Diversity of fate outcomes in cell pairs under lateral inhibition

Nara Guisoni1,2,*, Rosa Martinez-Corral1,*, Jordi Garcia-Ojalvo1,‡ and Joaquín de Navascués3,‡

ABSTRACT
Cell fate determination by lateral inhibition via Notch/Delta signalling has been extensively studied. Most formalised models consider Notch/Delta interactions in fields of cells, with parameters that typically lead to symmetry breaking of signalling states between neighbouring cells, commonly resulting in salt-and-pepper fate patterns. Here, we consider the case of signalling between isolated cell pairs, and find that the bifurcation properties of a standard mathematical model of lateral inhibition can lead to stable symmetric signalling states. We apply this model to the adult intestinal stem cell (ISC) of Drosophila, the fate of which is stochastic but dependent on the Notch/Delta pathway. We observe a correlation between signalling state in cell pairs and their contact area. We interpret this behaviour in terms of the properties of our model in the presence of population variability in contact areas, which affects the effective signalling threshold of individual cells. Our results suggest that the dynamics of Notch/Delta signalling can contribute to explain stochasticity in stem cell fate decisions, and that the standard model for lateral inhibition can account for a wider range of developmental outcomes than previously considered.

KEY WORDS: Lateral inhibition, Notch-Delta signalling, Symmetric cell division, Intestinal stem cells, Neutral competition

INTRODUCTION
The Notch/Delta signalling pathway is one of the main regulators of cellular differentiation during development and adult tissue maintenance (reviewed by Artavanis-Tsakonas et al., 1999; Ehebauer et al., 2006; Koch et al., 2013). It often drives mutually inhibitory interactions between cells, acting as a gate for differentiation. This mode of action has been termed lateral inhibition, and has been the object of experimental study as well as mathematical formalisation for decades (see, for example, Othmer and Scriven, 1971; Collier et al., 1996; Sprinzak et al., 2011; Petrovic et al., 2014). Quantitative models of lateral inhibition usually involve a field of cells expressing initially similar amounts of the receptor Notch and its membrane-bound ligand Delta. Delta trans-activates Notch in neighbouring cells and Notch, once activated, reduces in turn the ability of the cell to signal through Delta, leading to a state of mutual repression. This symmetry (and cell fate equivalence) is eventually broken by enforced biases and/or stochastic variation in Notch/Delta levels (Collier et al., 1996; Plathé, 2001; reviewed by Simpson, 1997) resulting in extended fine-grained spacing patterns (Othmer and Scriven, 1971; Collier et al., 1996; see also Shaya and Sprinzak, 2011) that have been experimentally characterized in depth in real developmental systems (reviewed by Greenwald, 1998; Arias and Stewart, 2002). By contrast, little attention has been paid so far to the effect of lateral inhibition in isolated cell pairs, beyond the trivial expectation that symmetry breaking will eventually take place, leading to cells taking opposing fates (see, for example, Collier et al., 1996; Rouault and Hakim, 2012). However, there has been no formal investigation of whether alternative steady states are possible, perhaps due to the lack of an experimental model to relate it to.

The cellular homeostasis of the adult Drosophila midgut (Fig. 1A-D) can provide this experimental scenario, as in this tissue Notch/Delta signalling occurs mostly in isolated pairs of cells (Ohlstein and Spradling, 2006; de Navascués et al., 2012; Goulas et al., 2012). The intestinal lining of the fly is maintained by intestinal stem cells (ISCs), which divide to both self-renew and provide committed progenitors (Fig. 1B). Progenitors specialise in producing either nutrient-absorbing enterocytes or secretory enteroendocrine cells (Guo and Ohlstein, 2015; Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Zeng and Hou, 2015). The precursors of enterocytes, called enteroblasts (EBs), are frequently found forming pairs with ISCs (Ohlstein and Spradling, 2006; Michelli and Perrimon, 2006; Bardin et al., 2010; de Navascués et al., 2012) (Fig. 1C). These pairs are thought to result from an earlier division of an ISC and subsequent fate allocation by Notch signalling, before a new division or terminal differentiation event takes place (Goulas et al., 2012; de Navascués et al., 2012). Importantly, ISC divisions in the enterocyte lineage result in asymmetric fate (one ISC and an EB), symmetric self-renewal (two ISCs) or differentiation (two EBs), which globally result in balanced, homeostatic proportions (de Navascués et al., 2012) (Fig. 1D). This mode of tissue maintenance, whereby the balance between stem cell self-renewal and differentiation is achieved at the population level rather than within every stem cell lineage, is termed neutral competition (Klein and Simons, 2011) and is found in a growing number of self-renewing adult tissues (Simons and Clevers, 2011). Although no molecular mechanism has been fully elucidated so far for any case of neutral competition, in the fly gut it has been proposed to arise from lateral inhibition mediated by Notch/Delta (de Navascués et al., 2012), a pathway known to define the fate of the ISC offspring (Ohlstein and Spradling, 2006; Michelli and Perrimon, 2006; Bardin et al., 2010).

