Int. J. Dev. Biol. 55: 945-952
doi: 10.1387/ijdb.113343su

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The *drumstick* gene acts cell-non-autonomously and triggers specification of the small intestine in the *Drosophila* hindgut

SARDER N. UDDIN[1,2], MASAHIRO YANO[1] and RYUTARO MURAKAMI*[,1]

[1]*Department of Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, Japan and* [2]*Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh*

ABSTRACT An odd family gene *drumstick (drm)* encodes a zinc finger protein, and is necessary for the development of the small intestine, an anterior domain of the ectodermal hindgut of *Drosophila melanogaster*. However, mechanisms that specify the small intestine, as well as gene regulatory pathways leading to transcriptional activation of *drm*, are still unclear. We found that *drm* is expressed in two different tissues abutting the anterior end of the hindgut primordium, that is, the posterior-most region of the midgut (endoderm) and basal portion of the Malpighian tubules. A small intestine marker gene, *unpaired (upd)*, begins to be expressed at the anterior-most region of the hindgut primordium that abuts the basal portion of Malpighian tubules, and the *upd*-positive region expands, resulting in a short tube during stages 11-13. The small intestine develops in both of the mutant embryos, *serpent (srp)* and *Krüppel (Kr)*, that lack the *drm*-positive midgut or Malpighian tubules, respectively, while it fails to develop in the *Kr srp* double-mutant embryos that lack both of the *drm*-positive tissues. These results demonstrate that *drm* expressed in the abutting tissues cell-non-autonomously induces development of the small intestine in the hindgut primordium, probably by deploying some extracellular signaling factor. *drm* expression in the posterior gut region disappears and the small intestine fails to form in *tailless (tll)* mutant embryos, whereas over-expression of *tll* causes expansion of *drm* expression throughout the midgut, inducing a longer small intestine. These results indicate that *drm* is activated under the control of *tll* and triggers development of the small intestine cell-non-autonomously through some extracellular signaling.

KEY WORDS: *Drosophila, drumstick, unpaired, cell-non-autonomous, small intestine*

Introduction

The digestive tract is an evolutionarily conserved and structurally simple organ composed of an epithelial tube surrounded by visceral muscles of mesodermal origin. The *Drosophila* gut consists of three major parts: ectodermal foregut, endodermal midgut and ectodermal hindgut. Each of these gut parts are subsequently subdivided into a number of distinct domains characterized by specific gene expression pattern (Murakami *et al*., 1994). These features make the *Drosophila* gut an attractive organ for the study of regional differentiation (Skaer, 1993; Hoch and Pankratz, 1996; Lengyel and Liu, 1998; Murakami *et al*., 1999; Lengyel and Iwaki, 2002). All the gut epithelia originate from anterior and posterior terminal domains of the cellular blastoderm, which form a continuous tube after invagination from both terminals. Fate decision of the both terminal domains is controlled by a gene regulatory system, the terminal system (reviewed in Nüsslein-Volhard *et al*., 1987; Skaer, 1993; Murakami *et al*., 1999). Initially, graded concentrations or activities of maternal morphogens are established, with maximum peaks at both anterior and posterior termini of the egg (Greenwood and Struhl, 1997; Martin *et al*., 1994; Savant-Bhonsale and Montell, 1993; Sprenger and Nüsslein-Volhard, 1992), which leads to activation of the two earliest zygotic genes *tailless* (*tll*) and *huckebein* (*hkb*) in a nested pattern (Brönner and Jäckle, 1991; Pignoni *et al*., 1990). *brachyenteron* (*byn)*, a *brachyury* ortholog, is essential for specifying hindgut primordium (Kispert *et al*., 1994; Murakami *et al*., 1995; Singer *et al*., 1996). In the posterior terminal region of the cellular blastoderm, *byn* is activated by *tll,* and repressed by a *hkb* target gene *srp*, thus being restricted to a region spanning 10-15%

Abbreviations used in this paper: ap, anal pads; arm, armadillo; bowl, brother of odd with entrails limited; byn, brachyenteron; ct, cut; drm, drumstick; EL, egg length; hg, hindgut/hindgut primordium; hkb, huckebein; knrl, knirps-related; Kr, Krüppel; li, large intestine; lin, lines; mg, midgut/ midgut primordium; mt, Malpighian tubules; otp, orthopedia; rec, rectum; si, small intestine; srp, serpent; st, stage; tll, tailless, upd, unpaired; wg, wingless; WT, wild-type.

***Address correspondence to:** Ryutaro Murakami. Department of Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, Yamaguchi 753-8512, Japan. Tel/Fax: +81.83.933.5696. e-mail: ryu@yamaguchi-u.ac.jp

Accepted: 4 August 2011. *Final, author-corrected PDF published online:* 2 January 2012. *Edited by:* Makoto Asashima

ISSN: Online 1696-3547, Print 0214-6282

egg length (EL) of the early cellular blastoderm, and the *byn*-positive region eventually forms the hindgut and anal pads (Reuter and Leptin, 1994; Rehorn *et al.*, 1996; Murakami *et al.*, 1999; Lengyel and Iwaki, 2002). During embryonic development, the hindgut primordium is subdivided into several domains characterized by specific gene expression and ultrastructure (Hoch and Pankratz, 1996; Pankratz and Hoch, 1995; Murakami and Shiotsuki, 2001; Takashima and Murakami, 2001). A major anterior domain is the small intestine, then, the large intestine follows. The large intestine is further subdivided into a dorsal and a ventral domains that are demarcated by a one-cell-wide boundary cell domain. The rectum domain arises posterior to the large intestine, and the anal pads form the posterior orifice of the gut tube. There are several studies on mechanisms of the subdivision of hindgut primordium: Wg signal emanated from the anal pads induces the rectum (Takashima and Murakami, 2001); boundary cells are specified by Notch signaling activated at the interface between the dorsal and ventral domains of the large intestine (Takashima *et al.*, 2002; Iwaki *et al.*, 2001; Fuβ and Hoch, 2002). These results imply importance of cell-to-cell interactions mediated by extracellular signal molecules in the process of subdividing hindgut into several domains. The small intestine has a couple of features similar to the rectum both in the ultrastructure and gene expression pattern: apical surface of the epithelium shows no trait of absorptive function, being covered by a thick cuticular layer; the epithelial tube is surrounded by a well-developed sphincter muscles (Murakami and Shiotsuki, 2001); both the small intestine and rectum express *hedgehog (hh)* and *knirps-related (knrl)* (Hoch and Pankratz, 1996; Fuβ *et al.*, 2001; Takashima and Murakami, 2001; Iwaki *et al.*, 2001; Lengyel and Iwaki, 2002; Green *et al.*, 2002; Johansen *et al.*, 2003). However, while the rectum is induced by Wg signaling, the small intestine is independent of Wg (Takashima and Murakami, 2001). Odd-family genes, *drumstick (drm)* and *bowl*, as well as Wnt-pathway related gene *lines (lin),* are involved in the process of fate decision of the small intestine (Iwaki *et al.*, 2001; Lengyel and Iwaki, 2002; Green *et al.*, 2002; Johansen *et al.*, 2003; Hatini *et al.*, 2005). *drm* is essential for the development of small intestine: *drm* mutant embryos fail to develop small intestine, while over-expression of

