A Left/Right Asymmetric Neuronal Differentiation Program Is Controlled by the *Caenorhabditis elegans* LSY-27 Zinc-Finger Transcription Factor

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ABSTRACT Functional diversification across the left/right axis is a common feature of many nervous systems. The genetic programs that control left/right asymmetric neuron function and gene expression in the nervous system are, however, poorly understood. We describe here the molecular characterization of two phenotypically similar mutant *Caenorhabditis elegans* strains in which left/right asymmetric gene expression programs of two gustatory neurons, called ASEL and ASER, are disrupted such that the differentiation program of the ASER neuron is derepressed in the ASEL neuron. We show that in one mutant strain the LIM homeobox gene *lim-6* is defective whereas in another strain a novel member of a nematode-specific, fast-evolving family of C2H2 zinc-finger transcription factors, *lsy-27*, is mutated, as revealed by whole-genome sequencing. *lsy-27* is broadly and exclusively expressed in the embryo and acts during the initiation, but not during the maintenance phase of ASE asymmetry control to assist in the initiation of *lim-6* expression.

LEFT/RIGHT asymmetric gene expression patterns in the nervous system of invertebrate and vertebrates species have been described and are generally thought to be the foundation of the striking functional lateralization of many nervous systems (Hobert *et al.* 2002; Sun *et al.* 2005; Sun and Walsh 2006; Taylor *et al.* 2010). Yet it is not well understood how left/right gene expression patterns are regulated. In the nematode *Caenorhabditis elegans*, a class of putative chemoreceptors of the GCY family are expressed in a left/right asymmetric manner in a bilateral pair of functionally lateralized gustatory neurons, called ASEL and ASER (Yu *et al.* 1997; Ortiz *et al.* 2006). These *gcy* genes are required for the left/right asymmetric processing of chemosensory information by the two ASE neurons (Ortiz *et al.* 2009). Genetic mutant screens have revealed a number of genes (called "*lsy* genes" for *l*aterally *sy*mmetric) that control the left/right asymmetric expression of *gcy* genes (Sarin

et al. 2007). Phenotypic analysis of these mutants has revealed several distinct types of asymmetry mutants. In class I mutants, the *gcy* expression profile of the ASER neuron completely converts to that of the ASEL neuron ("2 ASEL" mutants). In class II mutants, the opposite occurs ("2 ASER" mutants; *e.g., die-1* as shown in Figure 1A). In class III mutants, both ASEL and ASER *gcy* receptors are lost. In class IV mutants, the ASER-specific *gcy* genes are derepressed in ASEL, but the ASEL-specific *gcy* genes remain unaffected; or vice versa, ASEL-specific *gcy* genes are derepressed in ASER, but ASER-specific *gcy* genes remain unaffected (Sarin *et al.* 2007). Either the ASEL or ASER neurons therefore exist in a "mixed" state in class IV mutants (Figure 1A). Due to their more limited phenotypic effects, class IV genes would be expected to work downstream of class I and class II genes, and indeed, the analysis of the expression of class IV genes in class I or II mutant backgrounds confirmed this notion (Johnston *et al.* 2005, 2006) (Figure 1A).

Class IV genes are essential for the appropriate function of the ASE neurons. This was first demonstrated through a detailed phenotypic analysis of animals that lack the ASEL-expressed *lim-6* LIM homeobox gene and that therefore display a class IV phenotype in which ASEL-expressed *gcy* genes are unaffected, but ASER-expressed *gcy* genes are
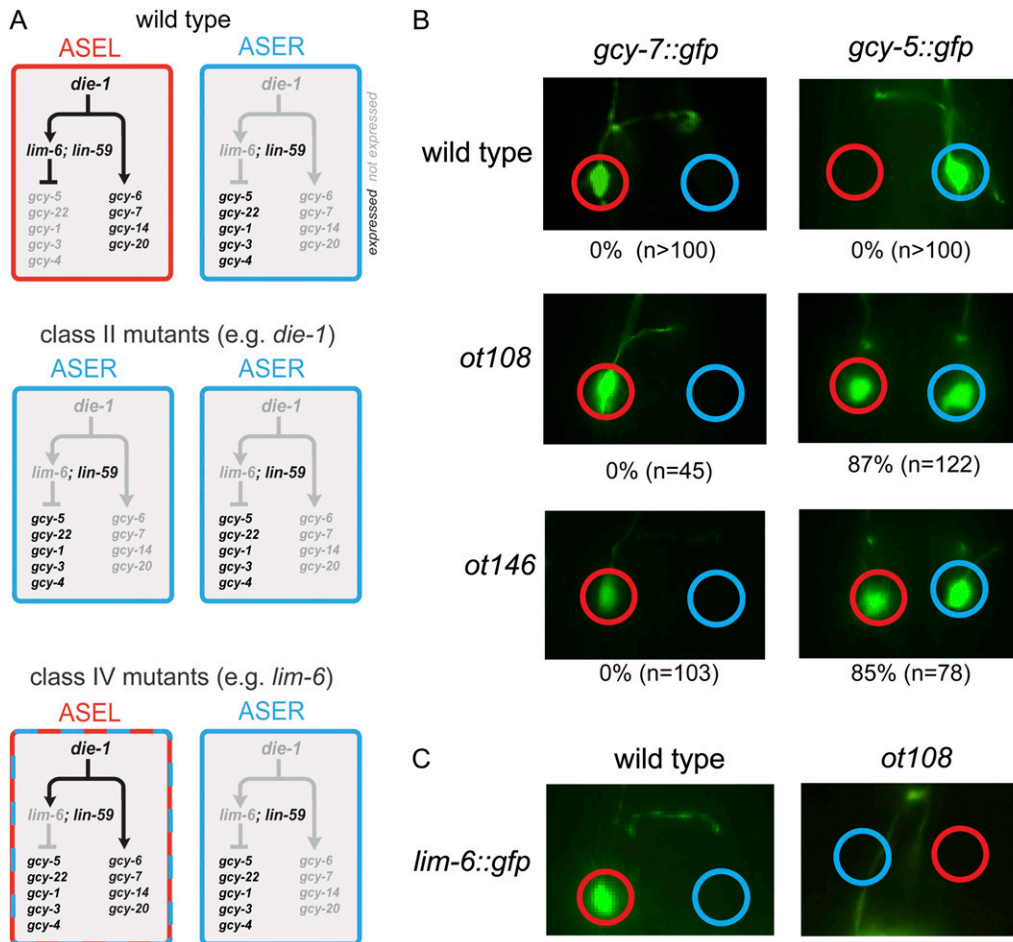
Figure 1 *lsy* genes and mutant phenotypes. (A) A simplified version of the genetic pathway that controls left/right asymmetry in the ASE neurons. Loss of *die-1*, a Zn-finger transcription factor, results in a class II Lsy phenotype (in which ASEL fate markers are lost and ASER fate markers are gained in ASEL), and loss of *lim-6*, a LIM homeobox gene, results in a class IV Lsy phenotype (in which ASER fate markers are gained in ASEL, but ASEL fate markers unaffected) (Hobert *et al.* 1999; Chang *et al.* 2004). Loss of broadly expressed *lin-59*, a histone methyltransferase, also results in a class IV Lsy phenotype (Sarin *et al.* 2007, 2010). (B) Effect of *lsy-27(ot108)* and *lim-6 (ot146)* mutant alleles on ASEL/ASER asymmetry markers. *otIs3 (gcy-7::gfp)* labels ASEL and *ntIs1(gcy-5::gfp)* labels ASER. The phenotype is quantified in Table 1. (C) *ot108* also affects *lim-6::gfp (otIs114)* expression. In 57.4% of animals, *lim-6::gfp* fails to be expressed, and in 27.7% of animals, expression is visible but weaker than in wild type (*n* = 47).

