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Reduced cell number in the hindgut epithelium disrupts hindgut left–right asymmetry in a mutant of *pebble*, encoding a RhoGEF, in *Drosophila* embryos

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ABSTRACT

Animals often show left–right (LR) asymmetry in their body structures. In some vertebrates, the mechanisms underlying LR symmetry breaking and the subsequent signals responsible for LR asymmetric development are well understood. However, in invertebrates, the molecular bases of these processes are largely unknown. Therefore, we have been studying the genetic pathway of LR asymmetric development in *Drosophila*. The embryonic gut is the first organ that shows directional LR asymmetry during *Drosophila* development. We performed a genetic screen to identify mutations affecting LR asymmetric development of the embryonic gut. From this screen, we isolated *pebble* (*pbl*), which encodes a homolog of a mammalian RhoGEF, Ect2. The laterality of the hindgut was randomized in embryos homozygous for a null mutant of *pbl*. Pbl is a multi-functional protein required for cytokinesis and the epithelial-to-mesenchymal transition in *Drosophila*. Consistent with Pbl's role in cytokinesis, we found reduced numbers of cells in the hindgut epithelium in *pbl* homozygous embryos. The specific expression of *pbl* in the hindgut epithelium, but not in other tissues, rescued the LR defects and reduced cell number in embryonic *pbl* homozygotes. Embryos homozygous for *string* (*stg*), a mutant that reduces cell number through a different mechanism, also showed LR defects of the hindgut. However, the reduction in cell number in the *pbl* mutants was not accompanied by defects in the specification of hindgut epithelial tissues or their integrity. Based on these results, we speculate that the reduction in cell number may be one reason for the LR asymmetry defect of the *pbl* hindgut, although we cannot exclude contributions from other functions of Pbl, including regulation of the actin cytoskeleton through its RhoGEF activity.

1. Introduction

In many animals, body structures show directional left–right (LR) asymmetry, which is determined genetically (Levin, 2004; Okumura et al., 2008). The mechanisms of LR axis determination in certain vertebrate species are well-studied (Burdine and Schier, 2000; Hamada et al., 2002; Tabin and Vogan, 2003; Tabin, 2006; Hirokawa et al., 2006). For example,

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in mouse, nodal flow, which is a leftward flow of extra-embryonic fluid in the node, produces the first LR axis information (Hirokawa et al., 2006; Shiratori and Hamada, 2006). The nodal cassette, a genetic cascade composed of *nodal*, *lefty*, and *Pitx2*, is activated downstream of the nodal flow and promotes the subsequent LR asymmetrical morphogenesis (Burdine and Schier, 2000). However, recent studies suggested that the mechanisms of LR asymmetrical development diverged evolutionarily even among vertebrates (Bergmann et al., 2003; Shibazaki et al., 2004; Levin and Palmer, 2007). For example, in amphibians, the first cue for LR asymmetric development is an LR asymmetric proton flux at the early stage of cleavage (Adams et al., 2006). In contrast, the molecular mechanisms of LR axis formation in invertebrates remain largely unknown, although some important clues have been obtained from nematodes and snail (Asami et al., 2008; Bergmann et al., 2003; Shibazaki et al., 2004). To understand such mechanisms in invertebrates, we have been studying LR asymmetric development in *Drosophila melanogaster* (Taniguchi et al., 2007a; Okumura et al., 2008).

Drosophila has several LR asymmetric organs, including the gut, brain, spermiduct, and genital plate (Gleichauf, 1936; Hayashi and Murakami, 2001; Ligoxygakis et al., 2001; Adám et al., 2003; Pascual et al., 2004; Baum, 2006). These organs show stereotypic LR asymmetry, and their LR inversion is very rare (Adám et al., 2003; Gleichauf, 1936; Hayashi and Murakami, 2001; Pascual et al., 2004). Among these organs, the embryonic gut is the first to form an LR asymmetrical morphology (Hayashi et al., 2005). Previous studies have identified many genes and signaling pathways involved in the LR asymmetrical development of the *Drosophila* embryonic gut, including *Myosin31DF* (*Myo31DF*), *single-minded*, *Drosophila E-Cadherin* (*DE-Cad*), *Myosin II*, the Rho GTPase family, canonical Wnt signaling, and JNK signaling (Hayashi and Murakami, 2001; Ligoxygakis et al., 2001; Hozumi et al., 2006; Maeda et al., 2007; Taniguchi et al., 2007b, 2011; Okumura et al., 2010; Kuroda et al., 2011).

