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 laterally symmetric) 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

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

Upon isolation of \textit{ot108} mutant animals in our original \textit{Lsy} screen (Sarin et al. 2007), we noted that \textit{ot108} fails to complement the derepression of ASER fate in the ASEL phenotype of a mutation in the \textit{die-1} Zn-finger transcription factor, an inducer of \textit{lim}-6 expression in \textit{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 \textit{ot108} to be an allele of \textit{die-1} (Sarin et al. 2007). However, our subsequent analysis revealed no mutation in the \textit{die-1} locus of \textit{ot108} mutant animals, and, moreover, the \textit{ot108} mutant phenotype could not be rescued with a genomic piece of DNA that rescues a canonical \textit{die-1} allele (data not shown). Subsequent chromosomal linkage analysis showed that \textit{ot108} is linked to chromosome V, while \textit{die-1} maps to chromosome II. After mapping \textit{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 \textit{F47H4.1} (Figure 2A and Figure S2). \textit{F47H4.1} is a member of C2H2 Zn-finger transcription factors with several paralogs in \textit{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 \textit{ot108} is phylogenetically conserved (Figure S2). The only gene in this family that had been previously characterized is the \textit{ham}-2 transcription factor, which is involved in \textit{C. elegans} HSN motor neuron specification (Baum et al. 1999).

Both a fosmid spanning the entire \textit{F47H4.1} locus plus neighboring genes and a genomic piece of DNA containing 2.6 kb upstream of \textit{F47H4.1} and the \textit{F47H4.1} locus (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 \textit{ot108} is phylogenetically conserved (Figure S2). The only gene in this family that had been previously characterized is the \textit{ham}-2 transcription factor, which is involved in \textit{C. elegans} HSN motor neuron specification (Baum et al. 1999).

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### \textbf{Table 1} \textit{Lsy} phenotypes of \textit{lim}-6 and \textit{lsy-27}

| % animals with the following phenotypes (at 25°C) | 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 | 100   |
| \textit{lim-6}(n2073)                          | 0             | 0               | 0                | 0               | 0               | 0               | 0   | 0     |
| \textit{lim-6}(ot146)                          | 0             | 0               | 0                | 0               | 0               | 0               | 0   | 0     |
| \textit{lsy-27}(ot108)                         | 0             | 0               | 0                | 0               | 0               | 0               | 0   | 0     |
| \textit{lsy-27}(tm593)                         | 0             | 0               | 0                | 0               | 0               | 0               | 0   | 0     |
| \textit{lsy-27}(tm593)                         | 0             | 0               | 0                | 0               | 0               | 0               | 0   | 0     |
| ASER marker (gcy-5::gfp; nts1)                 |               |                 |                  |                 |                 |                 |    |       |
| Wild type                                      | 0             | 0               | 0                | 0               | 100             | >100            | 0   | 0     |
| \textit{lim-6}(n2073)                          | 0             | 0               | 89               | 0               | 6               | 82              | 94  |
| \textit{lim-6}(ot146)                          | 0             | 0               | 51               | 0               | 44              | 90              | 56  |
| \textit{ot146}(n2073)                          | 0             | 0               | 39               | 0               | 13              | 122             | 87  |
| \textit{lsy-27}(ot108)                         | 0             | 0               | 62               | 0               | 38              | 117             | 62  |
| \textit{lsy-27}(tm593)                         | 0             | 0               | 6                | 0               | 91              | 31              | 9   |
| \textit{ot108}                                 | 0             | 0               | 6                | 0               | 100             | 56              | 0   |

\textit{ot108} is an altered function allele

The \textit{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 \textit{tm593} animals, which do not contain any of the DNA-binding Zn-fingers (see File S1). We were therefore surprised to note that the \textit{Lsy} phenotype of the \textit{tm593} deletion allele is notably milder than the \textit{ot108} missense allele in terms of both expressivity and penetrance (Table 1). We
therefore considered the possibility that \textit{ot108} (which is recessive) is an altered function allele (Table 1). We tested this possibility by removing \textit{lsy-27} gene activity in \textit{ot108} mutant animals using RNA interference (RNAi) directed against \textit{lsy-27}. We found that RNAi treatment completely reverted the \textit{ot108} phenotype (Table 2), suggesting that it is indeed altered \textit{lsy-27} function that explains the \textit{ot108} phenotype.

