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Notch Signaling Controls Cell Fate Specification along the Dorsoventral Axis of the *Drosophila* Gut

Bernhard Fuß and Michael Hoch[1]
Universität Bonn
Institut für Zoophysiologie
Abteilung Entwicklungsbiologie
Poppelsdorfer Schloss
D-53115 Bonn
Germany

Summary

Background: Gut formation is a key event during animal development. Recent genetic analysis in chick, mice, and *Drosophila* has identified Hedgehog and TGFß signals as essential players for the development of the primitive gut tube along its anterior-posterior (AP) axis. However, the genetic programs that control gut patterning along its dorsoventral (DV) axis have remained largely elusive.

Results: We demonstrate that the activation of the Notch receptor occurs in a single row of boundary cells which separates dorsal from ventral cells in the *Drosophila* hindgut. *rhomboid*, which encodes a transmembrane protein, and *knirps/knirps-related*, which encode nuclear steroid receptors, are Notch target genes required for the expression of *crumbs*, which encodes a transmembrane protein involved in organizing apical-basal polarity. Notch receptor activation depends on the expression of its ligand Delta in ventral cells, and localizing the Notch receptor to the apical domain of the boundary cells may be required for proper signaling. The analysis of gene expression mediated by a Notch response element suggests that boundary cell-specific expression can be obtained by cooperation of Suppressor of Hairless and the transcription factor Grainyhead or a related factor.

Conclusions: Our results demonstrate that Notch signaling plays a pivotal role in determining cell fates along the DV axis of the *Drosophila* hindgut. The finding that Notch signaling results in the expression of an apical polarity organizer which may be required, in turn, for apical Notch receptor localization suggests a simple mechanism by which the specification of a single cell row might be controlled.

Introduction

The morphological processes involved in the development of the gastrointestinal tract of animals are highly similar [1, 2]. In mouse and chicken embryos, gut formation is initiated by the formation of two open-ended tubes at opposite sites of the embryo. The tubes are generated by the invagination of the endodermal layer in an anterior-ventral position and later in a posterior-ventral region. The gut tubes then grow and extend toward each other until they meet and fuse around the yolk stalk [3]. In *Drosophila*, gut formation is also initiated with gastrulation by the invagination of cells at the anterior-ventral and posterior-dorsal region of the embryo to give rise to the foregut and the hindgut primordial tubes, respectively. The midgut forms inbetween these tubes by fusion of an anterior and a posterior primordium [4–6]. As the gut tubes form, visceral mesoderm is recruited to surround the invaginating gut epithelia. The primitive gut tube of vertebrates and invertebrates is initially regionalized along its AP axis into three broad domains: the foregut, the midgut, and the hindgut. Ultimately, these domains are further subdivided, and derivative organs, such as the lungs, pancreas, or liver in vertebrates and the proventriculus or the Malpighian tubules in *Drosophila*, are specified [1, 2, 4]. The similarity of the morphological processes during gut formation is paralleled by the function of evolutionarily conserved regulators of gastrointestinal development. The analysis of mouse and *Drosophila* mutants and ectopic expression studies in the chick have identified Hedgehog and Decapentaplegic/BMP signals to be essential for the development of the foregut and hindgut and the regionalization of the gut along its AP axis [7–15]. In contrast, very little is known about the genetic programs that control gut morphogenesis and cellular differentiation along its dorsoventral (DV) axis.

Notch signaling mediated by the Notch family of transmembrane receptors is an evolutionarily conserved cell interaction mechanism that controls cell fate decisions through local interactions during animal development ([16–20] for reviews). Genetic and molecular studies have suggested that the activation of the *Drosophila* Notch receptor occurs by interaction with its partially redundant transmembrane ligands Delta and Serrate. Genetic mosaic studies have revealed that they affect the activity of the Notch receptor in cells which are adjacent to the ligand-expressing cells [20]. Depending on the level of ligand expression, their interaction may be either agonistic or antagonistic [21], and signaling may therefore also depend upon a given competence of cells to receive the Notch signal. Ligand binding results in a proteolytic intracellular processing of Notch [19] and gives rise to the Notch intracellular domain fragment (N^{icd}). N^{icd} is released from the membrane and translocates to the nucleus where it interacts with Suppressor of Hairless [Su(H)], a ubiquitously expressed DNA binding protein. DNA-bound complexes containing both Su(H) and N^{icd} are thought to activate in cooperation with other transcriptional activators the transcription of Notch target genes [22]. Here we show that Notch signaling specifies cell fate along the DV axis of the *Drosophila* hindgut. Notch receptor activation and the induction of Notch target genes occurs in a single row of boundary cells which separates dorsal from ventral cells in the hindgut.

[1] Correspondence: m.hoch@uni-bonn.de

Results

Distinct Cell Types Arise along the DV Axis of the Large Intestine

The regionalization of the hindgut tube involves the formation of three major subregions: the small intestine, which localizes to the anterior end of the hindgut; the large intestine, which represents the middle part and the rectum, its posterior part [1, 10]. The formation of the small intestine and the rectum was shown to depend on Hedgehog and Wingless activities which coordinate morphogenesis and cell differentiation in the hindgut [10, 11, 15, 23, 24]. The steroid receptor-encoding genes *knirps* and *knirps-related*, which are expressed in banded expression domains in the small intestine and the rectum, were recently shown to be target genes of the Hedgehog and Wingless signaling pathways required for restricting endoreduplication cycles to the middle part of the hindgut, the large intestine [15].

