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Article

Early Embryonic Programming of Neuronal Left/Right Asymmetry in *C. elegans*

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Summary

Background: Nervous systems are largely bilaterally symmetric on a morphological level but often display striking degrees of functional left/right (L/R) asymmetry. How L/R asymmetric functional features are superimposed onto an essentially bilaterally symmetric structure and how nervous-system laterality relates to the L/R asymmetry of internal organs are poorly understood. We address these questions here by using the establishment of L/R asymmetry in the ASE chemosensory neurons of *C. elegans* as a paradigm. This bilaterally symmetric neuron pair is functionally lateralized in that it senses a distinct class of chemosensory cues and expresses a putative chemoreceptor family in a L/R asymmetric manner.
Results: We show that the directionality of the asymmetry of the two postmitotic ASE neurons ASE left (ASEL) and ASE right (ASER) in adults is dependent on a L-/R-symmetry-breaking event at a very early embryonic stage, the six-cell stage, which also establishes the L/R asymmetric placement of internal organs. However, the L/R asymmetry of the ASE neurons per se is dependent on an even earlier anterior-posterior (A/P) Notch signal that specifies embryonic ABa/ABp blastomere identities at the four-cell stage. This Notch signal, which functions through two T box genes, acts genetically upstream of a miRNA-controlled bistable feedback loop that regulates the L/R asymmetric gene-expression program in the postmitotic ASE cells.
Conclusions: Our results link adult neuronal laterality to the generation of the A/P axis at the two-cell stage and raise the possibility that neural asymmetries observed across the animal kingdom are similarly established by very early embryonic interactions.

Introduction

Despite the overall bilaterally symmetric nature of most animal body plans, several important exceptions from complete L/R bilateral symmetry exist. In most animals, organs such as the heart, stomach, and spleen are present on one side only. The nervous system of most animals is also largely bilaterally symmetric, yet several striking examples of L/R asymmetry exist on the functional level and, to a much lesser extent, also on the neuroanatomical level and the molecular level [1–8]. How the few known morphological and gene-expression lateralities in the nervous system relate to functional laterality is not understood.

Several mechanisms seem to be involved in determining the laterality of visceral asymmetries in vertebrates, including gap-junction communication, ion flux, and nodal cilia [9]. There is some evidence that neuroanatomical asymmetry is linked to these mechanisms [10]. However, human patients with inversus totalis show reversal of visceral asymmetries, but functional asymmetries of the brain, such as language dominance, remain intact in these patients [11, 12]. It therefore remains unclear how functional asymmetries of morphologically bilaterally symmetric structures are established within the nervous system and how they relate to visceral asymmetries.

The nervous system of *C. elegans* is largely bilaterally symmetric, yet L/R asymmetry of morphologically bilaterally symmetric neuron pairs is apparent at the level of both function and gene expression (reviewed in [1]). One striking example of L/R asymmetry is the ASEL/ASER gustatory neuron pair (Figure 1A). ASEL and ASER are morphologically bilaterally symmetric, displaying similar axodendritic morphology and synaptic connectivity [13]; moreover, both neurons display largely symmetric gene-expression programs (www.wormbase.org). However, ASEL and ASER sense distinct water-soluble molecules, thereby allowing the animal to discriminate between different chemosensory inputs [14]. This functional asymmetry is correlated with a directional asymmetry in the expression of a small number of genes, including several putative chemoreceptors of the GCY family; some members of this family are expressed in ASEL, thereby defining the "ASEL fate," and some are expressed in ASER, thereby defining the "ASER fate" (Figure 1A) [15, 16]. The adoption of ASEL versus ASER fate is layered on top of a largely bilaterally symmetric differentiation program and therefore represents an excellent paradigm for studying the generation of molecular and functional asymmetry in a morphologically bilaterally symmetric neuronal structure.

The terminal ASEL or ASER fates develop from a hybrid precursor state. Shortly after their birth, both ASE cells coexpress markers that later become restricted to either ASEL or ASER (Figure 1A) [17]. A bistable regulatory circuit, composed of several miRNAs and transcription factors, then ensures that ASER fate is turned off in ASEL and ASEL fate is turned off in ASER (Figure 1A) [17]. Genetic removal of components of this regulatory circuit convert the asymmetric ASE neurons into a symmetric state in which, depending on the genetic manipulation, both cells either adopt the ASEL fate or the ASER fate [17–19].

The late, postmitotic switch from an equipotent, hybrid state to either the ASEL or ASER fate suggests that at this late stage a specific signal induces the switch. However, whereas many bilateral neuron pairs descend from symmetric lineages, the ASE neurons descend from asymmetric lineages (Figure 1B) [1, 20]. This raises

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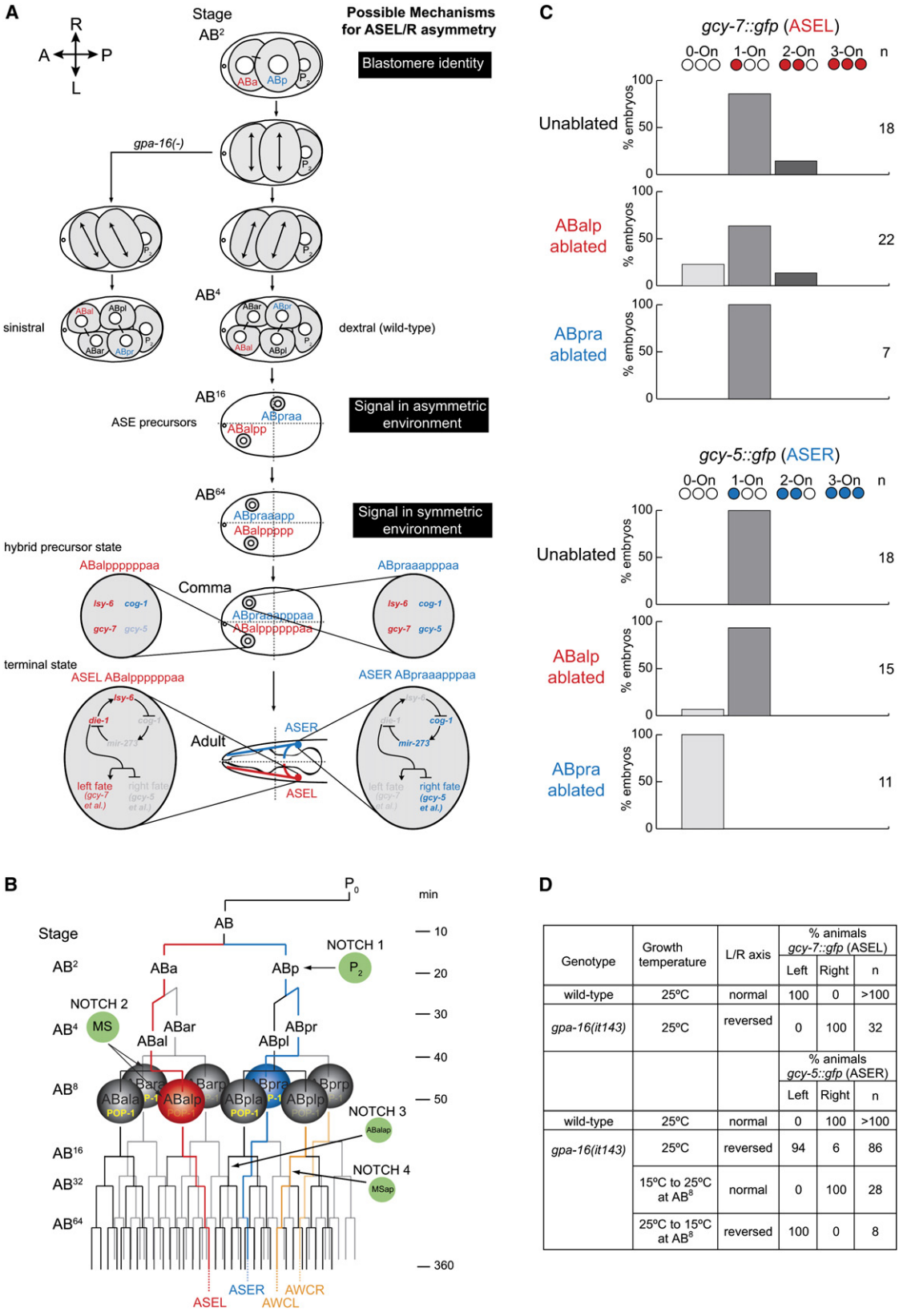

Genotype	Growth temperature	L/R axis	% animals *gcy-7::gfp* (ASEL)		
			Left	Right	n
wild-type	25ºC	normal	100	0	>100
gpa-16(it143)	25ºC	reversed	0	100	32
			% animals *gcy-5::gfp* (ASER)		
			Left	Right	n
wild-type	25ºC	normal	0	100	>100
gpa-16(it143)	25ºC	reversed	94	6	86
	15ºC to 25ºC at AB^8	normal	0	100	28
	25ºC to 15ºC at AB^8	reversed	100	0	8

Figure 1. Asymmetric Embryonic Origins of ASEL and ASER—Blastomere Identity or Asymmetry Signal?

