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RESEARCH ARTICLE

Redundancy and cooperation in Notch intercellular signaling

Juan C. Luna-Escalante[1,*,‡], Pau Formosa-Jordan[1,§] and Marta Ibañes[1,2,¶]

ABSTRACT

During metazoan development, Notch signaling drives spatially coordinated differentiation by establishing communication between adjacent cells. This occurs through either lateral inhibition, in which adjacent cells acquire distinct fates, or lateral induction, in which all cells become equivalent. Notch signaling is commonly activated by several distinct ligands, each of which drives signaling with a different efficiency upon binding to the Notch receptor of adjacent cells. Moreover, these ligands can also be distinctly regulated by Notch signaling. Under such complex circumstances, the overall spatial coordination becomes elusive. Here, we address this issue through both mathematical and computational analyses. Our results show that when two ligands have distinct efficiencies and compete for the same Notch receptor, they cooperate to drive new signaling states, thereby conferring additional robustness and evolvability to Notch signaling. Counterintuitively, whereas antagonistically regulated ligands cooperate to drive and enhance the response that is expected from the more efficient ligand, equivalently regulated ligands coordinate emergent spatial responses that are dependent on both ligands. Our study highlights the importance of ligand efficiency in multi-ligand scenarios, and can explain previously reported complex phenotypes.

KEY WORDS: Mathematical modeling, Notch signaling, Lateral induction, Lateral inhibition

INTRODUCTION

The Notch signaling pathway, which is conserved across metazoans, is responsible for coordinating cell fate decisions among directly interacting cells (see Artavanis-Tsakonas et al., 1999; Ehebauer et al., 2006; Greenwald and Rubin, 1992 for reviews). This pathway orchestrates the development of different organs, tissues and cells, such as the sensory organs in vertebrates (Chitnis, 1995), the eye imaginal disk in *Drosophila* (Roignant and Treisman, 2009), the pancreas (Apelqvist et al., 1999) and intestine (VanDussen et al., 2012) in mice, and immune cells (Radtke et al., 2004) in humans. Furthermore, the Notch signaling pathway also plays a relevant role in different human diseases and cancer (Mašek and Andersson, 2017; Nowell and Radtke, 2017).

The Notch signaling pathway is involved in the mediation of two distinct coordinated cell fate decisions: lateral inhibition and lateral induction. In lateral inhibition, a cell inhibits adjacent cells from adopting the same cell fate (Cabrera, 1990; Goriely et al., 1991; Heitzler and Simpson, 1991; Simpson, 1990; Sternberg, 1988). This is important during development, for example, so that each specified hair cell in sensory organs is surrounded by supporting cells (Daudet and Lewis, 2005; Neves et al., 2013; Petrovic et al., 2014; Pickles and van Heumen, 2000; Zine et al., 2000), and so that only a few select cells become neurons in vertebrate retinae (Formosa-Jordan et al., 2013; Harris, 1997; Livesey and Cepko, 2001; Marquardt and Gruss, 2002). On the other hand, lateral induction occurs when a cell induces neighboring cells to adopt the same cell fate (Adam et al., 1998; Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010; Neves et al., 2011). This type of decision underlies the specification of prosensory patches during development of the inner ear in vertebrates (Daudet and Lewis, 2005; Hartman et al., 2010; Neves et al., 2011), the differentiation of the lens fibers in rats (Saravanamuthu et al., 2009), muscle differentiation in mice (Manderfield et al., 2012), as well as embryonic endocrine epithelial-mesenchymal transitions and oncogenic transformation (Timmerman et al., 2004).

Notch is a transmembrane receptor that binds to membrane-bound DSL ligands (Delta and Serrate/Jagged/Lag-2 ligands) of neighboring cells. This type of interaction is known as a trans-interaction (see Fortini, 2009; Kopan and Ilagan, 2009 for reviews). After binding, cleavage of the intracellular domain of Notch (hereafter referred to as the signal) translocates to the nucleus where it regulates the transcription of a battery of genes, including proneural repressor genes, which in turn regulate the expression of the DSL molecules. As such, Notch signaling can ultimately regulate the transcription of its own ligands in either a repressive or an inductive manner. For example, by activating the proneural repressor genes, Notch signaling can repress the transcription of Delta in the ommatidial crystal of *Drosophila* (Lubensky et al., 2011), and Delta-like 1 (Dll1) and Jagged2 (Jag2) in the developing inner ear of vertebrates (Kiernan et al., 2001; Neves et al., 2011). In contrast, Jagged1 (Jag1) and Delta-like 4 are Notch ligands that are induced by Notch signaling in the inner ear of vertebrates (Kiernan et al., 2001; Neves et al., 2011) and in angiogenesis (Benedito et al., 2009), respectively.

Our understanding of Notch-mediated lateral communication has typically been conceived through the action of a single type of ligand activating a single type of receptor (Fig. 1). In this case, lateral inhibition arises naturally when Notch signaling represses the ligand (Collier et al., 1996) (Fig. 1C). A cell expressing the ligand trans-activates Notch signaling in an adjacent cell, which, in turn, inhibits the production of this same ligand (Fig. 1C). Equivalent cells that mutually communicate through such lateral inhibition can become distinct upon amplifying small differences early on, and drive a periodic pattern of two cell types within a tissue, as previously shown by mathematical modeling (Collier et al., 1996) (Fig. 1A). These two cell types correspond to two distinct signaling states (Sprinzak et al., 2010): a sending cell (S), which strongly produces the ligand; and an adjacent receiving cell (R), which has

[1]Departament de Física de la Matèria Condensada, Universitat de Barcelona, Barcelona 08028, Spain. [2]Universitat de Barcelona Institute of Complex Systems (UBICS), Universitat de Barcelona, Barcelona 08028, Spain.
*Present address: Department of Developmental and Stem Cell Biology, Institut Pasteur, Paris 75015, France. ‡Present address: Laboratoire de Physique Statistique de l'ENS, Paris 75005, France. §Present address: Sainsbury Laboratory, University of Cambridge, Cambridge CB2 1LR, UK.

¶Author for correspondence (mibanes@ub.edu)

M.I., 0000-0002-7913-7936

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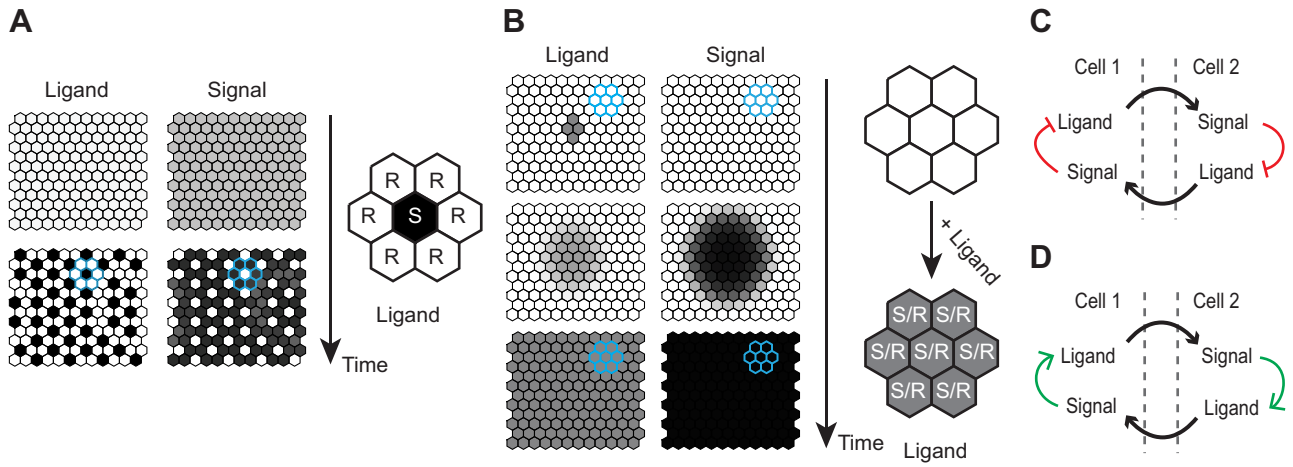

Fig. 1. One ligand-mediated spatial coordination of cell fates, where the regulation of the ligand by Notch signaling dictates the type of lateral communication. (A,C) Lateral inhibition. (B,D) Lateral induction. (A,B, left) Computational ligand and signaling activities in each cell (hexagon) of a tissue are depicted in linear grayscale from white (lowest activity) to black (highest activity). For clarity, the ligand and signaling activities are plotted separately in two tissue layouts, although in reality they correspond to the same one. (A) Pattern formation from equivalent cells for the regulatory interactions in C. (B) Ligand propagation for the regulatory interactions in D. The ligand is transiently and exogenously activated in the four central cells (top), and propagates (middle) to drive the transition of the whole tissue to another state (bottom). (A,B, right) Ligand activity cartoon (grayscale) and signaling states (letters) of the seven cells outlined in blue in the corresponding left panels. Signal-sending cells (S) have ligand activity. Signal-receiving cells (*R*) have high signaling activity. Signal sending and receiving cells (S/R) have both high ligand and signal activity. + Ligand: the transient and local exogenous addition of ligand. Parameter values are r_d=100 and as defined in the Materials and Methods. (C,D) Regulation between the ligand and signal of two adjacent cells (arrows indicate induction and blunt arrows indicate inhibition).

high Notch signaling activity (Fig. 1A). In contrast, lateral induction arises when the ligand activates the signal in adjacent cells causing increased ligand production therein (Fig. 1D). Thus, cells communicating through lateral induction reach the same cell fate and signaling state, acting as both sending and receiving (S/R) cells (Boareto et al., 2015; Daudet and Lewis, 2005; Hartman et al., 2010; Jiménez et al., 2017; Matsuda et al., 2012; Neves et al., 2011; Petrovic et al., 2014) (Fig. 1B).