While studying the role of the *kni* and *knrl* genes which act redundantly during hindgut development [15], we observed that both genes are also coexpressed in the large intestine, from germ band extension stage onward in two rows of lateral cells (20 ± 1) on each side of the tube (Figures 1A, 1B, and 1G). This expression is maintained until late stage 16. In the lateral cell rows, *kni* and *knrl* are coexpressed with the *rhomboid* (*rho*) gene (Figures 1C, 1D, and 1G) that encodes a transmembrane protein involved in epidermal growth factor receptor (EGFR) signaling [25, 26]. *rho* gene expression in the lateral cells appears slightly earlier than *kni*/*knrl* gene expression (compare Figures 1A and 1C) and is also maintained until late stage 16. At the transitions to the small intestine and the large intestine, *rho* is expressed in two circular expression domains (Figure 1D). The transmembrane protein and apical polarity determinant Crumbs (Crb) [27, 28] becomes strongly upregulated in the lateral cell rows after germ band extension stage and displays an unusual cellular distribution. Whereas in dorsal and ventral cells of the hindgut Crb is located at the apical cell margins, Crb is localized to the entire apical domain of the lateral cell rows, and expression is maintained until the end of embryogenesis (Figures 1E and 1F). Similarly, Discs lost [29], another apical polarity organizer, is located to the entire apical cell surface in these cells (data not shown).

Using various cell shape and cell polarity markers, such as the septate junction markers Fas III, Neurexin IV, and Discs lost [29], we were able to determine that the cells of the lateral cell rows show a flat and long-shaped morphology and that these cells separate homogenous cell populations in the dorsal and the ventral halves of the large intestine (Figures 2A and 2B). The cells of the lateral cell rows can thus be considered boundary cells separating dorsal from ventral cells in the large intestine. The dorsal cells, which are big and columnar, express the homeodomain protein Engrailed (En) from extended germ band stage onward until late embryogenesis (Figures 2C and 2D) [24, 30]. In contrast, the ventral cells, which are small and cuboidal, display expression of Delta from extended germ band stage onward until late embryogenesis (Figures 2E and 2F). Double immunostainings reveal that En expression in the dorsal half of the large intestine is adjacent and nonoverlapping to the *kni*/*knrl*/*rho* expression domains in the boundary cells (Figure 2G). Similarly, the Delta expression domain in the ventral half is adjacent to it, although we cannot exclude coexpression at a low level in the boundary cells (Figure 2H). In summary, dorsal cells express En (Figures 2I and 2K); boundary cells *kni*/*knrl*, *rho*, *crb*, (Figures 2J and 2K) and ventral cells express Delta (Figure 2K).

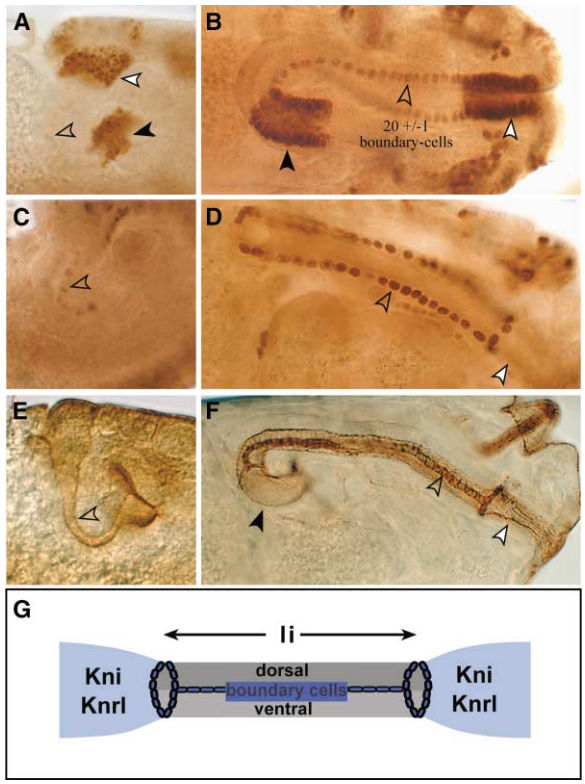

Figure 1. Localized Gene Expression in Two Lateral Rows of Cells on Each Side of the Hindgut

(A, C, and E) Antibody stainings of wild-type embryos at germ band extension stage and at stage 16 (B, D, and F). The white arrowhead marks the developing rectum, the black arrow the small intestine, and the open arrow the boundary cells of the hindgut. (A and B) Kni protein expression. Note that Knrl is expressed identically to Kni [15]. (C and D) Rho expression visualized by anti-β-Gal immunostainings of a *rholacZ* line. Note that *rho* is expressed in the lateral cell rows and in two rings of cells at the transition to the small intestine and the rectum. (E and F) Crb expression. Note the upregulation and the apical localization of Crb in the lateral cell rows and in the two rings at the transitions to the small intestine and the rectum (F). (G) Schematic illustration of the expression domains along the AP and the DV axis of the hindgut. li, large intestine.

Notch Signaling Is Required to Establish the Boundary Cell Fate

To investigate the role of the genes expressed in the large intestine, we performed lack- and gain-of-function studies. In amorphic *Notch* and *Delta* mutant embryos, *kni*/*knrl* (Figures 3A and 3B), *rho* (Figure 3C), and high levels of Crb expression on the apical plate are absent in the large intestine, and the boundary cell fate is not established. In contrast, ventral cell morphologies are