(A) Illustration indicating the embryonic positioning of the ASEL (red) or ASER (blue) precursor blastomeres from the AB^2 stage to adulthood and the possible mechanisms for the generation of ASE L/R asymmetry. At early stages, all cells are shown, whereas at later stages, only the ASE precursor cells are shown for clarity. Also shown is the asymmetric positioning of the AB^4 blastomeres (randomized in *gpa-16* mutants), the genes expressed in ASEL (red) and ASER (blue) at the hybrid precursor stage, and the bistable feedback loop that regulates postmitotic ASE L/R

the alternative possibility that ASE asymmetry may be a consequence of the lineage history of these neurons, meaning, it may depend on the identity of the asymmetric blastomeres from which ASEL and ASER descend.

Blastomere identity is determined by a well-defined set of cellular interactions in the early embryo ([21–24] and reviewed in [25]). Because of the skewed axis of two blastomere cell divisions, embryonic AB blastomeres are positioned in a L/R asymmetric manner at the AB^4 or six-cell stage (Figure 1A). The asymmetry of cell positions in the six-cell embryo generates specific invariant cell contacts within the embryo and thus allows a series of several defined Wnt and Notch signaling events to pattern the fates and lineages of the AB^8 blastomeres (Figure 1B) [21, 26–32].

In light of this prior knowledge, we envisage two models for the generation of ASE asymmetry in *C. elegans* (schematically illustrated in the right column in Figure 1A). The fundamental difference between these models is the timing of the asymmetry inducing event.

(1) The first model ("signaling model" or "late model") involves a specific external asymmetry signal occurring relatively late in embryogenesis. Because the asymmetric positioning of the early blastomeres leads to the asymmetric positioning of the ASE precursors during embryogenesis, this signal could be received at a stage when the ASE precursors are asymmetrically positioned (Figure 1A). Alternatively, signaling could occur at a later stage after the ASE precursors have adopted bilaterally symmetric positions in the embryo (Figure 1A). Such a late signal would be in accord with the observation that directly after their birth, the ASE neurons appear to be bilaterally symmetric and only switch to a L/R asymmetric state later in embryogenesis.

(2) The second model ("blastomere identity model" or "early model") takes into account that ASEL and ASER are derived from distinct blastomeres (Figure 1B) and proposes that ASE asymmetry is dependent on early blastomere identity (Figure 1A). In this case, ASE asymmetry would be dependent on the cell autonomous and nonautonomous factors that initially program the identity of individual blastomeres in the early embryo; the asymmetry of ASEL and ASER would therefore be entirely independent of later embryonic signaling events. An interesting implication of this model would be that the lineages that generated the ASEL and ASER neurons need to memorize such an early blastomere-identity-determining event via some kind of "asymmetry mark" that is recalled at a much later stage after both neurons are born and have passed through a bilateral, symmetric precursor state.

These two models impart on a fundamental issue in the development of asymmetry during ontogeny and across phylogeny. As recently discussed [33], one school of thought argues that asymmetry is phylogenetically and ontogenetically derived from a bilaterally symmetric ground state that is modified by superimposed signaling events (exemplified by the "signaling model" we described above). An alternative view postulates that bilaterality is imposed onto an evolutionary ancient, nonbilateral body plan and that asymmetries within a bilateral body plan represent a remnant of the ancient asymmetry [33]. This alternative view is particularly attractive if one considers the development of the *C. elegans* embryo, in whose course bilateral symmetry is indeed imposed on an essentially L/R asymmetric early embryo. The "blastomere-identity model" therefore implies that adult ASE laterality derives from an ontogenetically asymmetric state.

In this paper, we have made use of the ability to reprogram the fate of individual embryonic blastomeres by using both cellular ablations and mutant analysis to distinguish between the two possible models. Our data provides strong support for the blastomere-identity model.

Results

ASE L/R Asymmetry Is Dependent on Embryonic Asymmetry and Is Not Specified by an Independent Chiral Mechanism or by ASEL-ASER Communication

After their birth, both ASE neurons pass through a hybrid precursor state before acquiring either the ASEL or ASER terminal state [17]. An intuitive model would be that ASE asymmetry is determined at this postmitotic state when the switch from hybrid to terminal fate occurs. In an analogy to lateral signaling mediated by Notch/*lin-12* in vulval patterning [34] or to calcium-mediated lateral signaling in AWCL/R odorant-receptor choice [35], one could envision that the ASEL and ASER cells signal to each other to ensure that both cells adopt distinct cellular fates after they have passed through the hybrid precursor state. To address this possibility, we undertook laser-ablation experiments in which we removed precursors of either ASEL or ASER and thereby completely prevented the generation of either of these cells. By using *gfp*-based cell-fate markers

asymmetry. Although expression of terminal ASE cell-fate markers (*gcy* genes) is not observed until between 1.5- to 3-fold stages, it is likely that the hybrid precursor stage starts around the time when the ASE cells are born and is therefore illustrated schematically at the comma stage.
(B) Lineage diagram indicating the four Notch signals and the asymmetric segregation of POP-1 that function to diversify the fates of the AB blastomeres. ASEL (red) and ASER (blue) descend from bilaterally asymmetric lineages as opposed to the bilaterally symmetric origins of other bilateral neuron pairs, such as AWCL/R (orange).
(C) ASEL and ASER do not affect each other's fate. ASEL was removed by ablating the ABalp blastomere, and ASER was removed by ablating the ABpra blastomere. L/R fate was monitored with transgenic animals expressing chromosomally integrated *gcy-7^{prom}::gfp (otIs3)* and *gcy-5 ^{prom}::gfp (ntIs1)* at about the 3-fold to L1 stage. At this stage, *gcy-7,* which is expressed in ASEL and ASER during the embryonic hybrid precursor state, is about to become restricted to ASEL; therefore, wild-type embryos display a mixture of either one cell expressing *gcy-7* (ASEL in mature state) or both cells (ASEL and ASER in hybrid precursor state) [17]. Later stages in which *gcy-7* would be restricted exclusively to ASEL could not be scored because of the arrested development of manipulated embryos.
(D) Effect of reversing the L/R body axis on ASE L/R asymmetry, as assessed in *gpa-16* mutants. *gcy-7^{prom}::gfp (otIs3)* and *gcy-5 ^{prom}::gfp (ntIs1)* expression was scored in adults.