derepressed in ASEL (Figure 1A) (Hobert *et al.* 1999). Such mutant animals are unable to discriminate between ASEL- and ASER-sensed chemosensory cues (Pierce-Shimomura *et al.* 2001).

lim-6 is not the only gene with such a function. Three mutants retrieved from a previous large-scale mutagenesis screen for the asymmetry mutants *ot104, ot108*, and *ot146* (Sarin *et al.* 2007) display a phenotype similar to *lim-6* (Figure 1B and Table 1). *ot104* was found to be an allele of the ubiquitously expressed ASH1-type histone methyltransferase *lin-59* (Sarin *et al.* 2010), but the *ot108* and *ot146* alleles had not previously been molecularly characterized. We present their characterization in this Note.

ot146 is an allele of the LIM homeobox gene *lim-6*

ot146 mutant animals are viable and fertile and display no obvious morphological abnormalities. Their class IV Lsy phenotype is recessive. Due to its failure to complement what turned out to be a very unusual allele, called *ot101*, of the zinc (Zn)-finger transcription factor *che-1*, a terminal selector of ASEL and ASER neuron fate (Etchberger *et al.* 2009), we had assumed that *ot146* was located on chromosome I, where *che-1* is located (Sarin *et al.* 2007). However, subse-

quent mapping placed *ot146* on chromosome X, where the *lim-6* locus resides. We find that *ot146* contains a C83Y change in the second LIM domain of *lim-6* (supporting information, Figure S1). The mutated cysteine residue is 100% conserved in all LIM domains and is essential for the structural integrity of a LIM domain through the coordination of a Zn ion (Kadrmas and Beckerle 2004). The *ot146* allele fails to complement the *lim-6* null allele *nr2073*, and its Lsy phenotype is rescued by a genomic piece of DNA that contains the *lim-6* locus (Table 2). We conclude that *ot146* is an allele of *lim-6*. This is the first *lim-6* allele retrieved from our mutant screen [the only previously characterized *lim-6* allele, *nr2073*, is a reverse engineered allele (Hobert *et al.* 1999)].

ot108 affects a member of a C2H2 Zn-finger protein family

Like *lim-6* mutant animals, *ot108* mutant animals show derepression of the ASER marker *gcy-5* in ASEL, while *gcy-7* expression in ASEL is unaffected (Figure 1B and Table 1). Other than the Lsy phenotype, *ot108* mutants animals are viable and fertile and display no obvious morphological abnormalities. Aside from the effect of *ot108* on *gcy-5*

Table 1 Lsy phenotypes of *lim-6* and *lsy-27*

	% animals with the following phenotypes (at 25°):							
	ASEL only (%)	ASEL > ASER (%)	No expression (%)	ASEL = ASER (%)	ASEL < ASER (%)	ASER only (%)	*n*	% Lsy
	ASEL marker (*gcy-7::gfp; otIs3*)							
Wild type	100	0	0	0	0	0	>100	0
lim-6(nr2073)	100	0	0	0	0	0	35	0
lim-6(ot146)	100	0	0	0	0	0	103	0
lsy-27(ot108)	100	0	0	0	0	0	45	0
lsy-27(tm593)	100	0	0	0	0	0	66	0
	ASER marker (*gcy-5::gfp; ntIs1*)							
Wild type	0	0	0	0	0	100	>100	0
lim-6(nr2073)	0	0	0	89	5	6	82	94
lim-6(ot146)	0	0	0	8	77	15	78	85
ot146/nr2073	0	0	0	0	56	44	50	56
lsy-27(ot108)	0	0	0	39	48	13	122	87
lsy-27(tm593)	0	0	0	0	62	38	117	62
ot108/tm593	0	0	0	3	6	91	31	9
ot108/+	0	0	0	0	0	100	56	0

expression, *ot108* animals also show a significant loss of *lim-6* expression in ASEL, thereby providing an explanation of the *lim-6*-like phenotype of *ot108* mutant animals (Figure 1C).

