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A novel mechanism underlying axon guidance phenotypes: Indirect disruption of embryonic axon guidance by unspecified cells.

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Abstract

During characterization of the *Netrin* locus, we uncovered a background maternal effect mutation in which a small number of presumptive mesoderm cells fail to express the mesoderm determinant *twist* (*twi*). In contrast to *twi* mutants in which the mesoderm cells adopt alternative cell fates, the affected cells did not appear to differentiate. The cells failed to invaginate with other mesodermal cells and remained at the central nervous system (CNS) midline physically blocking cell migration and axon outgrowth. Due to low penetrance we were unable to map the mutation, but propose that the gene is required for coordination of gene expression throughout the cells of a tissue. We believe that the phenotype represents a novel way for cell fate alterations to disrupt axon guidance, distinct from alterations in neuronal or target cell identity.

Introduction

Like all animals, early development of *Drosophila* embryogenesis is controlled by gene products deposited by the mother in the oocyte (Tadros and Lipshitz, 2009). One of the first zygotic genes to be activated is the *twist* (*twi*) gene, a basic helix-loop-helix (bHLH) transcription factor required for mesodermal cell fate (Thisse *et al*., 1987). In *twi* mutants, the presumptive mesoderm fails to invaginate during gastrulation and adopts alternative cell fates, mainly neurectodermal (Leptin and Grunewald, 1990; Rao *et al*., 1991). The failure of a large number of cells to invaginate leads to an increase in the length of the embryo which, as the embryo is constrained by the egg membranes, leads to twisting of the embryo giving the mutant its name (Simpson, 1983).

The *Drosophila* central nervous system (CNS) is formed by the juxtaposition of neurectodermal tissue from opposite sides of the embryo after the presumptive mesoderm has

invaginated (Leptin, 1999). The site of invagination becomes the CNS midline, an axis of bilateral symmetry. The midline is a source of attractive and repulsive cues that guide axons and cell migration during CNS development (Evans and Bashaw, 2010). Proteins of the Netrin family primarily act as attractive signals. *Drosophila* has two *Netrin* genes, *NetA* and *NetB*, with deletion of both leading to decreased axon crossing of the CNS midline (Mitchell *et al*., 1996; Harris *et al*., 1996; Brankatschk and Dickson, 2006). Here we describe a cell fate alteration that disrupts CNS formation, not by altering neuronal or target cell identity, but producing apparently unspecified cells during gastrulation that persist to sterically hinder cell migration and axon outgrowth.

Materials and Methods

Drosophila Stocks and Genetics

The *KG03586* transposon and transposase stocks were obtained from the Bloomington *Drosophila* stock center. Excision of the *KG03586* insertion was by standard techniques.

Immunohistochemistry

Immunohistochemistry was performed as described in Patel (1994). The anti-Singleminded antibody was obtained from S. Crews. *In situ* hybridizations were performed as described in Kopczynski *et al*. (1996).

Results and Discussion

The *NetA* and *NetB* genes appear to be largely redundant, with functional differences originating primarily in expression patterns (Mrkusich *et al*., 2010; Matthews and Grueber, 2011). Deletion of either gene alone has no discernible effect on CNS formation (Brankatschk and Dickson, 2006), so we were intrigued by subtle alterations to axon and midline cell position present in a stock with a transposon insertion in *NetB* (Figure 1). The *KG03586* transposon is inserted in a 55 kb intron of *NetB*, suggesting it might be altering *NetB* activity in an unusual manner. *NetB* mRNA expression was reduced or absent in embryos mutant for the *KG03586* transposon. An imprecise excision of the transposon restored *NetB* expression, but unexpectedly the CNS phenotype remained (Figure 1D). Analysis of the site of the *KG03586* transposon revealed a small gene, *CG32595*, which we named *hog* (Newquist *et al*., 2013). The CNS phenotype failed to appear when *KG03586* was crossed to a deficiency for the region, or when transgenic RNAi for the *hog* gene was expressed in the female germline (*hog* is maternally deposited in the egg). We conclude that the phenotype is in the background of the *KG03586* transposon. The phenotype occurs at low penetrance and disappeared from our stocks over time, preventing mapping.