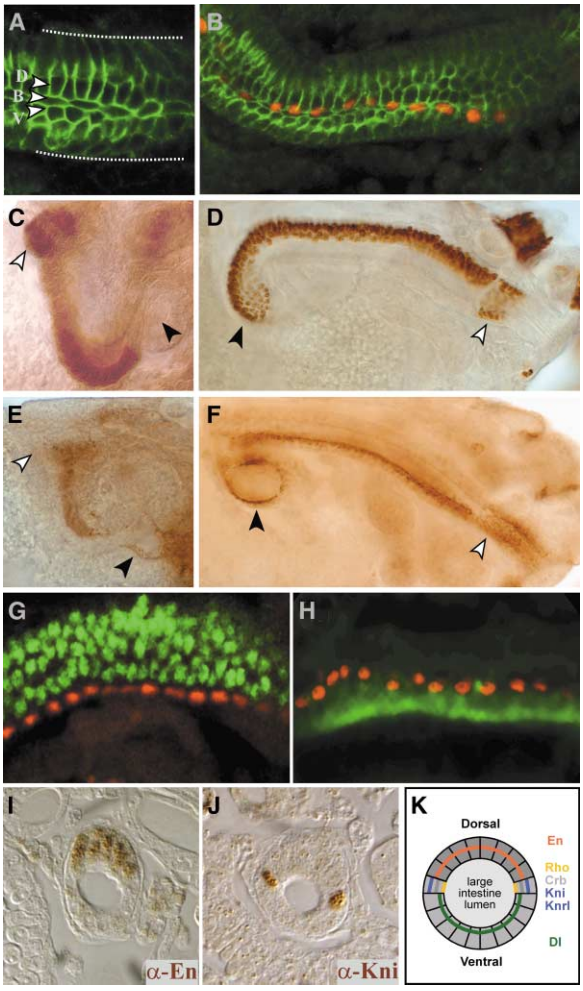

Figure 2. Expression Patterns of DV Regulators in the Large Intestine

(A) Anti-FasIII antibody staining visualizing the three distinct cell types along the DV axis of the large intestine by confocal microscopy. The boundary cells ("B") are flat and long shaped, whereas the dorsal cells ("D") are big and columnar. In contrast, the ventral cells ("V") are small and cuboidal. (B) Anti-FasIII; anti-βGal double immunostaining showing *rho* expression in the boundary cells. (C and E) Antibody stainings of wild-type embryos at germ band extension stage and (D, F, G, and H) at stage 16. Arrowhead designation as in Figure 1. (C and D) En is expressed on the dorsal half of the large intestine. (E and F) Delta is expressed on the ventral half. Note that both En and Delta are expressed in circular expression domains in the large intestine and the rectum. (G) Anti-En; anti-Kni double immunostainings demonstrating that the En expression domain in dorsal cells is adjacent and nonoverlapping to the Kni expression domain in the boundary cells. (H) Anti-Delta; anti-Kni double immunostainings showing that the Delta expression domain in ventral cells is adjacent to the Kni expression domain in the boundary cells. (I and J) Transverse sections of the large intestine, immunostained with anti-En ([I]; dorsal cells) and anti-Kni ([J]; boundary cells) antibodies. (K) Schematic illustration of a transverse section of the large intestestine showing a summary of the expression patterns.

normal in *Notch* or *Delta* mutant embryos, and En expression and dorsal cell fates are unchanged (data not shown). This indicates that Notch signaling is required to establish the boundary cells but not for dorsal or ventral cell fates. To further test this, we performed gain-of-function experiments using the UAS/Gal4 system [31]. As driver lines, we used the G445.2 or the 14-3-fkhGal4 strains which mediate ubiquitous gene expression in the developing hindgut from the extended germ band stage onward until late stage 16 [15]. In order to ectopically activate the Notch signaling pathway, we used flies carrying the Notch intracellular domain fragment, N^{icd}, under the control of UAS sequences. Expressing N^{icd} ubiquitously in the hindgut results in an ectopic induction of *kni* (Figure 3D) and of *rho* (Figure 3E). In addition, the cellular localization of the Crb protein is affected in these embryos. In dorsal and ventral cells of the large intestine of wild-type embryos, Crb is localized to the apical cell margins, whereas it is localized to the entire apical plates of the boundary cells (Figure 3F). In the embryos, in which N^{icd} is ectopically expressed, Crb protein is found on the apical plates of all the hindgut cells; in addition, we find it in high concentrations in vesicles, especially on the baso/lateral sides of the cells (Figure 3G). A similar but less intensive ectopic expression of Crb can also be induced if both Kni and Rho are coexpressed in all the hindgut cells, suggesting that *crb* may be a downstream effector gene of Kni/Knrl and Rho activities (Figure 3H). This is consistent with the analysis of rho^{7M}; *Df(3L)* ri^{XT1} mutants [*Df(3L)* ri^{XT1} is a deficieny encompassing the *kni* and *knrl* transcription units] in which the expression of *crb* in the boundary cells is strongly reduced (data not shown). In summary, these results suggest that *rho*, *kni/knrl*, and Crb are target genes which are activated in response to Notch signaling in the boundary cells (Figure 3I).

kni/knrl and *rho* Are Regulated Independently of Each Other

To investigate the relationship between *rho* and *kni/knrl* in the boundary cells, we studied the expression of the genes in the respective mutants. *rho* expression is still present in *kni* mutants and *Df(3L)* ri^{XT1} mutants (Figure 4A). Similarly, *kni* and *knrl* expression are maintained in amorphic rho^{7M} mutants or EGF receptor mutants, such as *faint little ball* (*flb*). In flb^{IK35} mutants, the hindgut tube is much shortened due to a reduction of the cell number. However, we still find banded expression of both genes in the small intestine and the rectum along the AP axis of the hindgut, and we find expression in a few cells in the large intestine region (Figure 4B). Ectopic expression of *rho* using the corresponding UAS-effector line combined with a driver line that mediates ubiquitous expression in the hindgut did not result in ectopic *kni/knrl* gene expression and vice versa. This points toward *rho* and *kni/knrl* being regulated independently of each other.