Upon isolation of *ot108* mutant animals in our original Lsy screen (Sarin *et al.* 2007), we noted that *ot108* fails to complement the derepression of ASER fate in the ASEL phenotype of a mutation in the *die-1* Zn-finger transcription factor, an inducer of *lim-6* expression in ASEL (a class II gene that also results in the loss of ASEL fate) (Figure 1A). Due to this lack of complementation, we had therefore initially considered *ot108* to be an allele of *die-1* (Sarin *et al.* 2007). However, our subsequent analysis revealed no mutation in the *die-1* locus of *ot108* mutant animals and, moreover, the *ot108* mutant phenotype could not be rescued with a genomic piece of DNA that rescues a canonical *die-1* allele (data not shown). Subsequent chromosomal linkage analysis showed that *ot108* is linked to chromosome V, while *die-1* maps to chromosome II. After mapping *ot108* to the right arm of chromosome V using conventional SNP mapping (Wicks *et al.* 2001), we subjected the strain to whole-genome sequencing using an Illumina GAII genome analyzer (Sarin *et al.* 2008) and analyzed the data with MAQGene (Bigelow *et al.* 2009). Sequencing parameters and results are summarized in Table S1. In brief, within the genetically defined interval, we detected 22 sequence variants predicted to affect protein-coding genes (missense, non-sense or splice-site mutations). Nineteen of these variants were found in other whole-genome sequencing data sets that our lab has generated and were therefore considered background variants, leaving three protein-coding alterations. One of these alterations is a Ser-to-Leu change in the predicted C2H2 Zn-finger transcription factor F47H4.1 (Figure 2A and Figure S2). F47H4.1 is a member of C2H2 Zn-finger transcription factors with several paralogs in *Caenorhabditis*

elegans and orthologs in other nematode species, but no apparent orthologs outside nematodes (Figure 2B and Figure S2). All members of this family contain three closely clustered C2H2 Zn fingers at the N terminus of the protein, but no other recognizable domains. The serine residue that is mutated in *ot108* is phylogenetically conserved (Figure S2). The only gene in this family that had been previously characterized is the *ham-2* transcription factor, which is involved in *C. elegans* HSN motor neuron specification (Baum *et al.* 1999).

Both a fosmid spanning the entire F47H4.1 locus plus neighboring genes and a genomic piece of DNA containing 2.6 kb upstream of F47H4.1 and the F47H4.1 locus (Figure 2A) rescue the *ot108* mutant phenotype (Table 2). Animals carrying a deletion allele of F47H4.1, *tm593* (kindly provided by the *C. elegans* knockout facility at Tokyo Women's Medical University School of Medicine) (Figure 2A), also display a class IV Lsy phenotype (Table 1). Also, like *ot108* animals, *tm593* animals are viable and fertile and display no obvious morphological abnormalities. Taken together, we conclude that it is the mutation in F47H4.1 that results in the class IV Lsy phenotype of *ot108* mutant animals, and we therefore called this gene *lsy-27* (Table S3 shows an updated numbering of *lsy* genes).

ot108 is an altered function allele

The *tm593* deletion allele is a molecular null, as confirmed by RT-PCR analysis, which revealed that only very short (<37 amino acids), truncated forms of the protein are generated in *tm593* animals, which do not contain any of the DNA-binding Zn-fingers (see File S1). We were therefore surprised to note that the Lsy phenotype of the *tm593* deletion allele is notably milder than the *ot108* missense allele in terms of both expressivity and penetrance (Table 1). We

Table 2 Transformation rescue and RNAi analysis

Genotype	Lsy phenotype[a] (%)	Wild-type phenotype (%)	n
Wild type	0	100	>100
lim-6(ot146)	85	15	78
ot146; otEx3859 (Ex[lim-6 fosmid::yfp; rol-6(d)])	0	100	41
lsy-27(ot108)	86.9	13.1	122
lsy-27(ot108); lsy-27(RNAi)	2.5	97.5	200
lsy-27(ot108); empty vector (RNAi)	86.8	13.2	111
lsy-27(RNAi)	0	100	71
lsy-27(ot108); Ex[lsy-27transl::gfp], line #1[b]	18.2	81.8	44
lsy-27(ot108); Ex[lsy-27transl::gfp], line #2	17.2	82.8	87
lsy-27(ot108); Ex[fosmid], line #1	5.6	94.4	18
lsy-27(ot108); Ex[fosmid], line #2	9.1	90.9	44
lsy-27(ot108); Ex[lsy-27fosmid::yfp], line #1[b]	0	100	70
Genotype as above but array not transmitted from parental generation[c]	0	100	11
lsy-27(ot108); Ex[lsy-27fosmid::yfp], line #2	0	100	54
Genotype as above but array not transmitted from parental generation	21.1	78.9	19

The *ot108* and *ot146* control data are repeated from Table 1 for comparison purposes. RNAi experiments were done by feeding, using standard protocols with a double-stranded RNA clone obtained from Geneservice.

[a] Scored as a *gcy-5* reporter (*ntIs1* or *otIs220*) derepressed in ASEL in first eleven rows or loss of *lim-6::gfp* (*otIs114*) in remaining four rows.

[b] All expression constructs are shown in Figure 2A. See File S1 for details on the generation of the reporter constructs.

[c] Arrays contain the *elt-2::gfp* injection marker. Animals derived from *elt-2::gfp(+)* parents that have lost this array as assessed by lack of intestinal *gfp* expression were scored.

therefore considered the possibility that *ot108* (which is recessive) is an altered function allele (Table 1). We tested this possibility by removing *lsy-27* gene activity in *ot108* mutant animals using RNA interference (RNAi) directed against *lsy-27*. We found that RNAi treatment completely reverted the *ot108* phenotype (Table 2), suggesting that it is indeed altered *lsy-27* function that explains the *ot108* phenotype.