Here, we explore the capacity of a standard model of lateral inhibition acting in pairs of interacting cells to result in steady states with different signalling states (either symmetric or asymmetric) co-existing in the tissue. We find that this is indeed possible, provided signalling thresholds vary across cell pairs. Next, we turn to the Drosophila midgut and find that the tissue displays high variability of contact area between pairs of ISC/EB cells, which can be...
Cell pair type frequencies correlate with Notch activity

In the Drosophila midgut, Notch-negative cells correspond to ISCs, and Notch-positive cells to EBs. The Notch activity reporter $GBE-Su(H)$ is hardly expressed above background levels in ISCs (Ohlstein and Spradling, 2007; J.d.N., unpublished), and hence our choice of a low-threshold value, $N_{thr} = 0.1$. Symmetric positive pairs in the model will equate to an event of symmetric differentiation (EB/EB), symmetric negative pairs to symmetric inhibition (ISC/ISC), and asymmetric pairs to asymmetric ISC fate (ISC/EB) (Fig. 2A). If this was the case, we would expect the relative frequencies of these pairs to correlate with overall Notch activity levels, as has been shown with the balance of the entire fate profile. Moreover, our model can reproduce the distribution of fate outcomes from the contact area distribution determined experimentally.

Our results expand the repertoire of possible outputs of a system governed by lateral inhibition, and connect this mode of signalling with a mode of stem cell-based tissue maintenance (neutral competition) that is highly relevant in adult tissue homeostasis and tumourigenesis (Simons and Clevers, 2011; Vermeulen et al., 2013; Baker et al., 2014), and whose molecular regulation is poorly understood.

**RESULTS**

Lateral inhibition can result in stable, opposing symmetric signalling states

We study the steady-state behaviour of a standard model of lateral inhibition for the case of two cells (see Materials and Methods). For fixed $r$ and $h$, the steady states of this system depend on two parameters, $a$ and $b$ (the dimensionless activation and inhibition thresholds, respectively; see Materials and Methods), which we allow to vary across the population of cell pairs. We then calculate the equilibrium state of the system in this two-dimensional parameter space, according to the resulting signalling profile: asymmetric (one cell positive for Notch activation and the other one negative), symmetric positive or symmetric negative for Notch activation (Fig. 1C). Thus, for a population of cell pairs with variable activation or inhibition thresholds ($a$ and $b$), the three possible signalling state profiles occur (Fig. 2A, see Fig. 2B-E for a comparison of the dynamic evolution of examples of the three profiles, with Fig. 2B corresponding to the parameter values from Collier et al., 1996). The three signalling state profiles can be found within a relatively short range of parameter values (Fig. 2A).

Importantly, this scenario does not change qualitatively when considering a wide range of threshold values for Notch activity classification, as defined in the Materials and Methods section ($0.001 \leq N_{thr} \leq 0.8$; Fig. S1B-E), or higher cooperativity values in Notch signalling or Delta inhibition [$r, h = (2, 5)$ or $(5, 2)$] (Fig. S1F,G). However, we found that cooperativity was necessary: for $r = 1, h = 1$, the heterogeneous (asymmetric) steady states are lost (Fig. S1H). This requirement of cooperativity is in agreement with other theoretical works on lateral inhibition (Sprinzak et al., 2011) as well as with *in vitro* estimates for $r$ of 1.7 (Sprinzak et al., 2010).