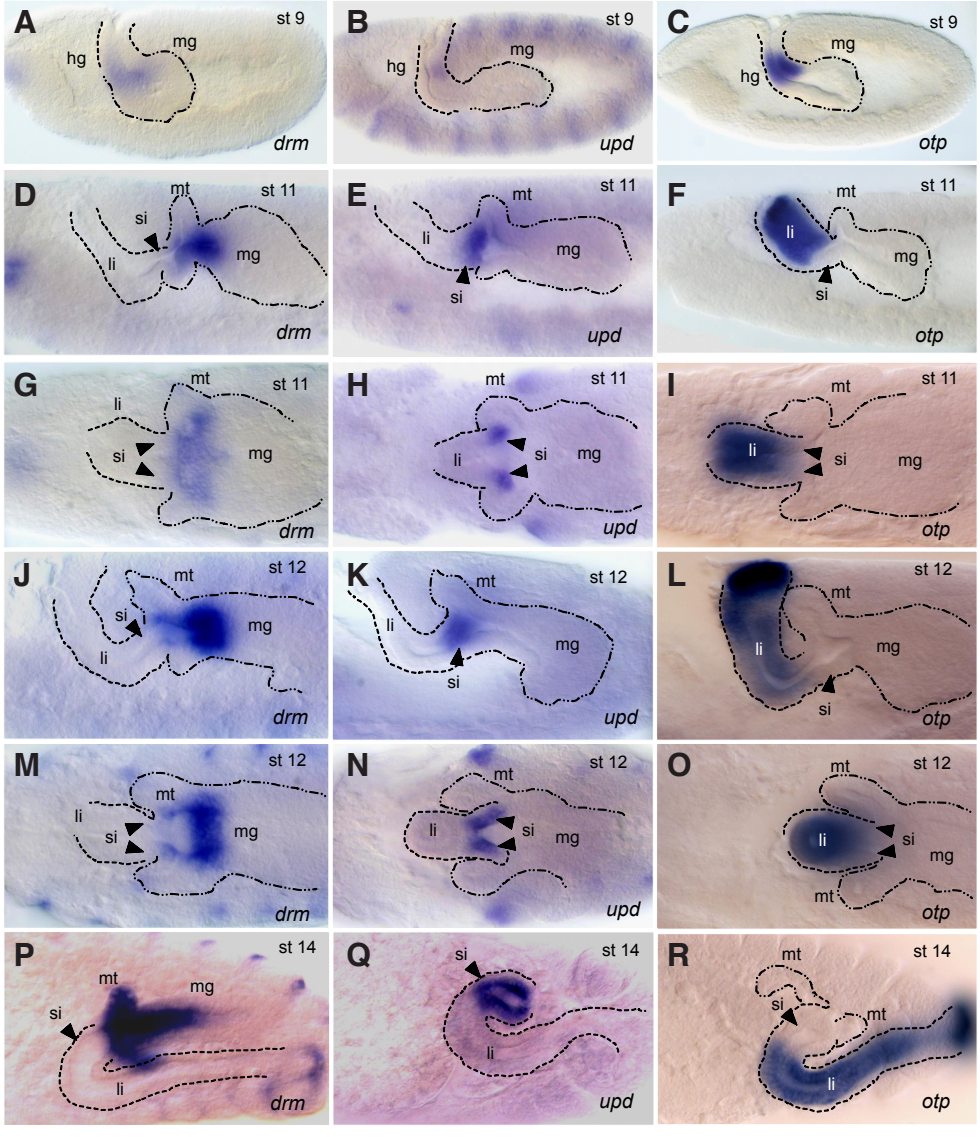

Fig. 1. Expression patterns of *drm, upd* and *otp* mRNAs during development of the small intestine in wild-type embryos. *In all panels, anterior of the embryos is to the left. Embryonic stages and detected mRNAs are indicated at the top right and bottom right of the panels, respectively.* **(A-C)** *Stage 9 embryos, lateral views:* **(A)** *Weak and diffuse* drm *signal in the hindgut (hg) and midgut primordium (mg) is detected.,* **(B)** *No* upd *signal appears yet.,* **(C)** otp *begins to be expressed in the hindgut primordium.* **(D-I)** *Stage 11 embryos:* **(D-F)** *Lateral views, and* **(G-I)** *dorsal views.,* **(D,G)** drm *expression becomes distinct in the posterior-most region of midgut and basal portion of the Malpighian tubules, not in the small intestine domain (si, arrowheads).,* **(E,H)** upd *is expressed as bilateral spots representing developing small intestine domain (arrowheads).,* **(F,I)** otp *signal is disappearing in the small intestine (arrowheads).* **(J-O)** *Stage 12 embryos:* **(J-L)** *Lateral views and* **(M-O)** *dorsal views.,* **(J,M)** drm *expression becomes restricted to the posterior-most region of midgut and basal portion of the Malpighian tubules.,* **(K,N)** upd *positive region expanded posteriorly, partially becoming tubular.,* **(L,O)** *The small intestine (arrowheads) is negative for* otp *signal.* **(P-R)** *Stage 14 embryos, lateral views:* **(P)** drm *is exclusively expressed in the posterior-most region of midgut and basal portion of the Malpighian tubules.,* **(Q)** upd *is expressed exclusively in the small intestine.,* **(R)** otp *is expressed in the large intestine. Abbreviations: hg, hindgut/hindgut primordium; li, large intestine; mg, midgut/midgut primordium; mt, Malpighian tubules; si, small intestine.*

drm transforms most of the hindgut primordium into small intestine. However, both upstream and downstream regulatory pathway of *drm* is still unclear. In the present study, we show that *drm* is activated under the control of *tll*, and, acts cell-non-autonomously in the process of specifying small intestine, probably deploying some extracellular signaling factor.

Results

drumstick (drm) is expressed in the posterior-most region of the midgut and basal portion of the Malpighian tubules, but not in the small intestine during late stages

Weak and rather diffuse signal of *drm* is detectable in the invaginating proctodeum as early as stage 7. During stage 9 and 10, *drm* expression is still weak and diffuse, and the positive region apparently includes the future hindgut and posterior-most region of the midgut (endoderm) (Fig. 1A). *unpaired* (*upd)*, a marker gene of the small intestine, is not expressed in the hindgut region at these stages (Fig. 1B). On the other hand *orthopedia* (*otp)*, a homeobox gene specifically expressed in the large intestine, is first expressed in the hindgut primordium at stage 9 (Fig. 1C). At stage 11, the expression pattern of *drm* becomes distinct, with *drm*-positive area being restricted to the posterior-most region of the midgut and basal portion of the buds of Malpighian tubules (Figs. 1 D,G). When viewed dorsally, *upd* signal first appears at this stage as bilateral spots abutting the buds of Malpighian tubules (Fig. 1H). Concomitantly, *otp* signal in the anterior-most region of the hindgut primordium becomes very weak (arrowheads in Figs. 1 F,I). During stage 12, the *upd*-positive region expands posteriorly and medially, becoming tubular (Figs. 1 K,N), and border between the *upd*-positive small intestine and *otp*-positive large intestine becomes clear (Figs. 1 K,L,N,O). During late stage 12 and 13, the *upd*-positive domain eventually forms a complete tube. At stage 14, all the domains in and around the small intestine can be recognizable by specific gene expression: *drm* for the posterior-most region of midgut and basal portion of the Malpighian tubules (Fig. 1P); *upd* for the small intestine (Fig. 1Q); *otp* for the large intestine (Fig. 1R). These observations indicate that *drm* is expressed in tissues abutting anterior end of the hindgut primordium after stage 11.