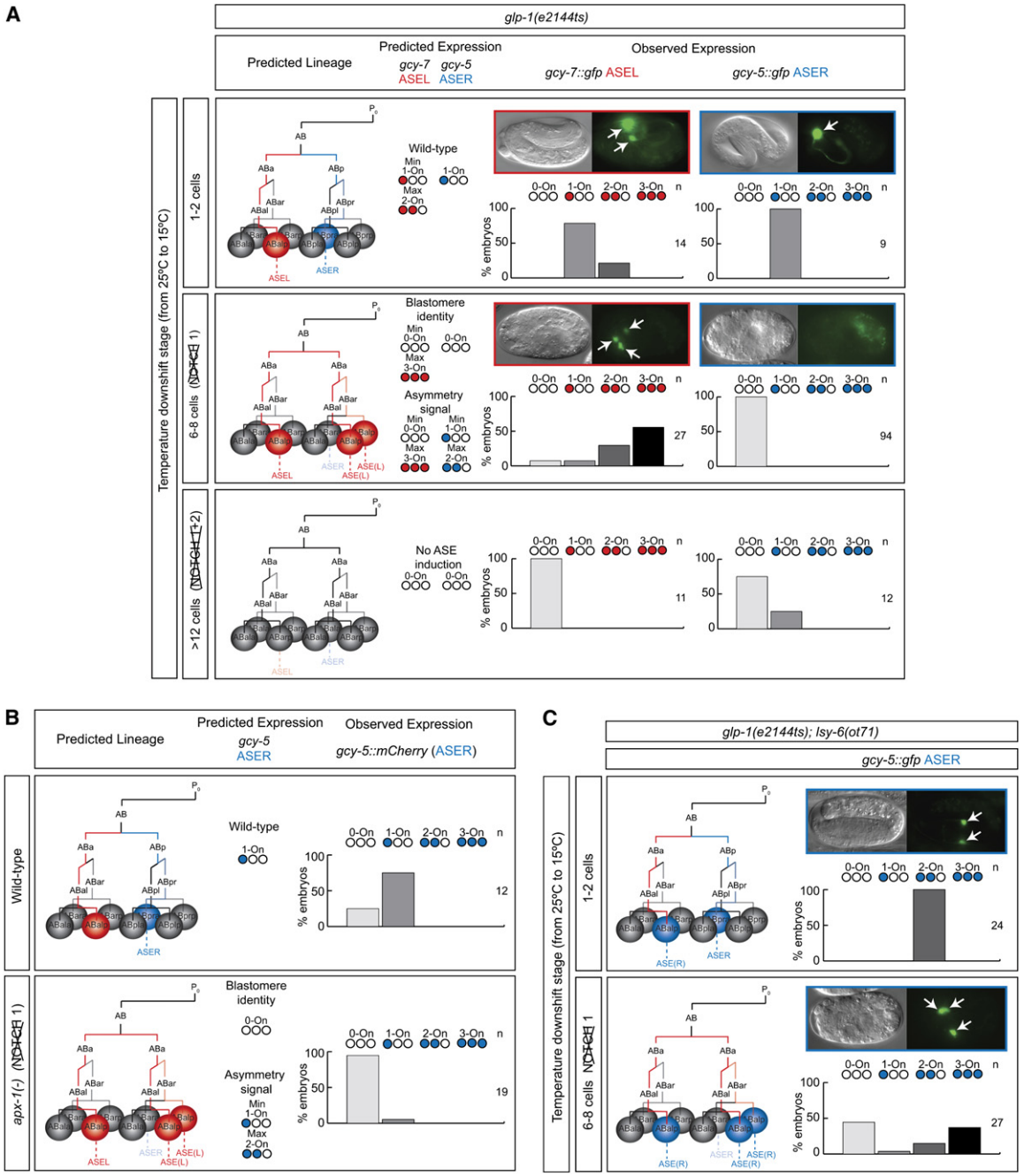

Figure 2. ASE Cells Generated from Ectopic Right-Sided ASEL Lineages Do Not Express ASER Markers

Each horizontal panel in this and subsequent figures illustrate from left to right the predicted lineage and then the predicted expression of *gcy-7* and/or *gcy-5* depending on the mechanism of ASE L/R asymmetry generation or the possible lineage origins of ectopic cells. To the right of the predicted expression the actual number of cells observed expressing *gcy-7^{prom}::gfp (otIs3)* and *gcy-5^{prom}::gfp (ntIs1)* or *gcy-5^{prom}::mCherry (otEx2332)* is indicated and can be easily compared with the different predictions on the left. In all cases, ASEL lineages and *gcy-7* expression are indicated in red and ASER lineages and *gcy-5* expression are indicated in blue. A crossed-out "Notch" indicates that the genetic or cellular ablation causes a disruption of one of the several Notch interactions indicated in Figure 1B. Animals were scored as late as their viability allows.

(A) Expression of *gcy-7* and *gcy-5* at the 3-fold to L1 stage in control embryos (upper panel), those in which the first Notch signal has been prevented (middle panel) and those in which the first and second Notch signals have been prevented (lower panel) with a temperature-sensitive allele of the Notch receptor *glp-1(e2144)*. When embryos are grown from the one- to two-cell stage at the permissive temperature of 15°C, this strain shows the wild-type pattern of a maximum of two cells expressing *gcy-7* and only one cell expressing *gcy-5* (upper panel). When embryos grown at the nonpermissive temperature of 25°C from the one- to two-cell to greater than 12-cell stage and then shifted to 15°C, no ASE lineages are formed and no expression of *gcy-7* or *gcy-5* is observed in the majority of embryos (lower panel). Preventing specifically the first Notch signal by growing the embryos at the nonpermissive temperature until the six-cell stage and then shifting the embryos to 15°C generates two ectopic ASEL lineages. In these embryos, a maximum of three cells expressing *gcy-7* is observed (as mentioned in Figure 1B, in the embryo *gcy-7* initially marks all ASE cells because of its expression in both ASEL and ASER in the hybrid precursor state [17]), and no expression of *gcy-5* is observed.

that monitor ASER and ASEL fate, respectively (see the Supplemental Experimental Procedures available with this article online for details on markers), we find that ablation of ABalp (the precursor to ASEL; Figure 1B) has no effect on the adoption of the ASER fate (Figure 1C). Vice versa, ablation of ABpra (the precursor to ASER; Figure 1B) has no effect on the adoption of the ASEL fate (Figure 1C). ASEL and ASER therefore adopt their terminal fate independent of one another.

ASE L/R asymmetry could also be determined by a nonautonomous signal that instructs either of the two cells to become different from one another after they are born at the precomma stage. For a L/R asymmetric signal to arise in a largely bilaterally symmetric precomma-stage embryo, one could envision a model proposed by Brown and Wolpert in which a chiral "F molecule" (where the F visually represents an inherent chirality of the molecule) is aligned along the anterior-posterior and dorsal-ventral axes [36]. Through its inherent asymmetry, the alignment to the other two axes could establish the L/R axis. Such a molecule could act in a number of ways, for example, to ensure the L/R asymmetric transport of a secreted signaling molecule. If ASE L/R asymmetry is determined by such an F molecule, one would expect that it affects ASE L/R asymmetry independently of the initial invariant asymmetry of the *C. elegans* embryo at the six-cell stage because it would be dependent only on the inherent chirality of the F molecule. If, in contrast, ASE L/R asymmetry is linked to the asymmetry of the early embryo, then a reversion of this early asymmetry would also reverse ASE L/R asymmetry.

We tested these possibilities by using a specific genetic manipulation. In the wild-type embryo, a mitotic spindle rotation generates an asymmetric embryo at the six-cell stage (Figure 1A). In *gpa-16(it143)* mutants, this spindle rotation is randomized, and this can generate animals in which visceral L/R asymmetries of the adult are reversed [37]. We find that in *gpa-16(it143)* mutants that produce axis-reversed adults, the expression of the ASEL marker *gcy-7* and the ASER marker *gcy-5* is also reversed (Figure 1D).

To rule out that the effect of *gpa-16* on ASE L/R asymmetry is a reflection of a role of the gene much later in development that is independent of the spindle-rotation process at the 6-cell stage, we made use of the temperature-sensitive nature of the *gpa-16(it143)* allele. Abrogating *gpa-16* function after the AB^8 stage has no effect on ASEL/ASER asymmetry in the nonreversed animals (Figure 1D). These results (1) argue against an involvement of an F-molecule-related mechanism and (2) demonstrate that the sidedness of ASE asymmetry is coupled to the six-cell embryonic asymmetry generated by the specific spindle rotation. Because the six-cell embryonic asymmetry also determines visceral asymmetries, the sidedness of these two types of asymmetries (neuronal and visceral) is therefore linked.

ASEL Fate Depends on Early Blastomere Identity

The result with axis-reversed embryos shows the coupling of early embryonic asymmetry and ASE L/R asymmetry, but it does not distinguish between the late signaling and the early blastomere identity models mentioned above (Figure 1A). To address these possibilities more directly, we used cellular and genetic ablations to reprogram the fate of individual embryonic blastomeres.

Through the manipulation of early Notch signals, previously known to control blastomere identity (Figure 1B) (reviewed in [32]), we first conducted a series of experiments to generate (1) a blastomere that produces an ectopic ABpraaapppaa lineage (represents ASE from right embryonic lineage) on the left side of the embryo and to generate (2) a blastomere that produces an ectopic ABalpppppppaa lineage (represents ASE from left embryonic lineage) on the right side of the embryo. If ASE L/R asymmetry is specified by a late asymmetry signal, then we would predict that the location of the ectopic ASE blastomere would be paramount in determining its fate; i.e., ectopic left-sided ASE cells will acquire an ASEL fate, and ectopic right-sided ASE cells will acquire an ASER fate. If, however, ASE L/R asymmetry is specified as a consequence of blastomere identity, then an ectopic ABpraaapppaa lineage on the left side of the embryo will still acquire an ASER fate because its lineage history is identical to that of the endogenous ASER and vice versa.