In a biological system, the existence of three possible signalling state profiles would be equivalent to having three different cell fate combinations across a population of initially uncommitted cell pairs interacting through Notch/Delta, with the specific fate combination of a given cell pair depending on the sensitivity to Delta activation and Notch inhibition of the pair. To investigate the potential of this lateral inhibition model, incorporating variable activation and inhibition thresholds, to describe a real biological system, we turned to the *Drosophila* midgut.
populations of ISCs and EBs (Biteau et al., 2008; de Navascués et al., 2012). Indeed, heterozygous conditions for the null allele N55e11 lead to more ISC-ISC pairs and fewer EB-EB pairs relative to the wild type, whereas heterozygosis for the hyperactive, Dl-dependent l(1)NB mutation favours EB-EB pairs at the expense of ISC-ISC pairs, the presence of which is negligible (Fig. 1E). To ensure that these differences are due to variations in Notch activity, rather than to the genetic background of the different mutant chromosomes, we carried out a serial knock-down of Notch in ISCs and EBs. To achieve this, we used the GeneSwitch system (Osterwalder et al., 2001) with the driver da-GS, which is largely specific to ISCs and EBs in the intestine (Fig. S2A; Nicolas Buchon, personal communication), and increasing concentrations of the inducer RU486. In these experiments, the flies share the same genetic background, and differ only in the amount of RU486 present in the food. Indeed, we find that mild knock-down of Notch (to a level that does not yet lead to ISC-like tumour growth; Fig. S2B-F) increases the number of ISC-ISC pairs, at the cost of ISC-EB and EB-EB pairs, in a dose-dependent manner (Fig. 1F). The baseline fractions of the control for the induction experiment (‘mock’ bars in Fig. 1F) are different from the control for the mutants (‘N+/−’ bars in Fig. 1E), even if at the overall level the total numbers of ISCs and EBs are balanced (Fig. 1G,H; de Navascués et al., 2012), highlighting the importance of controlling for genetic background in these experiments. From these data, we conclude that the frequency of undifferentiated pair types are a good readout of the strength of Notch signalling.

Cell contact area as modulator of activation threshold

To relate the model to real tissue, we need first to consider how the dimensionless parameters a and b are related to biological features displaying variability across undifferentiated (esg+) cell pairs. We assume that biochemical processes intrinsic to the cell, such as protein degradation rates (δD and δN), the maximal biosynthesis rates (α and β), and the threshold of Delta inhibition by Notch in the same cell (KD), will not be highly variable among cells with a common developmental identity. However, the threshold of Notch activation by neighbouring Delta (KN) depends directly on the interaction between the two cells, which could be variable for different pairs of cells. For example, owing to spatial heterogeneity of cell packing, the contact area between cell pairs could be substantially different from pair to pair. Indeed, tissue images reveal that undifferentiated cells in nests show irregular shapes and variable contact area (Fig. 3A). In the Notch/Delta system, the amounts of Notch and Delta are usually limiting, and this seems to hold true for the adult Drosophila gut, where haploinsufficiency has been described (Biteau et al., 2008; de Navascués et al., 2012; see also Fig. 1E-H). Therefore, it is expected that variations of ∼2-fold or more in contact area would lead to significant changes in the levels of Notch activation. This is captured in the model by expressing the dimensionless activation threshold a in terms of the contact area, following Eqn 7 in the Materials and Methods. Specifically, we assume that a is inversely related with the contact area (the larger the contact area, the easier it is for Delta to activate Notch, and thus the smaller the activation threshold). As shown in
Fig. 2A (see also Fig. S3), variation in a best allows for heterogeneity in steady-state levels of Notch activity and therefore in fate choice. From this, we hypothesize that variation of contact area (or any other biological feature correlating with the threshold of Notch activation) is likely to allow the diversity in fate outcome predicted by the model.

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We could not find any strong pattern in the variations of Notch or Delta immunodetection intensity within confocal planes, and it would seem that both are only slightly enriched at the boundary between two undifferentiated cells (Fig. 3E,F). This indicates that Notch and Delta concentrations are largely independent of the position at the membrane along the cell perimeter, and in particular along the contact between esg+ cells. Moreover, the localisation of both Notch and Delta along the apical-basal axis of the cells is also largely homogeneous (Fig S5E,F). This is manifest in the small variation in the average amounts of Notch and Delta between different optical planes (Fig. S5B,C, left panels), and in the narrow distribution of mean values per plane, with low values of coefficients of variation per plane, of Notch intensity values.
(Fig. S5B,C, right panels). Therefore, the contact area between cells is a good approximation to the total amount of Notch and Delta proteins available for signalling.

