. & Hobert, O. (2004) *Nature* **430,** 785–789.
6. Johnston, R. J. & Hobert, O. (2003) *Nature* **426,** 845–849.
7. Ambros, V. (2004) *Nature* **431,** 350–355.
8. Kim, K. & Li, C. (2004) *J. Comp. Neurol.* **475,** 540–550.
9. Palmer, R. E., Inoue, T., Sherwood, D. R., Jiang, L. I. & Sternberg, P. W. (2002) *Dev. Biol.* **252,** 202–213.
10. Ishihara, T., Iino, Y., Mohri, A., Mori, I., Gengyo-Ando, K., Mitani, S. & Katsura, I. (2002) *Cell* **109,** 639–649.
11. Thomas, R. (1978) *J. Theor. Biol.* **73,** 631–656.
12. Arkin, A., Ross, J. & McAdams, H. H. (1998) *Genetics* **149,** 1633–1648.
13. Greenwald, I. (1998) *Genes Dev.* **12,** 1751–1762.
14. Ferrell, J. E., Jr. (2002) *Curr. Opin. Cell Biol.* **14,** 140–148.
15. Monod, J. & Jacob, F. (1961) *Cold Spring Harbor Symp. Quant. Biol.* **26,** 389–401.
16. Tyson, J. J., Chen, K. C. & Novak, B. (2003) *Curr. Opin. Cell Biol.* **15,** 221–231.
17. Hasty, J., McMillen, D., Isaacs, F. & Collins, J. J. (2001) *Nat. Rev. Genet.* **2,** 268–279.
18. Smolen, P., Baxter, D. A. & Byrne, J. H. (2000) *Neuron* **26,** 567–580.
19. Briscoe, J., Pierani, A., Jessell, T. M. & Ericson, J. (2000) *Cell* **101,** 435–445.
20. Dasen, J. S., Liu, J. P. & Jessell, T. M. (2003) *Nature* **425,** 926–933.
21. Karp, X. & Greenwald, I. (2003) *Genes Dev.* **17,** 3100–3111.
22. Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D. & Alon, U. (2002) *Science* **298,** 824–827.
23. Lee, T. I., Rinaldi, N. J., Robert, F., Odom, D. T., Bar-Joseph, Z., Gerber, G. K., Hannett, N. M., Harbison, C. T., Thompson, C. M., Simon, I., *et al.* (2002) *Science* **298,** 799–804.
24. Bolouri, H. & Davidson, E. H. (2002) *Bioessays* **24,** 1118–1129.
25. Mangan, S. & Alon, U. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 11980–11985.
26. Edlund, T. & Jessell, T. M. (1999) *Cell* **96,** 211–224.
27. Achard, P., Herr, A., Baulcombe, D. C. & Harberd, N. P. (2004) *Development (Cambridge, U.K.)* **131,** 3357–3365.
28. Grosshans, H., Johnson, T., Reinert, K. L., Gerstein, M. & Slack, F. J. (2005) *Dev. Cell* **8,** 321–330.