Closer examination of the CNS in the *KG03586* background revealed large rounded cells (Figure 1D), which did not stain with neuronal or midline cell markers. These cells appeared to be disrupting midline patterning and axon crossing. The maternal deposition and early disappearance of the *hog* mRNA suggested that the cells might arise as the result of a gastrulation defect. The *twi* gene is normally expressed in future mesoderm cells in a broad ventral band (Figure 2A). We examined *twi* expression in *KG03586* mutants and found that the *twi* mRNA pattern showed single or small groups of cells lacking *twi* expression (Figure 2B). As *twi* is required for mesodermal cells to invaginate, we examined the pattern of Single-minded (Sim) protein staining at the end of gastrulation. Sim marks the CNS midline which is created by invagination of the mesoderm. In *KG03586* mutants, we noticed that the regular array of Sim positive cells was occasionally

interrupted by unstained cells (Figure 2C,D). These cells lie slightly dorsal to the Sim positive cells, suggesting they may have attempted to invaginate. In *twi* mutants, there is a brief but ineffectual attempt at invagination (Leptin and Grunewald, 1990; Ip *et al*., 1994).

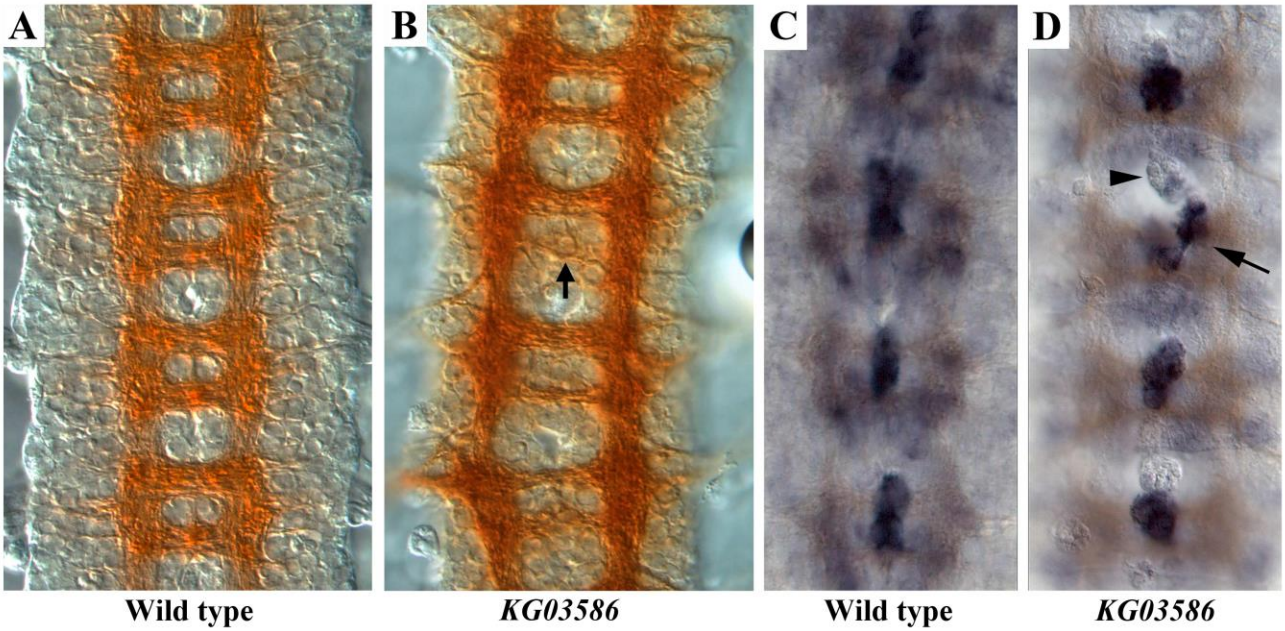

Figure 1. CNS phenotypes of *hog* mutants. Stage 16 (A,B) and stage 15 (C,D) embryos stained with monoclonal antibody BP102, which recognizes all CNS axons (brown), and an *in situ* hybridization probe against NetB (blue; C,D). (A) Wild type. The CNS axon scaffold is visible as a repeated ladder-like pattern. (B) *KG03586* mutant embryo. This is a strong example in which a posterior commissure (arrow) is almost completely missing. (C) Wild type. Strong NetB expression at the CNS midline can be seen in a regular repeated pattern. Additional neuronal expression can be seen lateral to the midline, mainly out of the plane of focus. The CNS axon scaffold is out of focus. (D) *KG03586* mutant embryo. The arrow indicates a cluster of *NetB* expressing midline glia that are mispositioned away from the underlying anterior commissure. Immediately above the midline glia is an abnormal cluster of cells (arrowhead). Similar cells can be seen in the lowest, but not the middle, segment. They are also absent from the wild type embryo in panel C.