We note that Arm largely parallels Notch and Delta localisation at the membrane (Fig 3D; Fig. S5D) but shows a stronger enrichment at the boundary (Fig. 3D), in agreement with previous reports (Maeda et al., 2008). Incidentally, these results also reveal that neither Arm nor Notch nor Delta are restricted to the apical domain in the midgut epithelium, and instead can be found in similar amounts along the apical-basal axis of the membrane in ISCs and EBs (Fig. S5D-F). This situation contrasts with Arm and Notch distribution in other Drosophila epithelia (Tepass and Hartenstein, 1994; Tepass et al., 2001; Sanders et al., 2009).

Taken together, our results suggest that both the Notch receptor and its ligand Delta are randomly and homogeneously distributed in the cell membrane, which suggests that measurements of membrane contact area may be relevant to the dynamics of Delta-Notch signalling as a proxy for the activation threshold $a$ in our model.

**Correlation of contact area values and cell fate profiles**

We have shown that contact area can be used as a measure of the amount of Notch and Delta available for interaction. Therefore, our model predicts that contact area should correlate with the patterns of symmetric and asymmetric fates. Indeed, we observe increasing average contact area in ISC-ISC (7.67 $\mu$m$^2$), ISC-EB (13.28 $\mu$m$^2$) and EB-EB (14.79 $\mu$m$^2$) cells (Fig. 4A). The contact area in ISC-ISC pairs was significantly lower than in the other two cell-pair types (with $P$-values of 0.002 and 0.005 when compared with ISC-EB and EB-EB pairs, respectively), whereas the latter could not be statistically distinguished from each other ($P=0.5$). We must also remark that EB cells increase in size after they progress from their original ISC state (Fig. 4B and Fig. S6B), which complicates our interpretation of the correlation between fate and contact area, especially in EB-EB pairs. This fact, together with the statistical similarity between ISC-EB and EB-EB pairs mentioned above, led us to focus on the distinction between ISC-ISC and ISC-EB, filtering the results coming from the latter cell-pair type based on the size of the EB cells (see below).

We measured the contact areas in 480 pairs of esg$^{-}$ cells with both symmetric (ISC-ISC, $n=74$) and asymmetric (ISC-EB, $n=406$) fates. The data show that contact area in these pairs is highly variable, ranging from just around 1 $\mu$m$^2$ to over 40 $\mu$m$^2$ (Fig. 4C). This degree of variability (of more than one order of magnitude) indicates that contact area has the potential to be a regulatory mechanism of the system (through its influence on the dimensionless activation threshold $a$).

Next, we classified measurements of contact area according to the fate profile of their corresponding cell pair and compared their values (Fig. 4D). We found that the cumulative distributions of contact areas in ISC-ISC and ISC-EB pairs are clearly separated. On average, the contact area between two ISCs (11.59±0.73 $\mu$m$^2$; mean± s.e.m.) is clearly smaller than that between an ISC and an EB (17.68±0.42 $\mu$m$^2$) ($P=8\times10^{-7}$). To verify that this correlation is not simply a consequence of EBs being larger, we compared the distributions of contact areas between ISC-ISC pairs and different subsets of ISC-EB pairs, which included only the pairs where the EB member was smaller than a given size limit (1×, 0.75× and 0.5× the maximum ISC size). The effect of the thresholding on the distribution of cell sizes (measured in terms of the projected areas) is shown in Fig. 4E. As the threshold decreases, the average size of the filtered EBs becomes progressively smaller, until it can no longer be statistically discriminated from the distribution of ISC sizes (for the subset with size limit equal to half the maximum ISC size, $P=0.07$). However, the corresponding contact area distributions of these subsets of ISC-EB pairs are all strikingly similar, with values systematically larger than those of ISC-ISC pairs at a high level of significance (maximum $P$-value=2×10$^{-4}$) (Fig. 4F). This is in good agreement with the fact that the cell size of the pair members and their contact area are not correlated (Fig. S6C,D).