En Prevents the Activation of Notch Target Genes in Dorsal Cells

To study whether En, which is expressed in the adjacent dorsal cells, contributes to the boundary cell fate, we examined the expression of *kni/knrl*, *rho*, and *crb* in *en* mutants and in *en; invected* double mutants (en^{E}), since it was shown previously that *en* and *invected* can act redundantly [32, 33]. Whereas the expression of the Notch target genes remained unchanged in *en* mutants, it was absent in the large intestine of *en; invected* double

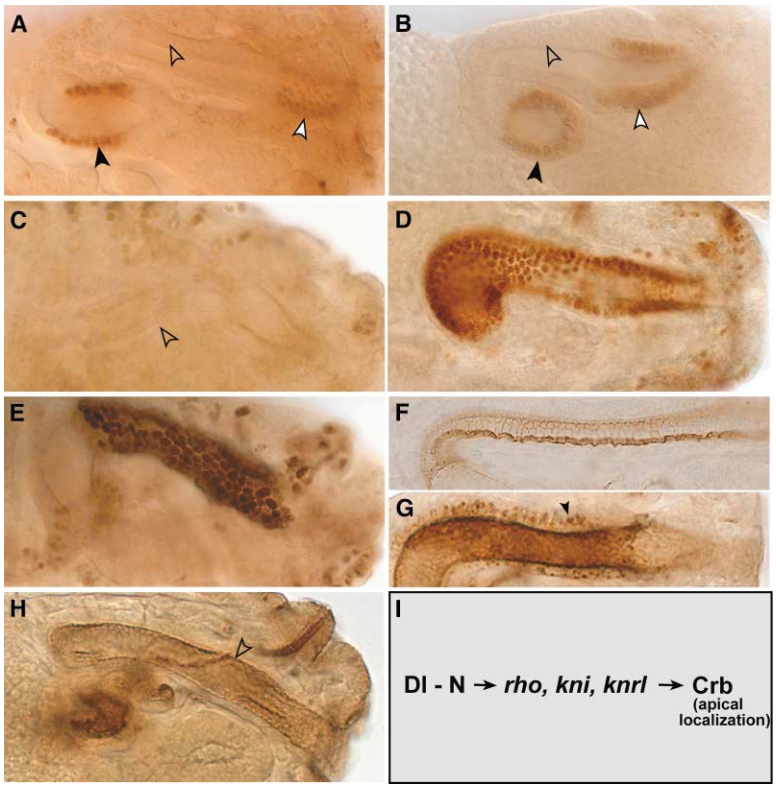

Figure 3. The Boundary Cell Fate Requires Notch Signaling Activity

Arrowheads in (A)–(H) mark the small intestine (black), the boundary cells (open), and the rectum (white) of stage 16 embryos. Kni (or Knrl, data not shown) expression is lost in (A) *Notch*^{55E11} and (B) *Delta*^{9P} mutants. Similarly, *rho* monitored by the *rholacZ* pattern expression is lacking, and the boundary cell fate is not established (C). In 14-3*fkh*-Gal4::UAS-N^{ICD} flies in which Notch signaling is activated ubiquitously in the hindgut, Kni (D), Knrl (data not shown), and Rho (E) are ectopically expressed in the large intestine. Similarly, Crb is ectopically expressed in the apical plate regions of all the hindgut cells (compare expression in wild-type in [F] with expression upon ectopic Notch signaling in [G]). Vesicles containing high levels of Crb protein are marked by a black arrow. (H) 14-3*fkh*-Gal4::UAS-Rho;UAS Kni embryos that display ectopic expression of both Rho and Kni in the hindgut. Crb is also induced at high levels in the hindgut (compare [H] to [F]). (I) Summary diagram showing that *rho*, *kni*, and *knrl* are Notch target genes induced in the boundary cells. These genes are required, in turn, for the induction of Crb in DV boundary cells in which the protein is localized to the apical membrane domain.

mutants (Figure 4C). Morphological studies indicate that the dorsal and the boundary cell fates are not established in these mutants, and the large intestine seems to consist entirely of the ventral cell fates (Figure 4D). To investigate the cause for this effect, we studied the expression of Delta in these mutants and found that it is expressed ubiquitously in the large intestine (Figure 4E). These data indicate that a boundary between Delta expressing and nonexpressing cells is required for Notch receptor activation. Ectopic expression of En in the large intestine using the 14-3 fkh driver and UAS-En effector lines results in a repression of *kni*/*knrl* and *rho* gene expression (Figure 4F). This indicates that En bears the potential to act as a negative regulator of Notch target genes (Figure 4H). We note that, upon ectopic activation of Notch signaling in the entire hindgut by expressing N^{icd}, En is repressed on the dorsal side of the large intestine, thus allowing ectopic activation of Notch target genes (Figure 4G).

Notch Signaling Is Confined to the Boundary Cells

In order to investigate whether Notch signaling in the large intestine of wild-type embryos is activated beyond the boundary cells but actively repressed dorsally and ventrally, we used flies that carry the chimeric Notch receptor/transcription factor fusion construct N-Gal4/VP16 [34] and determined the range of Notch signaling. Upon heat shock, this fusion protein, which is membrane bound, becomes ubiquitously expressed in the embryo. In cells in which the Notch receptor is activated by ligand binding, the intracellular Gal4-VP16 transcription factor moiety is cleaved off and is able to subsequently activate reporter gene expression in cells that carry a UAS-*lacZ* construct. The β-Gal expression pattern of such embryos reflects the range of Notch signaling. When we performed anti-β-Gal stainings of embryos that were heat shocked and carried the N-Gal4/VP16 and UAS-*lacZ* constructs, we observed β-Gal expression exclusively in the lateral boundary cells of the large intestine (Figures 5A–5C), demonstrating that Notch signaling is restricted to the boundary cells only. To further test this, we used flies carrying a *lacZ*-reporter construct in which multiple Su(H) binding sites from the *Enhancer of Split m8* gene are combined with binding sites for the transcription factor Grainyhead (Grh) [35]. In cells, in which Notch signaling is active and Grh is expressed, Su(H) cooperates with Grh to yield high levels of reporter gene expression, whereas reporter gene expression is repressed in cells in which Notch is inactive [35]. Determining the activity pattern of this construct in the hindgut using anti-β-Gal antibody stainings demonstrates that activation of the reporter gene occurs exclusively in the boundary cells of the large intestine (Figure 5D), consistent with the N-Gal4/VP16 data.