We propose that the failure of the unidentified cells to invaginate represents a novel mechanism for indirectly disrupting axon guidance: the creation of physical barriers for migrating cells and growing axons by a failure of cells to migrate to their correct locations. The observed phenotype would probably not be obvious if the unidentified cells did not persist at the CNS midline, a critical organizing center for CNS formation. We speculate that rather than adopting an alternative cell fate as in *twi* mutants, these cells appear to stall in the differentiation process, remaining morphologically distinct and failing to express mesodermal or CNS markers. The rounded morphology is suggestive of an undifferentiated state, but we have been unable to identify a marker for undifferentiated embryonic cells in *Drosophila* to confirm this. The phenotype contrasts with *twi*

mutants in which mutant cells adopt more lateral cell fates. The disruptions to the *twi* mRNA pattern suggest that the gene product is either directly involved in *twi* transcription, or is required for the coordination of transcription throughout the presumptive mesoderm (Lagha *et al*., 2013). It is also possible that the cells may not be able to respond to signals from other mesoderm cells as has been described for the community effect (Gurdon *et al*., 1993). The phenotype is somewhat reminiscent of axon regeneration in the injured spinal cord in which a scar of glial tissue imposes a barrier to axonal regeneration (Rolls *et al*., 2009).

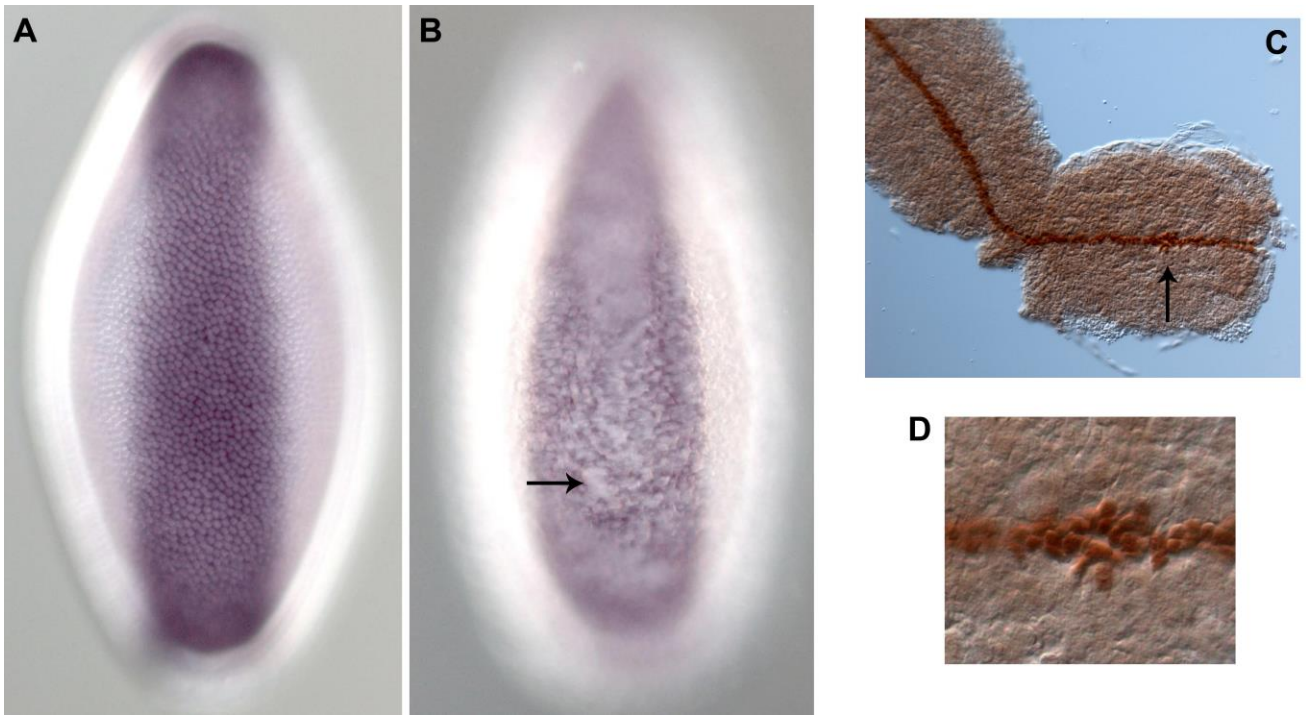

Figure 2. *twi* expression is affected by *hog* mutants. (A,B) Embryos stained with an *in situ* hybridization probe for *twi*. (C,D) Embryo stained with an antibody against Single-minded (Sim). (A) Wild type late stage 5 embryo showing a contiguous sheet of cells expressing *twi*. (B) Early stage 6 *hog* mutant embryo showing gaps in the *twi* mRNA pattern (arrow). Gastrulation has initiated so the sheet of *twi* expressing cells is slightly indented. (C) Stage 9 *hog* embryo showing the future CNS midline as a continuous line of Sim positive cells with a disruption near the posterior end (arrow). (D) Higher magnification view of the embryo in C, showing the disrupted region of Sim staining. A non-staining cell can be partially seen in the center of the disrupted region and other midline cells have been displaced laterally.

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