However, *in vivo*, the scenario is much more complicated, with communication through the Notch signaling pathway depending on numerous ligands and receptors. In this way, the various ligands can be antagonistically or equivalently regulated with respect to one another (Benedito et al., 2009; Gama-Norton et al., 2015; Kiernan et al., 2005; Okigawa et al., 2014; Petrovic et al., 2014; Preuße et al., 2015; Ye et al., 2017; Zine, 2003; Zine et al., 2000). Different Notch ligands typically activate the same canonical pathway such that the final process of lateral communication is the result of the combination of all signals (Fig. S1). Therefore, it is not always easy to predict the overall lateral communication that arises when multiple ligands activate Notch signaling. Along these lines, mathematical modeling has been useful in guiding our understanding of specific situations in which ligands are antagonistically regulated by Notch signaling (Boareto et al., 2015; Petrovic et al., 2014).

A factor to take into account when considering multiple Notch ligands is that each type can drive signaling with a characteristic efficiency (Benedito et al., 2009; Petrovic et al., 2014; Yang et al., 2005). Some ligands are so inefficient (hereafter named weak ligands) that even in high quantities they only weakly activate Notch signaling. For example, Jag1 activates Notch signaling less efficiently than Delta-like 4 (Dll4) in both angiogenesis (Benedito et al., 2009) and mouse haematopoietic stem cell development (Gama-Norton et al., 2015), and less efficiently than Delta-like 1 (Dll1) in chick inner ear development (Petrovic et al., 2014). When Notch signaling is activated by several ligands, differences in signaling efficiencies enable the cell to fine-tune the overall level of Notch signaling. In other words, multiple ligands do not just result in saturated activation. For example, whereas overexpression of a strong ligand can result in an increase in Notch signaling, overexpression of a weak ligand – when a strong ligand is also present – can drive a reduction in Notch signaling if the two ligands bind to the same type of Notch receptor (Petrovic et al., 2014). This type of modulation has been reported in both angiogenesis (Benedito et al., 2009) and inner ear development (Petrovic et al., 2014).

Differences in efficiency between Notch ligands can be attributed to various factors that drive distinct consequences. For example, binding between a ligand and a receptor of the same cell (cis-interaction) can prevent signaling through mutual inactivation (cis-inhibition) (Boareto et al., 2015; Fiuza et al., 2010; Formosa-Jordan and Ibañes, 2014; LeBon et al., 2014; Sakamoto et al., 2002; Sprinzak et al., 2011, 2010). Other much less studied factors that are expected to drive differences in ligand efficiencies are trans-interactions (Yang et al., 2005). For example, the various types of receptor-ligand complexes that are created through trans-interactions are all processed differently and likely lead to distinct levels of Notch signaling (Formosa-Jordan and Ibañes, 2014; Yang et al., 2005). The effect of overexpressing a weak ligand in a cell that also expresses a strong ligand depends on which factor causes the ligand to be weak. If the ligand is weak due to cis-inhibition, then its overexpression makes the cell better at sending but poorer at receiving signals (Sprinzak et al., 2010). In contrast, if the ligand is weak due to poor processing of trans-interactions, then its overexpression can cause the cell to become even worse at sending signals (Petrovic et al., 2014).

Previously, we have studied the effects of Jag1, a Notch-activated ligand that elicits weak signaling through trans-interactions, during avian inner ear development (Petrovic et al., 2014). Mathematical modeling predicted that this type of ligand, which drives lateral induction when acting alone, can facilitate patterning by lateral inhibition when acting together with a strong ligand that is repressed by Notch signaling (Petrovic et al., 2014). In this

Development (2018) 145, dev154807. doi:10.1242/dev.154807

way, Jag1 changes its role: it drives Notch signaling in adjacent cells when acting in isolation, but through competitive inhibition, reduces signaling to adjacent cells when a strong ligand is present. Modeling also predicted that the inefficiency of the weak ligand enables the transition from the prosensory state to the hair cell specification stage through activation of the strong ligand (Petrovic et al., 2014).

As exemplified above, theoretical modeling has been useful for understanding the complexity of Notch signaling. Here, we pinpoint common principles behind overall Notch signaling when several ligands with different efficiencies due to the distinct processing dynamics of trans-interactions (i.e. not those affected by cis-inhibition) are at play. To unveil such common principles, we evaluate a wide variety of scenarios where ligands are either equivalently or antagonistically regulated by Notch signaling, and where ligands share or do not share limiting resources like the Notch receptor. Our results suggest that efficiency differences arising from alternative processing of trans-interactions can make Notch signaling more flexible: not only can signaling responses be enhanced, but through the combination of existing elements, new emergent responses can be created to promote evolvability.

RESULTS

The model

We selected a modeling approach that strongly facilitates the theoretical study of spatially coordinated responses of multiple interacting cells across the parameter space. The tissue is modeled as a two-dimensional array of hexagonal cells (Fig. 1) in which we search for two types of coordinated responses – lateral inhibition and lateral induction. With respect to lateral inhibition, we searched for periodic patterning of distinct cell fates arising from equivalent cells (see linear stability analysis in the Materials and Methods). For outcomes of lateral induction, we chose the most restrictive definition (Matsuda et al., 2012; Petrovic et al., 2014), which states a ligand that is transiently and exogenously activated locally within a tissue propagates and drives the transition of the whole tissue from one stable state to another stable state, both being composed of entirely equivalent cells (i.e. both states are homogeneous and linearly stable under small perturbations) (see linear stability analysis in the Materials and Methods).

Our model is based on the activity dynamics of two ligands (*d* for ligand1 and *z* for ligand2), each of which activates Notch signaling (*s*) in adjacent cells through trans-interactions, and whose production is in turn regulated by this same signaling (see Materials and Methods). For the purpose of studying solely the effect of distinct ligand efficiencies in trans-activation, our model does not include any cis-interactions. Our model, which extends the model of Collier et al. (1996) by including a second ligand, is a simplified version of the model of Petrovic et al. (2014). Thus, in our model, the signaling activity of a cell changes in function of the average activity of each ligand over all adjacent cells (trans-interactions), and the ligand activity changes according to the signaling activity within the same cell (see Materials and Methods, Eqns 1-6).

Without loss of generality, the maximal activities of ligand1 and ligand2, as well as the maximal stationary signaling activity driven by ligand1 in isolation, were set to 1 (see Materials and Methods). To account for differences in trans-interaction ligand efficiencies, we set ligand2 to drive a maximal stationary signaling activity of value ε when it is the only ligand acting. Therefore, when $\varepsilon=1$, the two ligands are equally efficient, whereas when $\varepsilon<1$, ligand2 is a weak ligand and ligand1 is a strong ligand.

We defined the affinity of a ligand for the Notch receptor together with its maximal production rate in one parameter, r_l, hereafter, named trans-interaction strength (see Materials and Methods). Thus, r_d and r_z are the trans-interaction strengths for the two ligands *d* and *z*, respectively. The parameter $\varepsilon_r \equiv r_z/r_d$ defines the relative strength of the trans-interactions mediated by ligand2 (*z*) compared with those mediated by ligand1 (*d*). When we refer to overexpression of ligand1 or ligand2, it means we have set high values for r_d or r_z, respectively.

Trans-activation of Notch signaling depends on both the trans-interaction strengths (r_d, r_z) and the trans-interaction efficiencies of the ligands (1 and ε, respectively, for ligand1 and ligand2) (see Materials and Methods). In addition, we considered two different scenarios of signal trans-activation (Fig. S1): ligands that use independent resources to signal (i.e. a different receptor type each), and ligands that share common resources (i.e. the same type of receptor) (see Materials and Methods, Eqns 5 and 6). These distinct scenarios were modeled such that the presence of a weak ligand makes the cell a better sending cell only if the weak ligand signals through its own specific receptor (see Materials and Methods). In contrast, as in the model of Petrovic et al. (2014), if the weak ligand shares resources with the strong ligand, then it can cause a reduction in signaling to adjacent cells. In such a case, the ligand is known as a partial agonist (see Materials and Methods).

Two antagonistic ligands that signal with different efficiencies and share limited resources cooperate to drive enhanced responses

Previously, we created a model based on a scenario in which a weak Notch-activated ligand shares resources with a strong Notch-repressed ligand (Petrovic et al., 2014) (Fig. 2A). In such a model, we found that the presence of the weak ligand facilitates pattern formation through competitive inhibition with the strong ligand (Petrovic et al., 2014). As expected, the model presented in the current study yields similar results when considering these two types of ligands (Fig. 2B). When only the strong ligand is present, it induces patterning unless overexpressed (Fig. 2B) (Collier et al., 1996). If overexpressed, patterning arises only when the strong ligand acts together with a weak ligand (Fig. 2B). In this way, both ligands cooperate to drive patterning, albeit only when the ligands share resources like the Notch receptor (Fig. S2).

