Left-right symmetry breaking in mice by left-right dynein may occur via a biased chromatid segregation mechanism, without directly involving the Nodal gene

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INTRODUCTION

It is crucial for multicellular development that cells possess a memory system, which ensures stable inheritance of acquired developmental states during development of tissues and organs of an organism. The field of epigenetics studies this cellular memory system, and “epigenetic” is often defined as “mitotically heritable changes in gene expression that do not involve modulation of the primary DNA sequence.” For development, it is equally important that cells are able to change their acquired developmental state and differentiate along evolutionarily defined lineage paths. A crucial question is how epigenetic information can be changed and passed from the alternate alleles of the "left-right" (lrd) gene during the left-right axis establishing cell division. Here, asymmetry development would be ultimately governed by the chirality of the cytoskeleton and the DNA molecule. Our model predicts that randomization of chromatid segregation in lrd mutants should produce embryos with 25% situs solitus, 25% situs inversus, and 50% embryonic death due to heterotaxia and isomerism. Here we confirmed this prediction by using two distinct lrd mutant alleles. Other than lrd, thus far Nodal gene is the most upstream function implicated in visceral organs laterality determination. We next tested whether the Nodal gene constitutes the lra1 gene hypothesized in the model by testing mutant’s effect on 50% embryonic lethality observed in lrd mutants. Since Nodal mutation did not suppress lethality, we conclude that Nodal is not equivalent to the lra1 gene. In summary, we describe the origin of 50% lethality in lrd mutant mice not yet explained by any other laterality-generating hypothesis.

Keywords: laterality development, left-right dynein, asymmetric cell division, DNA strands differentiation, selective chromatid segregation
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Asymmetric cell division and body laterality determination

FIGURE 1 | Strand-specific imprinting in diploid and haploid organisms. (A) Hypothetical asymmetric cell division according to our strand-specific imprinting and selective segregation (SSIS) model. Only one pair of homologous chromosomes is illustrated. Lagging versus leading strand DNA replication epigenetically differentiates an important developmental gene on sister chromatids, $\text{ON}$ in one and $\text{OFF}$ in the other. A segregator, such as left-right dynein, "sorts" sister centromeres/chromatids according to their replication history in G2, causing selective segregation of older Watson template strands into specific daughter cell, and older Crick template strands into the other daughter cell (named WW:CC segregation). Hence, asymmetric DNA replication-coupled epigenetic chromatin modification and selective sister chromatid segregation in the parent cell can specify different developmental potentials to daughter cells (Klar, 1994). Symbols: W, template “Watson” strand; C, template “Crick” strand. Numbers 1–4 represent specific chromatids with respect to their strands’ constitution. (B) Illustration of how lagging-strand-specific imprinting explains the “1 in 4 granddaughters switching” rule in S. pombe mating-type switching. The mat1 locus efficiently switches $P$ and $M$ mating-type gene information by a cell cycle controlled DNA transposition mechanism. A replication terminator ensures unidirectional DNA replication of the mat1 locus, and lagging-strand DNA synthesis installs an imprint (indicated by star) in a sequence- and strand-specific manner in an unswitcable (Pu) cell. The imprint confers competence for switching at the mat1 locus only in the daughter cell inheriting the imprinted chromosome (Ps), which transposes opposite mating-type information copied from the silenced donor loci into the mat1 locus only in one of the sister chromatids (Klar, 2007).

We proposed that a similar mechanism might produce asymmetric cell divisions in diploid organisms by epigenetic means as well. First, strand-specific imprinting would epigenetically differentiate sister chromatids in S-Phase, and selective segregation of thus differentiated sister chromatids would create sister cells with different developmental fates. This model is called SSIS, and was initially developed by us to explain internal organ laterality development in vertebrates (Klar, 1994).