Restricting Notch Signaling to a Single Cell Row

In the *Drosophila* wing imaginal disc, the Notch receptor is activated along the border between dorsal and ventral cells, leading to the specification of cells that express Wingless and organize wing growth and patterning [36–38]. The range of Notch signaling is determined by the spatial and temporal expression pattern of its ligands, Delta and the transmembrane protein Serrate (Ser), and by the activity of the glycosyltransferase Fringe (Fng) [39–42]. Fng controls ligand selectivity of Notch and plays a major role in the Notch-dependent positioning of sharp compartment boundaries. It was shown to modify the glycosylation state of the receptor in the Golgi

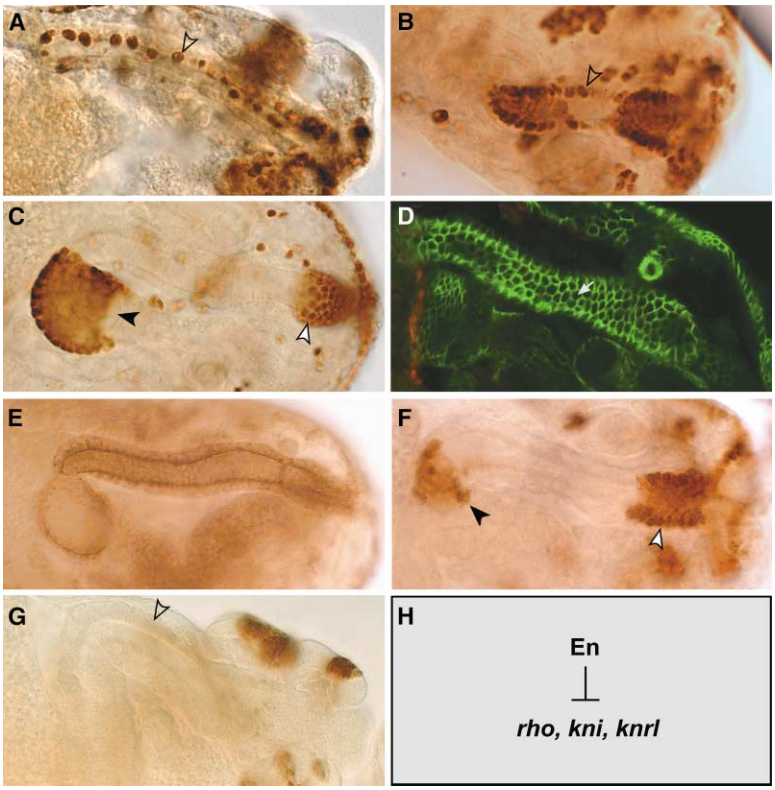

Figure 4. *kni* and *rho* Are Independently Regulated, and En Activity Is Required in Dorsal Cells

(A–G) Stage 16 embryos. (A) Expression of *rho* monitored by the *rholacZ* pattern still occurs in the boundary cells of kni^{FC13}(data not shown) and *Df (3L)ri* ^{XT1}, which are *kni;knrl* double mutant embryos (open arrow). (B) Anti-Kni antibody stainings of EGF receptor mutants flb^{IK35}. Note that the hindgut is strongly reduced due to a proliferation defect of the hindgut cells [55]. However, *kni* and *knrl* expression (data not shown) is still detectable in the remnants of the boundary cells (open arrow). (C) Anti-Kni antibody staining of en^{E} mutant embryos. Kni is still expressed in the small intestine and the rectum (black and white arrows) but is absent from the boundary cells. (D) β-Gal; anti-NrxIV double immunostaining of *rholacZ*; en^{E} homozygous mutants. Note that *rho* expression is lacking and that the large intestine consists of dorsal cell types (compare to Figures 6A–6F for wild-type). (E) Anti-Delta antibody staining of en^{E} mutant embryos. Delta is expressed ubiquitously in the hindgut. (F) Anti-Kni antibody stainings of G455*fkh*-Gal4::UAS-En embryos. Ectopic En expression in all the hindgut cells represses *kni* (and *knrl* and *rho*, data not shown) in the boundary cells of the large intestine. Note that the *kni* expression domains in the small intestine (black arrow) and the rectum (white arrow) are still present. (G) Ectopic Notch signaling in 14-3*fkh*-Gal4::$UASN^{ICD}$ embryos supresses En activity in dorsal cells (black arrow). (H) Exclusion of En from the boundary cells is a prerequisite for the activation of the Notch target genes *rho*, *kni*, and *knrl*.

complex, thereby lowering its sensitivity to Ser and raising its sensitivity to Delta [43–45]. To investigate whether Fng or Ser are also taking part in restricting Notch signaling to the boundary cells in the hindgut, we performed expression studies and lack- and gain-of-function analysis. In situ hybridization using a *Ser* antisense probe or β-Gal expression studies of a Ser-*lacZ* enhancer trap line show that *Ser* is not expressed in the large intestine of the hindgut (Figure 5E), and *Ser* mutants did show a normal hindgut. Furthermore, ectopically expressing Ser in all the hindgut cells had no effect on Notch target gene expression (data not shown). In contrast, Fng is expressed in boundary and dorsal cells as shown by double immunostainings of Fng and En (Figures 5F–5H). However, in amorphic fng^{80} mutants, misspecification of boundary cells occurs only at a low frequency (Figure 5I, compare magnifications in 5J to 5K), and ectopic expression of Fng using the 14-3 fkh driver and UAS-Fng flies did not induce an ectopic activation of the *kni*/*knrl* and *rho* genes in the hindgut (data not shown). These results indicate that Ser seems not to be required, and *fng* may play only a minor role in restricting Notch signaling to the boundary cells.