We also wanted to check that the differences in cell-pair types discussed above are not associated with differences in adhesion properties between ISCs and EBs, as could be suggested by the fact that Arm levels are enriched at the contact area between these cell pairs (Fig. 3D). For this, we measured the values of Arm at the boundary of ISC-ISC pairs and ISC-EB pairs. As we cannot distinguish which cell of the pair originates the Arm signal at the boundary (see Materials and Methods), we measured the signal assigned to ISCs in either ISC-ISC pairs or ISC-EB pairs, as an approximation to the strength of adhesion at the boundary of these cell pairs. We found a similar, moderate increment in Arm levels at the cell pair boundary in both ISC-ISC and ISC-EB pairs (Fig. S6E-F), indicating that adhesion properties are not fundamentally different between cell pair types (at least as far as adherens junctions are concerned). This fits well with the observation that the transcriptional profiles of ISCs and EBs are not particularly enriched with genes involved in cell adhesion (Dutta et al., 2015).

Taken together, these results indicate that a smaller contact area correlates with the ISC-ISC pair profile, and this is likely linked to a higher activation threshold of Notch in ISC-ISC pairs, rather than to changes in cell shape, size or adhesion properties in the ISC-EB pairs posterior to EB fate acquisition.

**Updating the model with area variation reproduces fate profile distributions**

This finding gives us biological justification to consider the activation threshold $a$ to be variable in the model (inversely proportional to the contact area), and to test the capacity of the model to produce the observed proportions of fate pairs in our various experimental conditions (Fig. 1E,F). To achieve this, we first generated a large sample of contact area values $A$, from a smooth kernel distribution based on the experimental data (Fig. 4C). To input values from $A$ into the model, we simply used Eqn 7 ($a(Ac/e)A$).

We then analysed the stable states of the model, obtaining the proportions of the three possible fate pairs resulting from $A$, for different values of $b$ and $c$.

In order to compare the fate distribution obtained from the model and the experimental datasets, we use the Kullback-Leibler relative entropy ($H$), which is a dissimilarity measure between two probability distributions (giving the value 0 if the distributions are equal; Kullback and Leibler, 1951). We found an excellent agreement between the proportions of EB-EB, EB-ISC and ISC-ISC pairs observed experimentally, and the distributions from the model for an extended range of values of $b$ and $c$, as indicated by the low values of $H$ between theoretical and experimental distributions (see Fig. S6G,H). Ranges of values for parameters $a$ and $b$ with good fit for each experimental condition are mapped to the model phase diagram in Fig. 4G (upper panel for Notch mutant and wild-type alleles; lower panel for RU486-induced Notch knock-down and the mock treatment), with corresponding pair frequencies (data and model) and parameter values ($c$, average $b$) given in Table 1 (with $H$ values ranging from 1.8×10$^{-4}$ to 1.6×10$^{-2}$). Focusing on the control datasets (Notch wild type and Notch mock knock-down, Fig. 4G), one finds that ISC-ISC pairs occur at the lowest values of contact area (largest values of $a$), in
good agreement with our experimental observations (Fig. 4A,D,F). In this region of parameter space, EB-EB and ISC-EB pairs are found at higher values of contact area ($a$), although we cannot distinguish statistically between the experimentally measured contact areas of these two cell-pair types (Fig. 4A). Importantly, we find that the regions of the parameter space where the model fits each of the mutant and knock-down experimental distributions correspond to parameter changes in line with the nature of the genetic perturbations. First, for all loss-of-function conditions of Notch ($\text{Notch}^{\text{knock-down}}$ and $\text{N55e11/}^+$ heterozygotes), the model can account for the observed pair frequencies with small shifts in the $b$ parameter and more substantial changes in the $a$ parameter (note the very different scale ranges for $a$ and $b$ in Fig. 4G). The shifts towards higher $a$ values can be explained as an increase in the $\text{KN}$ parameter (i.e. lower activation of Notch for a given Delta stimulus; see Eqns 5 and 7), which fits the nature of a loss-of-function condition. In the case of Notch gain of function, represented by the $\text{Nl1N-B/}^+$ hypermorphs, the model can reproduce the resulting pair frequencies by substantially reducing both $a$ (therefore decreasing $\text{KN}$, i.e. more Notch activation for the same Delta stimulus) and $b$ (which can be accounted for as an increase of $\alpha$, the maximal rate of Notch activation; see Eqn 7); again, both notions sit well with the nature of the $\text{l(1)NB}^{\text{ΔN/+}}$ allele (a Delta-dependent hypermorphic allele).
Our experimental observations thus agree with the model predictions, including both gain- and loss-of-function perturbations of Notch signalling. Hence, our results confirm that the contact area between pairs of cells can influence the fate outcome of Notch/Delta signalling in the Drosophila midgut (Fig. 4H), with a small contact area clearly favouring symmetric self-renewal.

