A novel *C. elegans* zinc finger transcription factor, *lsy-2*, required for the cell type-specific expression of the *lsy-6* microRNA

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Summary

The two *Caenorhabditis elegans* gustatory neurons, ASE left (ASEL) and ASE right (ASER) are morphologically bilaterally symmetric, yet left/right asymmetric in function and in the expression of specific chemosensory signaling molecules. The ASEL versus ASER cell-fate decision is controlled by a complex gene regulatory network composed of microRNAs (miRNAs) and transcription factors. Alterations in the activities of each of these regulatory factors cause a complete lateral cell-fate switch. Here, we describe *lsy-2*, a novel C2H2 zinc finger transcription factor that is required for the execution of the ASEL stable state. In *lsy-2* null mutants, the ASEL neuron adopts the complete ASER gene expression profile, including both upstream regulatory and terminal effector genes. The normally left/right asymmetric ASE neurons are therefore 'symmetrized' in *lsy-2* mutants. Cell-specific rescue experiments indicate that *lsy-2* is required autonomously in ASEL for the activation of ASEL-specifying factors and the repression of ASER-specifying factors. Genetic epistasis experiments demonstrate that *lsy-2* exerts its activity by regulating the transcription of the *lsy-6* miRNA in the ASEL neuron, thereby making *lsy-2* one of the few factors known to control the cell-type specificity of miRNA gene expression.

Key words: *C. elegans*, Chemosensory neurons, Laterality, Fate determination, MicroRNA

Introduction

Monod and Jacob proposed more than 40 years ago that the terminal differentiated state of a cell in a multicellular organism is determined and stabilized by feedback loops composed of gene regulatory factors (Monod and Jacob, 1961). The autoregulation of transcription factors has now indeed been recognized as a major feature of gene differentiation programs (Davidson, 2001; Edlund and Jessell, 1999).

We have recently identified a complex autoregulatory feedback loop that controls a cell-fate decision in the nervous system of the nematode *Caenorhabditis elegans* (Fig. 1A) (Chang et al., 2004; Chang et al., 2003; Hobert et al., 1999; Johnston and Hobert, 2003; Johnston et al., 2005). The two morphologically bilaterally symmetric taste-receptor neurons ASEL and ASER develop from a common ground state to express a number of features that are specific for ASEL versus ASER (Fig. 1A). These left/right asymmetric features include the expression of several putative chemoreceptors of the GCY family. For example, in adult animals, the *gcy-7* gene is exclusively expressed in ASEL, whereas the *gcy-5* gene is exclusively expressed in ASER (Fig. 1). The expression of these two terminally differentiated states requires the activity of a set of gene regulatory factors that interact with one another in a bistable, double-negative feedback loop (Johnston et al., 2005). In this loop, ASEL-specific inducer genes, including the *die-1* zinc finger transcription factor and the *lsy-6* miRNA, activate other ASEL-specific inducer and effector genes, while repressing ASER-inducers and ASER-effectors in the ASEL neuron. By contrast, in the ASER neuron, ASER-inducer genes, including the *cog-1* homeobox gene and the *mir-273* miRNA, activate ASER-inducers and effectors, while repressing the ASEL-inducing genes *die-1* and *lsy-6* (Fig. 1A).

What triggers the left/right asymmetric activity of the loop? Are there other, as yet unknown, factors that are components of the regulatory loop, and/or required for the loop to function appropriately? To address these questions, we have isolated and analyzed mutants in which the ASEL and/or ASER cell fates are not appropriately executed. We describe here one factor, *lsy-2*, that is required for the execution of the ASEL fate. *lsy-2* codes for a novel C2H2 zinc finger transcription factor that is not an integral part of the regulatory loop. Rather, *lsy-2* constitutes a permissive factor that is present in both ASEL and ASER, but is required specifically in ASEL for the execution of the ASEL fate. Furthermore, we show that *lsy-2* exerts its activity by regulating the expression of the *lsy-6* miRNA.

miRNAs are abundant gene regulatory factors that contribute to the generation of cellular and morphological diversity in a developing organism (Ambros, 2004). Like other gene regulatory factors that contribute to organismal complexity, many, if not most, miRNAs are expressed in a spatially and temporally tightly controlled manner (e.g. Wienholds et al., 2005), yet the mechanisms that control miRNA gene

expression are only beginning to be elucidated. Our identification of *lsy-2* as a regulator of the *lsy-6* miRNA therefore contributes to our understanding of the control of miRNA expression, and of the complex regulatory networks necessary for terminal cell-fate specification.

Materials and methods

Strains and transgenes

Previously described strains and transgene: N2 Bristol wild type (Brenner, 1974), CB4856 Hawaiian wild-type isolate (Hodgkin and Doniach, 1997), NL2099 *rrf-3(pk1426)II* (Simmer et al., 2002), *otIs3=Is[gcy-7^{prom}::gfp; lin-15 (+)]*, *ntIs1=Is[gcy-5^{prom}::gfp; lin-15 (+)]*, *otIs114=Is[lim-6^{prom}::gfp; rol-6(d)]* (Chang et al., 2003), *otIs151=Is[ceh-36^{prom}::rfp; rol-6(d)]* (Johnston and Hobert, 2003), *otIs160=Is[lsy-6^{prom}::gfp; rol-6(d)]* (Johnston et al., 2005), *syIs63=Is[cog-1::gfp]* (Palmer et al., 2002), *otEx1759=Ex[ceh-36^{prom}::gfp::die-1$^{3'UTR}$; rol-6(d)]* (Chang et al., 2004).

New transgenes: *otEx2044=Ex[ceh-36^{prom}::lsy-6; elt-2::gfp]*; *otEx1322* to *otEx1324* – three independent lines of *Ex[lsy-2^{prom}::gfp; rol-6(d)]*; *otEx1325* to *otEx1328*, *otEx1777* to *otEx1780*, and *otEx1790* – nine independent lines of *Ex[lsy-2::gfp; rol-6(d)]*; *otEx1945* to *otEx1947*, and *otEx1954* – four independent lines of *Ex[ceh-36^{prom}::lsy-2; elt-2::gfp]*.

DNA constructs and generation of transgenic strains

lsy-2^{prom}::gfp, lsy-2::gfp and *ceh-36^{prom}::lsy-2* were constructed using a PCR fusion approach (Hobert, 2002). The *gfp* constructs are shown in Fig. 4A. *lsy-2^{prom}::gfp* was generated by fusing 3 kb upstream (up to the preceding gene) of the *lsy-2* gene to the *gfp*-coding region. The construct was injected at 50 ng/μl together with *rol-6(d)* as an injection marker (100 ng/μl). *lsy-2::gfp* was generated by fusing 3 kb of the upstream region and the all of the exons and introns of *lsy-2* to the *gfp*-coding region and the *unc-54* 3′UTR. The construct was injected at 5 ng/μl together with *rol-6(d)* as an injection marker (100 ng/μl). *ceh-36^{prom}::lsy-2* was generated by fusing the 5 kb *ceh-36* promoter (Chang et al., 2003) to the *lsy-2* cDNA, including the coding region and the 3′ UTR. The construct was injected at 5 ng/μl together with *rol-6(d)* as an injection marker (100 ng/μl).