The development of bilateral asymmetry can be conceptually divided into three steps: First comes the initial symmetry-breaking event, usually ascribed to cellular amplification of a molecular chirality. This is followed by differential gene expression in cell fields on either side of the midline, which translates to step three, left/right (L/R) asymmetric organogenesis (Aw and Levin, 2009). For internal organ situs development in vertebrates, a great deal of molecular understanding has been achieved to decipher steps two and three, where many molecular pathways, seemingly conserved between model organisms, have been defined and well accepted (Nakamura and Hamada, 2012). For example, the TGF-β-related signaling molecule Nodal is conserved in all deuterostomes examined, and usually specifies the left body-side (Chea et al., 2005). Its activity is inhibited toward the midline by Nodal’s own transcriptional targets of the Lefty family of diffusible molecules, which represents a prime example of a reaction-diffusion mechanism (Nakamura et al., 2006; Muller et al., 2012). In contrast, identity of the symmetry-breaking event, the “first event,” that initiates mechanism, such that one out of four granddaughter cells switches cell type and expresses the mating-type opposite to that of the grandmother cell (Figure 1B). Genetic and biochemical analysis revealed that mating-type switching is controlled by lagging-versus leading-strand DNA replication at the mat1 locus. In particular, lagging-strand DNA synthesis installs an imprint at mat1 (most probably a two nucleotide long DNA:RNA hybrid from an incompletely removed Okazaki fragment), which initiates a double-strand break during the following S-Phase to start the DNA transposition event that underlies mat1 switching. Hence developmental asymmetry between sister cells can be traced back to double-helical structure of the mat1 gene and lagging- versus leading-strand synthesis of specific DNA strands in two consecutive cell divisions (Klar, 2007).
left-biased Nodal expression is controversial, because no unifying mechanism between vertebrate phyla has been identified to date (Vandenberg and Levin, 2009). Some vertebrates such as mice, frogs, and zebrafish are proposed to employ motile cilia during gastrulation at equivalent embryonic organizer regions, known as the node, gastrocoel roof plate, and Kupffer vesicle, respectively. Cilia’s beating is thought to either transport a morphogen leftwards in extraembryonic space (Nonaka et al., 1998), or induce asymmetrical calcium signaling in conjunction with mechanosensory cilia (McGrath et al., 2003). As a consequence, Nodal signaling is induced more strongly in left-sided neighboring tissues (lateral plate mesoderm in the mouse), and its autoregulatory feedback loop with Lefty molecules confers robustness to the signaling cascade (Nakamura and Hamada, 2012). This model is very attractive as it links the molecular chirality of the cilium and its building blocks to chirality of the developing embryo. However, several observations prominently question this model’s universality, and some data would rather support a role for nodal cilia during step two of bilateral asymmetry development, namely, asymmetric gene expression on either side of the midline. First, pigs, for example, undergo I/R axis development without motile nodal cilia, undermining a universal role for motile cilia in vertebrate and mammalian symmetry-breaking (Vandenberg and Levin, 2010). Second, in species that employ cilia, a number of genes that are required for proper nodal cilia motility and positioning are also expressed in non-ciliated cells at much earlier embryonic stages. Examples include planar cell polarity genes Vangl2 and Dvl2, insonin and left-right dynein (Joe and Levin, 2009). Thus, it is unclear whether these proteins exert their critical function in I/R axis development at the node. Third, mouse blastomere cells rearrangement has been shown to influence direction of embryonic turning, indicating that some aspects of laterality development certainly occur prior to gastrulation, and are independent of nodal cilia (Gardner, 2010). Last, both zebrafish and mouse mutants have been isolated, which show Kupffer vesicle or node ciliary defects but no L/R phenotypes, and vice versa (Vandenberg and Levin, 2010). Therefore, despite overwhelming evidence suggesting that cilia do have an important function in I/R asymmetry development in several species, they are unlikely to truly control initial symmetry-breaking in the embryo to generate I/R asymmetry (Tabin, 2005; Klar, 2008; Lobzin et al., 2012).

In 1959, Hummel and Chapman (1959) first described the recessive iv (inversus spurious) mutation, where 50% of homozygous mice develop situs inversus (i.e., mirror-image reversal of internal organs), and 50% have normal organ situs. Parental organ situs does not affect organ situs of the offspring, thus this mutation randomizes I/R asymmetry. More detailed analysis revealed high rates of heterotaxia (random and independent sidedness of internal organs) affecting both normal and situs inversus homozygous mutants at similar rates and severity (Layton, 1978). This suggests that in addition to its involvement in the first step of asymmetry development, the iv gene product is also needed in the second and/or third conceptual steps described above. Molecular cloning by Supp et al. (1997) showed that the iv mutation changed a highly conserved glutamic acid to lysine within the motor domain of a dynein heavy chain gene, which was thereafter named left-right dynein (Ivd). Ivd message was detected in blastocoely and (blastocyst-derived) ES cells, ventral node cells, and some ciliated embryonic and adult epithelia. It was classified as an anomalous dynein despite its obvious expression in many non-ciliated cell types. At the time the authors were not aware that node cells contain motile cilia, and even concluded that “… embryonic expression indicates that mechanisms other than ciliary movement are involved in I/R specification” (Supp et al., 1997). Later it was found that Node cells contained ciliated cells (Nonaka et al., 1998) whose motility is dependent on Ivd (Supp et al., 1999). Technically difficult studies further showed that beating nodal cilia created a leftward fluid-flow in extraembryonic space (Nonaka et al., 1998), and artificial fluid-flow reversal had a dominant effect on situs development in normal and Ivd mutant embryos (Nonaka et al., 2002). These data clearly highlight the node’s function as an embryonic organizer during I/R axis development. Whether the I/R asymmetry is truly established by nodal flow or whether this simply represents a “back-up” mechanism remains to be determined.