We noted that animals that carry one copy of the *ot108* allele and one copy of the *tm593* allele display a phenotype that is even milder than the phenotype of either allele alone (Table 1). One copy of the *ot108* allele alone is therefore not enough to induce the altered function activity, but perhaps may be enough to provide some wild-type gene activity, thereby alleviating the *tm593* phenotype. The need for sufficient *ot108* dosage is also illustrated by the fact that the phenotype of *ot108* mutant animals can be rescued through supplying wild-type copies of the locus (Table 1).

We considered the possibility that the complete removal of *lsy-27* in *tm593* animals may be mostly compensated for by *lsy-27* paralogs, while the *ot108* allele may interfere with the compensatory function of the paralogues. Through the use of deletion alleles of these loci (again kindly provided by the *C. elegans* knockout facility in Tokyo), we found that neither of the two most closely related *lsy-27* paralogs, *ztf-25* or *ztf-28*, either alone or in combination (*i.e.*, *ztf-25 ztf-28* double nulls) displayed a Lsy phenotype (Table S2). *ztf-28 lsy-27* double-null mutant animals also display no Lsy phenotype. *ztf-25 lsy-27* double mutants could not be built due to close linkage of the two loci, and we therefore needed to resort to RNAi. *lsy-27* RNAi in a *ztf-28 ztf-25* double-mutant background also did not result in a Lsy phenotype, but we note that even though *lsy-27* RNAi does suppress the *ot108* Lsy phenotype, it does not recapitulate the *lsy-27(tm593)*

phenotype (Table 2), thereby allowing no firm conclusion about a triple loss of function of all three *lsy-27* paralogs.

Expression pattern and timing of action of *lsy-27*

By recombineering *yfp* into the fosmid that contains the *lsy-27* locus and that rescues the *lsy-27* phenotype (Table 2), we generated a reporter with which we monitored *lsy-27* expression (Figure 2A). We find that *lsy-27* is expressed very broadly throughout the embryo (Figure 3A). Expression can already be observed in one-cell embryos and continues to about the comma stage, when expression starts to fade out (Figure 3A). By the comma stage, most neurons, including ASEL/ASER, have terminally divided and begun to terminally differentiate. No expression is observed after hatching in larvae or in adult animals. Through colocalizing expression of the *lsy-27* reporter with an ASE-specific mCherry reporter, we confirmed that *lsy-27* is expressed in both ASE neurons in the comma-stage embryo when ASE laterality is established. As assessed with translational *gfp* reporters that fuse the entire loci to *gfp*, the most closely related *lsy-27* paralog, *ztf-25*, displays an essentially indistinguishable broad, embryo-restricted expression pattern (Figure S3), while the more distant paralog *ztf-28* shows no expression in embryos and postembryonically is expressed only in the intestine (data not shown).

The expression pattern of *lsy-27* suggests an embryonic role for the gene. We sought to corroborate this notion by exploiting the observation that the *ot108* allele is strongly temperature sensitive (Figure 3B). At 25°, 87% of animals display a Lsy phenotype while 12% do at 15°. By altering *lsy-27* gene activity at different stages through temperature shifts, we find that *lsy-27* activity is required only during embryogenesis, but not during postembryonic stages (Figure
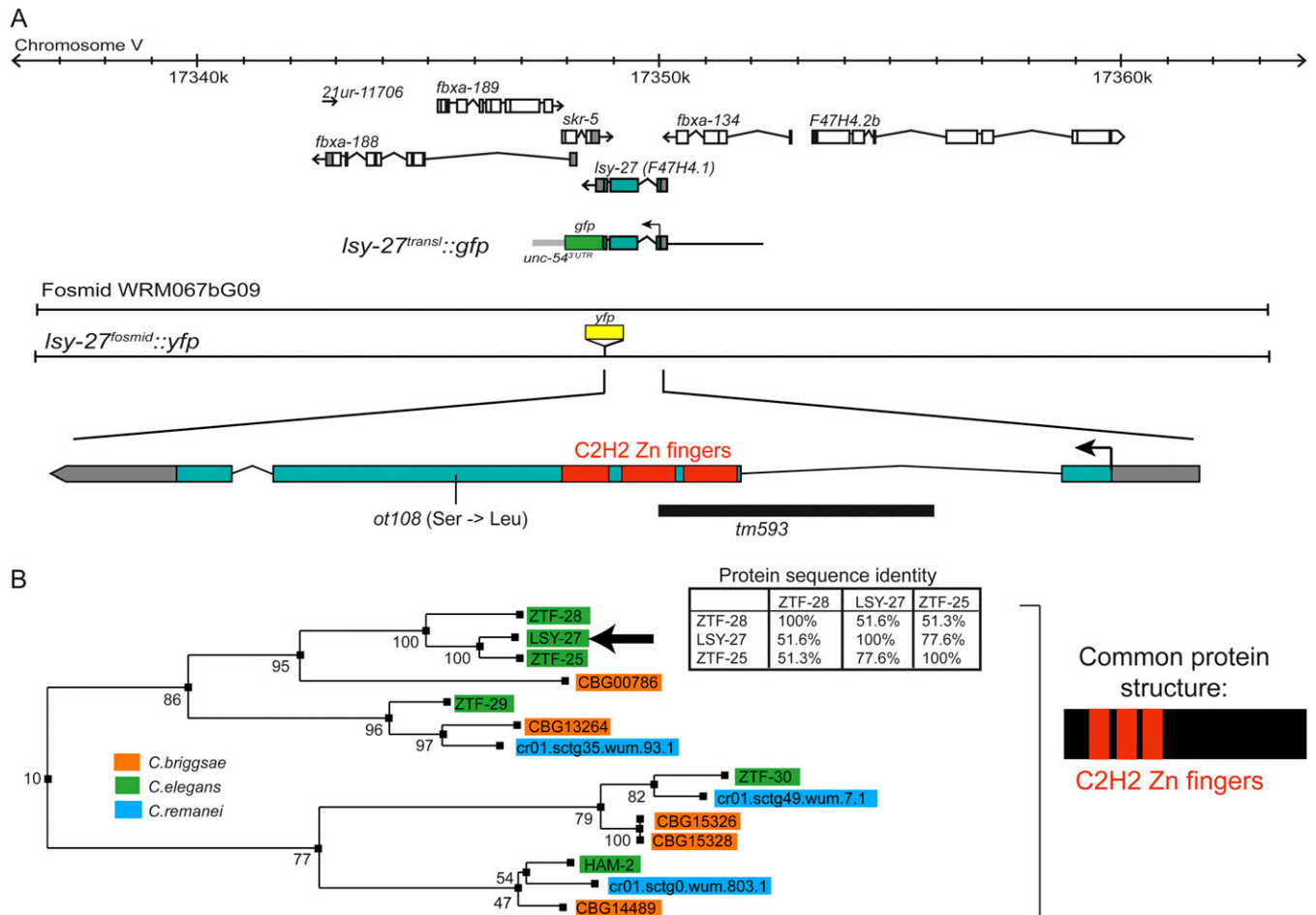
Figure 2 *lsy-27* is a new C2H2 Zn-finger protein. (A) Genomic position of *lsy-27* and rescue and reporter gene constructs. See File S1 for details on the generation of the reporter constructs. (B) The *lsy-27* gene family [modified from the TF317235 family tree generated by Treefam (http://www.treefam. org)] (Li *et al.* 2006).