When both the first and second Notch signals shown in Figure 1B are prevented with a temperature-sensitive allele of *glp-1*, the receptor for the first and second Notch interactions, no ASE lineages are formed because both ABalp and ABpra blastomere fates are absent [21] and no expression of two ASE-fate markers, *gcy-7* or *gcy-5*, is observed (Figure 2A, lower panel). However, if only the first Notch signal is disrupted, then two ectopic ABalpppppppaa lineages (ASE from left embryonic lineage) are generated because of the transformation of the ABplp and ABprp lineages into ABalp lineages; one of these ectopic lineages is located on the right side of the embryo (Figures 1B and 2A) [21]. Consistent with this notion, we observed up to three cells expressing *gcy-7* (this marks the hybrid precursor state in both ASEL and ASER, as well as the mature ASEL state), one being the endogenous ASEL cell and two representing the production of two ectopic ASE cells (Figure 2A, middle panel). No ASE cells in these manipulated embryos express *gcy-5*, suggesting that all the ASE cells have acquired an ASEL fate (Figure 2A, middle panel). Ablation of ABp in addition to elimination of the first Notch signal removes the two ectopic *gcy-7*-expressing cells, confirming that they are produced from the predicted lineage transformations (Figure S1A).

Corroborating the results with the Notch receptor *glp-1*, we find that the removal of its Delta-type ligand *apx-1*, which is responsible specifically for the first

(B) Expression of *gcy-5* at the 3-fold to L1 stage in wild-type embryos (upper panel) and maternal and zygotic *apx-1(zu183)* mutants. F2 embryos from *dpy* F1 progeny of an *apx-1(zu183) dpy-11 (e224)/nT1* strain carrying the *gcy-5^{prom}::mCherry (otEx2332)* array were analyzed (lower panel). Abrogating the function of *apx-1*, the Notch ligand specific for the first Notch signal, prevents this signal and thus generates two ectopic ASEL lineages (see text for details). The *gcy-5^{prom}::mCherry (otEx2332)* array (which had to be used because of the linkage of *ntIs1* and *apx-1*) is about 70% penetrant such that in wild-type embryos 70% of the embryos can be observed expressing *gcy-5* in a single cell at the 3-fold to L1 stage. (C) Execution of the ASEL fate, observed upon preventing the first Notch signal with the *glp-1(e2144)* temperature-sensitive allele (A), is prevented in *lsy-6* null mutants, leading to a switch from ASEL to ASER fate.

Notch induction [23, 38, 39], recapitulates the effects observed with the temperature-shift experiments of *glp-1* (Figure 2B).

The ectopic ASEL lineage produced upon disruption of the first Notch interaction requires the same genetic pathway that controls normal ASEL fate because removal of the first Notch signal in animals that lack the ASEL inducer *lsy-6* [17, 40] prevents the generation of the ectopic ASEL fate (Figure 2C).

Taken together, these results therefore provide the first indication that ASEL fate may be dependent on blastomere identity.

ASER Fate Depends on Early Blastomere Identity

In analogy to the ASEL lineage duplications, we also performed the reverse experiments and generated an ectopic ABpraaapppaa lineage (ASE from right embryonic lineage) on the left side of the embryo. To this end, we prevented the third Notch interaction (Figure 1B) by ablating ABal. As previously reported, this leads to a transformation of the ABpla lineage into the ABpra lineage [28], thereby (1) generating an ectopic ABpraaapppaa lineage (which normally produces ASER) on the left side of the embryo and (2) removing the endogenous ASEL lineage (Figure 3A, lower panel). After ABal ablation, despite the predicted loss of the ASEL cell, we still observe up to two cells expressing *gcy-7* (this marks the hybrid precursor state in both ASEL and ASER) at the 3-fold to L1 stage, suggesting that an ectopic ASE cell is indeed produced (Figure 3A, lower panel). However, we now observe two cells expressing the ASER-specific marker *gcy-5* (Figure 3A, lower panel), demonstrating that the ectopic ASE cell has acquired an ASER fate. These results were confirmed with independent ASEL- and ASER-fate markers (Figure S1C). We also confirmed the predicted lineage transformation by examining the expression of a marker that assesses the fate of the ASE cousin, which we find to be duplicated after ABal ablation, as expected (Figure S1C). Moreover, ablation of ABpl in addition to ABal removes the ectopic *gcy-5* expressing cell, confirming that the cell is produced from the predicted lineage transformation (Figure S1B).

Removing the zygotic expression of both *lin-12* and *glp-1*, the Notch receptors required for the third Notch signal also generates an ectopic ABpraaapppaa lineage on the left side of the embryo [41] (Figure 3B, lower panel). We find that *lin-12(n941) glp-1(q46)* double mutants indeed display an additional cell expressing *gcy-5* after hatching. This cell is situated on the left side and has projections identical to the endogenous ASER situated on the right side of the embryo (Figure 3B, lower panel). Together, this data shows that when an ectopic ABpraaapppaa lineage (ASE from right embryonic lineage) is produced on the left of the embryo, the ASE cell acquires an ASER identity. These results indicate that ASER fate is, like ASEL fate, a function of early blastomere identity.

ASE L/R Fate Develops Autonomously after AB^8-Cell Blastomere Identity Has Been Determined

To further examine the signaling model versus blastomere-identity model, we attempted to find a cell (or lineage) that when ablated would affect ASE L/R asymmetry. Such a finding would provide evidence for the signaling model. To this end, we performed cell ablations that remove entire lineages of cells situated in close proximity to the cells that generate the ASER or ASEL neurons. We found that no individual ablation has any effect on the expression of *gcy-5* and *gcy-7* unless it either removes the ASER or ASEL lineage or affects the second or third Notch interactions removing or duplicating ASE lineages (Figure S2). Moreover, if sequential ablations are performed in a manner that leaves only the ABalp blastomere (ASEL lineage) intact and correctly specified at the AB^8-cell stage, a single cell expressing *gcy-7* but not *gcy-5* is observed (Figure 4A, upper panel). This indicates that the ASE cell formed from the isolated ABalp blastomere acquires an ASEL fate. Vice versa, if all other lineages except the ABpra blastomere (ASER lineage) are ablated, then the single ASE cell produced now expresses *gcy-5* in addition to *gcy-7*, indicating that it has acquired an ASER fate (Figure 4A, lower panel).

This still leaves the possibility that a signal from within the ABalp or ABpra lineages is responsible for ASE laterality. This is particularly an issue until the AB^{32}-cell stage, from whereon the ABalp and ABpra lineage branches become bilaterally symmetric. To examine whether an asymmetry signal may exist within the ABalp or ABpra lineage before bilateralization at the AB^{32}-cell stage, we performed laser ablation with the two lineage branches and again found no effects on the expression of the ASEL or ASER fate (Figure S3). With the potential experimental caveat that cell ablations may not completely eliminate cell signals, these data indicate that no specific asymmetry signal may be required for generating ASE laterality. Instead, the L/R fate acquired by an ASE cell appears to be a function of its lineage history and the identity of the AB^8-cell blastomere from which it descends.

Differences in ABa/ABp Blastomere Identity Determine ASE L/R Asymmetry

We sought to examine the effect of reprogramming blastomere identity in a variety of distinct manners. A factor previously shown to be involved in establishing the different fates of the AB^8 blastomeres is the POP-1 protein, a Wnt-responsive TCF-1-like protein that is asymmetrically segregated in all anterior-posterior AB blastomere divisions starting at the AB^8 stage [27]. The ABpra blastomere, from which ASER forms, has high levels of POP-1, and the ABalp blastomere, from which ASEL forms, has low levels of POP-1 (schematically shown in Figure 1B). Genetic removal of *pop-1* does, however, not affect ASEL and ASER fate (Figure S4), and therefore, differences in POP-1 levels at the AB^8-cell stage cannot be responsible for determining the difference between ASEL and ASER.