Primer sequences (5′ to 3′)

lsy-2^{prom}::gfp

Primer A, GTTGAATCCGACTTCTTCAGGG;
Primer A*, GTTTCTAGCAATCTGGTTGTTG;
Primer B, CTAGAGTCGACCTGCAGGCCATGACAAAATTTGCCTCAGAC;
Primer C, AGCTTGCATGCCTGCAGGTCGACT;
Primer D, AAGGGCCCGTACGGCCGACTA;
Primer D*, GGAAACAGTTATGTTTGGTATATTGGG.

lsy-2::gfp

Primer A, GTTGAATCCGACTTCTTCAGGG;
Primer A*, GTTTCTAGCAATCTGGTTGTTG;
Primer B, CTAGAGTCGACCTGCAGGCAATCAACTGTGGTTCCATCATC;
Primer C, D and D*, as above.

ceh-36^{prom}::lsy-2

Primer A, CAAAAATGAGGCTACCAAG;
Primer A*, CAAAGTAGAGCACTGAGGGTG.
Primer B, CATTTCTTCTGGTTAGCATTTGTGCATGCGGGGGCAGG;
Primer C, CCTGCCCCCGCATGCACAAATGCTAACCAGAAGAAATG;
Primer D, GACTGCAAATGAGACAGTC;
Primer D*, GACGAAGACGACTCCATAG;

Primers were used as in PCR fusion reactions as previously described (Hobert, 2002).

RNA interference

RNAi was performed using a bacterial feeding protocol (Simmer et al., 2003). NGM agar plates containing 6 mM IPTG and 100 μg/ml ampicillin were seeded with bacteria expressing dsRNA, kindly provided by the Greenwald Laboratory. *otIs114; rrf-3* hermaphrodites at the L3/L4 stage were placed onto these plates and grown at 15°C. Adults were then transferred onto freshly seeded plates at 20°C, and their F1 progeny were scored for asymmetry and sterility defects. RNAi was performed in a *rrf-3(pk1426)* background because of its increased sensitivity to RNAi (Simmer et al., 2003).

Phenotypic analysis

Reporter transgenes were crossed into the respective mutant backgrounds. All animals were scored with an Axioplan 2 microscope. When needed, ASE neurons were unambiguously identified through the use of a transgene (*otIs151*) that expresses DsRedz bilaterally in ASEL and ASER, as well as in AWCL and AWCR, under control of the *ceh-36* promoter (Chang et al., 2003).

Results

lsy-2 is required for the execution of the ASEL neuronal cell fate

In order to better understand the mechanisms underlying the ASE left/right neuronal fate decision (Fig. 1A), we undertook a genetic screen to isolate mutants in which expression of the ASEL-specific *gcy-7* gene is disrupted. We exposed transgenic animals that contain the chromosomally integrated *gcy-7^{prom}::gfp* transgene *otIs3* to the mutagen ethylmethanesulphonate (EMS) (Brenner, 1974; Chang et al., 2003). F1 progeny of mutagenized L4 animals were singled out onto individual agar plates and the F2 progeny were screened for lateral symmetry (*lsy*) defects under a stereomicroscope equipped with a fluorescent light source. Screening through 12,200 haploid genomes, we identified a total of 10 mutant alleles that display a ‘two ASER’ phenotype in which the ASEL neuronal fate has switched to an ASER neuronal fate (see Fig. 1B for an example). One mutant, *ot71*, defines the *lsy-6* miRNA locus (Johnston and Hobert, 2003); three mutants, *ot69*, *ot74* and *ot78*, lie within the *lin-49* transcription factor locus (Chang et al., 2003); one mutant, *ot79*, defines the *ceh-36* homeobox locus (Chang et al., 2003); and five mutant alleles (*ot64*, *ot65*, *ot67*, *ot72* and *ot77*) define a novel, single genetic locus on the X chromosome. We focus here on the characterization of this novel locus, which we termed *lsy-2* (pronounced ‘*lousy two*’). In *lsy-2* mutants, ASEL no longer expresses the ASEL-specific terminal fate markers *gcy-6*, *gcy-7*, *lim-6* and *flp-4*, but rather expresses the normally ASER-specific terminal fate marker *gcy-5* (Fig. 1B, Table 1, data not shown). All *lsy-2* alleles are fully recessive and display a similar range of pleiotropies, including protruding vulva, abnormally migrating gonad arms and completely penetrant sterility.

lsy-2 encodes a C2H2 zinc finger protein

Using single nucleotide polymorphisms and three-factor mapping (Brenner, 1974; Wicks et al., 2001), we mapped *lsy-2* to a ~80 kb region on the X chromosome (Fig. 2). Because all previously cloned *lsy* genes coded for gene regulatory factors (Chang et al., 2004; Chang et al., 2003; Hobert et