3B). This contrasts with the continuous requirement of other *lsy* genes during postembryonic stages (O'Meara *et al.* 2010) and demonstrates that laterality control can be divided into initiation and maintenance phases.

The maternal loading of LSY-27 protein into oocytes as well as the embryonic focus of action also prompted us to ask whether *lsy-27* gene activity can be solely maternally supplied. Using transgenic *lsy-27* mutant animals that carry the germline-expressed *lsy-27* reporter fosmid, we assayed progeny that have lost the array and therefore contain only maternally supplied gene activity. In such animals, the Lsy phenotype is rescued (Table 2), corroborating maternal deposition of *lsy-27* gene activity.

Concluding remarks

We have described here a member of a nematode-specific C2H2 Zn-finger transcription factor family, *lsy-27*, which functions in ASE laterality control. The *lsy-27* mutant phenotype is similar to that of the ASEL-restricted LIM homeobox gene *lim-6*, as well as the ubiquitously expressed *lin-59* histone methyltransferase. We found that *lsy-27* not only

affects the terminal *gcy* gene markers in a manner similar to *lim-6*, but also affects *lim-6* expression. The embryo-restricted expression and function of *lsy-27* contrasts with the expression of *lim-6*, which is expressed continuously throughout the life of the ASEL neuron. We propose that the function of *lsy-27* is restricted to triggering the initial onset of *lim-6* expression. *lsy-27* may cooperate with ASEL-expressed *die-1* to trigger *lim-6* expression in the embryo. Once *lim-6* is turned on, *lsy-27* is no longer required to control laterality. This maintenance role is carried out by *die-1* (O'Meara *et al.* 2010) in conjunction with *lim-6*, which positively autoregulates (Johnston *et al.* 2005). Interestingly, *lsy-27* is not involved in conveying other *die-1* functions, such as the induction of ASEL fate markers (*e.g., gcy-7*), since those are affected only in *die-1*, but not in *lsy-27* mutants.

With the molecular identification of *ot108* and *ot146*, we have identified all but one gene retrieved from our large-scale screening of left/right asymmetry mutants (summarized in Table S4). Due to some adjustments in allele assignments as described here and elsewhere (Etchberger *et al.* 2009; Sarin *et al.* 2009; Flowers *et al.* 2010), we have recalculated saturation using various models (Sarin *et al.*
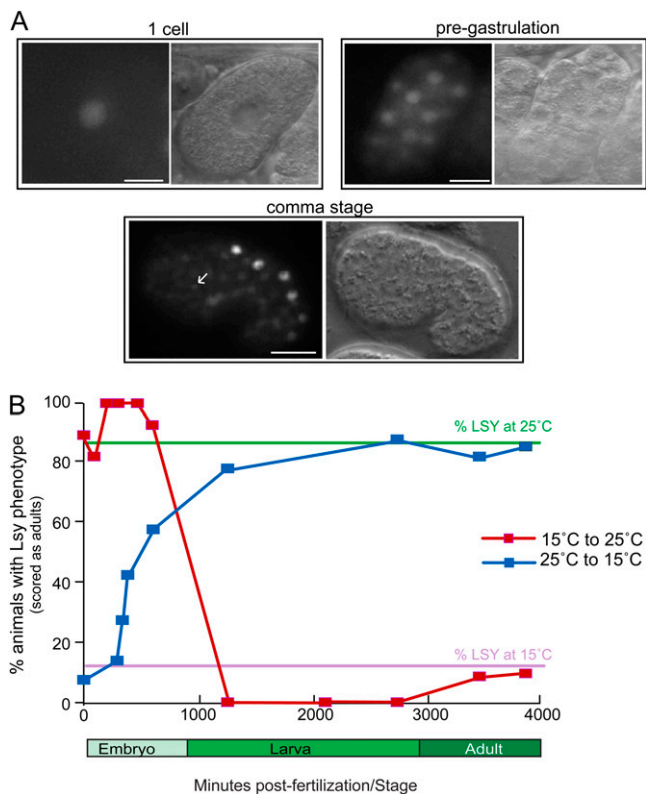
Figure 3 *lsy-27* is expressed and acts during the initiation but not during the maintenance phase of left/right asymmetry control. (A) Expression pattern of *lsy-27^fosmid^::yfp* (shown in Figure 2A) at different embryonic stages. The embryos appear slightly deformed as they are squished together in the gonad of an adult animal. The white arrow indicates the ASEL neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter *otIs232(che-1::mCherry)* (not shown). The *lsy-27^transl^::gfp* (shown in Figure 2A) shows a similar expression pattern except that, due to its failure to be expressed in the germline, we see only the onset of expression when zygotic gene expression starts in the early embryo. Bar, 10 μm. (B) Temperature-shift experiments with *ot108; ntIs1* animals indicate that *lsy-27* activity is required only during embryogenesis, but not during postembryonic stages. Animals were cultured for at least three generations at either 15° or 25°. Animals were analyzed by isolating two- to four-cell embryos and temperature shifts were performed at various developmental stages. All animals were scored as 3-day-old adults.