The ASEL/ASER lineages arise from ABa and ABp, raising the possibility that ASE asymmetry may be determined as early as the AB^2-cell stage as a function of the identity of the ABa or ABp blastomeres (Figure 1). We indeed noticed that in all our experiments, whenever ectopic ASE cells are generated from the ABa lineage, they acquire an ASEL fate, and whenever ectopic ASE cells are generated from the ABp lineage, they acquire an ASER fate, in an identical manner to the endogenous

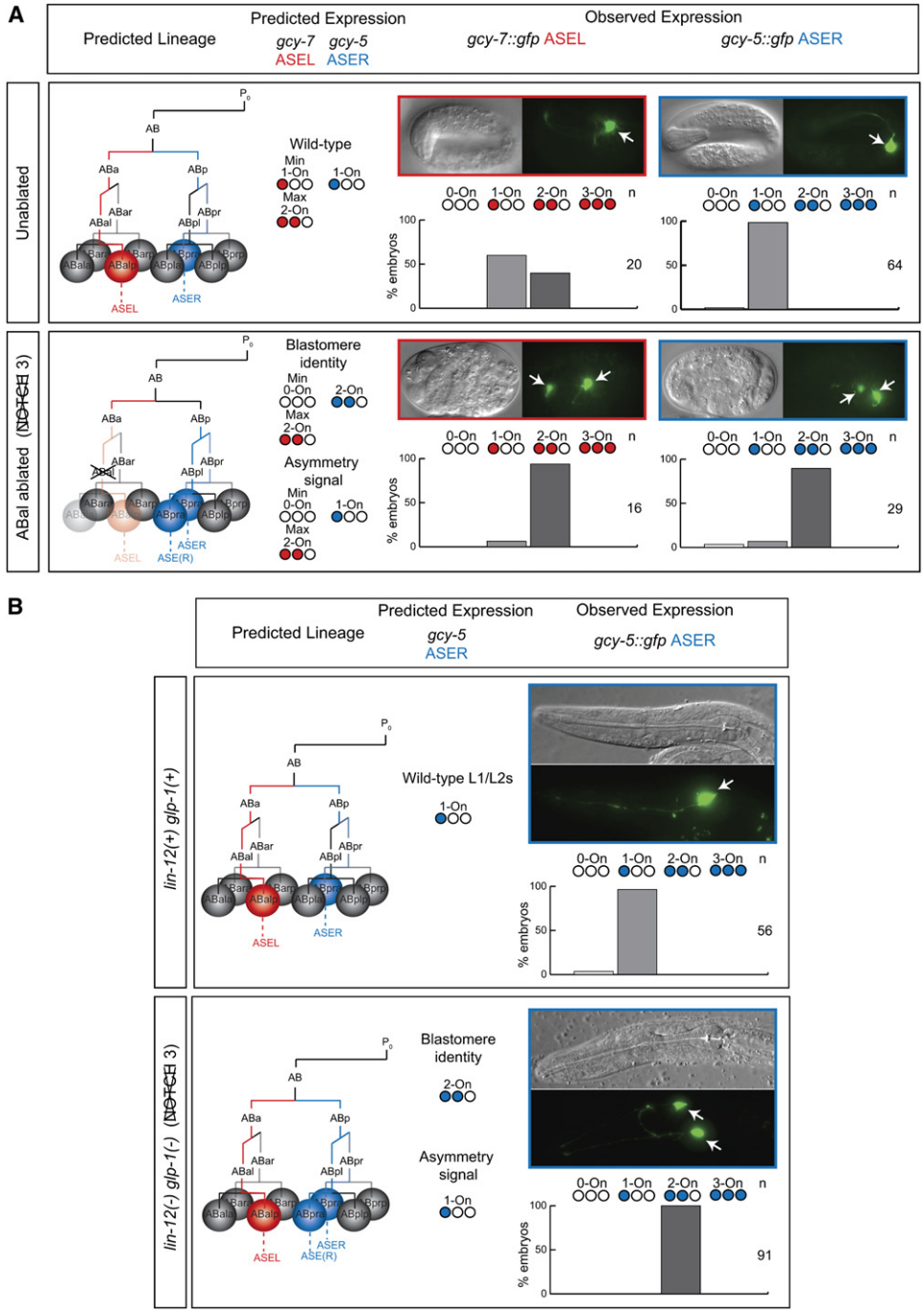

Figure 3. ASE Cells Generated from Ectopic Left-Sided ASER Lineages Still Express ASER Markers

(A) Expression of *gcy-7* and *gcy-5* at the 3-fold to L1 stage in unablated (upper panel) and ABal-ablated embryos (lower panel). The cross indicates the ablated blastomere, and the lighter shading the missing lineages. In unablated animals at the 3-fold to L1 stage, the ASE cells are still in the hybrid precursor state (Figure 1A), and therefore, one can observe a maximum of two cells expressing *gcy-7* in unablated embryos. The ASER marker *gcy-5* is not part of the initial hybrid precursor state, and therefore, one cell expressing *gcy-5* is observed. After ABal ablation, two cells expressing *gcy-5* are observed.

(B) Expression of the ASER marker *gcy-5* at an early larval stage in control *dpy unc* F1 progeny (upper panel) and *lag* F1 progeny (identified by twisted nose and loss of rectum) of a *lin-12(n941) glp-1(q46)/dpy-19(e1259) unc-69(e587)* strain (lower panel). Loss of the third Notch signal via the zygotic loss of both the Notch receptors *lin-12* and *glp-1* generates an ectopic ASER lineage on the left side of the embryo but leaves the endogenous ASEL lineage intact.

ASE lineages. This is particularly evident in *pop-1* mutants in which, because of several lineage transformations, a total of four ASE cells are generated (Figure S4). Laser ablations demonstrate that two of these cells derive from ABp and two from ABa (Figure S4). All those that are generated from ABa adopt the ASEL fate, whereas those generated from ABp generate the ASER fate (Figure S4).

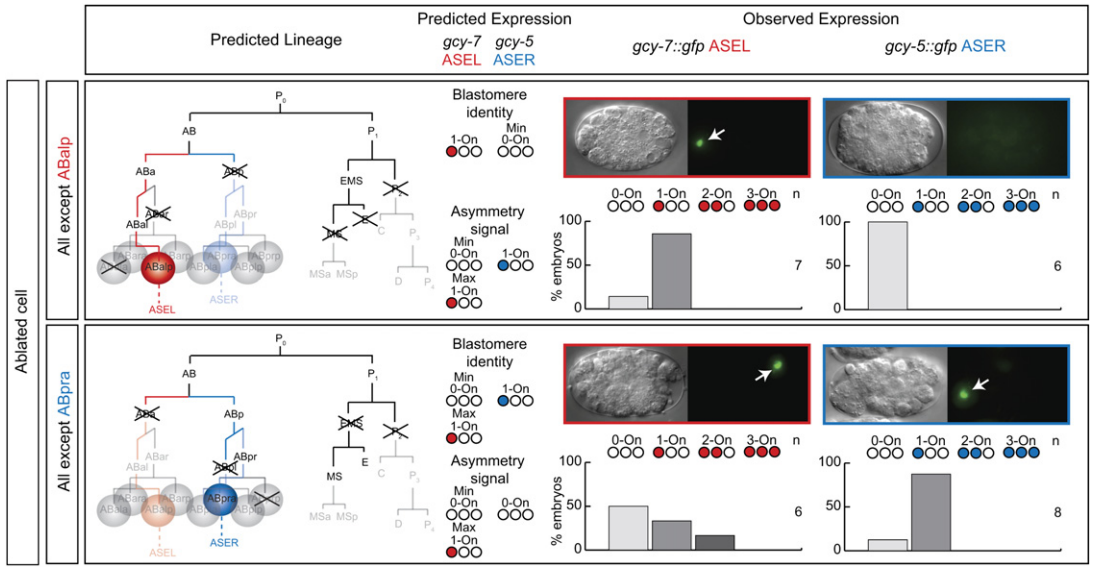

Figure 4. Isolated Blastomeres Adopt ASE L/R Asymmetry as Prefigured by Blastomere Identity

Expression of *gcy-7* and *gcy-5* at the 3-fold to L1 stage after the isolation of either the ABalp (ASEL lineage) blastomere (upper panel) or the ABpra (ASER lineage) blastomere (lower panel) by sequential ablations. The crosses indicate the ablated blastomeres, and the lighter shading the missing lineages. In the case of ABalp isolation, MS was ablated late in order to permit the second Notch signal that induces ABalp. After isolation of the ABalp blastomere, one cell expressing *gcy-7* is observed, but *gcy-5* expression is never observed. In contrast, both *gcy-7* and *gcy-5* expression is observed in a single cell after the isolation of the ABpra blastomere.