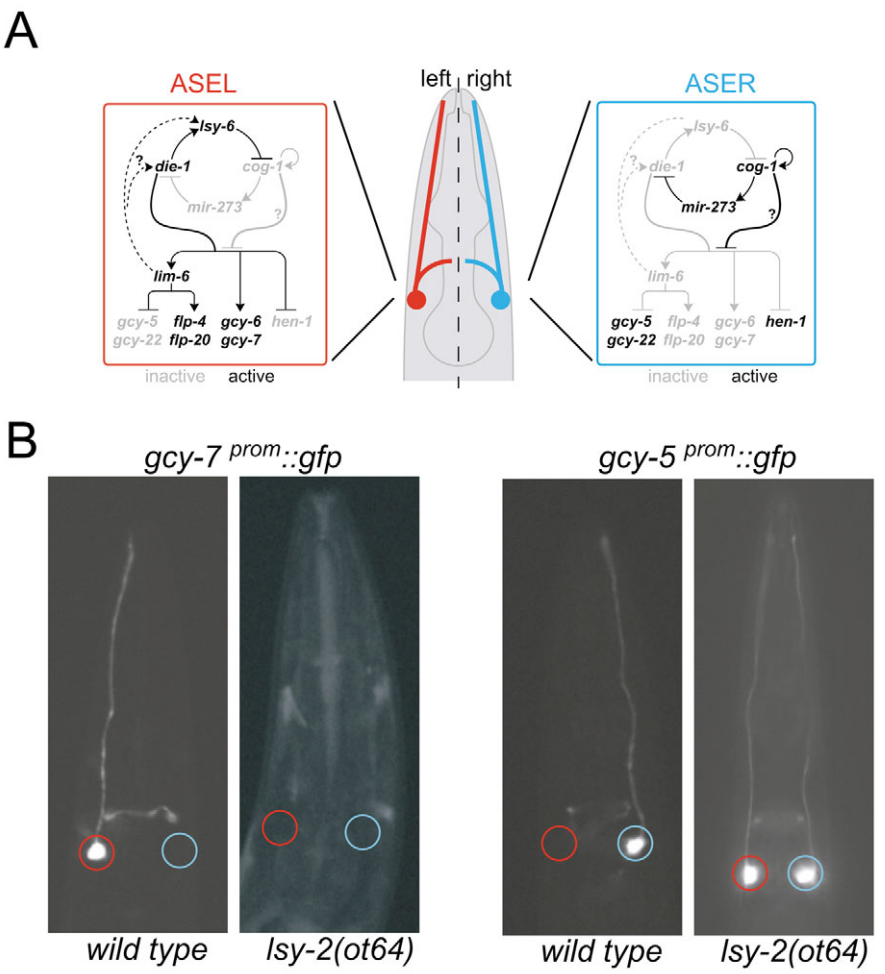

Fig. 1. The ASER versus ASEL fate decision in wild-type and *lsy-2* mutant animals. (A) Schematic representation of the bilaterally symmetric ASE gustatory neurons. Their bilaterality extends to cell position, axonal and dendritic morphology, synaptic connectivity (White et al., 1986) and the expression of a large number of bilaterally expressed genes (www.wormbase.org). The enlarged images illustrate the fate differences between ASEL and ASER, and provide a summary of the genetic regulatory network that controls the ASEL and ASER fates (Chang et al., 2004; Chang et al., 2003; Hobert et al., 1999; Johnston and Hobert, 2003; Johnston et al., 2005). The permissively acting factors *lin-49*, *unc-37* and *ceh-36* are not shown but are referred to in the Discussion. (B) In *lsy-2* mutant animals, ASEL-specific expression of *gcy-7*, assayed using a *gcy-7^{prom}::gfp transgene (otIs3)*, is lost and ASER-specific expression of *gcy-5*, assayed with a *gcy-5^{prom}::gfp transgene (ntIs1)*, is derepressed in ASEL. *lsy-2(ot64)* null mutant animals are shown. See Table 1 for quantification of the data and more alleles.

al., 1999; Johnston and Hobert, 2003) (R.J.J. and O.H., unpublished), we examined this genomic region for the presence of predicted gene regulatory factors. We noted one predicted protein, the F49H12.1 protein (Fig. 2), which contains a nuclear localization sequence and five zinc finger domains of the C2H2 type, characterized by a specific composition and spacing of zinc chelating cysteine and histidine residues (consensus C2H2 structure: $CX_{2\text{-}4}CX_{12}HX_{2\text{-}6}H$) (Iuchi, 2001). Although C2H2 zinc finger proteins can also bind RNA, the vast majority of these proteins are DNA-binding transcription factors (Iuchi, 2001).

The structure of the *F49H12.1* gene is confirmed by multiple, full-length EST clones (www.wormbase.org). We sequenced this gene in all available *lsy-2* mutant strains and found mutations in it in each of our five *lsy-2* strains. These mutations include two premature stop codons, two splice-site mutations and one missense mutation of an invariant cysteine residue in the second zinc finger motif (Fig. 3A; Table 1). The identical early stop alleles *lsy-2(ot64)* and *lsy-2(ot65)* were isolated from the same mutagenized population of animals, and possibly arose from one mutagenic event. A second pair of identical alleles, *lsy-2(ot67)* and *lsy-2(ot72)*, were isolated

Table 1. Lateral symmetry (*lsy*) defects observed in *lsy-2* mutants

		Ectopic of ASER fate in ASEL		Loss of ASEL fate in ASEL			
				Marker			
		gcy-5^{prom}::gfp (ntIs1)		*lim-6^{prom}::gfp (otIs114)*		*gcy-7^{prom}::gfp (otIs3)*	
Genotype	Type of mutation*	% Animals	*n*	% Animals	*n*	% Animals	*n*
Wild type	None	0	>100	0	>100	0	>100
lsy-2(ot64)	Nonsense†	100	48	100	27	100	36
lsy-2(ot67)	Splice donor	100	36	96	28	78	18
lsy-2(ot72)	Splice donor	96	49	91	32	67	24
lsy-2(ot77)	Missense	100	27	86	28	85	20

*See Fig. 3 for the location of the mutation in the protein-coding region.
†Putative null allele. Its molecular identity is the same as *ot65* (Fig. 3).

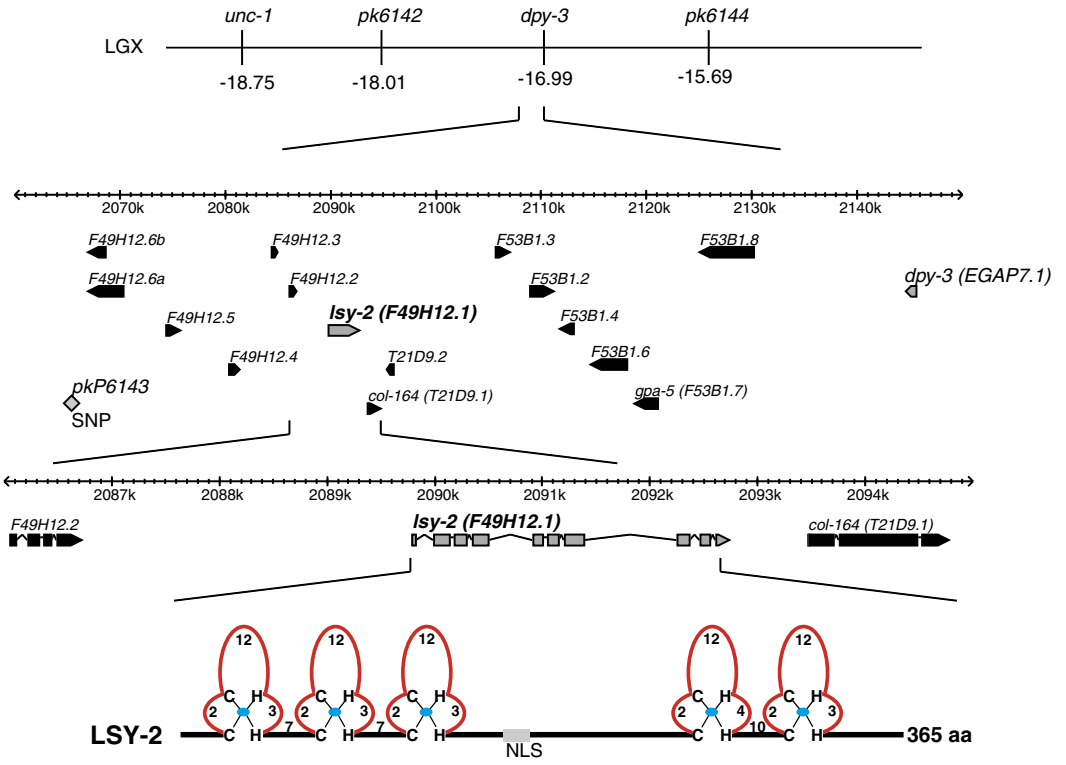

Fig. 2. Mapping of the *lsy-2* locus. (Top) A genetic map (not drawn to scale); (bottom) a physical map. *lsy-2* was mapped with the SNP markers *pkP6142* and *pkP6144* in the Hawaiian *C. elegans* isolate CB4856 (Hodgkin and Doniach, 1997; Wicks et al., 2001). An *unc-1 lsy-2 dpy-3* triple mutant was generated and used to three-factor map *lsy-2* between *pkP6143* (located on cosmid F49H12) and *dpy-3* (EGAP7.1). The blue dots in the schematic presentation of the LSY-2 protein (not drawn to scale) indicate Cys- and His-chelated zinc atoms; the numbers indicate the spacing between individual Cys and His residues. NLS, putative nuclear localization sequence.

from independently mutagenized populations of animals. Because of the early stop codon and its fully penetrant *lsy* and sterility phenotype, we consider the *lsy-2(ot64)* allele to be a putative null allele, and we used this allele for all of our ensuing genetic analyses.