We then analyzed the role of ligand-mediated feedback on pattern formation. As expected, we found that the strong ligand is able to mediate sufficient feedback to drive patterning (red area in Fig. 2B, defined in the Materials and Methods), a phenomenon that also occurs when the weak ligand is also present. Strikingly, the Notch-activated weak ligand (i.e. a ligand that, owing to its inefficiency, would not be able to drive any response by itself) can also mediate sufficient feedback to drive patterning, albeit concurrently with the strong ligand (gray area overlaps the red area, marked as striped in Fig. 2B and Fig. S2, see Materials and Methods).

Analysis of the stationary signal and ligand activities showed that patterning through strong and weak ligands that share resources can drive several distinct signaling states (Fig. 2C). When the weak ligand has a low trans-interaction strength ($\varepsilon_r << 1$, Fig. 2C bottom), cells with a high activity of the strong ligand become surrounded by cells with strong activity of the weak ligand and strong Notch signaling. This is in accordance with ligand expression patterns observed in the inner ear of chick embryos (Fig. 2D), and as previously modeled (Petrovic et al., 2014). While the cells activating the strong ligand are called sending (S) cells, herein we term the surrounding ones blocking and receiving (B/R) cells.

Development (2018) 145, dev154807. doi:10.1242/dev.154807

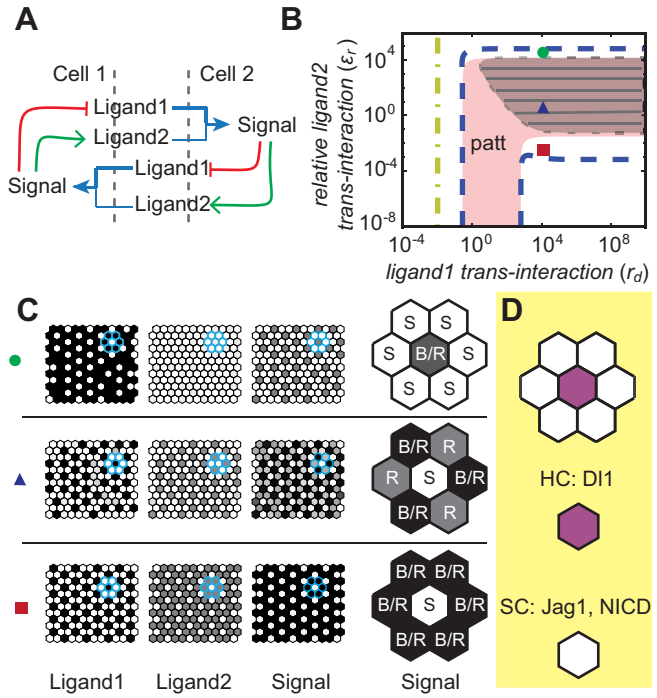

Fig. 2. Periodic patterns of signaling states driven by cooperative antagonistic ligands. (A) Regulatory interactions between ligands that share signaling resources (blue intersecting arrows), where ligand1 is the strong (thick blue line) and ligand2 is the weak (thin blue line) ligand. The strong ligand is repressed by the signal (blunt red arrow), whereas the weak ligand is activated (green arrow). (B) Parameter space region of spontaneous patterning (patt, enclosed by dashed blue lines, see Materials and Methods) for the regulatory interactions in A (ε=0.01). Patterning can be mediated by the strong ligand (red region, see Materials and Methods) or by both ligands acting cooperatively (white within patt, and striped area of superposition of gray and red regions). Colored symbols in the cooperative region indicate points in the parameter space analyzed in C. On the right of the dot-dashed line, the weak ligand is a partial agonist (see Materials and Methods). Spontaneous patterning can arise for high trans-interaction strengths of the strong ligand (r_d large, e.g. overexpressed), but requires the weak ligand (i.e. a range of ε_r values). In all figures, parameter values are as defined in the Materials and Methods. (C, left) Computational activity of the ligands and signal (shown in grayscale in separated tissue layouts) in the steady state for the three cooperative cases denoted by colored symbols in B (circle, ε_r=5 × 10^4; triangle, ε_r=1; square, ε_r=5 × 10^{-3}; all with r_d=10^4). (C, right) Signal activity cartoon (grayscale) and signaling states (letters) of the seven cells outlined in blue. Signal-blocking and -receiving cells (B/R) have sufficient activity of the weak ligand to reduce or prevent signaling to adjacent cells, and have high signal activity. (D) Cartoon of ligand expression and signal activity (NICD) in seven cells mimicking the chick inner ear. Dll1 is the strong ligand and Jag1 is the weak ligand, as reported and modeled by Petrovic et al. (2014). HC, hair cell; SC, supporting cell.

According to our definition, the blocking signaling state arises when there is activity of the weak ligand because it diminishes signaling to adjacent cells by the strong ligand. However, we find that a weak ligand with a strong trans-interaction strength drives distinct patterns of signaling, including signaling with three different states (Fig. 2C). Specifically, S cells can be in contact with both B/R and R cells (Fig. 2C, middle). While R cells exhibit intermediate signaling activities and do not significantly activate the weak ligand, B/R cells have high Notch and weak ligand activities.

In addition, we found that cooperation between weak and strong ligands has dynamic consequences for patterning. For example, patterning arises faster when there is cooperation between antagonistic strong and weak ligands than when there are two equal ligands (e.g. equally strong, both equally inhibited by Notch signaling) that do not share resources (Fig. S3 and Table S1, see Materials and Methods, and supplementary Materials and Methods). Therefore, co-expression of a weak ligand that is activated by Notch signaling can accelerate patterning (Fig. S3A) when compared with co-expression of an equally strong ligand that is repressed by Notch signaling and does not share common resources (Fig. S3B).

Subsequently, we analyzed the opposite scenario in which Notch signaling activates the strong ligand but represses the weak ligand (Fig. 3A). We set the strong ligand so that it could drive lateral induction on its own (Figs 1B, 3B). Exogenous application of this ligand on a few cells within an array of cells with almost no signaling or ligand results in ligand propagation and the sequential transition of all cells to a S/R state (Fig. 1B). In contrast, we set the weak ligand to be very inefficient and unable to drive any response on its own (Fig. 3B). Our analysis shows that when acting together, both ligands cooperate in such a way that the weak ligand facilitates lateral induction and enables transition under any trans-interaction strength or abundance of the strong ligand (Fig. 3B). This requires that the ligands share resources and signal with different efficiencies (Fig. S4). In such a cooperative case, cells can make the transition from a B state, in which high activity of the weak ligand strongly reduces signaling and therefore the strong ligand activity, to an S/R state that exhibits high levels of strong ligand and signaling (Fig. 3C). The reverse transition can also occur and each involves a distinct ligand that is propagated (Fig. S5). For example, when the

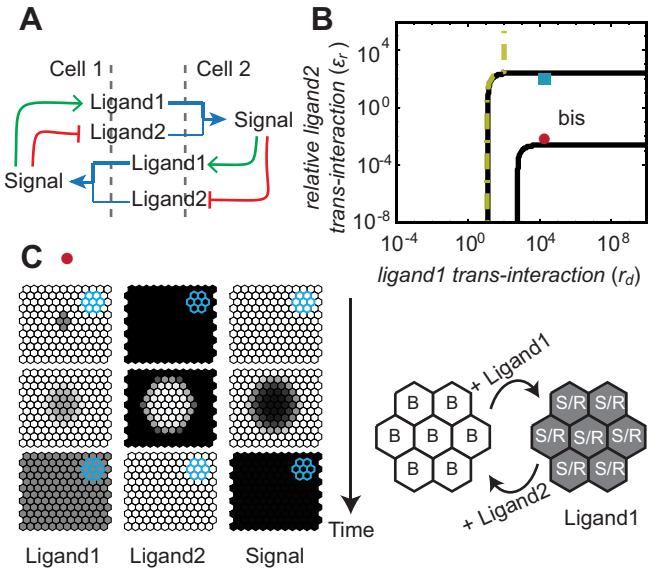

Fig. 3. Antagonistic ligands cooperatively drive ligand propagation. (A) Regulatory interactions between a strong (thick blue line) signal-activated ligand (green arrow) and a weak (thin blue line) signal-inhibited ligand (red blunt arrow). This regulation is the opposite of that depicted in Fig. 2A. Ligands share resources (blue intersecting arrows). (B) Parameter space region where lateral induction with bistability and ligand propagation (bis, enclosed by black continuous lines, see Materials and Methods) takes place for the regulatory interactions in A (ε=0.01). Lateral induction can occur even for high trans-interaction strengths of the strong ligand (r_d large, e.g. overexpressed), but requires the weak ligand (i.e. a range of ε_r values). On the right of the dot-dashed yellow line, the weak ligand is a partial agonist. (C) Ligand and signal activities over time for the parameter values indicated by the red dot in B (ε_r=10^{-2}, and r_d=10^4). The strong ligand is propagated when it is exogenously introduced in a local and transient manner. At relatively high weak-ligand trans-interaction strengths (square in B, ε_r=5 × 10^2), only the weak ligand can propagate, and thereby drives the opposite transition (Fig. S5). At any ε_r, only one ligand can propagate. (C, right) Cartoon of the strong ligand activity (grayscale) and signaling states (letters) of the seven cells outlined in blue.

Development (2018) 145, dev154807. doi:10.1242/dev.154807

trans-interaction strength of the weak ligand is low compared with that of the strong ligand ($\varepsilon_r << 1$), only the strong ligand can propagate and induce the transition from B to S/R (Fig. 3C). This also corresponds to the analogous case in which the weak ligand is absent (Fig. 1B). In contrast, when the weak ligand has a stronger trans-interaction strength ($\varepsilon_r >> 1$), the opposite transition becomes possible through the propagation of the Notch-repressed weak ligand (Fig. S5). This highlights the relevant and active role of the weak ligand in driving an unexpected outcome.