drumstick mutant embryos fail to form the small intestine while adjacent tissues remain largely intact

Above results suggest that *drm* works cell-non-autonomously in the development of small intestine. We examined effects of *drm* mutation on the small intestine and abutting tissues by use of region-specific probes to know what tissues are affected by the *drm* mutation.

knrl and *upd* have been used as marker genes of the small intestine (Figs. 2 A,C) (Harrison *et al*., 1998; Fuβ *et al*., 2001; Iwaki *et al*., 2001; Green *et al*., 2002; Johansen *et al*., 2003). In *drm* mutant embryos, hindgut is short, and there is no expression of *knrl* and *upd* in the anterior region of the hindgut (asterisks in Figs. 2 B,D), being consistent with the previous reports (Green *et al*., 2002; Johansen *et al*., 2003). To examine effects of *drm* mutation on the tissues abutting the small intestine, *in situ* hybridization was carried out using the following probes: *GATAe* probe for the endoderm/Malpighian tubules; *otp* probe for the large intestine; *cut* probe for the Malpighian tubules. In wild-type embryos, small intestine is clearly recognizable as a short tube negative for *GATAe*

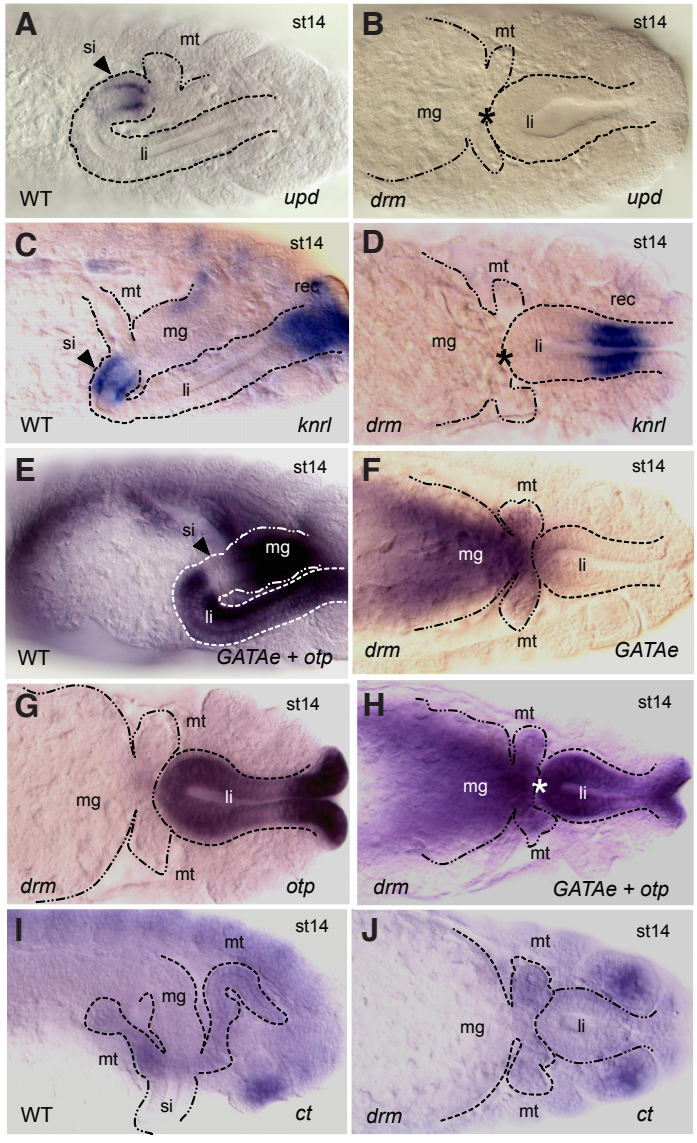

Fig. 2. *drm* mutant embryos fail to form small intestine while adjacent tissues remain largely intact. *In all panels, anterior of the embryos is to the left. Embryonic stages and detected mRNAs are indicated at the top right and bottom right of the panels, respectively, and mutant names are at the bottom left.* **(A,C,E,I)** *Wild-type embryos, and* **(B,D,F,G,H,J)** drm *mutant embryos:* **(A)** upd *is expressed in the small intestine of the wild-type embryo.* **(B)** upd *expression disappears (asterisk) in the* drm *mutant embryo.* **(C)** knrl *is expressed in the small intestine and rectum in the wild-type embryo.* **(D)** *In* drm *mutant, the small intestine does not form (asterisk), and* knrl *expression remains only in the rectum.* **(E)** GATAe-otp *double-staining of the wild-type embryo. The small intestine is recognized as a domain negative for both signals (arrowhead).* **(F)** *In* drm *mutant,* GATAe *is expressed in the midgut and the buds of Malpighian tubules.* **(G)** *In* drm *mutant,* otp *is normally expressed in the large intestine.* **(H)** GATAe-otp *double-staining of the* drm *mutant embryo. There is no gap between* GATAe *and* otp *expression domain, indicating that the small intestine is completely missing (asterisk).* **(I)** *Expression of* cut *(*ct*) in wild-type embryo. Malpighian tubules are positive for* ct. **(J)** *In the* drm *mutant embryo,* ct *is still expressed weakly in the Malpighian tubules. Abbreviations: li, large intestine; mg, midgut; mt, Malpighian tubules; rec, rectum; si, small intestine.*