We noted that animals that carry one copy of the \textit{ot108} allele and one copy of the \textit{tm593} allele display a phenotype that is even milder than the phenotype of either allele alone (Table 1). One copy of the \textit{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 \textit{tm593} phenotype. The need for sufficient \textit{ot108} dosage is also illustrated by the fact that the phenotype of \textit{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 \textit{lsy-27} in \textit{tm593} animals may be mostly compensated for by \textit{lsy-27} paralogs, while the \textit{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 \textit{C. elegans} knockout facility in Tokyo), we found that neither of the two most closely related \textit{lsy-27} paralogs, \textit{ztf-25} or \textit{ztf-28}, either alone or in combination (i.e., \textit{ztf-25 ztf-28} double nulls) displayed a \textit{Lsy} phenotype (Table S2). \textit{ztf-25 lsy-27} double-null mutant animals also display no \textit{Lsy} phenotype. \textit{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. \textit{lsy-27} RNAi in a \textit{ztf-25 ztf-28} double-mutant background also did not result in a \textit{Lsy} phenotype, but we note that even though \textit{lsy-27} RNAi does suppress the \textit{ot108} \textit{Lsy} phenotype, it does not recapitulate the \textit{lsy-27(tm593)} phenotype (Table 2), thereby allowing no firm conclusion about a triple loss of function of all three \textit{lsy-27} paralogs.

**Expression pattern and timing of action of \textit{lsy-27}**

By recombining yfp into the fosmid that contains the \textit{lsy-27} locus and that rescues the \textit{lsy-27} phenotype (Table 2), we generated a reporter with which we monitored \textit{lsy-27} expression (Figure 2A). We find that \textit{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 \textit{ASE/LASER}, 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 \textit{lsy-27} reporter with an \textit{ASE}-specific mCherry reporter, we confirmed that \textit{lsy-27} is expressed in both \textit{ASE} neurons in the comma-stage embryo when \textit{ASE} laterality is established. As assessed with translational \textit{gfp} reporters that fuse the entire loci to \textit{gfp}, the most closely related \textit{lsy-27} paralog, \textit{ztf-25}, displays an essentially indistinguishable broad, embryo-restricted expression pattern (Figure S3), while the more distant paralog \textit{ztf-28} shows no expression in embryos and postembryonically is expressed only in the intestine (data not shown).

The expression pattern of \textit{lsy-27} suggests an embryonic role for the gene. We sought to corroborate this notion by exploiting the observation that the \textit{ot108} allele is strongly temperature sensitive (Figure 3B). At 25°, 87% of animals display a \textit{Lsy} phenotype while 12% do at 15°. By altering \textit{lsy-27} gene activity at different stages through temperature shifts, we find that \textit{lsy-27} activity is required only during embryogenesis, but not during postembryonic stages (Figure

### Table 2 Transformation rescue and RNAi analysis

| Genotype | Lsy phenotype* (%) | Wild-type phenotype (%) | n |
|----------|--------------------|------------------------|---|
| Wild type | 0                  | 100                    | >100 |
| \textit{lim-6}(ot146) | 85                  | 15                     | 78 |
| \textit{ot146; otEx3859 (Ex[lim-6 fosmid::yfp; rol-6(d)])} | 0                  | 100                    | 41 |
| \textit{lsy-27(ot108)} | 86.9               | 13.1                   | 122 |
| \textit{lsy-27(ot108); lsy-27(RNAi)} | 2.5               | 97.5                   | 120 |
| \textit{lsy-27(ot108); empty vector (RNAi)} | 86.8               | 13.2                   | 111 |
| \textit{lsy-27(RNAi)} | 0                  | 100                    | 71 |
| \textit{lsy-27(ot108); Ex[lsy-27transl::gfp], line #1b} | 18.2               | 81.8                   | 44 |
| \textit{lsy-27(ot108); Ex[lsy-27transl::gfp], line #2} | 17.2               | 82.8                   | 87 |
| \textit{lsy-27(ot108); Ex[lsy-27transl::gfp], line #3} | 5.6               | 94.4                   | 18 |
| \textit{lsy-27(ot108); Ex[lsy-27transl::gfp], line #2} | 9.1               | 90.9                   | 44 |
| \textit{lsy-27(ot108); Ex[lsy-27transl::yfp], line #1b} | 0                 | 100                    | 70 |
| Genotype as above but array not transmitted from parental generationc | 0 | 100 | 11 |
| \textit{lsy-27(ot108); Ex[lsy-27transl::yfp], line #2} | 0 | 100 | 54 |
| Genotype as above but array not transmitted from parental generation | 21.1 | 78.9 | 19 |

The \textit{ot108} and \textit{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.