In light of these findings, we decided to evaluate whether the weak ligand could still promote the same responses even when the strong ligand was unable to drive them in isolation. For example, both patterning and ligand propagation between bistable states require strong nonlinearities in ligand dynamics. If the strong ligand does not present such nonlinearities, then it is unable to elicit a coordinated response on its own (i.e. a pattern, if repressed by Notch signaling; or ligand propagation, if activated by Notch signaling). We found that the presence of a very weak antagonistically regulated ligand that is not capable of driving a response on its own, can help promote a coordinated response (Fig. 4). This response corresponds to the type of response that would arise if the strong ligand exhibited more nonlinear dynamics. In addition, the feedback mediated by the weak ligand, in presence of the strong ligand, becomes the only feedback sufficient to drive the patterning response (gray area in Fig. 4B). Thus, two antagonistic ligands, that, when in isolation, cannot drive a response, can drive a response when cooperating together. Importantly, this only occurs if the ligands have distinct efficiencies and share resources.

Altogether, these results show that antagonistic strong and weak ligands that share resources can cooperate to drive responses that are characteristic of the regulation of the strong ligand. This is because by reducing signaling to adjacent cells, the weak ligand effectively mediates the same type of lateral regulation as the antagonistically regulated strong ligand (see Materials and Methods) (Petrovic et al., 2014).

Two equivalent ligands are redundant and drive a common response, but become detrimental upon overexpression

We next studied the case in which the two ligands are analogously regulated (i.e. both are either repressed or activated by Notch signaling) and drive Notch signaling with the same efficiency (i.e. ε=1). When both ligands are present and activate Notch signaling, they become redundant (i.e. the response can also be induced by just one of the two ligands) and drive the same response, independently of whether they share the same resources (Fig. 5A-C and Fig. S6A, B). When both ligands are repressed by Notch signaling they elicit a pattern formation response (Fig. 5B,C), whereas when both are activated by Notch signaling, ligands are propagated and cause cells to transition from a low ligand activity state to a higher ligand activity state (Fig. S6A,B). However, no response arises when either of the two ligands is overexpressed because Notch signaling becomes saturated (Figs S7A,B and S8A,B).

As expected, redundancy does not occur when one ligand is very weak (Fig. 5D,E, Fig. S6C,D). In addition, in such a case, overexpression of the weak ligand has very little effect compared with overexpression of the strong ligand when they do not share resources (Fig. 5D, Fig. S6C). However, when there is a single receptor type (i.e. shared resources), the strong ligand cannot drive a

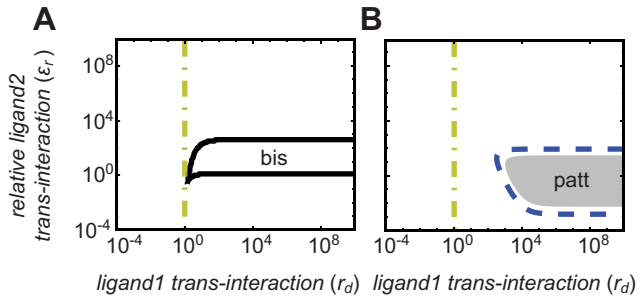

Fig. 4. The cooperation between antagonistic ligands enables a spatial coordination. Parameter space regions showing (A) bistability/ligand propagation (bis) and (B) spontaneous patterning (patt) for the regulatory scenarios described in Figs 3A and 2A, respectively, but with a strong ligand that cannot mediate a response when alone because of low nonlinearity (h_d=1). As in Figs 3 and 2, the weak ligand is too inefficient (ε=0.01) to drive a spatial coordination on its own (i.e. at $\varepsilon_r >> 1$ and $r_d << 1$) despite being highly nonlinear (h_z=4). When present together (within a range of ε_r), a spatial coordination emerges. On the right of the dot-dashed line, the weak ligand is a partial agonist. In B, the weak ligand-mediated feedback is sufficient to drive patterning when the strong ligand is also present (gray region, see Materials and Methods).

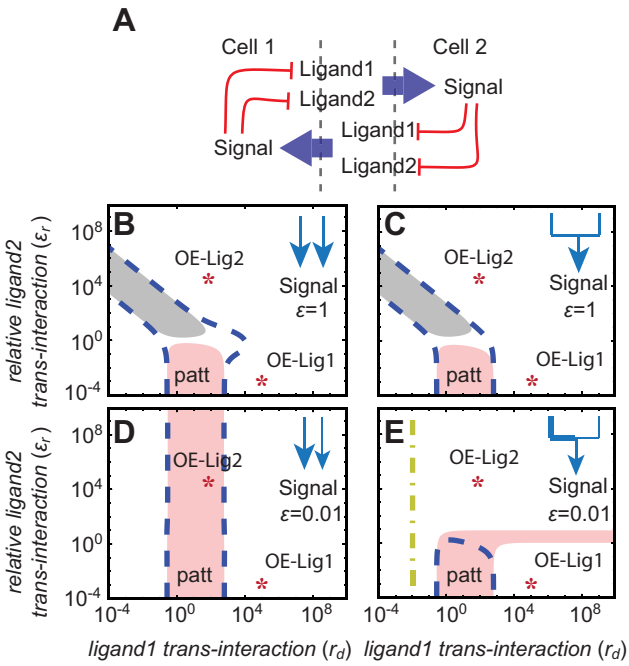

Fig. 5. Equivalent ligands can be redundant and even detrimental when overexpressed. (A) Regulatory interactions between signal-repressed (red blunt arrow) ligands. Blue arrow indicates signal activation by the two ligands. (B-E) Parameter space regions of spontaneous patterning (patt) for the regulatory interactions in A. Four different scenarios of ligand efficiency ε and resource use are represented. Intersecting and parallel arrows in the top right-hand corner of each panel indicate independent and shared signaling resources, respectively. Red and gray regions indicate where the feedback mediated by one ligand (red for the strong and gray for the weak) is sufficient to drive patterning. (B,C) Ligands redundantly drive patterning when they are equally efficient (ε=1). A pattern is not formed when either ligand is overexpressed (OE-Lig1 and OE-Lig2 for the strong and the weak ligand, respectively). (D,E) Ligand2 is a weak (ε=0.01) signaling ligand that does not mediate patterning when expressed in isolation. When both ligands are present and do not share resources (D), patterning mostly depends on the strong ligand. When ligands share resources, however (E), a pattern is not formed when the weak ligand is overexpressed (OE-Lig2). This phenomenon does not occur when ligands use independent resources (i.e. O.E-Lig2 in D is within the patt region). Ligand2 is a partial agonist only when it is weak and shares resources with the strong ligand (region on the right of the dot-dashed line in E). Analysis of phenotypes in the inner ear according to this regulatory scenario together with the action of a third ligand are shown in Fig. S9.

Development (2018) 145, dev154807. doi:10.1242/dev.154807

coordinated response when the weak ligand is overexpressed. This is true when both ligands are either repressed (Fig. 5E) or activated (Fig. S6D) by Notch signaling.

The responses observed in the scenarios in which ligands share common resources can provide explanations for the puzzling phenotypes that arise from mutations of Jag1, Jag2 and Dll1 during the specification and determination of hair cells in the mammalian inner ear (Kiernan et al., 2005; Liu et al., 2013; Zhang et al., 2000; Zine, 2003; Zine et al., 2000) (see Materials and Methods). These three ligands signal through only the Notch1 receptor despite Notch2 also being expressed (Kiernan et al., 2005; Zhang et al., 2000; Zine et al., 2000), and are distinctly regulated by Notch signaling. Whereas Jag1 is induced by Notch signaling, Jag2 and Dll1 are repressed (Daudet and Lewis, 2005; Hartman et al., 2010; Kiernan et al., 2005). It has been shown *in vivo* that Jag1 helps to mediate proper hair cell patterning (Zine et al., 2000). According to both Petrovic et al. (2014) and our current data (Fig. 2B), Jag1 should be a weak ligand. This would allow Jag1 to cooperate with a stronger ligand that is repressed by Notch to drive patterning. Analysis of a three-ligand scenario shows that, for this cooperation to emerge, Jag1 needs to be weaker than just one of the other two ligands (Fig. S9A). It has also been reported that Jag2 mutation disrupts inner hair cell (iHC) patterning, but not as much outer hair cell (oHC) patterning (Kiernan et al., 2005; Zhang et al., 2000). Our results suggest that Jag2 and Dll1 are similarly strong ligands in oHCs (albeit not necessarily equally strong), acting in a redundant manner (Fig. S9A), whereas Jag2 is stronger than Dll1 in iHCs, being required for patterning (Fig. S9B). Mutation of the glycosyltransferase lunatic fringe (Lfng) rescues the Jag2 mutant phenotype, suggesting that Lfng suppresses signaling by Dll1 (Zhang et al., 2000). In agreement, our results support that this rescue is to be expected when Lfng partially decreases Dll1 efficiency during iHC patterning (Fig. S9C) (see also supplementary Materials and Methods for further details). This partial change in efficiency would require that Notch signaling by Dll1 is altered after ligand-receptor binding (with no need for ligand-receptor affinity changes).