Apical Localization of Notch Correlates with Boundary Cell Fate

The above results suggest that the activation of the Notch receptor in the boundary cells of the hindgut is triggered by the binding of Delta, which is expressed at high levels in adjacent ventral cells. If Delta levels are uniform and this boundary condition is lost, as in en^{E} mutants, Notch signaling fails to occur. To further obtain insight into how the spatial control of Notch receptor activation is mediated, we determined the localization of the receptor using antibody stainings to Notch. In ventral and dorsal cells, Notch is expressed in the apical cell margins (Figures 6A–6C) [46], as can be demonstrated using coimmunostainings with Neurexin IV. However, in the boundary cells, the Notch receptor is positioned to the entire apical plate where it is colocalized with Crb or Discs-lost (Figures 6D–6H). To test whether the apical localization of the receptor is necessary for its signaling activity, we studied amorphic *crb* mutants in which the sorting of proteins to the apical domain of the cells is affected [28, 29]. In these mutants, we find a strong reduction of the number of boundary cells, although hindgut morphogenesis is only slightly affected (Figure 6I) [28]. In addition, the remaining boundary cells are mislocalized, and we often find two rows of cells instead of a single row as we find in wild-type (Figure 6J). Anti-Notch/anti-Kni double immunostainings of *crb* mutants demonstrate the reduction of apical Notch receptor localization in *crb* mutants (Figures 6G and 6K–6P). Furthermore, in cells in which the Notch receptor is not localized along the apical plate of the cells, the activation of Notch target genes fails to occur (Figures 6N–6P). These results indicate that apical localization of the receptor may be important for boundary cell fate determination.

Discussion

Notch signaling is a conserved mechanism in multicellular organisms to regulate the specification of cell types

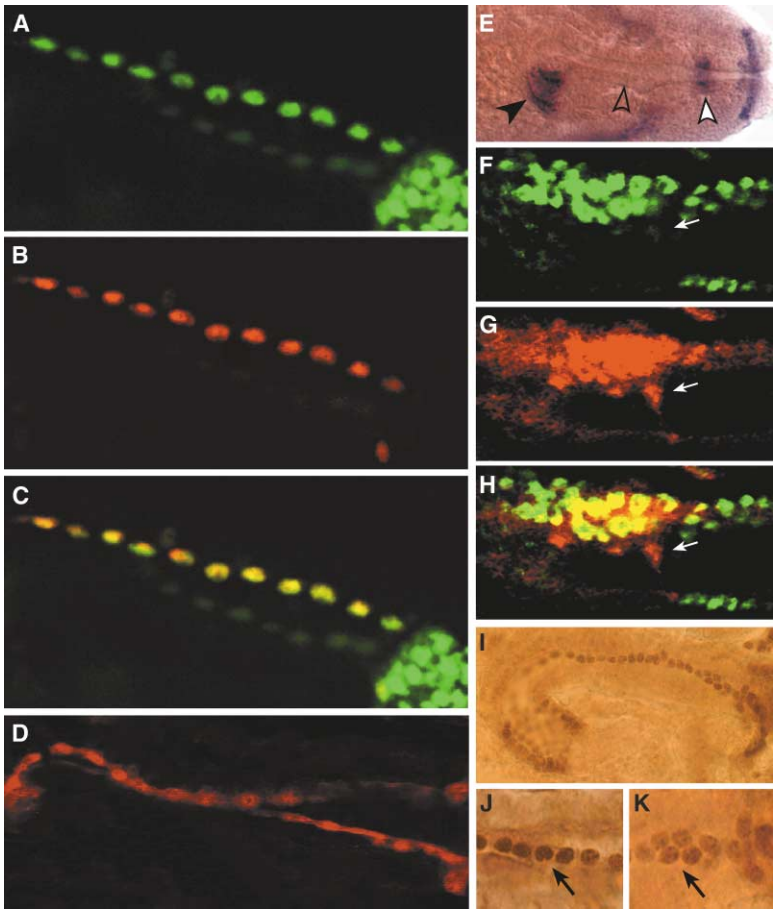

Figure 5. Range of Notch Signaling in the Large Intestine

(A–C) N-Gal4-VP16::UAS-LacZ embryos were heat shocked and double immunostained for Kni (green, [A]) and β-Gal (red, [B]). Colocalization of Notch activity and Kni expression in the boundary cells is visualized in the merge (C). (D) Anti β-Gal immunostaining of the Notch model response element that contains binding sites for Grainyhead and Su (H) [35]. Activation of reporter gene expression is restricted to boundary cells. (E) Serrate expression is absent from the large intestine (open arrow), visualized by in situ hybridization using a *Ser* riboprobe. Note that Ser is expressed in the small intestine (black arrow) and the rectum (white arrow). (F–H) Confocal images of a stage 16 *fnglacZ* embryo stained for anti-En (F), anti-βGal (G), and the merge in (H). Note that Fng is expressed in the boundary cells (white arrow) and colocalized with En in the dorsal half of the large intestine. (I) Anti-Kni antibody stainings of stage 16 fng^{80} mutants in which a misspecification of the boundary cells occurs at low frequency. Compare magnifications of wild-type in (J) and *fng* mutants in (K) (black arrows). Note that the boundary cells which are regularly spaced in a single row of cells in wild-type embryos are sometimes specified in two rows in fng^{80} mutant embryos.