To further confirm the molecular identity of *lsy-2*, we performed RNAi experiments and observed both the *lsy* phenotype and the sterility associated with all *lsy-2* mutant alleles (Table 2). Lastly, transformation rescue experiments, described in more detail below, further verified the molecular identity of *lsy-2* (Table 3).

Homologs of *lsy-2*

Nematode homologs

BLAST searches reveal an apparent *lsy-2* ortholog in the genomes of the related nematodes *C. briggsae* (Fig. 3A) and *C. remanei* (data not shown). In addition, we found a closely related *lsy-2* paralog in *C. elegans*, F52F12.4, which we have named *lsl-1* (pronounced '*lizzle one*') for *lsy-2* like (Fig. 3A). *lsl-1* is also highly conserved in *C. briggsae* (Fig. 3A). Among all of these nematode orthologs and paralogs, the individual C2H2 zinc fingers in the triple zinc finger cluster are significantly more conserved than the fingers in the double zinc finger cluster (Fig. 3A).

Table 2. RNA interference analysis of *lsy-2*

dsRNA	% Animals with loss of ASEL fate*	Sterility†	*n*
Mock	0	No	>100
lsy-2	32	Yes	73
lacZ‡	0	No	87

*Scored with *lim-6^{prom}::gfp (otIs114)*.
†Scored in the progeny of dsRNA-fed animals. 92% of *lsy-2(RNAi)* animals were sterile, compared with a completely penetrant sterility of all *lsy-2* mutant alleles.
‡Control.

Non-nematode homologs

Database searches with the *lsy-2* sequence using either the full-length protein or individual zinc fingers revealed no clear orthologs of *lsy-2* in non-nematode species. However, the existence of a tightly clustered, triple C2H2 zinc finger motif is reminiscent of the DNA-binding domain found in the

Table 3. Transgene rescue of the *lsy-2* mutant phenotype

Genotype	DNA on array	Line	*lsy* phenotype† % Animals	*lsy* phenotype† *n*	Sterility‡
Wild type*	None		0	>100	No
*lsy-2(ot64)**	None		100	48	Yes
lsy-2(ot64)	*lsy-2::gfp*	1	2	50	No
		2	28	36	No
		3	9	44	No
		4	8	48	No
		5	2	55	No
lsy-2(ot64)	*ceh-36^{prom}::lsy-2*	1	35	31	Yes
		2	46	24	Yes
		3	30	30	Yes
		4	46	28	Yes
lsy-2(ot64)	*ceh-36^{prom}::rfp*	1	100	29	Yes

*This control data is taken from Table 1.
†*lsy* phenotype scored: gain of ASER fate in ASEL or loss of ASEL fate in ASEL (see Table 1 for markers).
‡Scored by the ability of the strain to produce self-progeny. Note that the ability of an extrachromosomal transgene, which is normally silenced in germ cells, to rescue the sterility phenotype suggests that *lsy-2* functions outside of the germ cells to control fertility (e.g. in the somatic gonad).