* Scored as a \textit{gcy-5} reporter (\textit{ntIs114}) derepressed in \textit{ASE} in first eleven rows or loss of \textit{lim-6}: \textit{gfp} (\textit{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 \textit{elt-2}:: \textit{gfp} injection marker. Animals derived from \textit{elt-2}:: \textit{gfp(+)} parents that have lost this array as assessed by lack of intestinal \textit{gfp} expression were scored.
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. 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. 2009).
27fosmid::yfp pattern of the maintenance phase of left/right asymmetry control. (A) Expression of 27::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 two- to four-cell embryos 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 ASE neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter otIs232(che-1::mCherry) (not shown). The 27transl::gfp (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 ASE neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter otIs232(che-1::mCherry) (not shown). The 27transl::gfp (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 ASE neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter otIs232(che-1::mCherry) (not shown). The 27transl::gfp (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 ASE neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter otIs232(che-1::mCherry) (not shown).

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-27fosmid::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 ASE neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter otIs232(che-1::mCherry) (not shown). The lsy-27fosmid::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 ASE neuron shortly after birth based on colocalization with a bilateral ASE-specific reporter otIs232(che-1::mCherry) (not shown).

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Expression of lsy-27::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 two- to four-cell embryos at either 15°C or 25°C. 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.

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

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**Figure S1** Updated *lim-6* locus and the location of the *ot146* 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: GATCAGTGTTCCGGAGAGAG and *lim-6*-5′RACEdown: GGTCCTACTATATGTCGCG) 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.
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 PAHO 1991).
**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-25*<sup>trans::gfp</sup> expression pattern in midembryonic stages. Embryos are contained within the gonad of the mother.
Supporting Material and Methods

Genetic screen

The screen that uncovered *at146* and *at108*, 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:

\[ \text{otls}114 = \text{ls}[\text{lim-6}^{\text{prom}}::\text{gfp}; \text{rol-6}(d)] \]
\[ \text{otls}3 = [\text{gcy-7}^{\text{prom}}::\text{gfp}; \text{lin-15}(+)] \]
\[ \text{ntls}1 = [\text{gcy-5}^{\text{prom}}::\text{gfp}; \text{lin-15}(+)] \]
\[ \text{otls}220 = \text{ls}[\text{gcy-5}^{\text{prom}}::\text{mCherry}; \text{rol-6}(d)] \]
\[ \text{otls}151 = \text{ls}[\text{ceh-36}^{\text{prom}}::\text{DsRed}; \text{rol-6}(d)] \]
\[ \text{otls}232 = \text{ls}[\text{che-1}^{\text{prom}}::\text{mChopti}::\text{che-1}_{3}^{\text{UTR}}; \text{rol-6}(d)] \]

Rescue and expression constructs:

\[ \text{otEx}4280, \text{otEx}4281 = \text{two independent lines of Ex[fosmid WRM067BG09; elt-2::gfp]} \]
\[ \text{otEx}4400, \text{otEx}4401 = \text{two independent lines of Ex[lsy-27}^{\text{transi}}::\text{gfp}; \text{elt-2::gfp]} \]
\[ \text{otEx}4501, \text{otEx}4502 = \text{two independent lines of Ex[fosmid WRM067BG09::yfp; elt-2::gfp]} \]
\[ \text{otEx}4523 = \text{Ex[fosmid WRM067BG09::yfp]} \]
\[ \text{otEx}4337-4339 = \text{three independent lines of Ex[ztf-25}^{\text{transi}}::\text{gfp}; \text{rol-6}(d)] \]
\[ \text{otEx}3859 = \text{Ex[lim-6}^{\text{fosmid}}::\text{yfp}; \text{rol-6}(d)] \].