### DISCUSSION

We have considered a standard model of Notch/Delta-mediated lateral inhibition (Collier et al., 1996) and have investigated the effect of the trans-activation of Notch by Delta and the inhibition threshold of Delta by Notch signalling (here considered phenomenologically as the dimensionless thresholds $a$ and $b$, respectively) on the dynamics of lateral inhibition for a system of two cells. We find that, provided there is a degree of variability in contact areas between cell pairs, three different signalling states (and therefore fate combinations) can occur under the same conditions. This is a considerable expansion of the model, the use of which has so far mostly been centred on solutions that provide fine-grained (checkerboard) patterns. The model reproduces the signalling outcomes observed in the Drosophila intestine, which translate into differentiation versus self-renewal fates. Thus, our results provide a mechanism whereby ISC fate choice is regulated; inheritance of signalling components could result in variation in the capability of signal transduction in the population. It is interesting to consider that although shortly after division most of the ISC daughter cells display similar levels of Notch and Delta proteins (Ohlstein and Spradling, 2006), endosomes bearing the signalling molecule Sara display an inhomogeneous inheritance pattern (Montagne and González-Gaitán, 2014). It has recently been found that ISC divisions producing enteroendocrine cell precursors do seem to segregate Delta asymmetrically towards the precursor cell (Guo and Ohlstein, 2015), which suggests that ISCs switch between different types of cell division.

Understanding how Notch/Delta signalling results in stochastic cell fate patterns is of particular relevance in adult homeostatic tissues, as Notch signalling controls fate in many types of tissue stem cells (Koch et al., 2013). Moreover, many adult stem cells balance their fate via neutral competition by lateral inhibition between sibling cells. This provides an alternative explanation to the neutral competition of Drosophila adult ISCs, which has been proposed to arise from Notch/Delta-mediated lateral inhibition involving the offspring of non-related ISCs, coinciding in
space (de Navascués et al., 2012) and resolving 20% of the time in symmetric fate. Although the two proposals are compatible with each other, the latter faces the difficulty that ISC/EB nests rarely contain more than two cells (de Navascués et al., 2012). Moreover, we and others have found isolated pairs of ISCs or EBs frequently in the tissue (de Navascués et al., 2012; Goulas et al., 2012). Our model provides a potential explanation of how the offspring of a single ISC (pairs of Notch/Delta signalling cells) may reach a symmetric steady state, leading to symmetric self-renewal or differentiation.

It would be interesting to see how our model translates to a larger group of interacting cells, in particular in light of recent findings in the oesophageal epithelium. There, tissue is maintained by the neutral competition of basal progenitor cells (Doupé et al., 2012), and this competition is heavily influenced by Notch signalling, to the point that alterations in the pathway can lead to the fixation of mutant clones and poise the tissue for tumour initiation (Alcolea et al., 2014).

MATERIALS AND METHODS

The model: lateral inhibition mediated by Notch-Delta interaction

We consider that the rate of Notch activation in a cell is an increasing function of Delta concentration on its neighbour (signalling), and that the rate of Delta expression is a decreasing function of the level of activated Notch in the same cell (inhibition). We represent these interactions by means of a standard mathematical model of Notch/Delta signalling (Collier et al., 1996) we rewrite Eqns 1-4 in dimensionless form as:

\[ \frac{dN_1}{dt} = \alpha f(D_2) - \delta_N N_1, \]  
\[ \frac{dD_1}{dt} = \beta g(N_1) - \delta_D D_1, \]  
\[ \frac{dN_2}{dt} = \alpha f(D_1) - \delta_N N_2 \]  and  
\[ \frac{dD_2}{dt} = \beta g(N_2) - \delta_D D_2. \]  