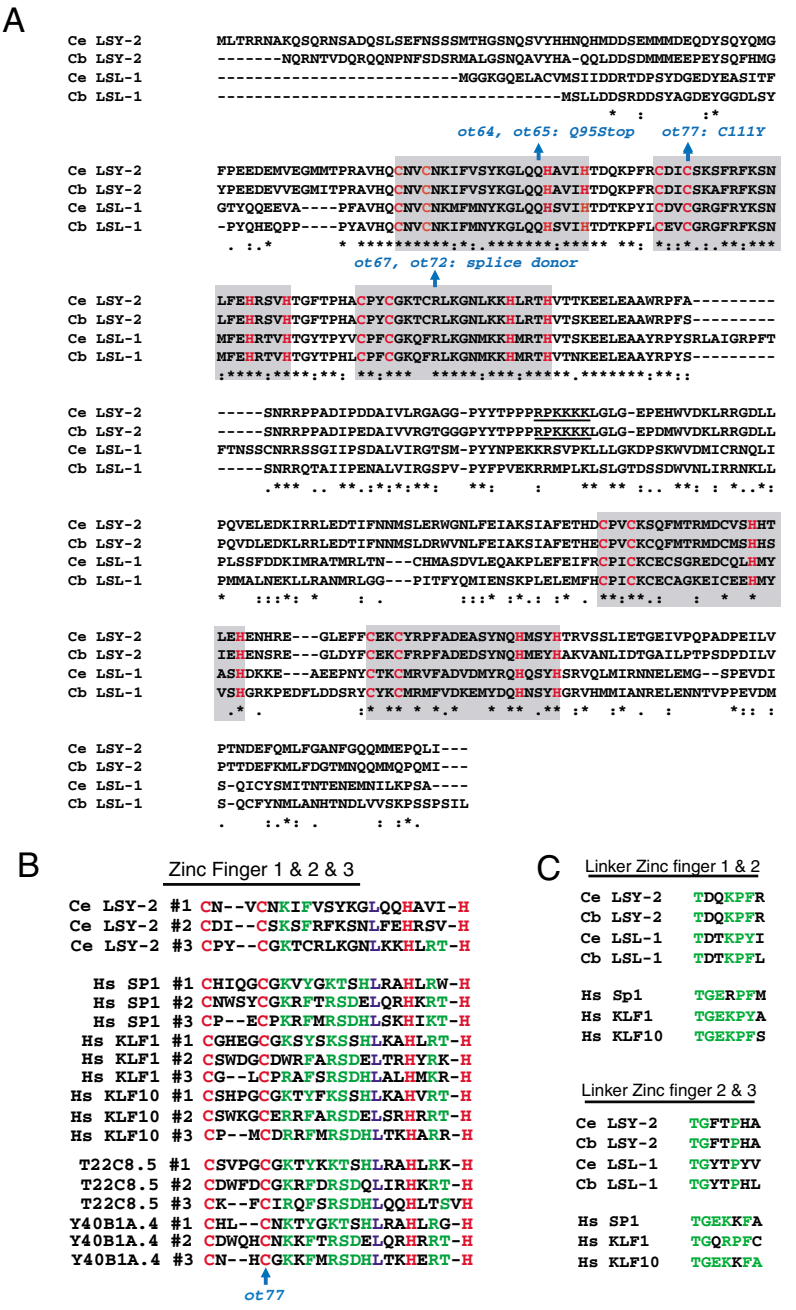

Fig. 3. *lsy-2* encodes a C2H2 zinc finger protein. (A) Sequence alignment of *lsy-2* and its paralog, *lsl-1*, and their *C. briggsae* orthologs. Conserved cysteines and histidines in the C2H2 fingers (grey boxes) are indicated in red. A putative nuclear localization sequence is underlined. Mutant alleles are indicated in blue. (B) Alignment of the first three C2H2 fingers of LSY-2 with representative members of the SP1/KLF-like family (Kaczynski et al., 2003) and two other SP1/KLF-like *C. elegans* proteins. These two proteins are the top hits when BLAST searching the *C. elegans* genome for human or *Drosophila* SP1-like proteins. Other KLF-like proteins have previously been noted in worms (Oates et al., 2001) and are not shown here. Residues in red and blue are 100% conserved; residues in green are partly conserved. Sequences of human proteins are from Kaczynski et al. (Kaczynski et al., 2003). (C) The linkers between C2H2 zinc fingers in the triple motif in LSY-2 have a similar length and share conserved amino acids with known transcription factors of the SP1/KLF family. One representative member of each of the three subgroups of human SP1/KLF proteins is shown.

SP1/KLF proteins, a family of transcription factors with wide-spread functions in growth and development (Kaczynski et al., 2003) (Fig. 3B). The importance of the triple zinc finger motif of LSY-2 is highlighted by the *ot77* allele, which contains a missense mutation in the second zinc finger that is predicted to affect DNA binding (Fig. 3A,B). This mutant causes an almost complete loss of gene function (Table 1). Apart from the zinc fingers themselves, the linker regions that connect the individual zinc fingers are conserved between the triple zinc finger motifs of LSY-2 and the SP1/KLF family (Fig. 3C). The linker region is important for appropriately spacing the contacts that the adjacent zinc fingers make with DNA, and this region is also engaged in direct contacts with the phosphate backbone via a conserved lysine residue (Iuchi, 2001; Pavletich and Pabo, 1991). Although many multiple-zinc finger proteins display a highly variable length of the linker region (Iuchi, 2001), the linker regions of LSY-2 and the SP1/KLF factors have a similar length and share several amino acids. However, searching the *C. elegans* genome sequence with human SP1 and *Drosophila* Sp1 proteins, or other KLF proteins, reveals several worm triple zinc finger proteins that have a higher similarity to SP1/KLF proteins than LSY-2 does (Oates et al., 2001) (data not shown). Taken together, our sequence analysis indicates that LSY-2 is likely to be a DNA-binding protein that is not broadly conserved, but is distantly related to the SP1/KLF-family of transcription factors.

lsy-2 is ubiquitously expressed and localizes to moving speckles in nuclei

In order to analyze the cellular focus of *lsy-2* activity, we generated a *lsy-2::gfp* reporter gene fusion in which the coding region for green fluorescent protein was fused to the *lsy-2* locus, including the full-coding region and its complete 5′ region to the next gene (Fig. 4A). Nine independent transgenic lines carrying the *lsy-2::gfp* extrachromosomal array displayed *gfp* expression in all cells and tissue types of the worm, including ASEL and ASER (Fig. 4B,C). Expression is first observed at the 50-cell stage, and persists throughout embryogenesis, larval stages and adulthood (Fig. 4B). Notably, LSY-2::GFP was localized to moving punctae in the nuclei (Fig. 4D), which is consistent with a role for *lsy-2* in regulating gene expression (see Discussion). This expression pattern relates to endogenous gene function, as *lsy-2::gfp* transgenes can rescue the sterility and *lsy* phenotype of *lsy-2* null mutants (Table 3). A similar ubiquitous expression pattern is observed in animals expressing *gfp* under the control of only the 5′ upstream region of the *lsy-2* locus (Fig. 4B).

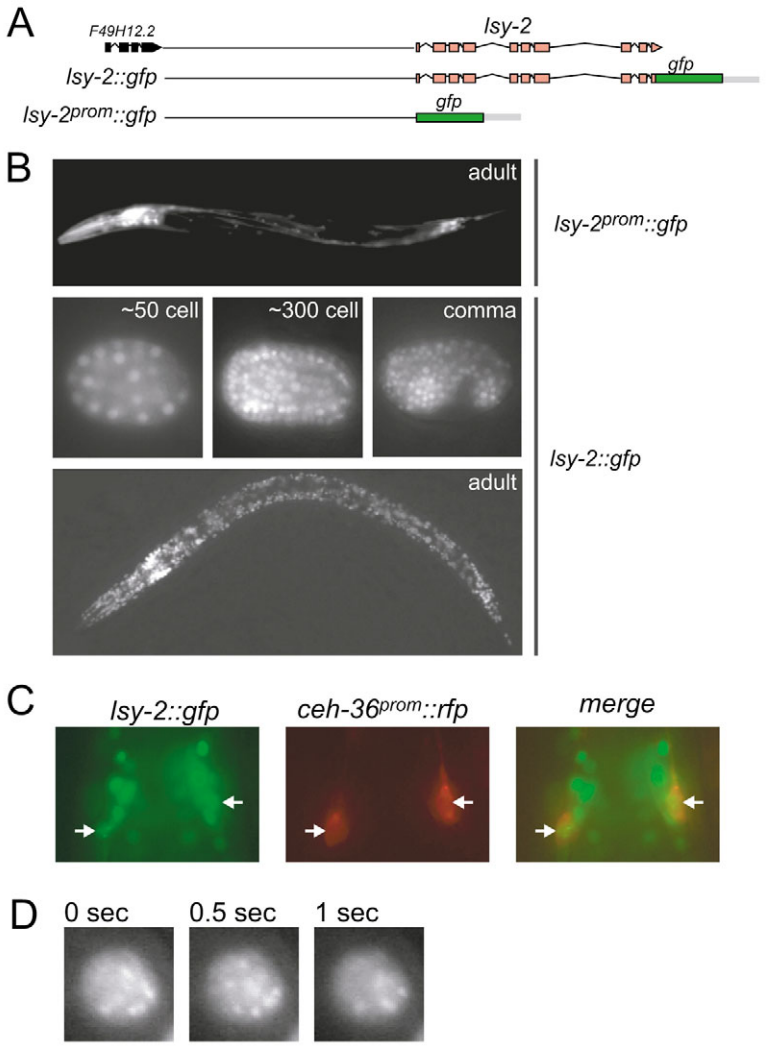

Fig. 4. *lsy-2* is expressed ubiquitously and localizes to moving speckles in the nucleus. (A) *lsy-2*^{prom}*::gfp* and *lsy-2::gfp* reporter gene constructs used in this study. The gray bar behind the green *gfp* sequence indicates the heterologous *unc-54* 3′UTR. (B) *lsy-2*^{prom}*::gfp* and *lsy-2::gfp* reporter gene constructs are ubiquitously expressed at different developmental stages. Nine independent *lsy-2::gfp* transgenic lines and three independent *lsy-2*^{prom}*::gfp* lines show similar expression patterns. (C) *lsy-2::gfp* is expressed in ASEL and ASER. ASEL and ASER are labeled with the *ceh-36*^{prom}*::rfp* transgene *otIs151*. Note that the *rfp* reporter is diffusely localized throughout the cytoplasm, whereas the *gfp* signal is in nuclear speckles. *lsy-2::gfp* rescues the *lsy-2* mutant phenotype (Table 3). (D) *lsy-2::gfp* is localized to moving nuclear speckles. Individual time frames of a movie, shot with Openlab Software at a time interval of half a second per frame (20 frames total), are displayed. The movie is available upon request.