Generation of expression constructs

*lsy-27}^{\text{transi}}::\text{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-S4_{3}^{\text{UTR}} (30ng/μl)*, using a standard PCR fusion protocol (Hobert 2002) and was coinfected with *elt-2::gfp* (50ng/μl) as injection marker. *ztf-25}^{\text{transi}}::\text{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-S4_{3}^{\text{UTR}} (30ng/μl)* and was coinfected with *rol-6(d) (50ng/μl)* as injection marker. *lsy-27}^{\text{transi}}::\text{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’):

\[ \text{lsy-27}^{\text{transi}}::\text{gfp}: \]
\[ \text{lsy-27_{translat}_A: CTGATACGAGTACGCGCATGGC} \]
\[ \text{lsy-27_{translat}_A*: GTCACCGCGGAAATGACATAC} \]
\[ \text{lsy-27_{translat}_B: AGTCGACTGAGACGACGCTCTCAATTTCTGAGCTGACGTGC} \]
\[ \text{lsy-27_{translat}_C: AGCTTGATGCTGCTCAGGTCG} \]
\[ \text{lsy-27_{translat}_D: AAGGGGCCGTACGGCCGACTA} \]
\[ \text{lsy-27_{translat}_D*: GGAACACGTGTTATTTGGTATA} \]
**ztf-25**

*ztf-25*transl.*::gfp:

ztf-25_translat_A: GAGCCACTATCCACGCAC
tzf-25_translat_A*: GAAATGCGTGAGTTGC
ztf-25_translat_B: AGTCGACCTGCAGGATGCAAGCTCTGCTCAACTTCCAGGTTGAAACGCTTC

C, D, D* same as above

**lsy-27**fosmid

*lsy-27*fosmid_Fwd: CCGTCGAGAGCAAATGATTTTCCAGAGACGCTCTCAAGGATAAAATAGGATGACTGAAGAGGAGAATTTCTCCAC
*lsy-27*fosmid_Rev: ataaacctgaacatcagttcataaggaggttaaaataggtaataataaaatagTTAttgtatagttcatccatgccatg

**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: CTCTCCCCCTTCACCAAC
  - tm593_external_rev: GCGTTGGAAGTGTGACGC

- tm560 genotyping primers:
  - tm560_external_fwd: GACAGTCCTGTTACTTGGTAG
  - tm560_external_rev: CAGGTGGAACCTGATCGT

- tm573 genotyping primers:
  - tm573_external_fwd: CCGAGCTGGATAGGGAG
  - tm573_external_rev: CAGGACAAGCAGGAAATC

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

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

Wild type: ...tttttgaagccccgctc [361 base pairs] tttgagaacttttttaaatctt...
tm593 ...tttttgaagccccgctc T tttgagaacttttttaaatctt...

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: CGTCTTACAGTCAGTCTC
F47H4.1_RT_B: GAATCATCTTTGCTCTGAG

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).
| Genotype | % of animals expressing gcy-5 (ntls1) at 25°C | n |
|----------|-------------------------------------------|---|
|          | ASEL=ASE | ASEL<ASE | ASER only |  |
| 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) | 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.
| Original assignment ¹ | Allele | novel assignment | Reference |
|----------------------|--------|-----------------|-----------|
| Isy-5                | ot37, ot240 | unc-37 | (FLOWERS et al. 2010) |
| Isy-14               | ot101  | che-1          | (ETCHBERGER et al. 2009) |
|                     | ot146  | lim-6          | this paper |
| Isy-16               | ot158  | nhr-67         | (SARIN et al. 2009) |
| Isy-17               | ot190  | nhr-67         | (SARIN et al. 2009) |
| Isy-18               | ot192  | fozi-1         | unpubl. data ² |
| Isy-19               | ot177  | Isy-12         | (O’MEARA et al. 2010) |
| die-1                | ot108  | Isy-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 incompatibilities between N2 Bristol and the Hawaiian 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 exclude the possibility that these two mutations arose from the same parent (“jackpot” mutation).
| 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)         |
| Class III    | no ASE/R fate | 1          | che-1           | C2H2 Zn finger TF | 24          | (ETCHBERGER et al. 2009; SARIN et al. 2007)    |
| specification|               |            |                 |                  |             |                                               |
| Class IV     | mixed fate in | 5          | fozi-1          | C2H2 Zn finger TF | 13          | (JOHNSTON et al. 2006; SARIN et al. 2007)       |
|              | ASEL or ASER  |            | lsy-20          | unknown          | 1           | (SARIN et al. 2007)                            |
|              | lin-59 (prev. |            |                 | SET domain       | 1           | (SARIN et al. 2010)                            |
|              | lsy-27        |            |                 | LIM homeobox     | 1           | (HOBERT et al. 1999), this paper               |
| Class V      | heterogeneous | 1          | nhr-67          | C4 Zn finger TF  | 7           | (SARIN et al. 2009)                            |
| phenotype    |               |            |                 |                  |             |                                               |

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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