2007) and retain our previous conclusion that the screen has not yet reached saturation. Future genetic screens are likely to provide further insights into the control of lateralized gene expression in the nervous system.

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GENETICS

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Figure S1 Updated *lim-6* locus and the location of the *o146* allele. Based on our own 5'RACE results (using the Invitrogen GeneRacer Kit and the following lim-6 gene specific reverse primers: lim-6-5'RACEup: GATCACTGTTCCGAGAGAG and lim-6-5'RACEupnest: GGTCCAAATCTACTATGTCGC) and based on conservation to other nematode orthologs, the gene annotation shown here updates previous Wormbase annotations of *lim-6* through the addition of two new exons (first two exons). This put previously identified *cis*-regulatory sites for *lim-6* expression - two CHE-1 binding ASE motifs and a presumptive autoregulatory motif (ETCHBERGER *et al.* 2009) - into the second intron of the gene, as indicated. The Cys to Tyr mutation in *ot146* affects an invariant residue in the second LIM domain.

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LSY-27      M-TSIHSP--------------VTRKEDTELKRPDLRGKFVCSSCSQNFQ
ZTF-25      M-TSILNP--------------VIRKEYSELKRPDLRGKFICASCGQHFQ
ZTF-28      MDTSDHKPLIDRFETEFAEKSSLLRKEESELKRPDLRGDFLCLSCGQNFK
            * **   .*                 : *** :*********.*:* **.*:*:

                ↓   ↓    ↓                   ↓   ↓  ↓
LSY-27      HSASLNRHRQLMHSNEHTCMMCERALNQKETIREHMRNEHNLAQVFTCGC
ZTF-25      HNASLNRHRRLLHGNEHTCMMCDRKLNAKETIRDHMRNEHNLFQVFTCGC
ZTF-28      HGASLNRHRKLVHSDEYTCMLCARKLYLKETVRDHMRNEHYLGQVYTCGC
            *.*******:*:*.:*:**:* * *   ***:*:****** * **:****

                 ↓   ↓   ↓
LSY-27      CNWTFASKRQLTEHTKCIQGTGAPGDTIPIAKSINAPGSLIQSTIQGTPP
ZTF-25      CNWSFSSKRQLSEHTKCIQGTGAPGDTIPIAKSCNAPGSLIQSTIQGTPP
ZTF-28      CNWTFSNKKALTEHAKAIQETGAPGDANPIAKSGNSPGSLLKSPIQRAG-
            ***:*:.*: *:**:*.** ******: ***** *:****::*.** :

                        L in ot108
LSY-27      VVKTGRKRPMGGSLSPSSSVS-TSISSRDASGSPP--------------P
ZTF-25      VVKTGRK--RGGSLSSSSSVS-TSISSRDVSGSPP--------------P
ZTF-28      -FKLNRL--LNKSLSPSTSNSTTSSSSRDASGSPQPELELKLEPEPEPEP
             .* .*     . ***.*:* * ** ****.****                *

LSY-27      TEEEAERKVLFDNAVDTILQSKFFTYQQITEVDTWVKIIESANTLADTLQ
ZTF-25      TEEEAERKVLFDNAVDTILQSKFFTYQQITEVDTWIKIIENANTLADTLQ
ZTF-28      NEEALEYKRFFNNAVDTILQRNFFTDQQITEVDTWVIIIESANALATALQ
            .**   * * :*:******** :*** *********: ***.**:** :**