lsy-2 is required in the postmitotic ASE neurons for correct cell-fate specification

As *lsy-2* is widely expressed, we tested whether *lsy-2* is required specifically in the mature ASE neurons to determine the stable ASEL cell fate. We fused the *lsy-2* cDNA to the transcriptional regulatory region of the *ceh-36* gene (‘*ceh-36*^{prom}’), which is active in two bilateral pairs of head sensory neurons, AWCL/R and ASEL/R (Chang et al., 2003; Lanjuin et al., 2003). In *lsy-2(ot64)* null mutant animals carrying *ceh-36*^{prom}*::lsy-2*, ASE laterality was at least partially restored

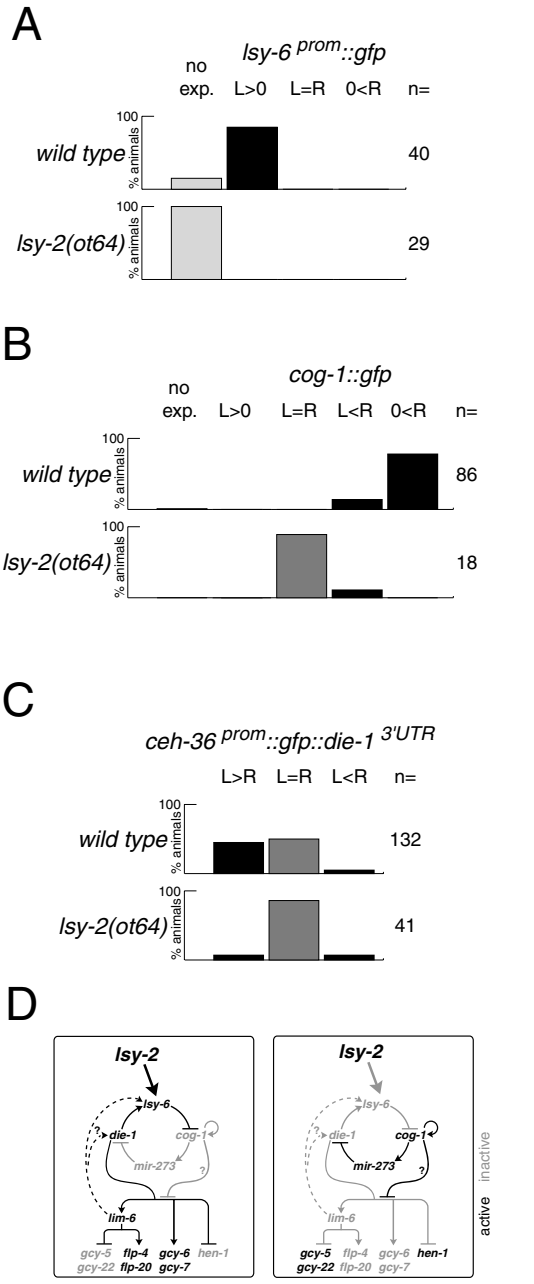

Fig. 5. *lsy-2* is required for proper expression of feedback loop regulators. (A-C) No exp., no expression in ASEL and ASER; L>R, greater expression in ASEL versus ASER; L>0, expression in ASEL only; L=R, equal expression in ASEL and ASER. (A) Expression of *lsy-6*, assayed with the *lsy-6*^{prom}*::gfp* transgene *otIs160*, is lost in the ASEL neuron of *lsy-2(ot64)* null mutant animals. (B) ASER-specific expression of *cog-1*, assayed with the *cog-1::gfp* transgene *syIs63*, is de-repressed in ASEL in *lsy-2(ot64)* null mutant animals. (C) *mir-273* mediated downregulation of the *die-1* 3′UTR in ASER, assayed with the *ceh-36*^{prom}*::gfp::die-1*$^{3'UTR}$ transgene *otEx1759*, is disrupted in *lsy-2(ot64)* null mutant animals. (D) Placement of *lsy-2* relative to the previously described bistable feedback loop in ASEL (left) and ASER (right). For a more detailed explanation of the regulatory interactions, see Johnston et al. (Johnston et al., 2005).

(Table 3). As microsurgical ablation of ASER or genetic ablation of AWCL/R has no effect on ASEL development (data not shown), we can conclude that it is the resupplied *lsy-2* gene activity in ASEL, rather than ASER, AWCL or AWCR, that is responsible for the rescue of the mutant phenotype. The *ceh-36* promoter is activated around the time that the ASE neurons are generated and therefore the rescue experiments also indicate that *lsy-2* is likely to function postmitotically. Other members of the regulatory loop controlling ASEL/R fate also function postmitotically (Chang et al., 2004; Chang et al., 2003; Hobert et al., 1999; Johnston and Hobert, 2003).

Because the provision of extra copies of the *lsy-2* gene in ASER with the *ceh-36^{prom}::lsy-2* transgene does not induce the ASEL fate in ASER, we can furthermore conclude that, even upon overexpression, *lsy-2* is not sufficient to induce ASEL fate. Together with the normal expression of *lsy-2* in both ASEL and ASER, we can conclude that *lsy-2* acts permissively rather than instructively to induce ASEL fate in ASEL.

lsy-2 acts upstream of the *lsy-6* miRNA

A bistable feedback loop consisting of the miRNAs *lsy-6* and *mir-273*, and their respective target transcription factors *cog-1* and *die-1*, is required for ASE laterality (Fig. 1A) (Johnston et al., 2005). Because the genetic removal of *lsy-2* causes a conversion of ASEL to the ASER stable state, we wanted to test what role *lsy-2* plays in the feedback loop. We first examined the effect of *lsy-2* on the ASEL-specific expression of the miRNA *lsy-6*. We found a complete loss of *lsy-6* expression in *lsy-2* null mutant animals (Fig. 5A). *lsy-6* represses the expression of the *cog-1* transcription factor in ASEL and, as a result, expression of *cog-1* is biased towards the right cell (Johnston and Hobert, 2003). As would be expected from a loss of endogenous *lsy-6* miRNA expression, *cog-1* expression is de-repressed in the ASEL neuron of *lsy-2* null mutant animals (Fig. 5B). The de-repression of *cog-1* in ASEL in *lsy-2* null mutant is functionally relevant and provides an explanation for the 'two ASER' phenotype of *lsy-2* mutants, as a reduction of *cog-1* gene activity in a *lsy-2* null mutant background significantly represses the adoption of the ASER fate in ASEL (Table 4).