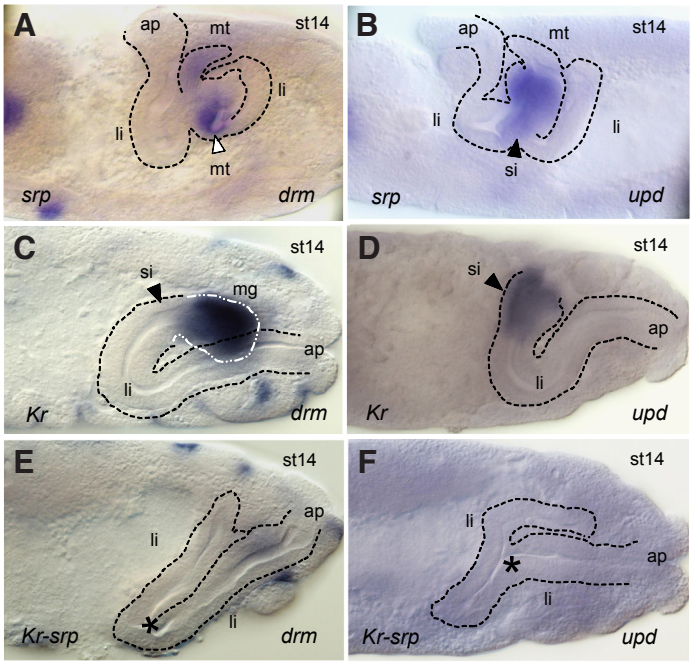

Fig. 3. Development of the small intestine in the mutants that lack *drm*-positive tissues. *In all panels, anterior of the embryos is to the left. Mutant names are indicated at the bottom left of the panels. Embryonic stages and detected mRNAs are at the top right and bottom right, respectively.* **(A,B)** srp *mutant embryos, in which prospective midgut transforms into large intestine.* **(A)** drm *is expressed in rudimentary Malpighian tubules (mt, arrowhead) between innate and supernumerary large intestine.* **(B)** upd*-positive small intestine tissue appears (arrow head), being intermingled with rudimentary Malpighian tubules.* **(C,D)** Kr *mutant embryos, in which Malpighian tubules do not develop.* **(C)** drm *is expressed in the posterior-most region of the midgut.* **(D)** upd*-positive small intestine develops.* **(E,F)** Kr srp *double-mutant embryos. Asterisks indicate approximate position of the boundary between innate and supernumerary hindgut.* **(E)** drm *expression disappears in the* Kr srp *double-mutant embryo.* **(F)** upd *expression also disappears in the* Kr srp *double-mutant embryo, indicating that the small intestine does not develop. Abbreviations: ap, anal pads; li, large intestine; mg, midgut; mt, Malpighian tubules; si, small intestine.*

and *otp* probes (Fig. 2E). In *drm* mutant embryos, expression of both marker genes remains largely intact (Figs. 2 F,G). It should be noted that buds of the Malpighian tubules form and express *GATAe* in the *drm* embryos (Fig. 2F). However, small intestine that is negative for both marker genes does not form, resulting in a continuous staining region positive for *GATAe-otp* mixed probes at the midgut-hindgut junction (asterisk in Fig. 1H). These results demonstrate that in *drm* mutant embryos, small intestine does not form, and also suggest that adjacent tissues that express *drm* in normal embryos remain largely intact. Actually, buds of Malpighian tubules express *cut (ct)*, a marker genes of the tissue, although the signals are weaker than those in wild-type embryos (Figs. 2 I,J). Regarding the posterior-most domain of the midgut, its macroscopic morphology in later embryonic stages looks slightly abnormal, with rather short convoluted portion in the posterior-most region, suggesting *drm* mutation affects development of this region. Although marker genes specific for this region in differentiated midgut are not available, *wg* is known to be expressed in the midgut-hindgut junctional region (mainly on the midgut side). We found that *wg* expression in this region disappears in *drm* mutant embryos (data not shown). This result implies a possibility that *drm* activity is mediated by Wg signaling, which we consider afterward (see Fig. 4).

Development of the small intestine in mutations that affect drumstick-positive adjacent tissues

Above results strongly suggest that *drm* induces small intestine cell-non-autonomously. *drm* is expressed in two different tissues, the posterior-most region of midgut and basal portion of the Malpighian tubules. Either one or both of the two *drm*-positive tissues are thought to be responsible for inducing small intestine. To address this issue, we examined mutants that fail to form each of the *drm*-positive tissues, the midgut epithelium and Malpighian tubules. *srp* is a key gene that specifies developmental fate of the midgut (endoderm). In *srp* mutant embryos, prospective anterior and posterior midgut domains transform into a portion of the ectodermal foregut and hindgut, respectively. Thus, prospective posterior midgut in *srp* embryos transforms into a supernumerary hindgut (mostly large intestine) (Abel *et al.*, 1993; Rehorn *et al.*, 1996; Reuter, 1994). In *srp* mutant embryos, *drm* expression is observed in a deformed epithelial tissue mass between the innate and supernumerary large intestine (Fig. 3A), which is assumed to represent rudimental Malpighian tubules. In the same region, strong *upd* expression is also detected (Fig. 3B), which is thought to represent rudimental tissues of the small intestine intermingled with rudimental Malpighian tubules. These results demonstrate that the

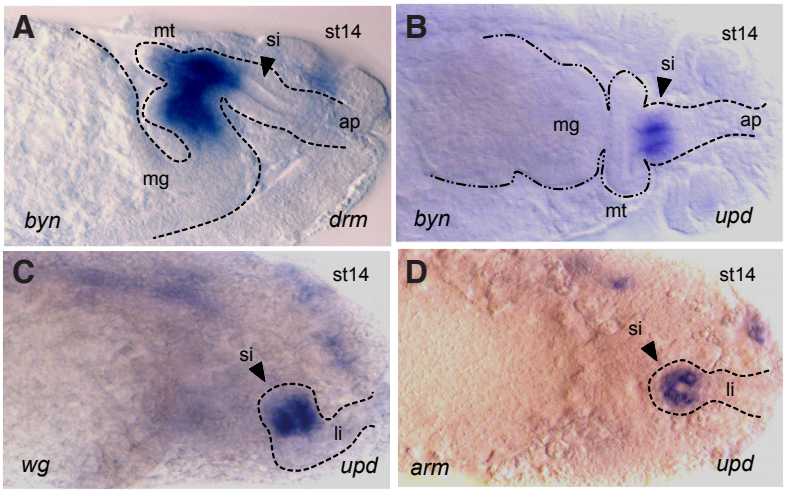

Fig. 4. *byn* and Wg signaling is not necessary for the development of small intestine. *In all panels, anterior of the embryos is to the left. Mutant names are indicated at the bottom left of the panels. Embryonic stages and detected mRNAs are indicated at the top right and bottom right, respectively.* **(A,B)** byn *mutant embryos.* **(A)** drm *is expressed in the posterior-most region of midgut and Malpighinan tubules in the* byn *mutant embryo.* **(B)** upd*-positive small intestine also developed (arrow head).* **(C)** wg *mutant embryo. The small intestine is recognized as* upd*-positive domain.* **(D)** arm *mutant embryo. The small intestine is recognized as* upd*-positive domain. Abbreviations: ap, anal pads; li, large intestine; mg, midgut; mt, Malpighian tubules; si, small intestine.*