If ASE L/R asymmetry is indeed determined by the ABa/ABp lineage difference, then it must be dependent on the first Notch interaction that specifies this lineage difference at the AB^2-cell stage (Figure 1B). As we have shown above, this is indeed the case (Figures 3A and 3B). The primary function of this Notch signal is to repress two T box transcription factors, *tbx-37* and *tbx-38*, in ABp but not ABa [42], raising the possibility that the difference in blastomere identity established by the presence or absence of *tbx-37* and *tbx-38* specifies ASE asymmetry. To test this possibility, we examined *tbx-37(zu467) tbx-38(zu463)* double mutants in which ABa is transformed to ABp. We find that when ABa is transformed to ABp, all ASE cells produced acquire the ASER fate (Figure 5A). Laser ablations confirm that the ectopic, ASER-fate-expressing cells indeed derive from the transformed ABa lineage (Figure 5B). Therefore, ASE asymmetry is established by the differences in ABa/ABp blastomere identity because of Notch-mediated, ABp-specific repression of *tbx-37* and *tbx-38* expression.

In all experiments described so far, ectopic ASE cells that descend from ABa acquire an ASEL fate and those from ABp acquire an ASER fate. However, through transformation of the fates of the AB^2, AB^8, or AB^{16} blastomeres, the ectopic ASE cells that are generated share exactly the same cleavage pattern, i.e., sequence of anterior-posterior and left-right divisions, as the endogenous ASEL lineages or ASER lineages. It is therefore unclear whether it is the specific cleavage pattern or a more general "memory" of the ABa versus ABp lineage difference that is responsible for ASE L/R asymmetry. To distinguish between these two possibilities, we examined animals lacking *ref-1*, a bHLH transcription factor expressed in several early lineages in both Notch-dependent and Notch-independent manners [43, 44]. Because of the ectopic expression of several amphid-neuron-fate markers, including ASEL-fate markers, it has been suggested that in *ref-1(ok288)* mutants, an ectopic blastomere is generated at the AB^{32}-cell stage [45]; however, the lineage history of this ectopic ASE neuron had not been examined. Through laser ablations of ABp, ABa, and ABa descendants, we determined that the ectopic ASEL neuron generated in *ref-1* mutants descends from the ABara blastomere (Figure 6A; data not shown). The ectopic ASEL cell therefore derives from the ABa lineage, consistent with ASE asymmetry being established by the ABa/ABp lineage difference, yet it does not share an identical sequence of divisions with the endogenous ASEL cell (ABalppp versus ABaraxx). Thus, it is more likely that the distinct identity of the ABa-versus-ABp blastomere rather than the precise sequence of cell divisions in this lineage is responsible for postembryonic ASE laterality.

Early Embryonic A/P Asymmetry Is Required for ASE L/R Asymmetry

Blastomere fate at the four-cell stage is specified at an even earlier stage via the asymmetric segregation of several cell-fate determinants that direct subsequent events including the restriction of Notch interactions to particular blastomeres. This asymmetric segregation occurs after the asymmetric anterior/posterior (A/P) division of the fertilized egg into the unequally sized large AB and small P_1 blastomeres. A group of genes known as the *par* genes regulate this process [46, 47]. Each *par* mutant affects the asymmetric segregation of cell-fate determinants in different ways [48]. In *par-2(it5)* mutants, the asymmetric segregation of some cell-fate determinants such as MEX-3, SKN-1, and PAL-1 is unaffected, yet an overproduction of ABa-derived pharyngeal cells is observed [48]. An ectopic production of

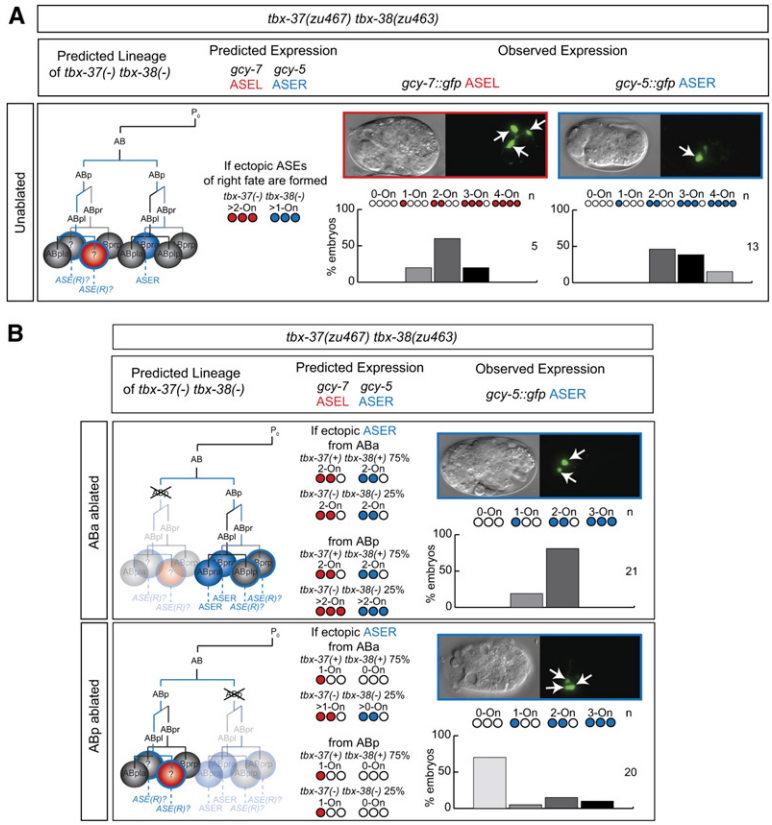

Figure 5. ABp-Specific Repression of *tbx-37* and *tbx-38* by the First Notch Interaction Specifies ASE L/R Asymmetry

(A) Expression of *gcy-7* and *gcy-5* at the 3-fold to L1 stage in *tbx-37(zu467) tbx-38(zu463)* double mutants. Because these mutants are embryonic lethal, arrested F1 progeny from a *tbx-37(zu467) tbx-38(zu463)/qC1* balanced strain were scored. In these mutants, because of the ABa to ABp transformation, ABala and ABarp are transformed to ABpla and ABprp, respectively and would not be expected to give rise to ASE cells. ABara and ABalp should be transformed to ABpra (ASE lineage) and ABplp, respectively. However, they still receive the second Notch induction from MS and execute an unknown lineage program [42], raising the possibility that one if not more ASE cells are formed from these lineages. In these mutants, three cells expressing *gcy-7* and three or four cells expressing *gcy-5* are observed. This is consistent with the production of ectopic ASE cells that because of the ABa to ABp transformation adopt an ASER instead of an ASEL fate.

(B) Ablations confirm that the two or three extra *gcy-5* expressing cells in *tbx-37(zu467) tbx-38 (zu463)* mutants do indeed descend from the transformed ABa lineage because they are missing after the ablation of the transformed ABa blastomere (this ablation also duplicates the ASER lineage of the left side) but not after ABp ablation. Because it was no longer possible to identify *tbx-37(zu467) tbx-38 (zu463)* mutants from among the ablated F1 progeny of the *tbx-37(zu467) tbx-38(zu463)/qC1* balanced strain, all ablated progeny were scored.

pharyngeal cells is also observed upon loss of the first Notch signal [21, 23, 29], suggesting that in *par-2* mutants ABp is transformed to ABa, perhaps because of the loss of the *apx-1/glp-1*-dependent signal that distinguishes ABa from ABp. If ABp is transformed to ABa in *par-2* mutants, we would predict a total of three ASE cells that all acquire ASEL fate with a loss of ASER fate. In these mutants, we indeed observe that a maximum of three ASE cells are formed; these cells rarely express ASER fate, thereby suggesting that ASER fate is indeed lost in these mutants (Figure 6B). These results, which are identical to a loss of the first Notch signal (Figures 3A and 3B), demonstrate that early embryonic A/P polarity, generated in part by the asymmetric cortical localization of PAR-2 at the one-cell stage, is required for the adult L/R asymmetry of the ASE neurons.