The cellular basis of LR asymmetrical morphogenesis in the *Drosophila* embryonic hindgut has been studied (Taniguchi et al., 2011). The hindgut first forms symmetrically, at stage 12 (Hayashi and Murakami, 2001; Taniguchi et al., 2011). It then rotates counterclockwise 90 degrees as viewed from the posterior end, and consequently forms an LR asymmetrical structure at stage 13 (Hayashi and Murakami, 2001; Taniguchi et al., 2011). Even before this rotation, the shape of the hindgut epithelial cells at the apical plane is LR asymmetric (Taniguchi et al., 2011). Because the three-dimensional structure formed by these cells cannot be superimposed on its mirror image, this LR asymmetric property is referred to as "planar cell-shape chirality" (PCC) (Taniguchi et al., 2011).

A PCC-like phenomenon was independently found for certain mammalian cells (Xu et al., 2007; Wan et al., 2011). Thus, PCC may be evolutionarily conserved, although whether the underlying mechanisms are homologous remains to be studied (Horne-Badovinac and Munro, 2011). In *Drosophila*, PCC formation probably depends on the asymmetrical distribution of DE-Cad at apical cell boundaries (Taniguchi et al., 2011). Furthermore, a computer simulation suggested that PCC can drive the counterclockwise 90-degree rotation of the embryonic hindgut (Taniguchi et al., 2011). In embryos homozygous for a *Myo31DF* mutant, the direction of hindgut rotation is inverted (Hozumi et al., 2006). In these embryos, the PCC is the mirror image of that in wild-type embryos, and the distribution of DE-Cad is also reversed (Taniguchi et al., 2011). These results suggested that PCC may be a cause of the counterclockwise rotation.

The rotation of the hindgut also depends on the actin cytoskeleton (Hozumi et al., 2006), and the organization and function of actin filaments are regulated by the Rho GTPase family (Hall, 1998). The suppression of Rho1 in the hindgut epithelium randomizes the LR asymmetry of the hindgut (Hozumi et al., 2006). In general, the activity of Rho1 is regulated positively by RhoGEF and negatively by RhoGAP (Symons and Settleman, 2000). A *Drosophila* homolog of the mammalian RhoGEF Ect2, Pbl (encoded by the *pebble* (*pbl*) gene), specifically binds to Rho1 (Prokopenko et al., 1999), and activates it in the cell cortex, via a complex it forms with Tumbleweed and Pavarotti (Somers and Saint, 2003). The activated Rho1 then acts through its effectors, Diaphanous and Citron kinase, to promote formation of the contractile actin–myosin ring (Somers and Saint, 2003). In addition, Pbl homologs play an evolutionarily conserved role in cytokinesis (Tatsumoto et al., 1999; Morita et al., 2005).

Furthermore, in *Drosophila*, *pbl* is required for the epithelial-to-mesenchymal transition (EMT) of the mesoderm and for mesoderm migration (Smallhorn et al., 2004; Schumacher et al., 2004). These two functions of Pbl can be separated genetically, indicating that Pbl is a multi-functional protein (Schumacher et al., 2004). For example, although Pbl is known as a RhoGEF, it is also required for the activation of Rac GTPase during mesoderm migration (van Impel et al., 2009).

However, although Rho1 and the actin cytoskeleton are known to be involved in asymmetric development, no role of Pbl in LR asymmetric development has been reported in any species.

2. Results

2.1. *Identification of $pbl^{fortune}$ as a mutant with defects in LR asymmetry of the embryonic hindgut*

To identify genes involved in LR asymmetric development of the *Drosophila* embryonic gut, we performed a genetic screen of mutants on the third chromosome (to be presented elsewhere). We established about 2000 ethyl methanesulfonate-induced mutant lines. Embryos homozygous for each mutation were examined for laterality defects of the gut. From this screen, we isolated a new allele of *pbl*, designated $pbl^{fortune}$, which affected the LR asymmetry of the hindgut (Fig. 1B–E). We found that about 20% of embryos homozygous for $pbl^{fortune}$ showed no laterality in their hindgut (Fig. 1B–E), which normally curves to the right at stage 13.

We then examined the trans-heterozygotes and homozygotes of other known *pbl* alleles for LR defects. pbl^{2} is a null allele (Prokopenko et al., 1999), and pbl^{3} is a strong loss-of-function allele (Prokopenko et al., 1999; Schumacher et al., 2004). We found that $pbl^{fortune}/pbl^{2}$, pbl^{2}/pbl^{2}, and pbl^{3}/pbl^{3} embryos showed LR inversion of the hindgut as well as the