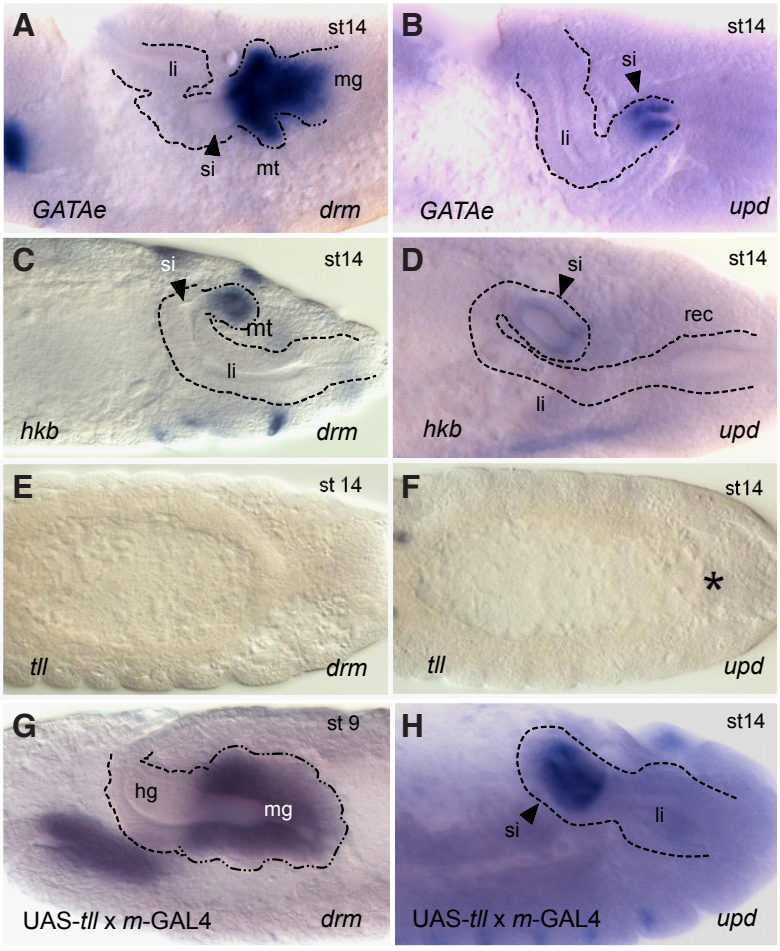

Fig. 5. *Tailless (tll)* is necessary for the *drm* expression. *In all panels, anterior of the embryos is to the left. Mutant names are indicated at the bottom left of the panels. Embryonic stages and detected mRNAs are indicated at the top right and bottom, respectively.* **(A,B)** GATAe *deficient embryos.* **(A)** drm *is expressed normally at the posterior-most region of midgut and Malpighian tubules.* **(B)** upd*-positive small intestine develops normally (arrowhead).* **(C,D)** hkb *mutant embryos.* **(C)** drm *is expressed in basal portion of the Malpighian tubules.* **(D)** upd*-positive small intestine develops normally (arrowhead).* **(E,F)** tll *mutant embryos.* **(E)** drm *expression is missing in the* tll *mutant embryo.* **(F)** *Expression of* upd *is abolished in the* tll *mutant embryo (asterisks).* **(G,H)** *Over-expression of* tll *with* maternal-*GAL4,* **(G)** drm *expression is ectopically induced throughout the midgut.* **(H)** *Expression domain of* upd *markedly expands (compare with that in Fig. 2A). Abbreviations: li, large intestine; mg, midgut; mt, Malpighian tubules; rec, rectum; si, small intestine.*

small intestine can develop in the absence of the midgut. We next examined *Krüppel (Kr)* mutant embryos, in which prospective region of the Malpighian tubules develops as a portion of the hindgut (Harbecke and Janning, 1989; Liu and Jack, 1992; Redemann *et al.*, 1988; Skaer, 1993). In *Kr* embryos, *drm* was found to be expressed in the posterior-most region of the midgut (Fig. 3C), and, *upd* expression also remains in the anterior-most portion of the hindgut (Fig. 3D). These results indicate that the small intestine can develop in the absence of either one of the two *drm*-positive tissues, the midgut or Malpighian tubules. In other words, either one of the *drm*-positive tissues may be sufficient for inducing small intestine. Thus, we generate *Kr srp* double-mutant embryos to eliminate both *drm*-positive tissues. In the double-mutant embryos, *drm* expression in the posterior gut tissues is completely abolished (Fig. 3E), and, *upd* expression is not detected (Fig. 3F). These results are summarized in Table 1, and they unequivocally demonstrate that it is the *drm*-positive tissues abutting hindgut primordium that induces small intestine, and that the action of *drm* is cell-non-autonomous.

The small intestine is specified independently of brachyenteron (byn) and wingless (wg) signaling

byn is a T-box gene orthologous to the vertebrate *brachyury* gene, and it has been recognized as a master gene in the hindgut development. In *byn* mutant embryos, hindgut does not develop, or, only a very short epithelial tube remains (Kispert *et al.*, 1994; Murakami *et al.*, 1995; Singer *et al.*, 1996). We found that the strongest *byn* allele (byn^{apro}) does not affect *drm* expression in the adjacent midgut and Malpighian tubules (Fig. 4A), and, unexpectedly, residual short epithelial tube expresses the small intestine marker *upd* (Fig. 4B) and *knrl* (data not shown). We also confirmed that a large intestine marker *otp* is abolished completely in *byn* embryos as was reported previously (data not shown). These results demonstrate that the small intestine can develop in the absence of *byn* activity, and it does not need adjacent large intestine. Next, we tried to identify extracellular signaling that mediates *drm* activity. Various Wnt family genes including *wingless* (*wg)* are expressed at the junctional region between midgut and hindgut, and we found that *wg* expression in the midgut-hindgut junction disappears in *drm* mutants (data not shown). However, the small intestine develops in *wg* mutant embryos (Fig. 4C). The small intestine also develops in *armadillo* (*arm*) mutant embryos (Fig. 4D). Moreover, forced-expression of active form of *arm* (*armS10*) does not affect development of the small intestine while entire large intestine transforms into rectum (data not shown). Thus, extracellular signaling factor that is supposed to mediate *drm* activity remains unknown. We then examined candidates of upstream genes that regulate *drm* expression.

TABLE 1

SUMMARY OF THE GUT PHENOTYPES OF THE MUTATIONS: *SRP*, *KR*, *KR SRP*, *DRM*

Mutation	Tissue formation	*drm* expression in each tissue
WT	Mg	+
	mt	+
	si	
srp	*no* mg	-
	mt	+
	si	
Kr	mg	+
	no mt	-
	si	
Kr srp	*no* mg	-
	no mt	-
	no **si**	
drm	mg (affected?)	-
	mt	-
	no **si**	

Abbreviations: mg, midgut; mt, Malpighian tubules, si, small intestine

drumstick is activated under the control of tailless (tll)

drm-positive midgut region, as well as the ectodermal small intestine, arises from a posterior region of the cellular blastoderm. In order to find gene regulatory pathway leading to the *drm* expression in posterior gut tube, we examined mutants of the genes that are expressed in posterior terminal region of the cellular blastoderm stage. *GATAe* is a GATA factor gene that is required for activating a large part of the genes expressed in the differentiated midgut epithelium (Murakami *et al.*, 2005; Okumura *et al.*, 2005). Embryos deficient for *GATAe*, however, were found to express *drm* in the posterior-most region of the midgut and in the basal portion of the Malpighian tubules (Fig. 5A), and the small intestine develops normally, as is proven with the *upd* expression (Fig. 5B). Mutation of a gap gene *hkb*, one of the earliest zygotic genes activated in the future endodermal region, was also examined. *drm* expression remains in the anterior end of the proctodeal invagination which corresponds to the region of Malpighian tubules (Fig. 5C), and the small intestine develops normally (Fig. 5D). *tll* is another gap gene expressed in the posterior blastodermal region, and its mutation is known to affects wide blastodermal region including future hindgut, Malpighian tubules as well as a posterior portion of the midgut (Brönner and Jäckle, 1991; Pignoni *et al.*, 1990). In *tll* mutant embryos, *drm* expression is not detected in any stages in the posterior gut region (Fig. 5E). At the same time, *upd* expression is not detected in the posterior gut region (Fig. 5F). The disappearance of *drm* expression in the posterior gut region may be indirect outcome of the drastic *tll* phenotype, since most of the posterior gut regions fail to form in *tll* mutants (Skaer, 1993). However, when *tll* is over-expressed by use of *maternal*-GAL4 (rom) driver, *drm* is ectopically induced throughout the endoderm (Fig. 5G), and, at the same time, the small intestine becomes larger (Fig. 5H). These results strongly suggest some essential role of *tll* in the activation of *drm* as well as in the induction of small intestine.