Here, \( N_1,2 \) represent the levels of Notch activity in cells 1 and 2, and \( D_1,2 \) are the corresponding degradation rates. The production terms for Notch (\( f \)) and Delta (\( g \)) are given by the Hill functions

\[ f(D) = \frac{D}{D + D_{th}} \]  
\[ g(N) = \frac{1}{1 + (N/K_N)^b}, \]  

where the first function represents the signalling effect of Delta on the neighbouring cell, and the second corresponds to the inhibition of Delta expression by activated Notch in the same cell. \( K_N \) is the threshold of Notch activation by neighbouring Delta, \( D_{th} \) is the threshold of Delta inhibition by Notch in the same cell, and the coefficients \( a \) and \( b \) represent the cooperative character of the two aforementioned processes. Similarly to (Collier et al., 1996) we rewrite Eqs 1-4 in dimensionless form as:

\[ \frac{dN_{1,2}}{dt} = \frac{D_{th,1}}{a + b N_{1,2}} - N_{1,2}, \]  
\[ \frac{dD_{1,2}}{dt} = \nu \left( \frac{1}{1 + (N_{1,2}/b)^b} - D_{1,2} \right). \]  

The parameter \( \nu \) is the ratio between the degradation rates of Delta and Notch, \( \delta_D/\delta_N \), \( a \) and \( b \) are the dimensionless thresholds for Notch activation by Delta in the neighbouring cell, and Delta inhibition by Notch in the same cell, respectively:

\[ a = \frac{K_N \delta_N}{\beta} = \frac{c}{A}, \quad b = \frac{K_D \delta_N}{a}. \]  

Steady states and cell fate identification

The system of Eqn 6 has a homogeneous steady state in which Notch and Delta have the same values in the two cells:

\[ N^* = f(D^*), \quad D^* = g(N^*). \]  

This state corresponds to a situation in which both cells in the pair have the same fate. The stability boundary of this homogeneous steady state can be calculated using standard methods (Collier et al., 1996), and is represented by a dotted line in Fig. 2A. Above this line the homogeneous state is stable. Below it, a heterogeneous stable steady state appears in which the values of Notch and Delta are different between the two cells:

\[ N_1^* = f(D_1^*), \quad D_1^* = g(N_1^*), \quad N_2^* = f(D_2^*), \quad D_2^* = g(N_2^*). \]  

In parallel with this classification of steady states, a cell is considered to be Notch positive when the level of Notch surpasses a certain threshold \( N_{th} \) (considered here to be 0.1), and Notch negative in the opposite case. In the case of the Drosophila midgut, a Notch-positive cell would correspond to an EB, and a Notch-negative cell to an ISC. In that way, a homogeneous steady state can represent either an ISC/ISC pair (symmetric Notch positive, blue region in Fig. 2A) or an EB/EB pair (symmetric Notch positive, green region in Fig. 2A). Most heterogeneous steady states, in turn, correspond to an ISC/EB pair (orange region in Fig. 2A), although heterogeneous states in which both values of Notch lie below (or above) the threshold \( N_{th} \) still represent symmetric ISC/ISC (or EB/EB) pairs. This is reflected in Fig. 2A through the difference between the stability boundary (dotted grey line) and the boundaries between the fate-pair domains (shown in colour code).

Dynamic behaviour

We investigate the temporal evolution of the model by solving numerically the system of Eqn 6. For this purpose, we use a finite difference approximation (two-stage Runge-Kutta; LeVeque, 2007). In our calculations, we are considering \( r=h=2 \) and \( \delta_N=\delta_D \), as in previous works (Collier et al., 1996; Sprinzak et al., 2010). Cells are considered initially negative for Notch activation (Ohlstein and Spradling, 2007; Guo and Ohlstein, 2015).

Drosophila culture and strains

Adult flies were raised in standard cornmeal medium, collected daily and maintained in fresh vials with added yeast (food replaced every 24-48 h). Untreated flies were dissected at 4-6 days of age. Mifepristone (RU486)-treated flies were treated as described above and transferred to RU486-containing medium at 3-8 days of age for 3 more days before dissection.

EBs were identified by co-expression of one of two enhancer trap reporters of the undifferentiated cell marker escargot (FlyBase: P{PTT-GG}esgT20212; Quiñones-Coello et al., 2007; P{lacW}esgT000666; Spradling et al., 2012). Embryonic stem cells were maintained in a standard 293T culture and strains were maintained in fresh vials with added yeast (food replaced every 24-48 h). Untreated flies were dissected at 4-6 days of age. Mifepristone (RU486)-treated flies were treated as described above and transferred to RU486-containing medium at 3-8 days of age for 3 more days before dissection.
et al., 1999) and one of two GBE-Su(H) synthetic reporters of Notch transcriptional activity (Bray and Furriols, 2001; de Navascues et al., 2012). ISC's were identified by expression of the esg reporter alone (Micchelli and Perrimon, 2006; Ohlslein and Spradling, 2006).