To further characterize the role of *lsy-2* in the bistable *lsy-6/cog-1/mir-273/die-1* feedback loop, we examined the effect of *lsy-2* on the *die-1* sensor gene *ceh-36^{prom}::gfp::die-1$^{3'UTR}$*. This sensor gene provides a means to observe the miRNA-mediated repression of *die-1* in ASER, conferred by *mir-273* and other, as yet unknown, miRNAs (Chang et al., 2004) (D. Didiano and O.H., unpublished). In *lsy-2* null mutant animals carrying the *die-1* sensor gene, differential expression of GFP is disrupted (Fig. 5C). Taken together, these results indicate that *lsy-2* is required for the proper execution of the ASEL cell fate that is controlled by the previously described bistable feedback loop.

Because components of the regulatory loop regulate the expression of one another (Fig. 1A), the effects of *lsy-2* on the expression of individual loop components could be caused either by *lsy-2* affecting the expression of only a single component of the loop, or, alternatively, by *lsy-2* independently affecting the expression of several components of the loop. We previously reported that *die-1* is the likely output regulator of the loop that directly or indirectly controls the expression of effector genes of the loop (i.e. of the terminal differentiation markers) (Johnston et al., 2005). This makes *lsy-6* the regulatory gene that is most distal from the effector genes (Fig. 1A). We first asked whether ectopic expression of *lsy-6* miRNA, which induces the ASEL fate in both ASE neurons (Johnston and Hobert, 2003), requires the activity of *lsy-2*. If

Table 4. Genetic epistasis analysis

Genotype	Inappropriate execution of ASER fate in ASEL: Measured as loss of expression of an ASEL fate marker in ASEL†	Inappropriate execution of ASER fate in ASEL: Measured by ectopic expression of an ASER fate marker in ASEL‡
Wild type*	0% (*n*>100)	0% (*n*>100)
*lsy-2(ot64)**	100% (*n*=36)	100% (*n*=48)
cog-1(ot28)	0% (*n*>100)	0% (*n*>100)
cog-1(ot28); lsy-2(ot64)	44% (*n*=41)	25% (*n*=36)

	Inappropriate execution of ASEL fate in ASER: Measured as ectopic expression of an ASEL fate marker in ASER†	Inappropriate execution of ASEL fate in ASER: Measured as loss of expression of an ASER fate marker in ASER‡
Wild-type*	0% (*n*>100)	0% (*n*>100)
*lsy-2(ot64)**	0% (*n*=27)	0% (*n*=48)
Ex[ceh-36^{prom}::lsy-6]	71% (*n*=42)	83% (*n*=60)
lsy-2(ot64); Ex[ceh-36^{prom}::lsy-6]	70% (*n*=37)	78% (*n*=40)

	Inappropriate execution of ASER fate in ASEL: Measured as expression of an ASEL fate marker in ASEL†	Inappropriate execution of ASER fate in ASEL: Measured as absence of expression of an ASER fate marker in ASEL‡
Wild-type*	100% (*n*>100)	100% (*n*>100)
*lsy-2(ot64)**	0% (*n*=27)	0% (*n*=48)
Ex[ceh-36^{prom}::lsy-6]	100% (*n*=42)	100% (*n*=60)
lsy-2(ot64); Ex[ceh-36^{prom}::lsy-6]	78% (*n*=37)	81% (*n*=40)

*Same data as in Table 1.
†Assayed with *lim-6^{prom}::gfp (otIs114)*.
‡Assayed with *gcy-5^{prom}::gfp (ntIs1)*.

lsy-2 is required for the expression of a loop component that acts downstream of *lsy-6* (i.e. anywhere between *lsy-6* and the effector genes), ectopic *lsy-6* would not be able to exert its ASEL-inducing effect in *lsy-2* null mutants. If the effect of ectopic *lsy-6* expression is unaltered in *lsy-2* null mutants, *lsy-2* would act upstream of *lsy-6*. If *lsy-2* acts on multiple components in the loop, intermediate effects might be expected. We find that the ASEL-inducing activity of ectopic *lsy-6* expression is virtually unaffected in *lsy-2* null mutants, as measured with two distinct cell fate markers (Table 4).

Another approach to examine the epistatic relationship of *lsy-2* and *lsy-6* is to determine whether the loss of ASEL fate observed in *lsy-2* null mutants can be rescued by the expression of *lsy-6* under the control of a heterologous, *lsy-2*-independent promoter in ASEL. Indeed, in most *lsy-2* null mutant animals examined, heterologously expressed *lsy-6* is able to restore the ASEL fate, as measured with two distinct cell fate markers (Table 4). *lsy-6* is in fact as efficient at restoring ASEL fate in ASEL in *lsy-2* mutants as it is at inducing ASEL fate when ectopically expressed in ASER (~80%; Table 4). The most parsimonious explanation of these observations is that *lsy-2* acts upstream of *lsy-6* to regulate ASE asymmetry (Fig. 5D).

lsy-6 is expressed in several neuron types besides ASEL, including labial sensory neurons and the PVQ ventral cord interneurons (Johnston and Hobert, 2003). Although *lsy-2* is expressed in all of these neuron types, *lsy-6^{prom}::gfp* expression is lost only in the ASEL neuron of *lsy-2* null mutants, and not in other head or tail neurons (data not shown). Like many transcription factor interactions with their target genes (e.g. Altun-Gultekin et al., 2001; Tsalik et al., 2003), the genetic interaction of *lsy-2* and *lsy-6* is therefore cell-type specific.

Discussion

The molecular function of LSY-2

We have described a novel C2H2 zinc finger protein that plays a permissive role in controlling the execution of the ASEL cell fate. Although *lsy-2* is ubiquitously expressed, its spatially and temporally restricted function in the ASEL/R cell fate decision indicates that *lsy-2* is a specific regulator of a select number of target genes in different cell types, rather than a global facilitator of gene expression. How does *lsy-2* regulate gene expression? The subnuclear localization of *lsy-2* to moving speckles suggests a role in splicing or transcriptional regulation. Nuclear speckles were initially characterized as 'supply houses' of splicing factors to sites of active transcription (Lamond and Spector, 2003). However, there is a growing body of evidence indicating that subnuclear structures may also contain specific core components and regulators of transcription. For example, nuclear speckle localization has been reported for transcription factors such as ALL-1/Trithorax, steroid receptor co-activator (SRC-1), steroidogenic factor 1 (SF-1) and Pnn/DRS (Alpatov et al., 2004; Amazit et al., 2003; Chen et al., 2004; Zeng et al., 1997). Although we cannot exclude a role for *lsy-2* as a splicing factor, the now well-established localization of at least some transcription factors to speckles, together with the triple C2H2 zinc finger motif architecture of LSY-2 that is shared with *bona fide* DNA-binding transcription factors, strongly suggests a role of LSY-2 in transcriptional regulation.