Discussion

drumstick is required cell-non-autonomously for specifying small intestine

An odd family gene *drm* is essential for the development of small intestine of the *Drosophila* ectodermal hindgut. *drm* mutant exhibits very short hindgut, and fails to form the small intestine, an anterior domain of the ectodermal hindgut. In contrast, the small intestine domain expands when *drm* is forced-expressed throughout the hindgut (Iwaki *et al.*, 2001; Lengyel and Iwaki, 2002; Green *et al.*, 2002; Johansen *et al.*, 2003). From extensive genetic analyses in their studies, Johansen *et al.,* (2003) proposed a gene regulatory model for the specification of the small intestine. In their model, *drm* that is expressed in the anterior of the hindgut primordium induces small intestine by suppressing the activity of widely-expressed transcription factor Lin. However, as is described in the RESULTS, we found that distinct *drm* expression was observed exclusively in the posterior-most region of the midgut and basal portion of the Malpighian tubules from stage 11 onward, but, not in the hindgut primordium itself. *Kr* mutant embryos lack the Malpighian tubules while the midgut remains intact. On the other hand, *srp* embryos lack the midgut while Malpighian tubules remain intact. Both of the single mutations do not affect initial development of the small intestine. However, *Kr srp* double-mutant embryos, which lack both of the *drm*-positive tissues, fail to form the small intestine. These results unequivocally demonstrate that *drm* works in the posterior-most region of the midgut and basal portion of the Malpighian tubules, and induces the small intestine in the adjacent hindgut primordium cell-non-autonomously. In this process, Drm is assumed to activate some extracellular signaling factor, and the latter acts on the hindgut primordium and triggers developmental program of the small intestine. Canonical Wnt signaling is not essential for this process, since *arm* mutant, which lacks common transcription factor of the Wnt signaling pathway, does not affect initial development of the small intestine. Furthermore, when constitutive active form of *arm* (*armS10*) was forced-expressed throughout the hindgut primordium, no marked change was observed in the small intestine (data not shown). Thus, signaling factor deployed in the specification of small intestine remains still unknown.

The *drm*-positive region of the midgut corresponds to the domain m13 that was recognized by its characteristic gene expression pattern in larva (Murakami *et al.*, 1994). This region, as is the case of other posterior midgut regions, gives rise to a reabsorptive tissue in larva (Bodensein, 1950). It remains to be elucidated whether this region possesses characteristic cytological features. In embryos, the region, together with the Malpighian tubules, may act as a signaling center that affects adjacent hindgut primordium under the control of Drm. In addition to the induction of small intestine, the *drm*-positive regions may possibly play some role in the differentiation of the posterior midgut, since our preliminary study revealed that *drm* mutation abolishes expression of a marker gene specific to a posterior region of the midgut.

upd-positive small intestine cells first appeared at stage 11 as bilateral spots abutting the buds of Malpighian tubules, and soon formed short tube during stage 12-13. There have been reports that prospective region of the small intestine stops cell division and DNA replication around stage 11 (Iwaki *et al.*, 2001; Fuβ *et al.*, 2001). In fact, approximate numbers of *upd*-positive cells were 16 per one side at stage 11, and increased only to 20-22 at stage 13-14 (data not shown). Thus, morphological change of the small intestine during stage 11-13 may be brought about mainly by cell-rearrangement and cell-shape changes of the *upd*-positive cells.

Upstream regulatory pathway of drumstick activation in the posterior gut region

In order to elucidate upstream gene regulatory pathway of the *drm* expression, we examined several mutants that show defects in the posterior gut region. Among the mutants investigated, *tll* was the only mutation that abolished *drm* expression. *tll* is one of the earliest zygotic genes that are expressed in both anterior and posterior terminal regions of the cellular blastoderm (Brönner and Jäckle, 1991; Pignoni *et al.*, 1990). Tissues including posterior gut regions around midgut-hindgut junction are impaired in *tll* mutant embryos. Thus, the above result that *drm* expression in the posterior gut region disappeared in *tll* mutant embryos does not necessarily mean that *drm* is activated under the control of *tll*. Rather, it could be an indirect effect resulting from strong defects caused in the prospective posterior gut region during early stages of development. However, forced-expression of *tll* throughout the embryo was found to cause ectopic expression of *drm* throughout the developing midgut, which strongly suggests that *drm* is activated under the control of *tll*. In normal development,

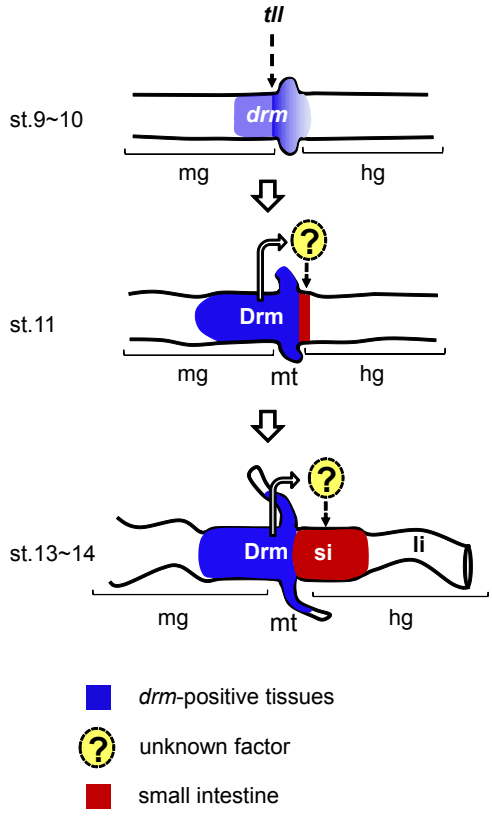

Fig. 6. A model of gene regulatory pathway for specification of the small intestine. drm *is activated under the control of* tll, *being expressed in the posterior-most region of midgut as well as in the basal portion of Malpighian tubules. Subsequently, Drm protein activates unknown extracellular signaling factor and the latter acts on the hindgut primordium and triggers specification of the small intestine at stage 11. Abbreviations: hg, hindgut; li, large intestine; mg, midgut; mt, Malpighian tubules; si, small intestine. The color codes are indicated at the figure bottom.*

drm expression is restricted only to the posterior-most region of midgut and basal portion of the Malpighian tubules from stage 11 onward. Some unknown factors, in conjunction with *tll*, may be involved in defining the spatial expression pattern of *drm* in these regions. A model of gene regulatory pathway leading to the specification of small intestine, which is deduced from the present and previous studies, is presented in Fig. 6.

Signaling pathway of the development of small intestine is still puzzling

In the present study, we revealed a few essential steps in the pathway of small intestine development. However, mechanisms of the specification of small intestine are still rather ambiguous and puzzling. In the model of Johansen *et al.,* (2003), *drm* suppresses activity of the ubiquitously-expressed transcription factor Lin, and, Lin suppresses activity of another odd-family protein Bowl in the absence of *drm* activity. Thus, *drm* activity eventually leads to the activation of Bowl, and the latter is essential for determining tissue identity of the small intestine. However, there is no direct evidence that demonstrates specific roles of Bowl in this process. All these circumstantial knowledges suggest that there may be some unknown factor that defines identity of the small intestine. Thus, most important issues to be solved is: extracellular signaling factor generated under the control of *drm*, and, gene that determines tissue identity of the small intestine.