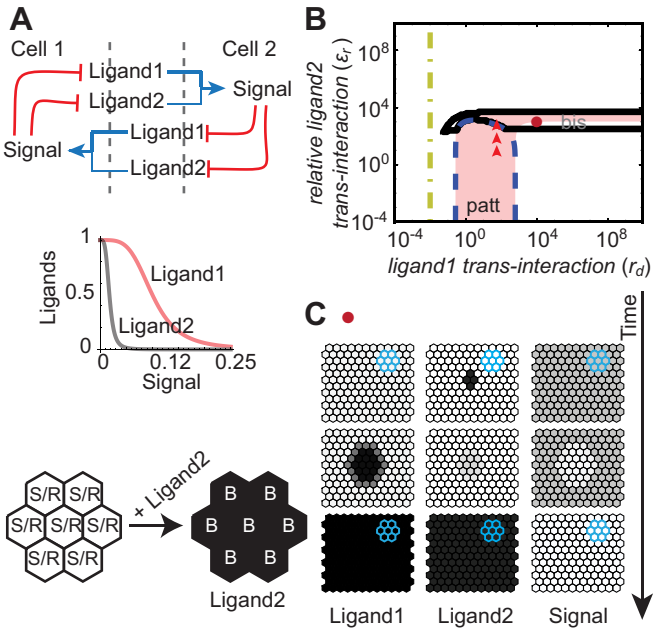

Fig. 6. Two Notch-repressed ligands can drive ligand propagation. (A) Regulatory interactions between a strong (thick blue line) and a weak (thin blue line, ε=0.01) ligand that induce signaling through shared resources (intersecting blue arrows) and are both repressed by Notch signaling (blunt red arrows). The weak ligand (ligand2) is repressed under lower signaling activity than the strong one (ligand1) (bottom, b_d=10^4, b_z=10^7). (B) Parameter space regions of spontaneous patterning (patt) and bistability/ligand propagation (bis) in the regulatory scenario depicted in A. When alone, the strong ligand drives patterning (patt). When present together with the weak ligand, lateral induction (bis) arises. Arrowheads indicate the transition between patt and bis upon changes in the trans-interaction of the weak ligand. In the red region, the strong ligand-mediated feedback is sufficient for patterning. On the right of the dot-dashed line, the weak ligand is a partial agonist. (C) Ligand and signal activities upon local exogenous introduction of the weak ligand in the four central cells, for r_d=10^4 and ε_r=10^3 (red dot in B). (C, left) Cartoon of the weak ligand activity (grayscale) and signaling states (letters) of the seven cells outlined in blue. Transition occurs from the S/R to the B state upon local exogenous induction of the weak ligand and its propagation. In the B state, activity levels of both ligands are high and the Notch signaling is blocked by the weak ligand.

Together, a strong and a weak ligand that are equivalently regulated can drive new emergent responses

The disruption that overexpression of a weak ligand causes when sharing resources with a strong equally regulated ligand is not due to signal saturation, but rather because the weak ligand opposes the actions of the strong ligand (Fig. 5E, Fig. S6D, see Materials and Methods). For example, an overexpressed weak ligand that (just like the strong ligand) is repressed by Notch signaling mediates its own induction in adjacent cells and thereby impedes patterning (Fig. 5E, see Materials and Methods). Conversely, when both ligands are activated by Notch signaling, the weak ligand disrupts ligand propagation via a type of lateral inhibition (gray region in Fig. S6D). We reasoned that there must be a regime in which these kinds of antagonistic lateral regulations are functional.

Although a very weak ligand that is repressed by Notch signaling cannot drive any relevant response on its own, we found that it can mediate fully functional lateral induction when acting with a stronger ligand that is also repressed by Notch (Fig. 6A,B). This functional lateral induction corresponds to a propagation of the weak ligand and the transition of all cells from one stable state to another stable state (Fig. 6C). This response requires that the weak ligand becomes repressed under lower signaling activity than the stronger ligand (i.e. $1/b_z<1/b_d$), thereby enabling mutual lateral induction between adjacent cells (Fig. 6A, Fig. S10). Such ligand propagation is an emergent response that cannot be driven by either ligand expressed in isolation, and is antagonistic to the expected response based on the regulation of each single ligand by Notch signaling.

In this kind of response, cells are simultaneously S/R cells and can transition to a B state through propagation of the weak ligand. In the S/R state, signal activity is sufficiently high to completely repress the weak ligand, but still low enough to only partially repress the strong ligand (Fig. 6C). After ligand propagation, signaling drops and repression of the two ligands is relieved. This corresponds to a B state because the weak ligand causes the neighboring cells to have a lower signaling activity compared with if only the strong ligand was expressed. During this transition, the signal decreases as the ligand propagates, and the transition does not occur if the strong ligand is locally increased. Thus, this response is mainly mediated by the weak ligand, because despite the presence of the strong ligand being a requirement, only the induction of the weak ligand (ligand2) drives this transition.

A robust emergent response also occurs when both the strong and weak ligands are activated by Notch signaling and share resources (Fig. 7A). In this case, weak ligand-mediated salt-and-pepper patterning arises (Fig. 7B,C). The weak ligand mediates an effective circuit of lateral inhibition that in turn drives patterning when the

Development (2018) 145, dev154807. doi:10.1242/dev.154807

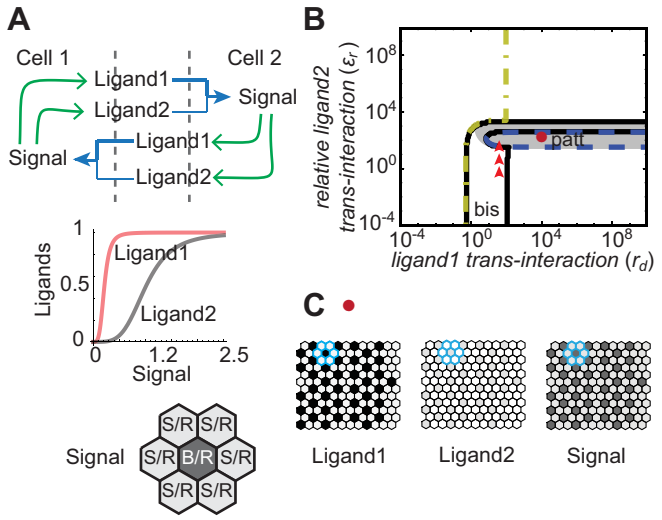

Fig. 7. Two Notch-activated ligands can drive periodic patterning. (A) Regulatory interactions between a strong (thick blue line) and a weak (thin blue line, ε=0.01) ligand that induce signaling through shared resources (intersecting blue arrows) and are both activated by Notch signaling (green arrows). The strong ligand (ligand1) is activated at a lower signal activity than the weak ligand (ligand2) (bottom, b_d=500, b_z=1). (B) Parameter space region of spontaneous patterning (patt) and bistability with ligand propagation (bis) for the regulatory scenario depicted in A. When alone, the strong ligand drives propagation upon exogenous application (bis). When both ligands are present, patterning (patt) arises. Arrowheads indicate the transition between patt and bis upon changes of the weak ligand trans-interaction. On the right of the dot-dashed line, the weak ligand is a partial agonist. The feedback mediated by the weak ligand is sufficient to drive patterning within the gray area. (C) Stationary ligand and signal activities (grayscale) for r_d=10^4 and ε_r=10^2 (circle in B). (C, left) Signal activity cartoon (grayscale) and signaling states (letters) of the seven cells outlined in blue. Blocking and receiving (B/R) cells have a high signaling activity that activates both ligands and accordingly drives reduced, but strong enough, signaling in adjacent cells (S/R).

strong ligand is activated at a lower signal activity than the weak ligand (Fig. 7A, Fig. S10B). This emergent response is characterized by two distinct signaling states in which B/R cells are surrounded by S/R cells with low (but not null) signaling and ligands levels (Fig. 7C). The B/R cells are receiving as they have a high signaling activity that activates both ligands, and are blocking because they express the weak ligand and hence drive lower signaling in neighboring cells. In comparison, the S/R cells are sending because they drive signaling in adjacent cells (i.e. in the B/R cells) through low but sufficient activity of the strong ligand, and are receiving because their signal activity is sufficiently high to activate the strong ligand but not the weak one.

These results demonstrate that robust emergent responses can arise when two ligands are equivalently regulated by Notch signaling. For this to occur, however, both ligands – one being strong and the other weak – need to be present as well as share the receptor. Furthermore, each ligand must also be regulated by Notch signaling at different signaling thresholds, and the dynamics of the weak ligand be characteristically nonlinear (Fig. S10C-F). In such a scenario, the emergent response is opposite to the response driven by the strong ligand when acting alone. Thus, this response is unexpected when considering the type of regulation that Notch signaling mediates on the two ligands.

DISCUSSION

Single ligand-mediated responses have been thoroughly computationally addressed, and it is well known that these responses can be highly dependent on how Notch signaling regulates the production of the ligand (Collier et al., 1996; Matsuda et al., 2012, 2015; Petrovic et al., 2014; Wearing et al., 2000; Webb and Owen, 2004). However, when additional regulations are taken into account, such as receptor activation by Notch signaling as in *C. elegans* development (Wilkinson et al., 1994), elaborate responses can arise from a sole ligand. For example, theoretical modeling predicts that periodic patterns can emerge when Notch signaling activates both the ligand and the receptor (Owen et al., 2000). Commonly, various different ligands are expressed within tissues and activate Notch signaling at the same time (Benedito et al., 2009; Gama-Norton et al., 2015; Kiernan et al., 2005; Petrovic et al., 2014; Preuße et al., 2015). Owing to nonlinear coupling between the regulatory circuits mediated by each ligand, mathematical modeling becomes a powerful tool for addressing what functional response can be expected when two ligands are co-expressed.