through local cell interactions [16–20]. We show that in the large intestine of the *Drosophila* hindgut a single row of boundary cells that forms in between En-expressing dorsal cells and Delta-expressing ventral cells is determined by Notch signaling (Figure 6Q). Unlike in wing imaginal disc development, Ser seems not involved, and the glycosyltransferase Fringe plays only a minor role for the proper positioning of the DV boundary in the large intestine. Our results rather suggest two major determinants that control where Notch signaling can occur in the hindgut: (1) the localization of Delta, which is expressed in ventral cells at high levels and not expressed (or at very low levels) in the adjacent boundary cells in which Notch signaling is eventually activated; (2) the Crb-dependent transport of the Notch receptor to the apical membrane domain of the boundary cells. How the initial En and Dl expression domains are set up in the large intestine is not known. We note, however, that the En expression domain in the hindgut primordium is initially broader in early stage 7 embryos and only subsequently refines to the dorsal cells, whereas Dl expression is confined to ventral cells from early stage 7 onward (our unpublished data). It is thus possible that Notch signaling in the boundary cells leads to a cell-autonomous repression of *en* expression, consistent with the repression of *en* upon N^{icd} overexpression. Our immunhistological studies show that the proper specification of boundary cells in *crb* mutants correlates with the apical localization of the Notch receptor. The finding that Crb expression in the boundary cells depends on Notch signaling suggests the possibility of a feedback loop that ensures proper receptor localization required for establishing the competence of the boundary cells to receive the Delta signal. We cannot exclude, however, that the failure of Notch signaling in *crb* mutants may also be caused by the mislocalization of other localized proteins. It is noteworthy that the apical side of the boundary cells faces the lumen of the hindgut. Activating Notch receptors along the entire apical plate of the boundary cells would therefore require also a secreted form of Delta. The extracellular domain of Delta has been found as a soluble product in the supernatant of *Drosophila* cultured cells and in embryonic extracts, and it was shown to arise by a proteolytic activity of the ADAM metalloprotease Kuzbanian [47–49]. Both soluble forms of Delta and Serrate are able to act as antagonists and agonists of the Notch pathway in vivo [50]. It is possible that such a form of Delta and/or additional apically localized factors are involved in binding and activating the Notch receptor locally in the boundary cells of the hindgut.

Our results further demonstrate that Notch signaling induces the expression of the *rho* and *kni/knrl* genes and that both components are required, in turn, for the expression of Crb. It has been suggested recently that Su(H) functions as a core of a molecular switch by which the transcription of Notch target genes is regulated [22, 35, 51]. In the absence of Notch signaling, Su(H) func-

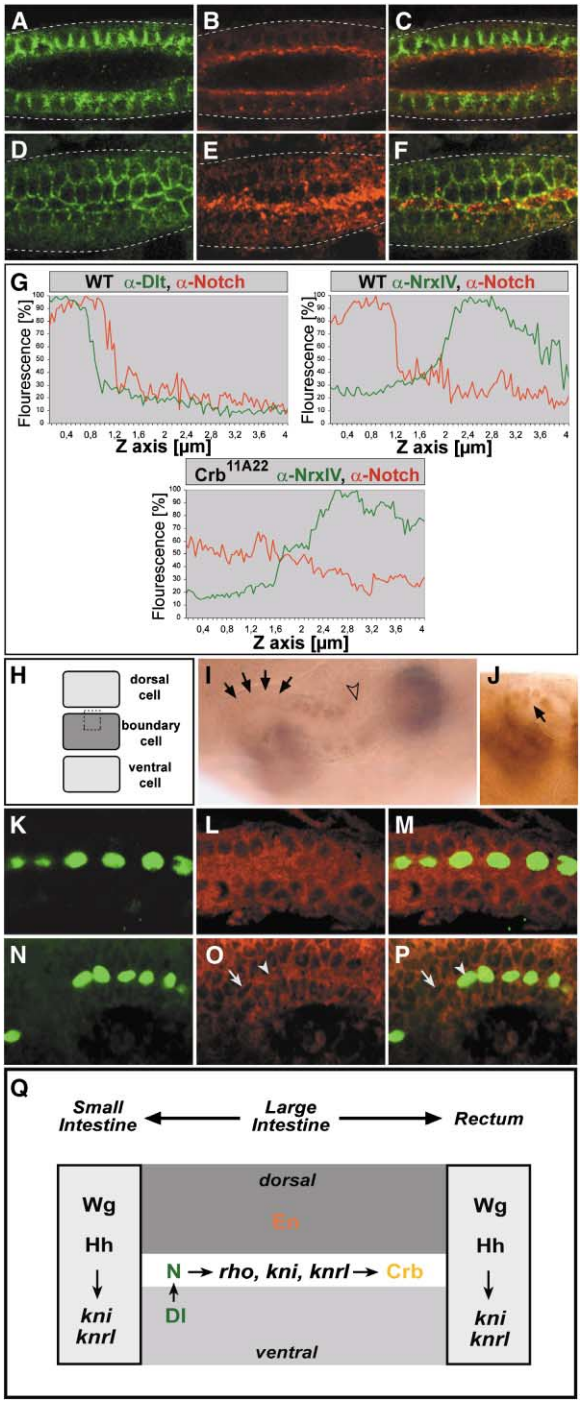

Figure 6. Notch Signaling in Boundary Cells and Apical Localization of the Notch Receptor