Materials and Methods

Fly strains

The following fly stocks are used: Oregon R as wild type for the analyzing normal development, *wgPY40* (hypomorphic mutant, Murakami *et al*., 1994); *byn*^{apro} (strongest allele, Murakami *et al*., 1995); *srp*^{2} (amorphic allele; Reuter, 1994); *hkb*^{A} (null allele); *Df(3R)sbd45* (deletion of *GATAe* locus); *arm4m* (null mutant of *arm*); *Df(2L)drmP1* (chromosomal deletion of *drm* locus); *Kr*^{2} (amorphic mutant); *tll*^{9}(null allele); *Kr*^{2} *srp* double mutant was generated by conventional crossings.

The following GAL4 and UAS strains were used for misexpression experiments: *byn*-GAL4 (for misexpression in the hindgut primordium); 48Y-GAL4 (for misexpression in the midgut); maternal-GAL4 (ubiquitous); UAS-*tll;* UAS-*wg;* UAS-*drm;* UAS-*armS10*. All these strains are provided by DGRC, Kyoto Stock Center and Bloomington Stock Center, if not mentioned specifically. The flies were raised at 24°C for collection of the embryos.

In situ hybridization

The following cDNAs or EST clones were used as template to synthesis DIG-labeled RNA probes: *upd* cDNA (Harrison *et al*., 1998, EST clone AT23111, also known as *outstretched, os* -flybase); *drm* cDNA (Green *et al.,* 2002, EST clone LD26791); *knrl* cDNA (Chen *et al.,* 1998); *otp* cDNA (Simeone *et al*., 1994); *GATAe* cDNA (EST clone LD08432); *ct* cDNA (EST clone RE08418); *wg* cDNA (Baker, 1987). The whole mount *in situ* hybridization was carried out as described by Tautz and Pfeifle, (1989). Staging of the embryos were done according to Campos-Ortega and Hartenstein, (1985).

Acknowledgements

We thank DGRC Kyoto Stock Center and Bloomington Stock Center for providing fly strains used in this study. This study is supported in part by Grants-in-Aid from Japan Society for the Promotion of Science (JSPS) (22570205) to R. M., and S. N. U. is supported by Japanese Government Scholarship.

References

ABEL, T., MICHELSON, A.M. and MANIATIS, T.A. (1993). Drosophila *GATA* family member that binds to *ADH* regulatory sequences is expressed in the developing fat body. *Development* 119: 623-633.

BAKER, N. (1987). Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila*, the spatial distribution of a transcript in embryos. *EMBO J* 6: 1765-1774.

BODENSTEIN, D. (1950). The postembryonic development of *Drosophila*. In *Biology of Drosophila.* (ed. Demerec, M.) pp.275-367. Wiley, New York.

BRÖNNER, G. and JACKLE, H. (1991). Control and function of terminal gap gene activity in the posterior pole region of the *Drosophila* embryo. *Mech Dev* 35: 205-211.

CAMPOS-ORTEGA, J.A. and HARTENSTEIN, V. (1985). In *The Embryonic Development of Drosophila Melanogaster*, Springer-Verlag, Berlin.

CHEN, C.K., KUHNLEIN, R.P., EULENBERG, K.G., VINCENT, S., AFFOLTER, M. and SCHUH, R. (1998). The transcription factor KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during *Drosophila* tracheal development. *Development* 125: 4959-4968.

FUSS, B. and HOCH, M. (2002). Notch signaling control cell fate specification along the dorsoventral axis of the *Drosophila* gut. *Curr Biol* 12: 171-179.

FUSS, B., MEISSNER, T., BAUER, R., LEHMANN, C., ECKARDT, F. and HOCH, M. (2001). Control of endoreduplication domains in the *Drosophila* gut by the *knirps* and *knirps-related* genes. *Mech Dev* 100: 15-23.

GREEN, R. B., HATINI, V., JOHANSEN, K.A., LIU, X. and LENGYEL, J.A. (2002). Drumstick is a zinc finger protein that antagonizes Lines to control patterning

and morphogenesis of the *Drosophila* hindgut. *Development* 129: 3645-3656.

GREENWOOD, S. and STRUHL, G. (1997). Different levels of Ras activity can specify distinct transcriptional and morphological consequences in early *Drosophila* embryos. *Development* 124: 4879-4886.

HARBECKE, R. and JANNING, W. (1989). The segmentation gene *Krüppel* of *Drosophila Melanogaster* has homeotic properties. *Genes Dev* 3: 114-122.

HARRISON, D.A., MCCOON, P.E., BINARI, R., GILMAN, M. and PERRIMON, N. (1998). *Drosophila unpaired* encodes a secreted protein that activates the JAK signaling pathway. *Genes Dev* 12: 3252-3263.

HITANI, V., GREEN, R.B., LENGYEL, J. A., BARY, S. J. and DINARDO, S. (2005). The Drumstick/Lines/Bowl regulatory pathway links antagonistic Hedgehog and Wingless signaling inputs to epidermal cell differentiation. *Genes Dev* 19: 709-718.

HAYES, S.A., MILLER, J.M. and HOSHIZAKI, D.K. (2001). *serpent*, a GATA-like transcription factor gene, induces fat-cell development in *Drosophila melanogaster. Development* 128: 1193-1200.

HOCH, M. and PANKRATZ, M.J. (1996). Control of gut development by *forkhead* and signaling molecules in *Drosophila. Mech Dev* 58: 3-14.

IWAKI, D.D., JOHANSEN, K.A., SINGER, J.B. and LENGYEL, J.A. (2001). *drumstick, bowl*, and *lines* are required for patterning and cell rearrangement in the *Drosophila* embryonic hindgut. *Dev Biol* 240: 611-626.

JOHANSEN K.A., GREEN, R.B., IWAKI, D.D., HERNANDEZ, J.B. and LENGYEL, J.A. (2003). The Drm-Bowl-Lin relief-of-repression hierarchy controls fore- and hindgut patterning and morphogenesis. *Mech Dev* 120: 1139-1151.

KISPERT, A., HERRMANN, B.G., LEPTIN, M. and REUTER, R. (1994). Homologs of the mouse *Brachyury* gene are involved in the specification of posterior terminal structures in *Drosophila, Tribolium,* and *Locusta. Genes Dev* 8: 2137-2150.

LENGYEL, J.A. and IWAKI, D.D. (2002). It takes guts: the *Drosophila* hindgut as a model system for organogenesis. *Dev Biol* 243: 1-19.

LENGYEL, J.A. and LIU, X.J. (1998). Posterior gut development in *Drosophila*: A model system for identifying genes controlling epithelial morphogenesis. *Cell Res* 8: 273-284.

LIU, S. and JACK, J. (1992). Regulatory interactions and role in cell type specification of the Malpighian tubules by the *cut, Krüppel,* and *caudal* genes of *Drosophila. Dev Biol* 150: 133-143.

MARTIN, J. R., RAIBAUD, A. and OLLO, R. (1994). Terminal pattern elements in *Drosophila* embryo induced by the torso-like protein. *Nature* 367: 741-745.