We chose a minimal phenomenological description that, albeit simple, can provide insights into experimental data as previously shown for similar approaches (Cohen et al., 2010; Petrovic et al., 2014). We focused on two main features. First, ligands can activate Notch signaling with different trans-activation efficiencies. Second, ligands share resources to signal (i.e. there is only one Notch receptor type) or not (i.e. there are as many receptor types as ligands). Additional complexities, such as receptor activation by Notch signaling or cis-inhibition, were not included. Our results show redundancy and cooperation in the multi-ligand scenario (Table 1, Fig. 8). Ligands that exhibit different efficiencies and share resources can cooperate. Cooperation requires the dynamics of the weak ligand to be nonlinear. Cooperation between ligands antagonistically regulated by Notch enables the weak ligand to enhance and even drive the type of coordinated response that is expected from Notch regulation of the strong ligand. In contrast, similarly regulated ligands can cooperate to drive new emergent tissue responses. For this to happen, the ligands must be similarly regulated by Notch signaling yet at different activity thresholds. Otherwise, equivalently regulated ligands can be redundant and even detrimental to one another.

We exemplified that redundancy, together with enhancement, might be able to explain the distinct differentiation phenotypes observed in the cochlea of the mammalian inner ear (Fig. S9). Moreover, our results on emergent responses (Fig. 7A,B) may apply to angiogenesis. In this process, two cell types (tips and stalks) arise and two Notch ligands, one strong (Dll4) and one weak (Jag1), have opposing roles (Benedito et al., 2009). Through ligand Dll4-mediated signaling, tip cells strongly activate Notch signaling, which suppresses the tip phenotype, in adjacent (stalk) cells (Benedito et al., 2009). Jag1 is expressed in stalk cells and competes with Dll4, reducing Notch signaling (Benedito et al., 2009). Dll4 is activated by Notch signaling, raising the issue of how tip/stalk selection arises. Our analysis suggests that the selection can be mediated by Notch signaling if Jag1 is activated by Notch (Pedrosa et al., 2015) at a higher Notch signaling activity than Dll4. In this scenario, the selection is an emergent response that requires both Jag1 and a stronger ligand (Dll4) (Fig. 7A,B). This could be consistent with the altered selection phenotype found when either ligand is mutated or when Lfng, which can enhance differences in efficiency between the ligands, is mutated (Benedito et al., 2009).

To account for signaling states that arise when several ligands are present and share resources but signal with different efficiency, we have introduced a blocking state (B) in which the weak ligand

Development (2018) 145, dev154807. doi:10.1242/dev.154807

Table 1. Spatial coordination (periodic pattern or ligand propagation) when one or two Notch ligands are co-expressed, as obtained from Eqns 1-6

Coordination	Efficiency	Regulation*	Resources‡	Comment
Pattern (e.g. Fig. 1A)	Strong	1 Repressed	-	Collier et al. (1996)
Pattern (e.g. Fig. 5B)	Equally strong	2 Repressed	Independent	Redundancy
Pattern (e.g. Fig. 5C)	Equally strong	2 Repressed	Shared	Redundancy
Pattern (e.g. Fig. 5D)	Weak and strong	2 Repressed	Independent	Weak ligand irrelevant
Pattern (e.g. Fig. 5E)	Weak and strong	2 Repressed	Shared	Weak ligand detrimental
Pattern (e.g. Figs 2 and 4B)	Weak and strong	2, Weak activated, strong repressed	Shared	Enhancement Petrovic et al. (2014)
Pattern (e.g. Fig. 7)	Weak and strong	2 Activated	Shared	Emergent
Propagation (e.g. Fig. 1B)	Strong	1 Activated	-	Matsuda et al. (2012)
Propagation (e.g. Fig. S6A)	Equally strong	2 Activated	Independent	Redundancy
Propagation (e.g. Fig. S6B)	Equally strong	2 Activated	Shared	Redundancy
Propagation (e.g. Fig. S6C)	Weak and strong	2 Activated	Independent	Weak ligand irrelevant
Propagation (e.g. Fig. S6D)	Weak and strong	2 Activated	Shared	Weak ligand detrimental
Propagation (e.g. Figs 3 and 4A)	Weak and strong	2, Weak repressed, strong activated	Shared	Enhancement
Propagation (e.g. Fig. 6)	Weak and strong	2 Repressed	Shared	Emergent

*Number of ligands and how Notch signaling regulates each one.
‡Whether the ligands signal through the same (shared) or distinct (independent) type of receptors.

diminishes signaling to adjacent cells. In this way, this state can describe the trans-inhibitory effect that can arise when different Notch ligands are at play (Benedito et al., 2009; Gama-Norton et al., 2015; Petrovic et al., 2014).

It is well known that the same regulatory interactions can drive distinct spatially coordinated outcomes. This can happen at different strengths of the interactions (i.e. different parameter values) or even at the same strengths (Corson et al., 2017; Lubensky et al., 2011; Palau-Ortin et al., 2015). A recent study focused on regulatory interactions that drive different coordinated responses depending on the basal level of activation of one element (Jiménez et al., 2017). It was found that this system does not necessarily decouple into two parts with each part performing one of the two coordinated responses, but, rather, one of the coordinated responses requires all the components (Jiménez et al., 2017). Our emergent states and enhanced responses fall into this category because they require all components and cannot be decoupled into submodules.

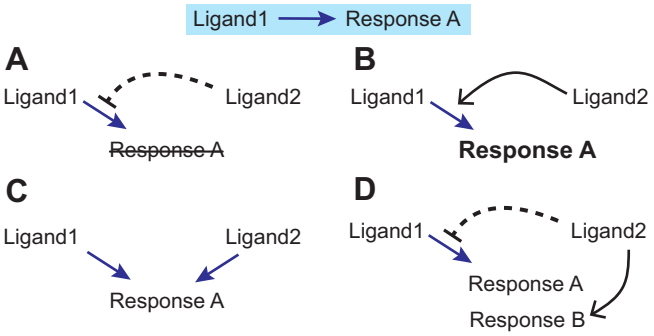

Fig. 8. The effect of two Notch-regulated ligands when they share signaling resources. The response mediated by a strong ligand (ligand1) – whether it be periodic patterning or ligand propagation (response A) – can be: (A) disrupted by a second ligand (ligand2, blunt arrow) when this ligand is overexpressed and equally regulated by Notch signaling (this effect is independent of the relative signaling efficiency of ligand2); (B) enhanced by ligand2 when ligands are antagonistically Notch regulated; or (C) redundantly driven by ligand2 when ligand2 is as efficient as ligand1 and equally regulated by Notch. (D) When both ligands are activated or inhibited by Notch signaling, and ligand2 is weak, a different emergent response can arise (response B). In this scenario, different responses are possible under different activity levels of ligand2.

During the development of vertebrate neural-sensory organs, there are transitions from lateral induction to lateral inhibition (Daudet and Lewis, 2005; Hartman et al., 2010; Millimaki et al., 2007; Petrovic et al., 2014; Zine, 2003). To begin with, a group of cells specifies a prosensory domain by lateral induction. Then, through lateral inhibition, some cells differentiate into sensory cells while the others differentiate into supporting tissue. From the classical paradigm of one ligand-mediated lateral regulation, it is expected that these transitions can only occur via the sequential non-overlapping action of single antagonistic ligands. In other words, first a ligand activated by Notch drives lateral induction, then after it disappears, a ligand repressed by Notch emerges and drives lateral inhibition. However, it has been reported that these transitions can also involve the co-expression of multiple ligands (Daudet and Lewis, 2005; Petrovic et al., 2014; Zine, 2003). For example, we have previously shown that this type of transition can occur through the activation of a stronger, Notch-repressed ligand on tissues that also express a weak, Notch-activated ligand (Petrovic et al., 2014). Here, our current results suggest two additional ways in which transitions between lateral induction and lateral inhibition can occur: (1) by modulating the strength of trans-interactions of two co-expressed ligands that are equally regulated by Notch, share common signaling resources and signal with different efficiencies (arrowheads in Figs. 6B, 7B); and (2) by modulating the relative thresholds of ligand regulation (arrowheads in Fig. S10A,B).

In general, animals have more types of ligands than receptors, and as such when the different types of ligands and receptors interact, they can signal with different strengths (Bray, 2006; D'Souza et al., 2010; Kopan and Ilagan, 2009; Lendahl, 1998). In addition, this fact increases the likelihood that multiple ligands compete for common receptors. Here, our theoretical results suggest that when ligands compete for signaling, existing responses can be enhanced and new functional emergent responses can even arise. Furthermore, these emergent responses are inconsistent with the responses that arise in the absence of competition. Thus, as competition between ligands can drive new responses by merely combining already existing elements, we propose that competition confers both a robustness and evolvability to Notch signaling. Ultimately, we expect that our theoretical analyses will help envisage future experiments and quantifications for unraveling the effects of distinct ligands of Notch signaling.