To address this question, our lab has started to generate a conditional allele for Ivd to discriminate between early cytoplasmic and later axonal (cilia) functions. A study from our lab has provided genetic evidence that Ivd does indeed have a functional role in non-ciliated cells (Armakolas and Klar, 2007). Liu et al. (2002) had engineered mouse ES cells lines, which allowed for selection of Cre/loxP-mediated mitotic recombinants between homologs. If recombination happens in G2, recombinated chromatids can either segregate together (Z segregation) or into different sister cells (X segregation). X segregants thereby acquire homogygosity of any heterozygous marker distal to the crossover site. Interestingly, centromere-proximal IoxP sites on chromosome 7 (DT1E9 cell line) always led to X segregation, whereas IoxP sites on chromosome 11 or further centromere-distal on chromosome 7, produced the usually expected random mix of X and Z segregants (Liu et al., 2002). Differentiation of DT1E9 ES cells to endoderm cells preserved the exclusive X segregation pattern, whereas neuroectoderm cells showed exclusive Z segregation. Three other in vitro differentiated cell types showed random patterns (Armakolas and Klar, 2006). We proposed that cell type-specific biased segregation patterns were due to selective chromatid recombination as well as selective segregation of chromosone 7 sister chromatids in mitosis. Remarkably, Ivd mRNA expression was evident in ES, endoderm, and neuroectoderm cells, and RNAs-mediated knockdown randomized segregation patterns, consistent with our SSIS model (Klar, 2008). In this model, Ivd would “sort” sister chromatids based on their replication history and selectively segregate sister centromeres to sister cells (Figure 1A). We propose that Ivd’s function in non-ciliated cells is to bias sister chromatid segregation of one or a specific set of chromosomes. By theory, this function is not confined to a single I/R axis establishing asymmetric cell division, but probably happens in other developmental contexts where asymmetric or strictly symmetric cell divisions occur. Additional support for this is provided by Ivd’s expression profile available on the gene annotation portal biogps.org, where Ivd shows high expression in hematopoietic stem cells (http://www.biogps.org/#goto). Here we tested developmental biology predictions of the SSIS model concerning the Ivd mutant. In a second experiment we tested whether
the Nodal gene comprises the “left-right axis development 1” (lra1) gene specified in the SSIS model.

**MATERIALS AND METHODS**

**MOUSE BREEDING AND HUSBANDRY**

Lrd-Neo-GFP mice were a kind gift from Dr. Martina Breuckner at Yale University, New Haven, CT. The iv stock (EM:02331) was purchased (live) from EMMA repository, Harwell, UK. Delta Nodal mice were a kind gift from Dr. Michael Kuehn, Frederick National Laboratory, MD. All mice were kept according to Animal Care and User Committee (ACUC) guidelines, Frederick National Laboratory, MD.

**GENOTYPING**

Between 3 and 4 weeks of age, tailclips were performed according to ACUC guidelines. Tails were digested by overnight incubation at 55°C in 200 μl of tail buffer (100 mM NaCl, 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5% (w/v) N-Lauroylsarcosine, 100 μg/ml Proteinase K). The solution was then diluted 1:1 with dH2O. 1 μl was used for PCR reactions. Lrd-Neo-GFP primers: wtF3: CICTGCAGAGACAGGCGC3′, wtR3: GCTTTGCCGCTGGTGGAGA, ivF3: CCGGTCCTAGGGCAAAGGTT, PCR: 95°C 2 min – 34× (94°C 20 s, 64.5°C 20 s, 72°C 30 s) 72°C 5 min, wt allele: 194 bp, targeted allele: 266 bp. Nodal Delta primers: F4299: CAGAGAGG-GGATTGGGTTTGCAG, R4457: GATCGGAACTCAGGAAC- GTCCGGATTGGGTTTGCAGAC, 1999 Tagal F: CCTAACCGAAACAAGCACTGGTCG, 1999 Tagal R: CACGGATCTCCAGGGCGGAGTC. 25 μl PCR product was digested with 25 U of Taq alpha I (NEB) in a 40 μl reaction, at 65°C for 45 min. The iv mutation destroys the Taq alpha I site in the PCR fragment. wt bands: 92 bp, iv band: 184 bp.