A sagittal section (A–C) and a lateral section (D–F) of the large intestine in wild-type embryos, stained for NrxIV (green) and Notch (red). Note the apical localization of Notch (A–C) along the entire apical domain of the boundary cells (E) as compared to its localization along the cell margins in adjacent dorsal and ventral cells (D–F). (G) Analysis of Notch receptor localization along the z axis in the boundary cells of wild-type embryos (upper panels), double-immunostained for Notch (red)/Discs lost (Dlt; green) and Notch (red)/NrxIV (green). The lower panel shows the Notch receptor localization along the z axis in the boundary cells of *crb* mutant embryos. Dlt is an apical marker and NrxIV a marker for the pleated septate junctions. The Z series covering a 4 μm thick layer were performed using a Leica TCSII confocal microscope, from the apical membrane of a DV border cell into basal direction. Localization of the scanning region is shown schematically in (H). (I and J) Anti-Kni immunostaining of crb^{11A22} mutant embryos. Loss of boundary cell specification is observed (arrows in [I]), and local duplications of the DV cell fate occur (arrow in [J]), although hindgut morphogenesis is only slightly affected [28]. (K–M) WT embryos were double immunostained for Kni ([K], green) and Notch ([L], red). The merge is shown in (M). Note the apical localization of the Notch receptor in the boundary cells of wild-type stage 15 embryos. In contrast, apical Notch receptor localization in the boundary cells of crb^{11A22} mutant embryos (N–P) is strongly reduced (arrowhead in [O]) and even lost in some regions of the hindgut (arrow in [O], compare to [L]). This correlates with a failure to express the Notch target genes *rho* or *kni* ([N], compare to [K]). (Q) Model for the AP and DV patterning of the *Drosophila* hindgut. Hedgehog and Wingless control *kni* and *knrl* expression in the small intestine (si) and the rectum (re) [15]. DV patterning occurs in the large intestine, in which three different cell types are established: Notch signaling determines the fate of the boundary cells, Delta is expressed in ventral cells, and En determines the fate of dorsal cells (see text). For the specification of the boundary cells, Delta, which is expressed in the ventral cells, interacts with the Notch receptor that is localized apically in the adjacent boundary cells. Receptor activation induces the expression of the Notch target genes *kni/knrl* and *rho*, which are required, in turn, for *crb* expression. Crb protein is expressed at high levels in the apical membrane domain of the boundary cells and may be required for proper Notch receptor localization. En, which is expressed in dorsal cells, has the potential to act as a negative regulator of Notch target gene expression and thereby contributes to establishing sharp expression boundaries.

tions as a repressor, and, in the presence of Notch signaling, Su(H) can cooperate synergistically with other transcriptional activators to induce transcription of target genes. The finding that boundary cell-specific reporter gene expression can be induced in the hindgut by using a model Notch response element that is composed of binding sites for Su(H) and the widely expressed activator Grainyhead [35] suggests the possibility that the localized activation of the *rho* and *kni/knrl* genes could rely on the same factors and the same molecular switch mechanism that has recently been proposed for this element and for Notch-dependent *atonal* and *single minded* expression [22, 35, 51, 52]. In evolutionary terms, the gut is most likely one of the most ancient organs that evolved in multicellular organisms [53, 54]. Consistently, the morphological processes involved in the development of the gastrointestinal tract of animals are highly similar [3, 7–10]. It remains to be shown whether or not the evolutionarily conserved regulators of the Notch signaling cascade also determine dorsoventral aspects of gut development in other animals, including vertebrates.

Conclusions

Our results provide evidence that Notch signaling in the *Drosophila* hindgut controls the fate of a single row of boundary cells separating the dorsal and ventral halves of the gut tube. Activation of the Notch receptor in the boundary cells is mediated by its ligand Delta that is expressed in adjacent ventral cells. The induction of Notch target genes activate the expression of the apical polarity organizer Crb, which may be required, in turn, for apical Notch receptor localization. Our findings identify a

simple mechanism that controls the specification of a single row of DV boundary cells in an animal gut.

Experimental Procedures

Drosophila Stocks

We used N^{55e11}, Dl^{9P} (amorphic allele), flb^{IK35}, rho^{7M}, en^{IL034}, *Df(2R) en*^{E}, *kni*^{FC13}, *Df(3L) ri*^{XT1} (deficiency encompassing both *kni* and *knrl* transcription units), Ser^{RX106}, fng^{80} (loss-of-function allele; gift of S. Cohen), crb^{11A22}, *rho lacZ*, and Oregon R fly stains.

Ectopic Expression Studies

Ectopic expression studies were performed at 29°C using the 14-3 fkh-Gal4 and the G455.2 Gal4 driver lines that mediate ubiquitous expression in the hindgut [15]. As UAS effector strains, we used UAS-Dl (M. Muskavitch), UAS-Ser (E. Knust), UAS-Fng (S. Cohen), UAS-Kni (R. Schuh), UAS-N^{ICD}, and $N^{hsGal4\text{-}VP16}$ (G. Struhl). $N^{hsGal4\text{-}VP16}$ embryos were collected after 2 hr at 25°C, incubated at 18°C for 14 hr, followed by a 2 hr 37°C heat shock and a 2–4 hr recovery period at 25°C prior to fixation.

Antibody Stainings and In Situ Hybridization

Fixation and immunostaining of whole-mount embryos was carried out as described previously [13] using the Vectastain ABC Elite-horseradish peroxidase system. To examine hindgut development, we used the following antibodies: mouse anti-En (1:100; Hybridoma Bank), mouse anti-Dl (1:200; M. Muskavitch), guinea pig anti-Kni (1:200; R. Schuh), rabbit anti-Neurexin IV (1:1000; Bhat et al.,1999), mouse anti-Fasciclin III (1:2000; septate-junction marker, D. Brower), anti-Crb (1:20; Wodarz et al., 1995), mouse anti-βGal (1:100, Promega); rabbit anti-βGal (1:200, Cappel). Fluorescent detection of protein expression was performed with $Alexa^{594}$-coupled anti-rabbit (1:200), $Alexa^{488}$-coupled anti-mouse (1:100; both purchased from Molecular Probes), and Cy3-coupled anti-guinea pig antibody (1:100; Dianova) antibodies, respectively. Confocal analysis was done with a Leica TCSII confocal microscope. The embryos were mounted into Araldite as described earlier [13]. Digoxygenin-labeled RNA antisense probes were generated by in vitro transcription of a *Ser* cDNA (kindly provided by E. Knust). Whole-mount in situ hybridization of *Drosophila* embryos was performed as described earlier [13].

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