LSY-27      RIKKSQKVKAEGPAVESKMIPEKHVKQEIE
ZTF-25      RINKTKKIKAEMPA----DKDVKRFKLEVE
ZTF-28      SYKKAQQIQPAEPA----MIPEKRIKPEIE
             :*::::::.  **        *:.* *:*
```

Figure S2 Sequence of LSY-27 and its two paralogs, ZTF-25 and ZTF-28. The C2H2 Zn fingers and the position of the *ot108* allele are indicated. The blue F and L residues can be found in the ~50% of all C2H2 Zn fingers (http://smart.embl-heidelberg.de/). The arrows indicate positions in the C2H2 Zn fingers thought to contact DNA (PAVLETICH and PABO 1991).

A

B

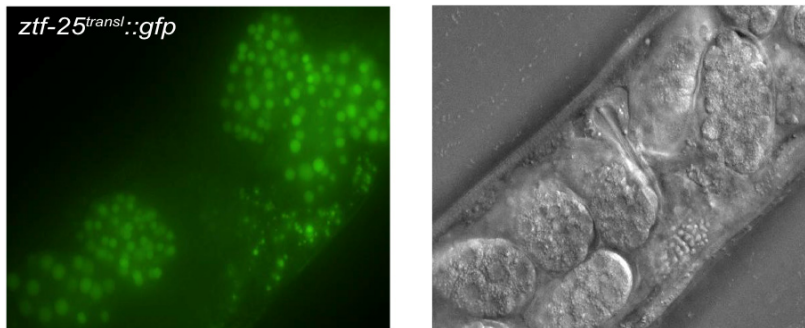**Figure S3** Expression pattern of the *lsy-27* paralog *ztf-25*. A: Gene and allele structure of *ztf-25*. A translational reporter was generating by fusing the genomic DNA containing 1.3 kb upstream of ATG to GFP and a heterologous 3'UTR. Note the proximity of *ztf-25* and its closest paralog, *lsy-27*. B: *ztf-5^transl^::gfp* expression pattern in midembryonic stages. Embryos are contained within the gonad of the mother.

<div align="center">

File S1

Supporting Information

</div>

Supporting Material and Methods

Genetic screen

The screen that uncovered *ot146* and *ot108*, as well as a large number of additional *lsy* genes has been described (SARIN *et al.* 2007). Since the appearance of that paper, the identities of several alleles originally characterized as novel genes have been resolved, including mutants described in this paper. We provide a summary of the updated gene designations and molecular identities in Table S3 and Table S4.

Transgenes

Transgenes that label ASEL and/or ASER fates:

otIs114 = Is[lim-6^{prom}::gfp; rol-6(d)]

otIs3= [gcy-7^{prom}::gfp; lin-15(+)]

ntIs1 = [gcy-5^{prom}::gfp; lin-15(+)]

otIs220 = Is[gcy-5^{prom}::mCherry; rol-6(d)]

otIs151 = Is[ceh-36^{prom}::DsRed; rol-6(d)]

otIs232 = Is[che-1^{promA}::mChopti::che-1_3'UTR; rol-6(d)]

Rescue and expression constructs:

otEx4280, otEx4281 = two independent lines of Ex[fosmid WRM067BG09; elt-2::gfp].

otEx4400, otEx4401 = two independent lines of Ex[lsy-27^{transl}::gfp; elt-2::gfp]

otEx4501, otEx4502 = two independent lines of Ex[fosmid_WRM067BG09::yfp; elt-2::gfp]

otEx4523 = Ex[fosmid_WRM067BG09::yfp]

otEx4337-4339 = three independent lines of Ex[ztf-25^{transl}::gfp; rol-6(d)]]

otEx3859 = Ex[lim-6^{fosmid}::yfp; rol-6[d]].

Generation of expression constructs

lsy-27^{transl}::gfp was generated by PCR-fusing the genomic locus of *lsy-27* including 2.6 kb of the upstream region to the *gfp* coding region and *unc-54_3'UTR* (30ng/μl), using a standard PCR fusion protocol (HOBERT 2002) and was coinjected with *elt-2::gfp* (50ng/μl) as injection marker. *ztf-25^{transl}::gfp* was generated by PCR-fusing the genomic locus of *ztf-25* including 1.3 kb of the upstream region to the *gfp* coding region and *unc-54_3'UTR* (30ng/μl) and was coinjected with *rol-6(d)* (50ng/μl) as injection marker. *lsy-27^{fosmid}::yfp* was generated by inserting yfp right before the stop codon of *lsy-27* in fosmid *WRM067BG09* (TURSUN *et al.* 2009).

The primer sequences for these constructs are as follows (from 5' to 3'):

lsy-27^{transl}::gfp:

lsy-27_translat_A: CTGATACGAGTACGGCATGGC

lsy-27_translat_A*: GTCACGCGCAAATGCATAC

lsy-27_translat_B: AGTCGACCTGCAGGCATGCAAGCTCTCAATTTCCTGCTTGACGTGC

lsy-27_translat_C: AGCTTGCATGCCTGCAGGTCG

lsy-27_translat_D: AAGGGCCCGTACGGCCGACTA

lsy-27_translat_D*: GGAAACAGTTATGTTTGGTATA

ztf-25[transl]::gfp:

ztf-25_translat_A: GAGCCACTATCCACGCAC

ztf-25_translat_A*: GAAATGCGTGGAGTTGC

ztf-25_translat_B: AGTCGACCTGCAGGCATGCAAGCTCTCAACTTCCAGCTTGAAACGCTTC

C, D, D* same as above

lsy-27[fosmid]::yfp:

lsy-27_fosmid_Fwd: *CCGTCGAGAGCAAGATGATTCCAGAGAAGCACGTCAAGCAGGAAATTGAGatgagtaaaggagaagaacttttcac*

lsy-27_fosmid_Rev:

ataaaactgaacatcagttgaatagggagttaaaataggaaataaaatagTTAtttgtatagttcatccatgccatg

Genotyping the *ztf-25, ztf-28* and *lsy-27* deletion alleles

We genotyped animals for the presence of the deletion alleles:

  tm593 genotyping primers:

tm593_external_fwd: CTCTCCCCTTCTCCACAAC

tm593_external_rev: GCGTTGGAGTGTGTCAGC

  tm610 genotyping primers:

tm610_external_fwd: GACAGTCCTGGTTACTTGGTAG

tm610_external_rev: CAGGTGGAACTGATCGTG

  tm573 genotyping primers:

tm573_external_fwd: CCGAGCTGGATAGGGAG

tm573_external_rev: CAGGACAAGCAGAGGAAATC

Molecular characterization of the *lsy-27(tm593)* allele

  We determined the precise nature of the *lsy-27(tm593)* deletion through Sanger sequecning and find that it is a 361 bp deletion with one T inserted instead :

Wild type: ...tttttggttaagagcccgctc **[361 base pairs]** tttgagaactttttcaagctt...

tm593   ...tttttggttaagagcccgctc **T** tttgagaactttttcaagctt...

  To analyze the transcriptional product made in *tm593* animals, we performed RT-PCR analysis with the Invitrogen Superscript one-step RT-PCR System with Platinum Taq Polymerase using primers located at the 5' and 3' end of the coding sequence:

