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Notch-, Wingless-, and Dpp-mediated signaling pathways are required for functional specification of *Drosophila* midgut cells

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Abstract

The mechanisms for cell fate determination have been extensively studied whereas little is known about the mechanism through which functional specificity is established. In the *Drosophila* midgut, copper cells provide an excellent model system to examine this mechanism. Copper is an essential element for the activity of a number of physiologically important enzymes including Cu/Zn-superoxide dismutase, cytochrome *c* oxidase, and dopamine-β-hydroxylase. *Drosophila* copper cells are involved in two distinct functions, i.e., copper absorption and acid secretion, which are visualized as a fluorescent signal and a color change of a pH indicator dye, respectively. Here we show that the absorptive function is established through two independent pathways, the Notch signaling pathway in adjacent interstitial cells and the Wingless signaling pathway in copper cells. Furthermore, the other function, acid secretion, is regulated through the Decapentaplegic and Wingless signaling pathways in interstitial cells. Our results clearly indicate that normal morphological development is insufficient for functional maturation, and that subsequent functional specification is achieved through several independent pathways. These results provide valuable insights into the molecular mechanism underlying functional specification.

Keywords: *Drosophila*; Notch; Wingless; Dpp; Gut; Copper; Proton; *dve*

Introduction

The *Drosophila* midgut consists of two germ layers, the visceral mesoderm and the endoderm. In the visceral mesoderm, four homeotic genes, *Sex combs reduced* (*Scr*), *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), and *abdominal-A* (*abd-A*), are expressed in non-overlapping domains in an order along the anterior–posterior axis similar to that in the epidermis. The direct target genes regulated by these homeotic genes are secreted signaling molecules such as *decapentaplegic* (*dpp)* and *wingless* (*wg*), which are expressed in the visceral mesoderm of parasegment (PS) 7 and PS8, respectively. Dpp and Wg maintain each other's expression and induce another secreted molecule, Vein (Vn), a ligand for epidermal growth factor receptor (EGFR). These molecules in turn undergo the feedback regulation of homeotic gene *Ubx*. This cross-regulatory feedback defines the precise pattern of *dpp* and *wg* expression in two adjacent parasegments. These secreted molecules diffuse into the underlying endoderm, and then induce morphogenetic events that ultimately compartmentalize the primordia into distinct sectors. Subsequently, cell fates are specified through the different thresholds of Dpp-, Wg-, and EGFR signaling to establish the functional organization of the midgut (reviewed in Bienz, 1997; Nakagoshi, 2005).

The middle midgut cells derived from the endoderm differentiate into four distinct types of cell: copper cells, interstitial cells, large-flat cells, and iron cells. Of these cells, copper cells provide an excellent model system to examine the mechanism of functional specification. Copper cells have invaginated microvillar membranes on their apical surface, which exhibit a typical banana shape, and are involved in two distinct functions, i.e., copper absorption and acid secretion, which are visualized as a fluorescent signal and a color change of a pH indicator dye, respectively. The guts of copper-fed first instar larvae exhibit

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orange–red fluorescence (copper fluorescence) on excitation with UV-light (Poulson and Bowen, 1952). It has been reported that this fluorescence reflects the absorption of copper and the formation of a copper–metallothionein complex (McNulty et al., 2001). When first instar larvae are fed with the pH indicator dye bromophenol blue (BPB), the gut lumen posterior to the copper cell region turns yellow (pH < 2.35) or green (2.35 < pH < 4) due to acid secretion whereas other regions remain blue (pH > 4) (Dubreuil, 2004; Dubreuil et al., 1998). These two functions are specified by the homeobox gene *labial* (*lab*), which is expressed in copper cells in response to Dpp, Wg, and Vn secreted from the adhering visceral mesoderm (Hoppler and Bienz, 1994; Hoppler and Bienz, 1995; Szüts et al., 1998). In *lab* mutants, the typical banana shape of invaginated microvillar membranes never develops and the two functions of copper cells are severely impaired (Dubreuil et al., 2001). Once the copper cell fate is determined by *lab*, it is maintained through the autoregulatory feedback (Chouinard and Kaufman, 1991; Tremml and Bienz, 1992), and subsequent functional development requires repression of the homeobox gene *defective proventriculus* (*dve*) (Nakagoshi et al., 1998). Expression of *dve* as well as *lab* mainly depends on Dpp signaling and also requires EGFR signaling mediated by Vn (Shirai et al., 2003). During embryogenesis, *dve* is expressed in four types of middle midgut cell, whereas it is repressed in copper cells of hatched first instar larvae. This repression requires the activity of Dve itself and that of Lab, and forced expression of *dve* impairs the copper absorptive function without affecting the morphological development of copper cells. Thus, temporally regulated *dve* repression is crucial for the functional development of copper cells (Nakagoshi et al., 1998). In wing discs, *dve* is also temporally repressed along the dorsal–ventral (D–V) boundary through Notch- and Wg-mediated signaling, and this repression is crucial for wing patterning (Nakagoshi et al., 2002). These signaling molecules such as Notch, Wg, and Dpp might be involved in the functional specification of copper cells as well as the nervous system (Aberle et al., 2002; Marqués et al., 2002; Packard et al., 2002; Presente et al., 2004). Here, we have examined the effects of these signaling molecules on the functional development of the *Drosophila* midgut. Our results indicate that two distinct functions, copper absorption and acid secretion, are regulated through independent signaling pathways.