**RESULTS**

A TEST OF A KEY PREDICTION OF THE SSIS MODEL

Our model makes several testable predictions for the phenotype of the lrd mouse mutant. First, randomization of sister chromatid segregation during the critical L/R axis establishing cell division should have three different outcomes: 25% WW:CC cell pairs leading to normal organ situs later in development, 25% CC:WW cell pairs, causing development of situs inversus, and 50% WC:WC cell pairs, causing severe developmental abnormalities incompatible with survival. The future L/R axis is set by cytoplasmic polarization and alignment of a single cell with respect to the anterior-posterior and dorsal-ventral body axes. Sister chromatids containing a hypothetical “leftness-encoding” left-right axis-establishing gene 1 (lra1) are epigenetically differentiated. Normally, left-right dynein would selectively segregate older Watson template strand-containing sister chromatids harboring the lra1 “ON” allele to the left body side, and older Crick template strand-containing sister chromatids harboring the lra1 “OFF” allele to the right body side as described in Figure 1A. Randomized segregation due to left-right dynein mutation will result in three different outcomes shown here: 25% WW:CC cell pairs, causing normal situs development, 25% CC:WW cell pairs, causing severe developmental situs abnormalities incompatible with survival, and 50% WC:WC cell pairs, causing death.

FIGURE 2 | SSIS-predictions concerning embryo situs and survival rates of lrd mutants. Proposed laterality-generating asymmetric cell division is randomized in the lrd mutant. The future L/R axis is set by cytoplasmic polarization and alignment of a single cell with respect to the anterior-posterior and dorsal-ventral body axes. Sister chromatids containing a hypothetical “leftness-encoding” left-right axis-establishing gene 1 (lra1) are epigenetically differentiated. Normally, left-right dynein would selectively segregate older Watson template strand-containing sister chromatids harboring the lra1 “ON” allele to the left body side, and older Crick template strand-containing sister chromatids harboring the lra1 “OFF” allele to the right body side as described in Figure 1A. Randomized segregation due to left-right dynein mutation will result in three different outcomes shown here: 25% WW:CC cell pairs, causing normal situs development, 25% CC:WW cell pairs, causing severe developmental situs abnormalities incompatible with survival.

The iv mutation destroys the Taq alpha I site in the PCR fragment. wt bands: 92 bp, iv band: 184 bp.
Lrd-Neo-GFP mice carry a GFP-lrd exon 1 fusion as well as a Neo cassette on the opposite strand of lrd intron 1. Since the Neo transgene is under the control of a very strong promoter and transcribed antisense to lrd, lrd transcription is effectively shut down and homozygous mutant mice are indistinguishable from true knockout mice: 50% of live animals exhibit situs inversus (McGrath et al., 2003). Several heterozygous intercrosses were set up and DNA from tails of 165 offspring was analyzed (Table 1). We detected 53 lrd+/− : 90 lrd−/− : 22 lrd+/+ animals. The SSIS hypothesis predicts 24 (165/7) of live-born mice to be lrd+/−. This is because 1/8 (half of 1/4 animals with −/− genotype) of the initial number of homozygous mutant mice is expected to live, 1/8 is expected to die and thus reduce the total number of mice that are available for analysis to 7/8. As a result, 1/8 of the initial mice correspond to 1/7 of observable mice. If lethality was not an issue, then ~41 mice (1/4 of 165) should have the lrd+/− genotype. Our observed number of 22 lrd+/− mice is statistically equivalent to the SSIS-predicted number of 23.57 (p-value of ~0.6, chi-square test).

Encouraged by the heterozygous cross results, we set up four iv−/+ X iv−/− crosses. The results are summarized in Table 2. Conventionally 1/2 of the offspring is expected to be iv−/−. However, if lethality affected 50% of the iv−/− mice, this fraction would be reduced to 1/3 among live animals. Analysis of 111 offspring revealed 74 iv−/− and 37 iv−/+ mice, which meets SSIS prediction exactly.

**Table 1 | Observed rates of allele frequencies: Lrd-Neo-GFP allele, lrd+/+ × lrd−/−.**

| lrd+/+ (%) | lrd−/− (%) | lrd+/− (%) |
|-----------|-----------|-----------|
| 53 (52)   | 90 (85)   | 22 (18)   |

**Table 2 | Observed rates of allele frequencies: iv allele, iv−/+ × iv−/−.**

| iv−/+ (%) | iv−/− (%) |
|-----------|-----------|
| 74 (67)   | 37 (33)   |

**DOES NODAL CONSTITUTE THE L4A1 GENE HYPOTHESIZED IN THE SSIS MODEL?**

According to the SSIS model, heterozygosity for the lra1 gene would prevent embryonic lethality in iv−/− embryos because heterotaxia or isomerism would not occur. Consequently, 50% would develop normal organ situs and 50% would develop situs inversus embryos with lra1+/−, iv−/− genotype (Figure 3A). We chose a candidate gene approach, and considered the Nodal gene as a likely candidate for the lra1 gene as it is the gene, other than iv, that functions most upstream in the L/R pathway. Nodal belongs to the TGF-β family of extracellular signaling molecules and has been shown to be amongst the earliest asymmetrically (left-sided) expressed molecules in a variety of species, ranging from snails to man (Nakamura and Hamada, 2012). Since Nodal is essential for mesoderm induction during gastrulation and the