Development (2018) 145, dev154807. doi:10.1242/dev.154807

MATERIALS AND METHODS

Model equations

The non-dimensional dynamics of the activities in any cell i of the tissue read

$$\frac{dl_i}{dt} = v_l(f_l(s_i) - l_i) \quad (1)$$

$$\frac{ds_i}{dt} = f_s(\langle d_i\rangle, \langle z_i\rangle) - s_i\,, \quad (2)$$

where t denotes time and Eqn 1 stands for the dynamics of a generic ligand activity l, which is substituted by d for the ligand1 and by z for the ligand2. The ligands and signal activities change according to a production term that is described by function $f_x(y)$ [or $f_x(y1,\ y2)$] and a linear degradation term. The function $f_x(y)$ [or $f_x(y1, y2)$] sets the regulation of the activity of x by y (or by $y1$ and $y2$). $\langle l_i\rangle \equiv (1/\omega)\sum_{j\in nn(i)} l_j$ is the average ligand activity in the ω=6 cells adjacent to cell i ($nn(i)$) in a two-dimensional array of perfect regular hexagonal cells. v_l is the ratio of the characteristic time scale dynamics of the signal over that of the ligand l. For simplicity, we assumed that this ratio is the same for both ligands (v_l=v for all l).

The regulation of the production of ligand activity by Notch signaling, $f_l(s_i)$, was set as an increasing $f_l \equiv f_+$ (s_i) or as a decreasing $f_l \equiv f_-$ (s_i) Hill function when the ligand is induced and when it is repressed, respectively:

$$f_l = f_+ \equiv \delta_l + (1-\delta_l)\frac{b_l s_i^{h_l}}{1 + b_l s_i^{h_l}} \quad (3)$$

$$f_l = f_- \equiv \frac{1}{1 + b_l s_i^{h_l}}\,. \quad (4)$$

In both cases, b_l^{-1/h_l} is the signal activity threshold for regulation of the ligand activity and h_l stands for the effective cooperativity of the process, which in this model is simplified to a single regulatory step. δ_l stands for the basal constant production of the ligand with respect to the maximal production (of value 1).

For signal production, $f_s(\langle d_i\rangle, \langle z_i\rangle)$, we used Michaelis-Menten regulation to account for graded signal trans-activation similar to previous reported experimental data (Sprinzak et al., 2010). When ligands share common limiting resources (i.e. there is only one receptor type and either ligand can bind to it to drive signaling) then $f_s \equiv f_{s,N}(\langle d_i\rangle, \langle z_i\rangle)$ with (Petrovic et al., 2014)

$$f_{s,N}(\langle d_i\rangle, \langle z_i\rangle) = \frac{r_d\langle d_i\rangle + \varepsilon\varepsilon_r r_d\langle z_i\rangle}{1 + r_d\langle d_i\rangle + \varepsilon_r r_d\langle z_i\rangle}\,. \quad (5)$$

When cells do not share common limiting resources, then $f_s \equiv f_{s,NN}$, as

$$f_{s,NN}(\langle d_i\rangle, \langle z_i\rangle) = \frac{r_d\langle d_i\rangle}{1 + r_d\langle d_i\rangle} + \varepsilon\frac{\varepsilon_r r_d\langle z_i\rangle}{1 + \varepsilon_r r_d\langle z_i\rangle}\,. \quad (6)$$

The trans-interaction strengths, r_d and r_z, are expected to depend on the affinity for Notch receptors and are proportional to the maximal production rates of ligands because d and z are normalized to maximal value being 1 (Formosa-Jordan and Ibañes, 2014). The relevant activity of each ligand is thus $r_d d$ and $r_z z$. Yet the activities of ligands shown in grayscale in the figures correspond to d and z.

For most of the presented results, unless otherwise stated, we used v=v_l=1 for both ligands, h_z=h_d=4 and δ_l=0.0001. We set b_l=1 (b_l=10^4) when the ligand is activated (repressed) by the signal to obtain ligand propagation (patterning) for each ligand acting in isolation.

To study the outcomes across the parameter space, we chose to perform linear stability analysis (LSA) of the homogeneous solutions, as described in the Materials and Methods subsections on LSA. This enables a vast analysis of the parameter space. Through LSA, we searched for those parameter space regions where there is a homogeneous stationary solution that is unstable with respect to inhomogeneous perturbations and a pattern can be expected to emerge (denoted as *patt* region and enclosed by blue dashed lines). We also evaluated the parameter space regions where there are two homogeneous states that are linearly stable (denoted as *bis* region and enclosed by black solid lines). These regions can sustain ligand propagation (i.e. the lateral induction phenotype). Numerical integration of the dynamics (see Materials and Methods in subsection 'Programs and simulation details') at specific parameter values was carried out to compute the ligand and signal activities over time of cells (depicted in figures with grayscale in hexagonal arrays) and to corroborate the LSA results (see supplementary Materials and Methods for further details).

Stationary homogeneous states

We used custom-made programs (C language, available upon request) to determine the homogeneous steady states of Eqns 1 and 2 [(ds/dt)=(dl/dt)=0, taking l=d for ligand1 and l=z for ligand2]. We drop the subindexes i given the homogeneity of the steady states. Implementing the bisection method for root finding, roots were computed for each case described above, i.e. ligands activated and/or repressed by the signal and ligands sharing or not common resources. Hereafter, the stationary solution for any variable x is named as x_0. On these homogeneous solutions, we performed linear stability analysis as described below.

Weak ligand that reduces signaling (partial agonist)

We determined the parameter regions where the weak signaling ligand, ligand2 (z), reduces the signaling in the stationary homogeneous state (region bounded on the left by a dot-dashed yellow line in the different parameter spaces diagrams shown in figures) by using the definition introduced by Formosa-Jordan and Ibañes (2014) and Petrovic et al. (2014): $R_{s,\langle z\rangle} = ((\partial/\partial\langle z\rangle)(\mathrm{d}s/\mathrm{d}t))|_{d_0,z_0} < 0$, where d_0 and z_0 are the non-dimensional values of ligand1 and ligand2 activities at the homogeneous steady state. This condition holds when, in the homogeneous state, the signaling activity in a cell is reduced upon the increase of ligand2 activity in all its surrounding cells. From $R_{s,\langle z\rangle}$ evaluated when ligands share common resources (Eqn 5), the above criterion can be straightforwardly rewritten as

$$\frac{r_d d_0}{1 + r_d d_0} > \varepsilon\,. \quad (7)$$

This expression indicates that a necessary condition for ligand2 to reduce signaling is to be a weak ligand (i.e. ε<1). It can be readily checked that when ligands do not share resources (Eqn 6). $R_{s,\langle z\rangle}$ is always positive, and hence ligand2 never reduces signaling. In case of multiple stable steady states, the role of ligand2 shown in the phase diagrams (dot-dashed yellow line) was calculated in the homogeneous stable state with low levels of the two ligands (Figs 6 and 7) and in the homogeneous state with low levels of ligand2 and high levels of ligand1 (Fig. 3A).

Effective lateral regulation

We defined $R_{x,y}$ as the partial derivative of the dynamics of species x with respect to species y, evaluated at the homogeneous stationary state. Hence, the sign of $R_{l,s}R_{s,\langle l\rangle}$ is used to determine the effective lateral regulation that the average ligand levels present in the neighborhood of a cell ($\langle l\rangle$) mediates on the ligand activity in such a cell, evaluated in the homogeneous state. In particular, it evaluates whether ligand activity effectively inhibits ($R_{l,s}R_{s,\langle l\rangle}$<0) or induces ($R_{l,s}R_{s,\langle l\rangle}$>0) the ligand activity in adjacent cells. According to dynamics in Eqns 1 and 2, these terms are $R_{l,s}R_{s,\langle l\rangle} = v_l(\partial f_l(s)/\partial s)(\partial f_s(\langle l\rangle)/\partial\langle l\rangle)$ evaluated in the homogeneous state. When there is only one ligand, $R_{s,\langle l\rangle} = (\partial f_s(\langle l\rangle)/\partial\langle l\rangle) > 0$ is always fulfilled, and hence the type of lateral regulation a ligand mediates is dependent only on how the signal regulates the ligand activity (i.e. on the sign of $R_{l,s} = v_l(\partial f_l(s)/\partial s)$). The same happens if there are two ligands that do not share resources, as both ligands always effectively activate the signaling ($R_{s,\langle d\rangle} = (\partial f_{s,NN}(\langle d\rangle,\langle z\rangle)/\partial\langle d\rangle) > 0$, $R_{s,\langle z\rangle} = (\partial f_{s,NN}(\langle d\rangle,\langle z\rangle)/\partial\langle z\rangle) > 0$). In contrast, when there are two ligands signaling differently and sharing limited resources, the weak ligand can exhibit a partial agonist behavior, as described in the previous subsection, so that the condition $R_{s,\langle l\rangle}$<0 would be fulfilled. Therefore, the sign of $R_{l,s}R_{s,\langle l\rangle}$ for a partial agonist ligand is opposed to the one it mediates when acting in isolation (i.e. when $R_{s,\langle l\rangle}$>0). In other words, the partial agonist mediates a lateral regulation of its ligand activity that is in opposition to the one it mediates when acting in isolation.