Delta levels were measured with the Delta^MO1468-GFP^1 transgenic fly (FlyBase: Ml[PT-GFSTF.1]Da^MO1468-GFP^1), which tags all three annotated Delta isoforms with GFP at the endogenous locus (Nagarkar-Jaiswal et al., 2015).

Genetic perturbation of Notch activity was performed: (1) by knockdown with da-Gr (FlyBase: P[da-GSAGAL.A]; Tricorre et al., 2009) and UAS-NotchRNAi (FlyBase: P[w[+mC]=UAS-N dsRNA.P]14E; Presente et al., 2002), and activation with mifepristone (RU486, Sigma), with mock treatment as control; or (2) by using the molecular null allele N^{Δ7} (Kidd et al., 1983) or the Delta-dependent, hypermorphic I^{(I)}N^{Δ} (FlyBase: N^{Δ7}; Lyman and Young, 1993; Brennan et al., 1997), with Oregon R as wild-type control.

**Immunohistofluorescence and imaging**

Immunohistofluorescence was performed essentially as described previously (Bardin et al., 2010) but with a heat-fixation step (Miller et al., 1989). This fixation method was essential for the robust immunodetection of Armadillo (Fang et al., 2016).

Primary antibodies used were: chicken anti-β-galactosidase (Abcam, ab9361, 1:200), rabbit anti-GFP (Abcam, ab6556, 1:200), sheep anti-Notch (Muñoz-Descalzo et al., 2011; 1:1000), anti-Arm (mAb, N2-7A1, 1:50) and anti-DI (mAb, C594.9B, 1:50). The N2-7A1 and C594.9B antibodies were obtained from the Developmental Studies Hybridoma Bank (NICHD and The University of Iowa). Secondary antibodies conjugated with Alexa fluorophores were from Invitrogen (A21203, A21071, A11039 and A11034, 1:500). Of note, immunodetection of GBE-Su(H)-lacZ expression in heat-fixed material was less sensitive than that of GBE-Su(H)-GFP:nls; this might have led to an underestimation of EB numbers, which in any case will have played against our hypotheses.

Confocal stacks were obtained in a Zeiss LSM 710 with an EC Plan-Neofluar 40× oil immersion objective (numerical aperture 1.3). For the quantification of membrane features, voxel sizes were 0.22×0.22×0.6, 0.21×0.21×1 or 0.18×0.18×0.6 (contact area), 0.16×0.16×0.7 µm (distribution of Notch and Armadillo), or 0.14×0.14×1 µm (distribution of Delta), with airy units adjusted so that there was negligible oversampling in Z, or not at all.

**Image analysis**

To measure contact area, stacks were analysed with a combination of ImageJ macros and python scripts, as follows. (1) All esg-GFP+ cells in the z-projection of the stack were manually identified. (2) Expression of the GBE-Su(H)-lacZ reporter was automatically thresholded in 3D to determine its expression status (positive or negative) in every esg+ cell, followed by a step of manual correction. (3) Nests of esg+ cells were automatically extracted, and the Pearson correlation and the Manders overlap). For each window, a normalised, average intensity measurement was obtained by interpolation from average normalized intensity values.

**Distribution along cell perimeter**

In each confocal plane, each membrane pixel position was assigned an angular value relative to the centroid by calculating its tangent arc (∏ depending on the quadrant). Thirty overlapping sliding windows (of 2π/15 rad with half window overlap) were delimited in each plane, and their pixel intensities were normalised and averaged.

**Distribution along the apical-basal axis**

Each cell was sliced in 10 overlapping angular windows (2π/5 rad with half overlap). For each window, a normalised, average intensity measurement was taken per confocal plane (i.e. along the apical-basal axis). Apical-basal positions were normalised from 0 to 1. Intensity data points along the apical-basal axis were obtained by interpolation from average normalized intensity values.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.137950.supplemental

**References**


USA: Oxford University Press.