![Figure 2](image-url)
We propose DNA’s chirality and its asymmetric mode of replication will be both heterozygotes as well. Nodal lethality of only heterozygous animals. We generated several males be heterozygous for the result of the cross described in. Thr eo u to fs e v e no fl i v em i c ew i l lb eo f 1/8 of initial mice correspond to 1/7 of observable (live) mice. As discussed above, the total number of observable mice to 7/8. As discussed above, (1/2 Nodal for Nodal is in fact the type (wt) blastocysts results in development of R/R isomers (Ub. and Li, 2002). Thus, Nodal is considered to encode “leftness.” If Nodal is in fact the 1 rat gene, the 50% lethality of lrd homozygous mutant embryos should be suppressed in Nodal1 heterozygotes according to our model (Figure 3A).

We determined whether heterozygosity for a null allele of the Nodal gene (Lowe et al., 2001) in iv crosses affected lethality ratios. Specifically, we determined whether the mutation suppresses 50% lethality of iv–/– mice described in Figure 2. Because the Nodal gene deletion is homozygous lethal, we therefore quantitated viability of only heterozygous animals. We generated several males heterozygous for iv and delta Nodal mutations, which we set up with iv–/–, Nodal–/– females. Therefore, half of all offspring will be heterozygous for delta Nodal allele. In this mating set up, several predictions concerning ratios of expected genotypes are made (Figure 3B). Should heterozygous Nodal mutation not influence lethality ratios, then 1/3 iv–/– individuals should be observed, just like the result of the cross described in Table 2. Accordingly, 50% will be heterozygous for the delta Nodal mutation, and 1/6 (1/3 x 1/2) will be both iv–/– and carriers of delta Nodal. If heterozygosity for Nodal rescues lethality in WC:WC segregants, then only 1/8 (1/2 x 1/2 x 1/2) of initial conceptuses will die, which reduces the total number of observable mice to 7/8. As discussed above, 1/8 of initial mice correspond to 1/7 of observable (live) mice. Three out of seven of live mice will be of iv–/– genotype, and 4/7 will be Nodal+/-, should this mutation suppress lethality in a subgroup of mice destined to die. Moreover, the ratio of iv–/– and Nodal–/– animals will now be not a simple product of their individual ratios, rather this genotype will be enriched, and is expected to occur at a 2:7 rate: 1/7 stems from WW:CC (or CC:WW) segregants and 1/7 from rescued WC:WC segregants (Figure 3B). The observed result of these crosses is summarized in Table 3. Amongst 202 offspring, we found 66 iv−/− and 103 Nodal+/- animals. Thirty-three mice were both iv−/− and Nodal+/-: These numbers do not support the Nodal gene being the hypothetical lrd gene. Rather, they show that lrd mutation causes 50% lethality in Nodal heterozygotes as well.

**DISCUSSION**

We propose DNA’s chirality and its asymmetric mode of replication as a potential source for installing binary imprints on the chromatin fiber, and selective segregation of thus differentiated sister chromatids to sister cells as a novel and largely uncharacterized molecular mechanism associated with asymmetric cell divisions. The lrd-dependent segregation bias of mouse chromosome 7 sister chromatids in mitotic recombination experiments involving ES cells, endoderm cells, and neuroectoderm (Armakolas and Klar, 2007) cells could represent a case for selective sister chromatid segregation. Even though direct evidence for this interpretation is still missing, it led us to further investigate the phenotype of the lrd mouse mutant. In our model lrd functions to “sort” and selectively segregate sister chromatids based on their replication history in a WW:CC fashion. This L/R symmetry-breaking asymmetric cell division would be oriented along the L/R axis, positional information for it would presumably come from polarized cytoskeleton (Vandenberg and Levin, 2009). Randomization of chromatid segregation in iv–/– mice would lead to 25% normal organ situs (WW:CC segregants), 25% situs inversus (CC:WW segregants), and 50% death (WC:WC segregants). The 50 situs solitus : 50 situs inversus distribution in lrd mutant live animals has been described in numerous studies, therefore we only focused on assessment of lethality ratios by studying Mendelian inheritance of lrd mutant alleles in appropriate genetic crosses. In order to eliminate potential allele-specific or genetic background-specific artifacts, we analyzed two distinct lrd null alleles that had been outbred onto mixed backgrounds. Both crosses revealed lrd homozygous mutant animals at rates 50% below Mendelian predictions. This result is consistent with our SSI hypothesis even though it does not provide definitive proof of it. Nearly all studies of mouse laterality stress the 50% situs solitus: 50% situs inversus phenotype of iv–/– mice and ignore the 50% lethality phenotype. Approximately 50% lethality was first noted by Layton (1978) in one of the earliest studies of iv mutant crosses. Our results presented here with iv confirmed the estimations of Layton (1978) and extended it to the newly made Lrd-Neo-GFP allele. One caveat for our SSI explanation is that the original iv allele might be a leaky missense mutation generating the observed effects. It was therefore important to investigate phenotypes of a different allele, which is why we used the second Lrd-Neo-GFP allele for our analysis. A third allele was investigated previously, but the analysis was very limited to draw conclusions regarding lethality (Supp et al., 1999). Unfortunately, that allele was not saved (M. Bruckner, personal communication).