A recent genome sequence analysis has revealed the presence of a conserved 8 bp motif located ~200 bp upstream of virtually all known nematode miRNA (Ohler et al., 2004). This observation suggested that a ubiquitously expressed transcription factor, such as *lsy-2*, might act through this motif to enable or facilitate global miRNA gene expression. However, we consider this possibility to be unlikely, as deletion of this motif does not affect the expression of *lsy-6* (data not shown). Loss of *lsy-2* also does not affect *lsy-6* expression in cells other than ASEL. A role for *lsy-2* as a permissive regulator of miRNA expression is also ruled out by the observation that none of the morphological defects that are associated with the loss of the *lin-4* or *let-7* miRNAs [*lin-4*: elongated, vulvaless (Ambros and Horvitz, 1984); *let-7*: lethal because of bursting vulva (Reinhart et al., 2000)] can be observed in *lsy-2* null mutants (data not shown). LSY-2 is therefore unlikely to be a general activator of miRNA gene expression.

In genetic terms, *lsy-2* exerts its function by regulating the cell-type specificity of *lsy-6* miRNA gene expression, thereby making *lsy-2* one of the very few factors known to be involved in the spatial control miRNA gene regulation. Although *lsy-2* is expressed throughout the nervous system, it nevertheless regulates *lsy-6* in only one of the three neuron classes that express *lsy-6*. Such cell-type specificity in regulatory interactions is a common theme in transcriptional regulation. One of the many prominent examples in *C. elegans* is the LIM homeobox genes that regulate specific target genes in some cell types but not in others, even though they are co-expressed (Altun-Gultekin et al., 2001; Tsalik et al., 2003).

Permissively acting factors in the bistable feedback loop

Our genetic screens have not only retrieved instructive, left/right asymmetrically expressed factors that are both required and sufficient to induce either the ASEL or ASER fate, but also permissive factors that control the ASEL/ASER fate decision (Chang et al., 2004; Chang et al., 2003; Johnston and Hobert, 2003). Instructive factors, such as *lsy-6*, *cog-1*, *mir-273* (in conjunction with other miRNAs; Dominic Didiano and O.H., unpublished) and *die-1*, are expressed asymmetrically in either ASEL or ASER and are not only required to induce either the ASEL or ASER fate, but are also sufficient to do so if misexpressed in the opposing cell. By contrast, permissive factors are expressed in both ASEL and ASER, and are therefore only required but not sufficient to induce the respective fate. As these permissive factors are not expressed in a left/right asymmetric manner, they are not intrinsic components of the bistable feedback loop shown in Fig. 1A and Fig. 5D, but are permissively required to confer the activity of left/right asymmetric factors in the loop. These permissive factors include the Groucho-like co-repressor UNC-37, the PHD/bromodomain protein LIN-49, the OTX-type homeodomain protein CEH-36 (Chang et al., 2003), and, as we describe here, LSY-2. *unc-37* is required for the execution of the ASER fate, whereas *lin-49*, *ceh-36* and *lsy-2* are required for the ASEL fate. What are the specific features of these permissively required factors and how may their cell-type specific activities be explained?

unc-37/Groucho

Based on the genetic interactions of *cog-1* and *unc-*

37/Groucho, and the presence of a Groucho-binding EH1 domain in the COG-1 protein, the cell-type specific activity of the ubiquitously expressed UNC-37 protein can be explained by its physical association with the ASER-specific COG-1 homeodomain protein (Chang et al., 2003). In a conceptually similar manner, the cell type-specific activity of UNC-37 in regulating VA motoneuron specification can be explained by its association with the VA motoneuron-specific homeodomain protein UNC-4 (Pflugrad et al., 1997).

ceh-36/Otx

This Otx-type homeobox gene is only expressed in two pairs of head neurons (Chang et al., 2003; Lanjuin et al., 2003), but its bilateral expression in ASEL and ASER still classifies the gene as a permissive factor required for ASEL cell fate. One potential model that may explain the cell-type specificity of *ceh-36* proposes that *ceh-36* activity in ASER is competed for by the ASER-inducing *cog-1* gene, which is exclusively expressed in ASER (Chang et al., 2003).

lin-49

This gene codes for a PHD/bromodomain protein that is required for the induction of the ASEL fate, as well as for the regulation of a variety of other cell fate decisions (Chamberlin and Thomas, 2000). We have previously shown that a complete loss of *lin-49* function in *lin-49* null mutant animals can be partially suppressed by lowering *cog-1* gene activity (Chang et al., 2003). This effect would support a role of *lin-49* upstream of *cog-1*. Consistent with this notion, *lin-49* mutants display a failure of downregulation of *cog-1* in ASEL in adult animals, a concomitant loss of *lsy-6* expression in ASEL, and, as a likely consequence of ectopic *cog-1* expression, a misregulation of the *die-1* 3′UTR (data not shown). These are phenotypes that closely resemble those observed in *lsy-2* mutants, and it is conceivable that both proteins act together to regulate the expression of *lsy-6* and other ASEL-inducing components of the feedback loop.

lsy-2

Of the above-mentioned cases, the case of *unc-37* provides the clearest example for how a bilaterally expressed, permissively acting factor can confer cell-specific activity through physical association with cell-specific, instructive regulatory proteins. By analogy to this case, a good candidate to confer functional specificity to *lsy-2* (and also *lin-49*, which acts in a genetically similar manner to *lsy-2*) is the ASEL-inducing zinc finger transcription factor *die-1*. Like *lsy-2*, *die-1* is required for the expression of the miRNA *lsy-6* (Chang et al., 2004). Genetically, the key difference between *lsy-2* and *die-1* is that *die-1* is left/right asymmetrically expressed and can act instructively; that is, it can induce ASEL fate if it is misexpressed in ASER. A common architectural feature of several well-characterized cis-regulatory regions is the presence of binding sites for both cell-type specifically expressed factors and broadly expressed transcription factors, such as, for example, SP1 (to which LSY-2 is distantly related) (e.g. Falvo et al., 2000; Xiao et al., 1987). Broadly expressed transcription factors appear to be required for baseline promoter activity, and functionally synergize with factors that provide spatiotemporal specificity. A similar scenario may hold true for LSY-2, which may synergize with DIE-1 to efficiently activate *lsy-6* expression. An ongoing analysis of the *lsy-6* promoter may identify cis-regulatory elements that could be directly targeted by DIE-1 and LSY-2 proteins.

The complexity of the ASEL versus ASER cell-fate decision

Factors that control the ASEL versus ASER cell fate decision and their regulatory interactions are summarized in Fig. 5D. Ongoing genetic analysis in our laboratory has uncovered even more factors that are involved in this cell fate decision. The gene regulatory network controlling the diversification of the ASE neurons therefore appears to be unusually complex at first sight. However, only if systematic and extensive genetic approaches similar to those that we have taken with the ASE neurons are applied to other neuronal fate decisions, can one assess whether such complexity in regulatory networks may be the rule or the exception.

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