Development (2018) 145, dev154807. doi:10.1242/dev.154807

Linear stability analysis

We performed linear stability analysis (LSA) (Murray, 2003) of each stationary homogeneous solution for a perfect two-dimensional hexagonal array with periodic boundary conditions as described by Formosa-Jordan and Ibañes (2009, 2014) and Petrovic et al. (2014). We evaluated the stability of the steady state to homogeneous and inhomogeneous perturbations and identified two opposing regimes described next. For two ligands, the sufficient and necessary condition for linear stability of the homogeneous solutions [i.e. Re(λ_Ω)< 0, where λ_Ω is the largest eigenvalue of the linearized system] reads

$$-\frac{1}{2}(1+v)+ \\ +\frac{1}{2}\sqrt{(1+v)^2+4(\Omega_{p,q}(R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s})-v)} \\ <0, \tag{8}$$

where we assumed v_l=v for both l ligands. $\Omega_{p,q}$ arises from the cell-to-cell coupling and reads $\Omega_{p,q}=\frac{1}{3}(\cos(2\pi p)+\cos(2\pi q)+\cos(2\pi(p-q)))$ $\in[-0.5, 1]$, with p and q referring to the fastest growing modes in the case of $Re(\Omega_{p,q})$>0. $R_{x,y}$ is the partial derivative of the dynamics of species x with respect to y [hence, $R_{x,y}=v_x(\partial f_x(y)/\partial y)|_{hss}$, with v_s=1] evaluated at the homogeneous steady state (*hss*). Homogeneous perturbations in space would correspond to $\Omega_{p,q}$=1, whereas inhomogeneous perturbations would involve $-0.5\leq\Omega_{p,q}<1$. The violation of the condition in Eqn 8 tells us that the fastest growing mode is $\Omega_{p,q}=-0.5$ when $(R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s})<0$, which gives the typical lateral inhibition periodicity, whereas the fastest growing mode would be $\Omega_{p,q}$=1 if $(R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s})>0$. Hence, to study linear stability across the parameter space to inhomogeneous and homogeneous perturbations, we set $\Omega_{p,q}=-0.5$ and $\Omega_{p,q}$=1, respectively. Notice that Eqn 8 depends on the lateral regulation that each ligand mediates on its activity $R_{l,s}R_{s,\langle l\rangle}$, i.e. on the strength and type of feedback each ligand drives (Collier et al., 1996).

LSA: linearly unstable homogeneous stationary solutions – patterning

According to the LSA described on a perfect array of hexagonal cells, the homogeneous stationary state is linearly unstable with respect to the inhomogeneous perturbation $\Omega_{p,q}=-0.5$ in the case of having two ligands (assuming that v_l=v) when

$$R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s}<-2. \tag{9}$$

Patterning may then arise. In the figures, the parameter space region in which this condition is fulfilled is enclosed by a blue dashed line and denominated as the patt region. Regions where patterning happens through non-linear instabilities are expected to be larger (Formosa-Jordan and Ibañes, 2009, 2014). The left-hand side of this condition is composed of two additive terms, each of which is the strength of the lateral regulation or feedback mediated by each ligand on itself (Collier et al., 1996). Regions where $R_{s,\langle d\rangle}R_{d,s}\leq-2$ hold are depicted in red and defined for simplicity as the regions where the ligand1 mediates a strong enough feedback to drive patterning. The analogous region for ligand2 ($R_{z,s}R_{s,\langle z\rangle}\leq-2$) is depicted in gray (the term $R_{z,s}R_{s,\langle z\rangle}$ is proportional to ε and hence the efficiency of signaling must be strong enough to linearly destabilize the homogeneous state). Each of these two terms, $R_{s,\langle d\rangle}R_{d,s}$ and $R_{s,\langle z\rangle}R_{z,s}$, is not independent of the presence of another ligand. Fig. 5E shows a region in red outside the dashed blue lines where, although ligand1 mediates a strong enough feedback to drive patterning ($R_{s,\langle d\rangle}R_{d,s}\leq-2$), patterning does not occur ($-2<R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s}$). These two inequalities involve necessarily that $R_{z,s}R_{s,\langle z\rangle}>0$, pinpointing that ligand2 mediates an effective lateral induction (despite its repression by Notch signaling, $R_{z,s}<0$).

The three ligands model and its LSA

For the study of three ligands (*d* for Jag2, *z*1 for Dll1 and *z*2 for Jag1, Fig. S9) in the context of mammalian inner ear development, we assumed that all ligands bind to the same and unique type of Notch receptor (Notch1), such that the signal dynamics reads:

$$\frac{ds_i}{dt}=\frac{r_{Jag2}\langle d_i\rangle+\varepsilon_{Dl1}r_{Dl1}\langle z1_i\rangle+\varepsilon_{Jag1}r_{Jag1}\langle z2_i\rangle}{1+r_{Jag2}\langle d_i\rangle+r_{Dl1}\langle z1_i\rangle+r_{Jag1}\langle z2_i\rangle}-s_i, \tag{10}$$

where ε_{Dl1} and ε_{Jag1} stand for the efficiencies of signaling of Dll1 and Jag1, respectively, with respect to Jag2 (which in isolation drives a maximal signal of value 1). The ligands dynamics (Eqn 1 for each ligand) use $f_l=f_+$ for Jag1 and $f_l=f_-$ for Jag2 and Dll1. The parameter values related to Jag1 dynamics were chosen such that: (1) Jag1 signaling efficiency is lower than that of Jag2; and (2) Jag1 expressed alone is able to drive bistability of homogeneous states and ligand propagation (δ_{Jag1}=0.0001, b_{Jag1}=1, $b_{Jag2}=b_{Dl1}=10^4$, all cooperativities h_l=4 and other parameter values as detailed in Fig. S9). For simplicity, we use *Dl*1 as subindex to refer to Dll1 ligand. To determine the linear stability of the homogeneous states of this three-ligand system, we used the criterion of Routh-Hurwitz (Murray, 2003). According to this (see supplementary Materials and Methods for further details), a sufficient condition for the homogeneous state to be linearly unstable, which enables the emergence of salt-and-pepper patterning, in this three-ligand system is (assuming v_l=v)

$$R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z_1\rangle}R_{z_1,s}+R_{s,\langle z_2\rangle}R_{z_2,s}<-2, \tag{11}$$

which defines the patt region enclosed by dashed blue lines in Fig. S9. Gray and red shaded regions in the phase diagrams shown in Fig. S9 indicate the regions where $R_{s,\langle l\rangle}R_{l,s}\leq-2$ for l=*Dl*1 and l=*Jag*2, respectively.

LSA: speed of the dynamics of patterning

A theoretical prediction of how fast patterning is expected to be achieved was computed through the exponential growing rate of the perturbation that grows the fastest over the homogeneous state. The calculation of this rate in the case of two ligands results in $\lambda_{-0.5}=(1/2)\left(-(1+v)+\sqrt{(1+v)^2-2(R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s}+2v)}\right)$. In the calculations depicted in Fig. S3, we assume v=1.

LSA: two linearly stable homogeneous states – bistability regimes

According to the LSA described above, by imposing the condition that neither homogeneous nor inhomogeneous perturbations of the stationary homogeneous steady state can grow over time in the linear regime, the following condition is obtained (assuming v_l=v):

$$-2\leq R_{s,\langle d\rangle}R_{d,s}+R_{s,\langle z\rangle}R_{z,s}\leq 1. \tag{12}$$

In the figures, the parameter space regions with two homogeneous stationary solutions that both fulfill the previous condition are enclosed by the solid black line and are denominated as bis regions. Hence, these are regions with two linearly stable homogeneous solutions. Transitions from one to the other can occur through nonlinear perturbations, such as those corresponding to exogenous application of high amounts of ligand.

Computation of regions within the parameter space

We used custom-made programs in C to evaluate the conditions set by LSA (conditions 9-11 defining patt and bis regions, and conditions defining red and gray regions), the theoretical growth rate obtained from LSA and the partial agonist condition across the parameter space, by defining a grid of parameter set values in logarithmic space. The results in the parameter space were plotted using custom-made Python scripts (version 2.7) with the matplotlib package (Hunter, 2007). These programs are available upon request.

Programs and simulation details

Numerical integration of the dynamics was performed by using NDSolve function of Mathematica 8.0 for a perfect hexagonal grid of 12×12 cells with periodic boundary conditions (custom-made program available upon request). Time step of integration was variable between 0.001 and 0.05 chosen by default by the Mathematica function. Integration was carried out to the final non-dimensional time 500. Initial conditions for each dynamic

variable x in each i cell was $x_i(t=0)=x_0(1+0.2\times(0.5-\eta))$, where x_0 is the stationary homogeneous solution and η is a random uniform number between 0 and 1. The signal and ligand activities are depicted in a linear grayscale with white being 0 and black being 1. Numerical integration of the dynamics was carried out to corroborate the patt and bis regions obtained through LSA results at some points of the parameter space and to evaluate the time for pattern formation in a point of the parameter space (see supplementary Materials and Methods for further details).

Programs and simulation details: ligand propagation

Transitions from a homogeneous state with low ligand activities to a homogeneous state with higher activities were allowed by imposing the low state as initial condition. Then, we selected a small cluster of four cells in the central region of the array and set their initial condition for the ligand as $l(t=0)=1$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.C.L.-E., P.F.-J., M.I.; Methodology: J.C.L.-E., P.F.-J., M.I.; Software: J.C.L.-E., P.F.-J.; Validation: J.C.L.-E.; Formal analysis: J.C.L.-E.; Investigation: J.C.L.-E.; Writing - original draft: J.C.L.-E., M.I.; Writing - review & editing: J.C.L.-E., P.F.-J., M.I.; Visualization: J.C.L.-E.; Supervision: M.I.; Project administration: M.I.; Funding acquisition: M.I.

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Supplementary information

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