We next sought to test another prediction of our model, namely that heterozygosity for the hypothetical lrd gene would rescue WC:WC segregants. The rationale therefore is that strand-specific imprinting of lrd would lead to conflicting (ON/OFF) lrd epi-allesles in both WC:WC sister cells. If one allele of lrd is a null allele (due to heterozygosity), then different epialleles cannot be conflicting anymore (Figure 3A). We chose a reverse genetics approach and tested the Nodal gene as a possible candidate for lrd. Analysis of ~200 offspring did not show a protective function for Nodal heterozygosity in lrd mutant animals; therefore, Nodal cannot be lrd. We did however confirm the 50% lethality phenotype of iv–/– genotype, indicating that lethality was not affected by Nodal gene dosage.

We have eliminated Nodal as a candidate for lrd, and its ActR2B receptor can also be disregarded, because a study from

**Table 3** Allele frequencies in offspring of iv–/– Nodal+/- x iv–/– Nodal+/- cross.

| Allele | n = 202 | Conv. expected | SSIS expected | Observed |
|--------|--------|----------------|---------------|---------|
| iv–/–  | 1/2 = 67.3 | 3/7 = 86.6 | 66 |
| Nodal+/- | 1/2 = 101 | 4/7 = 115.4 | 103 |
| iv–/– and Nodal+/- | 1/6 = 33.7 | 2/7 = 577 | 32 |

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En Li’s laboratory (Oh and Li, 2002) recorded situs ambiguous (pulmonary isomerism) in around 40% of 

\( ^{32} \text{P} \) ActZB+/- embryos. If ActZB were Iso1, then the SSIS model predicts occu-

rence of situs solitus and situs inversus only. The Nodal signaling pathway is highly complex and regulated by numerous factors on several levels (Schier, 2009). After Nodal precursor is acti-
vated by protein convertases and released into extracellular space, Lefty proteins limit its activity via a reaction-diffusion

mechanism. Nodal binding to activin receptors is assisted by dis-
tinct co-receptors, and sometimes Nodal binds in conjunction

with other TGF-β molecules as a heterodimer. Moreover, evi-
dence from zebrafish suggests tight post-transcriptional control of Nodal, Lefty and activin receptor gene expression by microR-

NAs (Schier, 2009). Given this level of complexity, a reverse genetic

cscreen is unfeasible to tackle the identity of Iso1 gene in the first

step of L/R asymmetry development.

Interestingly, two recent studies have suggested that the nema-
tode C. elegans employs SSIS mechanism during Neural asym-

metry development. A study from Michael Levin’s research group

provides genetic support that an SSIS-type asymmetric cell divi-
sion operates in olfactory neuron development, although the evidence has not been interpreted as such by the authors. The Levin

laboratory has a long-standing interest in vertebrate L/R axis devel-

opment, and has highlighted the role of the cytoskeleton in cellular polariza-
tion for years (Av and Levin, 2009; Vandoernberg and Levin, 2009, 2010). It had come to the authors’ attention that Arabidopsis

mutants affecting radial flower symmetry were mapped to alpha-
tubulin and a gamma-tubulin associated protein (Loebkin et al., 2012). Remarkably, introducing the same alpha-tubulin mutation into Xe-

nopus 1-cell embryos resulted in development of hetero-
taxia, and in cultured human HL-60 cells it disturbed the leftward bias (with respect to the nucleus-centrosome axis) of pseudopodia

protrusion. In addition, C. elegans “AWC” olfactory neural asym-

metry was also affected by mutating a tubulin homolog (TBA-9, 75% amino acid identity with Arabidopsis alpha-tubulin) at two

conserved amino acids. In wt worms, the AWC neuron is in

the “ON” state (AWC<sup>ON</sup>) on one body side and in the “OFF” state

(AWC<sup>OFF</sup>) on the other body side; sidedness is stochas-
tic (Chang et al., 2011). This developmental asymmetry can be visualized by introducing the “str-2p:GFP” fluorescent GFP con-

struct in the genome. Importantly, chromosomal integration site of the GFP transgene is irrelevant for faithful AWC<sup>ON</sup> versus