F47H4.1_RT_A: CGTCTATTCACAGTCCAGTCAC

F47H4.1_RT_B: GAATCATCTTGCTCTCGACG

  Individual band were then gel purified and Sanger sequenced. We detected three different transcripts of different length, all starting with the first, unaffected exon, but then reading in various different ways into the first intron before splicing into downstream exons. Each transcript contains premature stop codons and encode severely truncated versions of the protein (23 aa, 35 aa, and 37 aa long), none of which contain any of the DNA-binding Zn finger domains.

  Unexpectedly, we detected a PCR product with two primers that are entirely located within the deletion. We suspect that the deleted DNA has inserted elsewhere in the genome but emphasize that the RT-PCR analysis described above suggests that no functional product is present.

Table S1 Whole genome sequencing setting and results

Parameter	Setting/Results
Read length	75
Number of lanes on flow cells	3
Average coverage	31.5
Size of genetically defined interval	13 Mb
Total variants in interval compared to wild-type reference genome on right arm of LGV	961
Noncoding (Intergenic/intronic/silent/ncRNA/SNP)	939
Splice juction/missense/nonsense	22
Total variants minus strain background variants *	3

Variants were considered as background if they were also found in other WGS datasets from our lab (SARIN *et al.* 2010).

Table S2   Mutant analysis of *lsy-27* paralogues

Genotype	% of animals expressing *gcy-5 (ntIs1)* at			
	25˚C			
	ASEL=ASER	ASEL<ASER	ASER only	n
wild type	0%	0%	>100	>100
lsy-27(ot108)	39%	48%	13%	122
lsy-27(tm593)	0%	62%	38%	117
ztf-25(tm610)	0%	0%	100%	46
ztf-28(tm573)	0%	0%	100%	28
lsy-27(tm593); ztf-28(tm573)	0%	49%	51%	168
ztf-25(tm610); ztf-28(tm573)	0%	0%	100%	78
ztf-25(tm610); ztf-28(tm573); lsy-27(RNAi) [1]	0%	0%	100%	32

[1] Triple couldn't be built due to close linkage of *lsy-27* and *ztf-25*. Wild-type, *ot108* and *tm593* data is repeated from Table 1 for comparison purposes.

Table S3 Retired and novel *lsy* gene names

Original assignment [1]	Allele	novel assignment	Reference
lsy-5	ot37, ot240	*unc-37*	(FLOWERS *et al.* 2010)
lsy-14	ot101	*che-1*	(ETCHBERGER *et al.* 2009)
	ot146	*lim-6*	this paper
lsy-16	ot158	*nhr-67*	(SARIN *et al.* 2009)
lsy-17	ot190	*nhr-67*	(SARIN *et al.* 2009)
lsy-18	ot192	*fozi-1*	unpubl. data [2]
lsy-19	ot177	*lsy-12*	(O'MEARA *et al.* 2010)
die-1	ot108	*lsy-27*	this paper

[1] as shown in (SARIN *et al.* 2007)

[2] *ot192* was initially thought to be a distinct locus based on mapping results that were misleading likely due to incompatabilities between N2 Bristol and the Hawaian mapping strain. The *ot192* mutation is a C>T change that results in a premature stop codon in *fozi-1* (Q549Stop). The same mutation is found in *ot191* animals (SARIN *et al.* 2007) and we cannot excluse the possibility that these two mutations arose from the same parent ("jackpot" mutation).

Table S4   Final summary of mutant classes & genes

Mutant class	ASE phenotype	# of genes	Gene names	Molecular identity	# of alleles	Reference
Class I	"2 ASEL"	3	*cog-1*	homeobox	19	(CHANG *et al.* 2003; SARIN *et al.* 2007)
			unc-37	tsk. co-factor	4	(CHANG *et al.* 2003; FLOWERS *et al.* 2010; SARIN *et al.* 2007)
			lsy-22	tsk. co-factor	2	(FLOWERS *et al.* 2010)
Class II	"2 ASER"	7	*die-1*	C2H2 Zn finger TF	8	(CHANG *et al.* 2004; SARIN *et al.* 2007)
			lsy-2	C2H2 Zn finger TF	6	(JOHNSTON and HOBERT 2005; SARIN *et al.* 2007)
			lsy-12	MYST HAT	6	(O'MEARA *et al.* 2010; SARIN *et al.* 2007)
			lsy-6	miRNA	4	(JOHNSTON and HOBERT 2003; SARIN *et al.* 2007)
			lin-49	tsk. co-factor	3	(CHANG *et al.* 2003; SARIN *et al.* 2007)
			lsy-15	WD40	1	(SARIN *et al.* 2007), Poole et al. submitted
			ceh-36	Homeobox	1	(CHANG *et al.* 2003; SARIN *et al.* 2007)
Class III	no ASEL/R fate specification	1	*che-1*	C2H2 Zn finger TF	24	(ETCHBERGER *et al.* 2009; SARIN *et al.* 2007)
Class IV	mixed fate in ASEL or ASER	5	*fozi-1*	C2H2 Zn finger TF	13	(JOHNSTON *et al.* 2006; SARIN *et al.* 2007)
			lsy-20	unknown	1	(SARIN *et al.* 2007)
			lin-59 (prev. lsy-26)	SET domain	1	(SARIN *et al.* 2010)
			lim-6	LIM homeobox	1	(HOBERT *et al.* 1999), this paper
			lsy-27	C2H2 Zn finger TF	1	this paper
Class V	heterogeneous phenotype	1	*nhr-67*	C4 Zn finger TF	7	(SARIN *et al.* 2009)

This is an updated version of the mutant summary table from (SARIN *et al.* 2007). Mutants with <10% penetrance (e.g. *lsy-21*, (SARIN *et al.* 2007)) are not shown. Only class I to V *lsy* mutants are shown. Class VI do not affect ASE per se, but affect other cells. See also Table S3 for altered gene assignments, as compared to (SARIN *et al.* 2007).

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