Materials and methods

Fly strains

Oregon-R was used as the wild-type strain. The following GAL4 enhancer-trap lines were identified in the NP collection (Hayashi et al., 2002): *NP3012 and NP3123* (all midgut lines; on X), *NP3207 and NP3612* (interstitial cell lines; on X), and *NP3270 and NP3157* (copper cell lines; on II). These NP lines have a *yellow white* background. The following GAL4/UAS lines were used: *tsh-GAL4* (Shiga et al., 1996), *UAS-d.n.N* (Go et al., 1998), *UAS-dTCF*^{DN} (Cadigan et al., 1998), *UAS-dad* (Tsuneizumi et al., 1997), *UAS-NLS-lacZ (NZ20b23)* (Takamatsu et al., 2002), and *UAS-GFP.S65T* (Bloomington Stock Center). *Su(H)bs-lacZ* (Go et al., 1998), *E(spl)m8-lacZ* (Kramatschek and Campos-Ortega, 1994), *Dl*^{P1651}, and *Dl*^{bl5} (Mishra et al., 2001) were used as markers for Notch signaling. *lab-lacZ* (p6.0 *lab* 66A) was used as a copper cell marker (Chouinard and Kaufman, 1991). The interstitial cell marker *C5-2-7* and the enhancer-trap allele *dve*^{1} (*dve*^{1}*-lacZ*) were previously described (Nakagoshi et al., 1998).

Temperature-shift experiments

Temperature-sensitive alleles *wg*^{IL114} (Bejsovec and Martinez-Arias, 1991) and *N*^{ts1} (Shellenbarger and Mohler, 1975) were used to temporally inactivate these signaling pathways. Eggs collected during 4 h at the permissive temperature 18 °C (2 h at 25 °C) were shifted to the restrictive temperature 30 °C at various time points (after egg laying; AEL). Homozygous *wg*^{IL114} and transheterozygous *N*^{55e11}/*N*^{ts1} larvae were identified with a *yellow* marker and by the absence of *FM7actGFP* balancer chromosome, respectively. To examine the effect on *dve*^{1}*-lacZ* expression, the null allele *wg*^{CX4} was recombined with a *dve*^{1} chromosome. Eggs of transheterozygous *wg*^{IL114}/*wg*^{CX4} *dve*^{1} and *N*^{ts1}/Y; *dve*^{1}/+ were shifted to the restrictive temperature after 14 and 12 h AEL, respectively.

Immunohistochemistry

Embryos were stained with anti-β-gal monoclonal antibodies (1:100, Promega) and anti-GFP polyclonal antibodies (1:20, Molecular Probes) together with Biotin-, FITC-, or Cy3-conjugated secondary antibodies. The peroxidase reaction and immunofluorescence were performed as described (Nakagoshi et al., 1998). Confocal images were obtained with an OLYMPUS FluoView 300.

Copper cell morphology

Larvae were dissected in 1% glutaraldehyde/phosphate-buffered saline (PBS), washed with PBS several times, and then stained with 0.1% 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) for β-galactosidase activity. The guts were mounted in 80% glycerol and then the number of cells exhibiting typical features of an invaginated apical membrane (banana shape) was determined for the posterior-most 20 cells. The percentage of banana-shaped copper cells was scored in the control progeny (GAL4 line × Oregon-R) and test progeny (GAL4 line × UAS line). In the control progeny, the percentage of banana-shaped copper cells was >90%, which was taken to be normal (%). Normality was determined as follows: [normal (%) for test progeny]/[normal (%) for control progeny].

Acid secretion

Yeast paste containing 0.25% bromophenol blue (BPB) was fed to hatched larvae for >5 h (at 25 °C) or >15 h (at 18 °C). The larvae were then dissected in PBS, and the color of the gut lumen was immediately observed (see Supplementary Fig. 1). BPB turns yellow (pH < 2.35), green (2.35 < pH < 4), or blue (pH > 4) (Dubreuil et al., 1998). In the control progeny, the percentage of a yellow gut lumen was >80%, which was taken to be normal (%). Normality was determined as follows: [normal (%) for test progeny]/[normal (%) for control progeny].

Copper fluorescence

Yeast paste containing 0.25% BPB and 10 mM $CuSO_4$ was fed to hatched larvae for >5 h (at 25 °C) or >15 h (at 18 °C). The guts of dissected larvae were immediately mounted in PBS and excited with UV light using a DAPI filter (see Supplementary Fig. 1). The fluorescence was divided into three classes: strong fluorescence (normal), weak or dispersed fluorescence (weak), and no fluorescence (absent). In the control progeny, the percentage of strong fluorescence was >70%, which was taken to be normal (%). Normality was determined as follows: [normal (%) for test progeny]/[normal (%) for control progeny].

Statistical analyses

The significance of differences between the control and test progenies in gut function was analyzed with Mann–Whitney *U*-tests using Kaleidagraph