AWC<sup>OFF</sup> discrimination, indicating that the cause for asymmetric

GFP expression acts in trans for the transgene. The authors

chose this model system for body asymmetry development stud-

ies, because the AWC<sup>ON</sup> and AWC<sup>OFF</sup> cells show cytoskeletal polariza-
tion and asymmetric calcium signaling, which is sensi-
tive to the microtubule depolymerizing drug nocodazole (Chang et al., 2011). Overexpression of wt TBA-9 tubulin in transgenic

worms causes only mild laterality defects, with 82% of worms

displaying the normal AWC<sup>ON</sup>/AWC<sup>OFF</sup> phenotype. Overex-
pession of mutant TBA-9, in contrast, results in 42% normal

AWC<sup>ON</sup>/AWC<sup>OFF</sup> and 45% novel 2AWC<sup>ON</sup>/2AWC<sup>OFF</sup> “heterotaxic” phe-
notype. This roughly 50:50 distribution is consistent with a SSIS

mechanism operating in the mother AWC cell (Figure 4). We

hypothesize that one daughter inherits normally two AWC<sup>ON</sup> ep-

alleles and the other two AWC<sup>OFF</sup> epialleles are inherited by the

other daughter cell (Nodal<sup>CC</sup> segregation). Unlike our SSIS model

for mouse L/R axis development, this asymmetric distribution of

sister chromatids in the worm occurs irrespective of the L/R body

axis. We propose that introduction of mutated tubulin renders the

AWC cell’s cytoskeleton unable to direct selective chromatid seg-
gregation in mitosis, hence a novel WC/Segregation results at

50% frequency. A simple explanation for 2AWC<sup>ON</sup> phenotype in

WC/Segregation would be dominance of the AWC<sup>ON</sup> over the

AWC<sup>OFF</sup> epiallele.

A second study implicating an SSIS-like asymmetric cell divi-
sion in C. elegans neuronal asymmetry development has been recently published by Horvitz/Stillman laboratories (Nakano et al., 2011). Here, a GFP-reporter screen served to isolate mutants that

changed the paired asymmetric MI motor neuron/e3D epider-

mal cell pair to a symmetrical e3D cell pair on both sides of the

brain. Positional cloning identified a gain-of-function mutation in a histone chaper-

eone complex, which deposits higher nucleosome density. This

could represent an epigenetic imprint in itself, or serve to nucle-

ate covalent chromatin modifications. The latter seems somewhat

more likely, since the epigenetic imprint is transmitted through

several mitoses, as it is the MI/e3D great-grandmother cell

that directs development of distinct cell fates three cell divisions

later on. Like in our SSIS model, selective segregation of epige-

netically differentiated sister chromatids is an integral part of the

authors’ model. However, neither direct nor indirect evidence is

presented. Because genetic evidence suggests that mutated tubulin

(Lobkin et al., 2012) randomizes (the normally selective) chro-

matid segregation during an AWC<sup>ON</sup>/AWC<sup>OFF</sup> olfactory neuron asymmetry generating cell division, we propose to test whether

mutated TBA-9 also affects the MI/e3D neuronal asymmetry.

The SSIS model is conceptually based on three aspects: (i) dif-

ferential chromatin imprinting during inherently lagging versus

leading strand replication, (ii) one or several genes who’s expres-

sion is affected by this imprint, and (iii) a segregator that identifies

and “sorts” epigenetically differentiated sister chromatids by oper-

ating at sister centromeres in mitosis. We have presented genetic

evidence for (iii), namely that 

Acts as a segregator in a L/R axis

defining cell division in mouse. In contrast, Nakano et al. (2011)

have provided evidence for (i), but have not identified the segrega-

tor. If the segregator can be identified, C. elegans will be excellently

suited to use forward genetics to identify the gene or set of genes

(ii) that are imprint and selectively segregated, as outlined in

our previous, “Nodal<sup>CC</sup>” breeding experiment.

We have highlighted two studies that support a SSIS-type mech-

anism in the development of neuronal asymmetries in C. elegans.

Based on genetics of psychosis development in human carriers of

balanced chromosome 11 translocations, we have previously pro-

posed that a similar mechanism may operate during human brain

laterality development (Klar, 2004). Analogous to our model for body later-

ality development, brain laterality development would also initiate

with a single critical asymmetric cell division, where chromosome

...
11 sister chromatids are selectively segregated in a WW:CC fashion. If one chromosome 11 homolog is fused to the centromere of another chromosome not undergoing selective mitotic segregation, then WW:CC and WC:WC segregation for chromosome 11 are expected to occur at equal frequencies. Fifty per cent incidence of psychosis development in four different families with balanced chromosome 11 translocations support our hypothesis (Singh and Klar, 2007).

Whether asymmetric cell divisions elsewhere during normal tissue homeostasis employ a SSIS mechanism remains to be determined. If they do exist, then somatic chromosomal translocations could potentially randomize these asymmetric cell divisions and initiate tumorogenesis. An example would be a resting tissue stem cell that only enters the cell cycle upon tissue injury. It asymmetrically divides to produce a rapidly-proliferating transiently amplifying stem cell. This cellular asymmetry development would be controlled by asymmetric segregation of cytoplasmic determinants, but also by WW:CC segregation of epigenetically differentiated sister chromatids containing an AWC master-regulator gene in a WW:CC fashion, such that always a 1AWCON/1AWCOFF olfactory cell pair develops in each worm.