Discussion

Generation of ASE Asymmetry

We have used a series of cellular and genetic ablations to manipulate cell position and blastomere identity in the developing *C. elegans* embryo (summarized in Figure 7A). Conceptually, these experimental approaches are comparable to classic transplantation studies in vertebrates in which cell behavior is analyzed after grafting of cells into a new environment [49]. Based on our results, we propose the following scenario for the generation of ASE L/R asymmetry (Figure 7B): (1) The establishment of early A/P asymmetry, via the cortical localization of the PAR proteins, leads to an asymmetric division and asymmetric segregation of cell-fate determinants along the A/P axis, likely including the determinants required for the first Notch signal; (2) at the four-cell stage, the first Notch signal occurs in which P_2 signals to ABp and results in the downregulation of the *tbx-37* and *tbx-38* transcription factors in ABp but not ABa; (3) *tbx-37/tbx-38* trigger what we term a lineage-specific "asymmetry mark" that prefigures future ASE asymmetry; (4) *gpa-16*-dependent spindle rotation in ABa and ABp generates an asymmetric embryo at the six-cell stage, establishing the overall handedness of the adult and specifying the sidedness (but not asymmetry per se) of future ASE asymmetry; (5) subsequent asymmetric Notch interactions and ensuing cell migrations then act to superimpose bilateral symmetry on the developing embryo [50] and induce through as yet unknown means the eventual generation of two bilaterally symmetric ASE cells nine rounds of cell division later; and (6) the "asymmetry mark" is then interpreted by the postmitotic and initially bilaterally symmetric ASE cells to bias a bistable regulatory feedback loop such that the ASE cell from the ABa lineage acquires ASEL-specific features in addition to the ASE-generic, symmetric gene-expression program, and the ASE cell from the ABp lineage acquires ASER-specific features in addition to a ASE-generic symmetric gene-expression program (Figure 7B). We discuss individual aspects of this proposed sequence of events below.

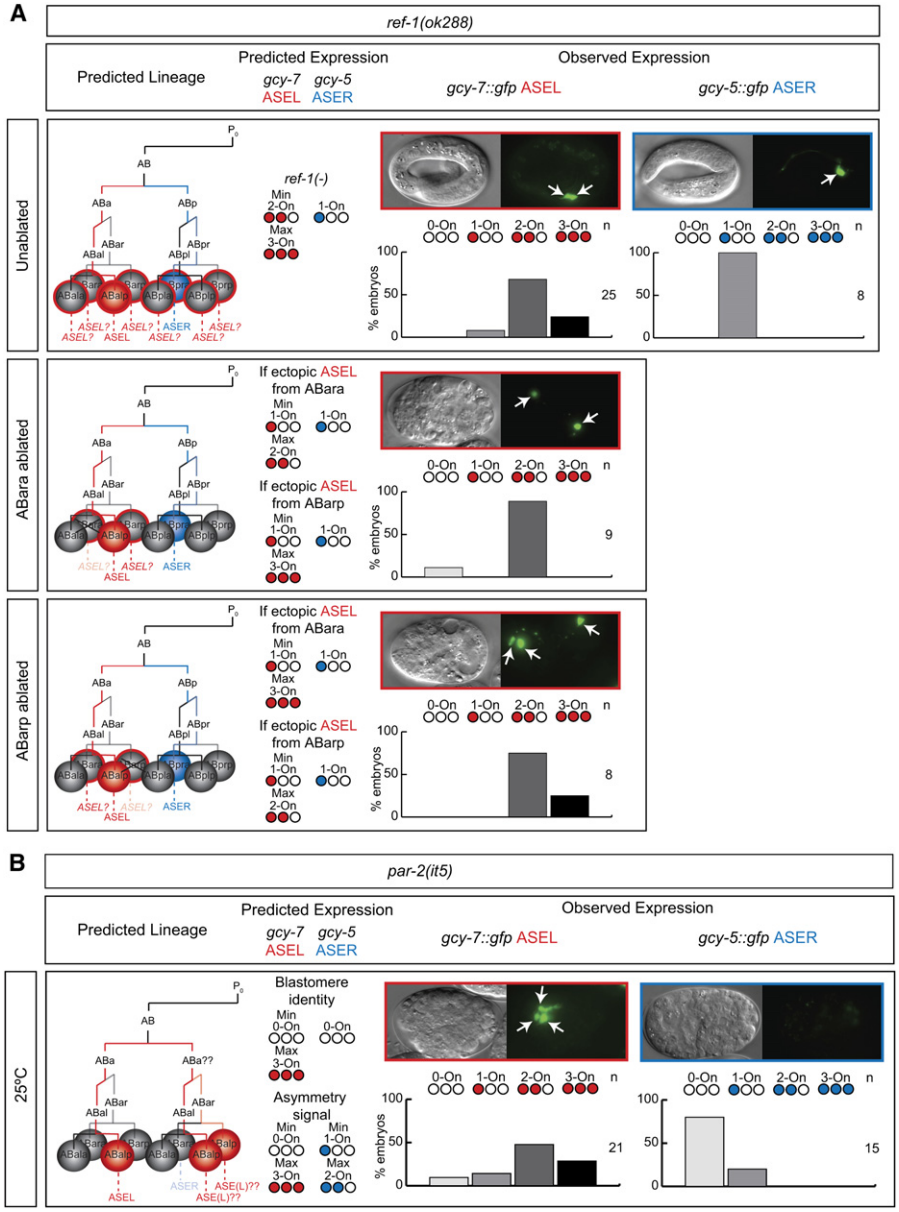

Figure 6. Effects of Manipulating Blastomere Identity in *ref-1* and *par-2* Mutants

(A) The ectopic ASEL cell in *ref-1(ok288)* mutants forms from the ABara blastomere. Expression of *gcy-7* and *gcy-5* at the 3-fold to L1 stage in unablated (upper panel), ABara-ablated (middle panel), and ABarp-ablated (lower panel) zygotic *ref-1(ok288)* mutants. Unablated *ref-1(ok288)* mutants show a maximum of three cells expressing *gcy-7*, two are shown on the right side and one on the left side. Only the normal ASER neuron expresses *gcy-5*, indicating the presence of an ectopic ASEL on the right side of *ref-1* mutants. Initial ablations demonstrated that the ectopic ASEL cell forms from the ABa lineage (data not shown). Further ablations demonstrate that the ectopic ASEL cell is removed after ablation of ABara but not ABarp, showing that it derives from the ABara lineage (middle and lower panels).

(B) Loss of A/P polarity at the one-cell stage in *par-2* mutants disrupts ASE L/R asymmetry. Expression of *gcy-7* and *gcy-5* at the 3-fold to L1 stage in *par-2(it5)* temperature-sensitive mutants at the nonpermissive temperature of 25°C. In these mutants, a maximum of three cells expressing *gcy-7* are observed, but the expression of *gcy-5* is almost never observed, indicating that early one-cell polarity is required for ASER cell fate.

The "Asymmetry Mark"—A Memory of Things Past

In spite of their distinct lineal origin, the ABalp blastomere (from which ASEL derives) and ABpra blastomere (from which ASER derives) execute a developmental program that results in the generation of two gustatory neurons, ASEL and ASER, that display a bilaterally symmetric differentiation program, resulting in similar morphology, synaptic connectivity, signaling properties, and molecular features. Even factors that eventually become asymmetrically expressed in either ASEL or ASER (e.g., *lsy-6*) are initially expressed in an apparently bilaterally symmetric manner [17]. In spite of these apparent similarities, newly born ASER and ASEL are already intrinsically different from one another. Specifically, we have shown here that a Notch signal at the four-cell stage determines a subaspect of ABa-versus-ABp blastomere identity that is responsible for the eventual L/R asymmetric activity of a miRNA-dependent