MURAKAMI, R. and SHIOTSUKI, Y. (2001). Ultrastructure of the hindgut of *Drosophila* larva, with spacial reference to the domains identified by specefic genes expression pattern. *J Morphol* 248: 144–150.

MURAKAMI, R., OKUMURA, T. and UCHIYAMA, H. (2005). GATA factors as key regulatory molecules in the development of *Drosophila* endoderm. *Develop Growth Differ* 47: 581-589.

MURAKAMI, R., SHIGENAGA, A., KAWAKITA, M., TAKIMOTO, K., YAMAOKA, I., AKASAKA, K. and SHIMADA, H. (1995). *aproctous*, a locus that is necessary for the development of the proctodeum in *Drosophila* embryos, encodes a homolog of the vertebrate *Brachyury* gene. *Roux's Arch Dev Biol* 205: 89-96.

MURAKAMI, R., SHIGENAGA, A., KAWANO, E., MATSUMOTO, A., YAMAOKA, I. and TANIMURA, T. (1994). Novel tissue units of regional differentiation in the gut epithelium of *Drosophila*, as revealed by P-element-mediated detection of enhancer. *Roux's Arch Dev Biol* 203: 243-249.

MURAKAMI, R., TAKASHIMA. S. and HAMAGUCHI, T. (1999). Developmental genetics of the *Drosophila* gut: specification of primordial, subdivision and overt-differentiation. *Cell Mol Biol* 45: 661-676.

NUSSLEIN-VOLHARD, C., FROHNHOFER, H. and LEHMANN, R. (1987). Determination of anteroposterior polarity in *Drosophila. Science* 238: 1675-1681.

OKUMURA, T., MATSUMOTO, A., TANIMURA, T. and MURAKAMI, R. (2005). An endoderm-specific GATA factor gene, *dGATAe*, is required for the terminal differentiation of the *Drosophila* endoderm. *Dev Biol* 278: 576-586.

PANKRATZ, M.J. and HOCH, M. (1995). Control of epithelial morphogenesis by cell signaling and integrin molecules in the *Drosophila* foregut. *Development* 121: 1885-1898.

PIGNONI, F., BALDARELLI, R. M., STEINGRIMSSON, E., DIAZ, R. J., PATAPOUTIAN, A., MERRIAM, J. R., and LENGYEL, J. A. (1990). The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor super family. *Cell* 62: 151–163.

REHORN, K.P., THELEN, H., MICHELSON, A.M. and REUTER, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila. Development* 122: 4023-4031.

REDEMANN, N., GAUL, U. and JACKLE, H. (1988). Disruption of a putative Cys-zinc interaction eliminates the biological activity of the *Krüppel* finger protein. *Nature* 332: 90-92.

REUTER, R. (1994). The gene *serpent* has homeotic properties and specifies endoderm versus ectoderm within the *Drosophila* gut. *Development* 120: 1123-1135.

REUTER, R. and LEPTIN, M. (1994). Interacting function of *snail, twist* and *huckebein* during the early development of germ layer in *Drosophila. Development* 120: 1137-1150.

SAVANT-BHONSALE, S. and MONTELL, D. J. (1993). *torso*-like encodes the localized determinant of *Drosophila* terminal pattern formation. *Genes Dev* 7: 2548-2555.

SIMEONE A., ROSARIA D`APICE, M., NIGRE, V., CASANOVA, J., GRAZIANI, F., ACAMPORA, D. and AVANTAGGIATO, V. (1994). *orthopedia*, a Novel Homeobox containing gene expressed in developing CNS of both *Mouse* and *Drosophila. Neuron* 13: 83-101.

SINGER, J. B., HARBECKE, R., KUSCH, T., REUTER, R. and LENGYEL, J. A. (1996). *Drosophila brachyenteron* regulates gene activity and morphogenesis in the gut. *Development* 122: 3707-3718.

SKAER, H. (1993). The Alimentary Canal. In: Developmental Biology of *Drosophila melamogester* (ed. Bate, M. and Martinez Arias, A.) pp.941-1012. Plainview, NY: Cold Spring Harbor Laboratory Press.

SPRENGER, F. and NUSSLEIN-VOLHARD, C. (1992). Torso receptor activity is regulated by a diffusible ligand produced at the extracellular terminal regions of the *Drosophila* egg. *Cell* 71: 987-1001.

TAKASHIMA, S. and MURAKAMI, R. (2001). Regulation of pattern formation in the *Drosophila* hindgut by *wg, hh, dpp, and en. Mech Dev* 101: 79-90.

TAKASHIMA, S., YOSHIMORI, H., YAMASAKI, N., MATSUNO, K. and MURAKAMI, R. (2002). Cell fate choice and boundary formation by combined action of *Notch* and *engrailed* in the *Drosophila* hindgut. *Dev Genes Evol* 212: 534-541.

TAUTZ, D. and PFEIFLE, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback. Chromosoma* 98: 81–85.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Hedgehog signalling is required for cloacal development in the zebrafish embryo
Caroline A. Parkin, Claire E. Allen and Philip W. Ingham*
Int. J. Dev. Biol. 53: 45 - 57 (2009)

Cdx2 specifies the differentiation of morphological as well as functional absorptive enterocytes of the small intestine
Hiroyuki Mutoh, Kiichi Satoh, Hiroto Kita, Hirotsugu Sakamoto, Hiroko Hayakawa, Hironori Yamamoto, Norio Isoda, Kiichi Tamada, Kenichi Ido and Kentaro Sugano
Int. J. Dev. Biol. (2005) 49: 867-871

Molecular analysis of the determination of developmental fate in the small intestinal epithelium in the chicken embryo
Hiroaki Hiramatsu and Sadao Yasugi
Int. J. Dev. Biol. (2004) 48: 1141 - 1148

Analysis of mouse eye development with chimeras and mosaics
J. Martin Collinson, Robert E. Hill and John D. West
Int. J. Dev. Biol. (2004) 48: 793-804

Terminal differentiation of palatal medial edge epithelial cells in vitro is not necessarily dependent on palatal shelf contact and midline epithelial seam formation
Toshiya Takigawa and Kohei Shiota
Int. J. Dev. Biol. (2004) 48: 307-317

Physiological and induced apoptosis in sea urchin larvae undergoing metamorphosis.
Maria C Roccheri, Cinzia Tipa, Rosa Bonaventura and Valeria Matranga
Int. J. Dev. Biol. (2002) 46: 801-806

mgm 1, the earliest sex-specific germline marker in Drosophila, reflects expression of the gene esg in male stem cells.
Adrian Streit, Luca Bernasconi, Pavel Sergeev, Alex Cruz and Monica Steinmann-Zwicky
Int. J. Dev. Biol. (2002) 46: 159-166

Early establishment of epithelial apoptosis in the developing human small intestine.
P H Vachon, E Cardin, C Harnois, J C Reed and A Vézina
Int. J. Dev. Biol. (2000) 44: 891-898

Intestinal fatty acid binding protein gene expression reveals the cephalocaudal patterning during zebrafish gut morphogenesis.
M André, S Ando, C Ballagny, M Durliat, G Poupard, C Briançon and P J Babin
Int. J. Dev. Biol. (2000) 44: 249-252

5 yr ISI Impact Factor (2010) = 2.961