Embryos transgenic for mutated (but not wild-type) tubulin developed either 1AWCON/1AWCOFF or 2AWCON olfactory neuron cells at a roughly 50–50 frequency. We explain this result by the SSIS model due to randomized chromatid segregation during the critical AWCON/AWCOFF neuron generating cell division due to the tubulin mutation.

We suggest that somatic chromosomal translocations in tissue stem cells could affect biased segregation of sister chromatids,
and change strictly asymmetrically dividing stem cells to stem cells that undergo symmetrical cell divisions in terms of epige-
netic imprints on differentiated sister chromatids distal to the
translocation breakpoint.

Curiously, a 1992 study published in The Lancet (Sandson et al., 1992) found a correlation of aberrant brain laterality devel-

opment and breast cancer. Right-handed breast cancer patients and healthy controls were subjected to computer tomographic
brain scans. Eighty-two per cent of control subjects showed left
hemispheric dominance, whereas in the breast cancer group this
number was reduced to 51%. Although this study should be
cautiously interpreted until replicated elsewhere, it certainly sug-
gests that brain laterality- and breast cancer-development share a
generic common pathway (Klar, 2011). We suggest that this path-
way controls asymmetric cell divisions during embryonic brain
development, and during cell turnover in the lactiferous duct
upon periodic hormonal growth stimulation. Hence, improv-
ing our understanding of vertebrate laterality development could
eventually impact on cancer prevention and treatment.

Taken together, 50% lethality phenotype in the hbd mouse

mutant supports predictions made by the SSIS model for laterality
development. Here, hbd is a cellular mechanism that selec-
tively segregates epigenetically differentiated sister chromatids
coming their replication history with respect to a cytoskeleton-
based early L/R axis (Klar, 2008; Vandenberg and Levin, 2010;
Lobzin et al., 2012). The overwhelming majority of studies on
hbd in the mouse have focused on its role in conferring nodal cilia
motility. This is understandable, since genetics of spontaneous and
targeted mouse mutations affecting laterality development have
generally pointed to a central role for motile nodal cilia. Moreover,
the earliest known molecular L/R asymmetries appear after node
formation in the mouse. In chicken and Xenopus, in contrast, ear-
lier asymmetries involving Gap-junctional communication (Levin
and Mercola, 1999), H+/K+ ATPase activity (Levin et al., 2002),
and serotonin signaling (Fukumoto et al., 2003) have been iden-
tified. As many of the studies on earlier asymmetry determinants
in frogs and chicken involved embryo-exposure to pharmacolo-
gical inhibitors, mouse embryo-culture protocols will need to
vastly improve until replication can even be considered. In this
regard it is noteworthy that one of the leading laboratories for
mouse embryos in vitro culture has recently tested the relation-
ship of nodal cilia emitted force and asymmetry development in
several mouse mutants affecting cilia biogenesis and motility (Shi-
nobara et al., 2012). Surprisingly, the authors found that as few as
two motile nodal cilia were sufficient to break bilateral symme-
try. These data are rather compatible with the “2-cilia hypothesis,”
which was initially postulated by the Hirokawa, Brucecker, and
Tabin laboratories (Okada et al., 1999; McGrath et al., 2003; Tabin
and Vogan, 2003). Here, mechanical force exerted by beating nodal
cilia is read out by mechanosensory cilia, which are associated with
polarity-2 (a calcium release channel) to induce left-sided cal-
cium signaling. Hence loss of polarity-2 is predicted to ablate
the calcium and Nodal signaling altogether. Pennekamp et al. (2002)
indicated loss of Nodal expression in the majority of polarity-2
deficient embryos; however the Nodal downstream target Pitx2
showed bilateral expression. Clearly how body laterality is ini-
tially developed, whether in visceral organs or in the nervous
system, remains controversial thus far. Further work is needed to

differentiate if any of the prevailing hypotheses can satisfactorily
explain body laterality development. The SSIS model is simple to
understand in that an asymmetric cell division constitutes the root
cause of development. In this model, developmental decisions are

made through particulate matter consisting of ON/OFF epigenetic
states of gene expression of developmentally important gene(s).
Thus, in addition to acting as genetic material, DNA strands
can provide the basis for evolution, cancer and development
(Furusawa, 2011).

AUTHOR CONTRIBUTIONS
Amar J. S. Klar and Stephan Sauer designed experiments, Stephan
Sauer carried out experiments and collected data, Stephan Sauer
and Amar J. S. Klar interpreted the data and wrote the manuscript.

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