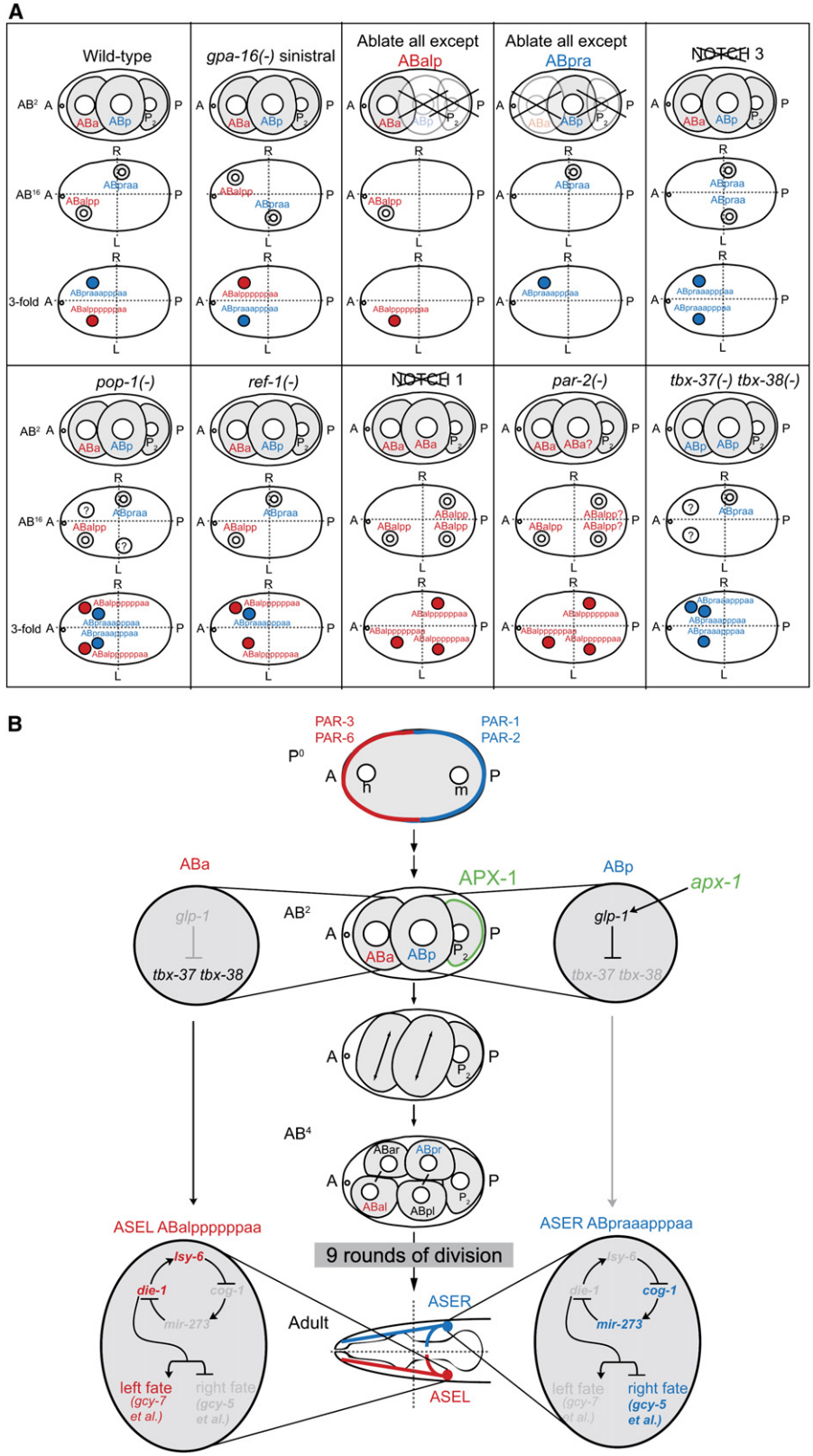

Figure 7. Summary and Model for the Specification of ASE L/R Asymmetry by Early Embryonic Blastomere Identity

(A) Summary of the results presented in this work. At early stages, all cells are shown, whereas at later stages only the ASE precursor cells are shown for clarity.

(B) Model for the specification of ASE L/R asymmetry and the sidedness of this asymmetry.

bistable regulatory feedback loop that functionally diversifies the left and right postmitotic ASE cells nine rounds of division later (Figure 7B). In light of the fact that at least one, and possibly both of the effectors of the first Notch signal, TBX-37 and TBX-38, are very short-lived proteins [42], they can not directly regulate the expression of the postmitotic trigger of the L/R diversification process, the miRNA *lsy-6*, because this gene is only expressed at around the stage when ASE neurons are born. We therefore suggest that one function of *tbx-37* and *tbx-38* at the four-cell stage is to establish a lineage-intrinsic "asymmetry mark" that imparts a memory of the distinct lineage origins of the ASE cells. This asymmetry mark is an integral component of early blastomere identity and "survives" the overall bilateralization process that symmetrizes the whole *C. elegans* embryo, including the ASEL and ASER lineage [50], so that it could be eventually interpreted in postmitotic ASE cells, i.e., long after symmetrization of the embryo is complete. It remains to be seen whether this subaspect of blastomere identity, i.e., the asymmetry mark, is dedicated for control of ASE asymmetry or whether it generates other asymmetries within the embryo, e.g., in other sublineages of the ASE-lineage branches.

tbx-37 and *tbx-38* may establish an "asymmetry mark" by triggering a sequential cascade of stage-specific transcription-factor codes or may impart some kind of a heritable epigenetic mark, perhaps on the chromatin level, in either the ASEL or ASER lineage. After the ASEL and ASER cells are born, this mark may then either facilitate or attenuate the onset of expression of a component of the bistable feedback loop (such as *lsy-6*), which is fated to be turned on initially in both ASEL and ASER. A slight L/R bias in the expression levels of a bistable loop component would be amplified through the feedback architecture of the system so that the ASEL and ASER cells are eventually locked into their stable states. An early epigenetic mark would be globally present in all descendants of the blastomere (ABa versus ABp) in which the mark is established. Indeed, whenever ectopic ASE cells are formed from different lineages, they acquire a left or right fate depending on their ABa/ABp lineage origin. This suggests that the "asymmetry mark" is present in all ABa- or ABp-derived cells for interpretation once a postmitotic ASE cell is formed.

Early Programming of L/R Asymmetries

Several mechanisms have been shown to be involved in determining the directionality of visceral asymmetries [9]. It has remained unclear, however, what the earliest symmetry-breaking event is and how conserved these mechanisms are between species [33, 51]. Moreover, although these mechanisms influence the sidedness of asymmetry, in most cases disruption of these mechanisms leads to randomization of asymmetry; they do not prevent asymmetry itself, suggesting the mechanisms that establish asymmetry are yet to be discovered [9, 51].

Our results suggest that in *C. elegans*, some functional neural asymmetries may be specified very early in embryogenesis by mechanisms that bias terminal cell-fate decisions in a lineage-specific manner. Although the sidedness of ASE asymmetry is dependent on the same mechanisms that establish visceral asymmetry in the worm (*gpa-16*-dependent asymmetric spindle rotation), this is restricted only to placement and establishment of the ASE lineages and cells rather than the functional divergence of these cells in terms of L/R asymmetric cell fate. We propose that as a consequence of the A/P asymmetric lineage origins of the ASE neurons and the asymmetric segregation of maternal cell-fate determinants along the A/P axis, an "asymmetry mark" is established early in embryogenesis for control of a postmitotic neuronal asymmetry. Our studies therefore reveal a nonanticipated link between the A/P and L/R axis.

Similar lineage-dependent mechanisms could establish functional asymmetries in other organisms. It is becoming increasingly clear, that, in an analogy to *C. elegans,* determinative prepatterning mechanisms within the embryo also exist in vertebrates. For example, the establishment of the D/V axis of *Xenopus*, like that of the invertebrates *Drosophila* and *C. elegans*, occurs shortly after fertilization and is dependent on the asymmetric segregation of cell-fate determinants [47, 52]. The L/R axis is also established early in *Xenopus* and is intimately linked to the establishment of the D/V axis [53]. Moreover, in *Xenopus*, various mRNAs and proteins are asymmetrically localized during the first few cleavages [54–56], and lineage specific injection of *Vg1* mRNA in early *Xenopus* blastomeres reverses L/R asymmetry [57, 58]. It is therefore conceivable that very early embryonic patterning events may prefigure later L/R asymmetric developmental decisions in vertebrates, in a similar manner to the case presented here in *C. elegans*. Our observation of early embryonic asymmetries predetermining adult lateralities also conforms to the view that lateralities in vertebrates may not be superimposed de novo on a bilaterally symmetric ground state but are rather reflections of nonbilaterality that may have preceded general bilaterality [33].

Experimental Procedures

The following mutant alleles were used: LGI, *gpa-16(it143), pop-1(zu189)*; LGII, *ref-1(ok288)*; LGIII, *glp-1(e2144)ts, glp-1(q46), lin-12(n941), par-2(it5), tbx-37(zu467) tbx-38(zu463)*; and LGV, *apx-1(zu183), lsy-6(ot71).* Experimental details about temperature shift experiments with the *glp-1(e2144)* allele are described in the Supplemental Experimental Procedures. The following balancers were used: *nT1(IV;V)*, *hT1(I;V)* and *qC1(III).* The following transgenes were used for assessment of cell fate: *otIs3: Is[gcy-7^{prom}::gfp;lin-15(+)], otIs114: Is[lim-6^{prom}::gfp;rol-6(d)], ntIs1: Is[gcy-5^{prom}::gfp;lin-15(+)], otEx2332: Ex[gcy-5^{prom}::mCherry;rol-6(d)], otEx2260: Ex[gcy-22^{prom1}::gfp;rol-6(d)], kyIs104: Is[str-1::gfp;lin-15(+)].* More details about these markers are provided in the Supplemental Experimental Procedures.

Laser ablations were performed in accordance to standard protocols, as described in detail in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Experimental Procedures and five figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/23/2279/DC1/.

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