Qualitative Crosstalk Analysis of Wnt and Notch Signaling in Mammalian Skin

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Abstract. The *Qualitative networks* framework allows to construct discrete executable models of biological systems. An efficient symbolic algorithm can be used to verify that all stable states of such a model are consistent with a set of requirements derived from the laboratory experimental data. Here we consider the crosstalk between the Notch and Wnt pathways that play a key role in the regulation of cell proliferation and differentiation. We extend the analysis of our model of interaction between these pathways in mammalian skin. By further constraining the requirements from the model, we formulate a hypothesis that the ligand Jagged, which is part of the Notch pathway, is a downstream target of Wnt signaling. This prediction has been recently validated experimentally by Estrach *et al.*.

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1 Introduction

The construction and analysis of models that mimic biological processes is an important method for gaining a high-level understanding of complex biological systems. Such computational models consist of an algorithm that mimics the behavior of a biological system. Like computer programs, such models can be executed. Furthermore, a variety of formalisms originally developed for the engineering and validation of computer systems have been applied to the analysis of these executable models (reviewed in [1]). Model checking [2] has been used to exhaustively verify the consistency of all possible executions of a model with respect to the experimental data based on which the model was proposed [3, 4]. This approach has been recently used to build an executable model of cell fate determination during *C. elegans* vulval development (see [5] for a description of the insights and their validation).

In our previous work [6], we have introduced the *Qualitative networks* framework for building executable models of biological systems, and proposed a methodology for their computational analysis. Qualitative networks are an extension of Boolean networks [7, 8] to discrete variables that allow to model a richer set of behaviors. We propose that the set of infinitely visited states of a Qualitative network correspond to the stable states of the biological system, and present an efficient symbolic algorithm for verifying that all such states adhere to a set of specifications derived from laboratory experimental observations. If this is not the case, we iteratively improve the model by adding new hypotheses. New hypotheses that lead to a model that better matches the experimental observations are potential new biological insights, and candidates for experimental validation. Using this approach, we build a model of the crosstalk between the Notch and Wnt signaling pathways in mammalian skin. In this work, we further analyze this model, and show how it can be used to predict new modes of interaction that were not known at the time the model was conceived.

The Notch and Wnt pathways are key players in the regulation of cell proliferation and differentiation. Alterations in their function have been linked to several types of cancer [9, 10]. Wnt signaling (reviewed in [11]) is based on the inhibition of β-Catenin degradation. Wnt is a short range signaling molecule that binds to receptors of the Frizzled family. This binding leads to the phosphorylation of Dishevelled (DSH), which in turn inhibits the action of Axin. Axin is part of the degradation complex in which the glycogen-synthase kinase-3β (GSK3β) phosphorylates β-Catenin, which leads to the degradation of β-Catenin. Therefore the inhibition of Axin allows β-Catenin to accumulate and travel to the nucleus where it activates the Tcf/Lef DNA-binding proteins. This leads to the activation of the target genes of Wnt signaling. Notch signaling is based on the Notch receptor protein, which is composed of an extracellular and a cytoplasmic parts located on either side of the cell membrane. Ligands expressed on the surface of an immediate neighbor cell leads to the cleavage of the Notch protein. The intracellular domain of the receptor (Notch-IC) travels to the nucleus, where it changes the role of the CSL transcription factor from an inhibitor to an activator. This leads to the activation of the transcription of the target

genes of Notch signaling. One of the targets of Notch signaling, p21 is a negative transcription regulator of Wnt [12], thus creating an interaction between both signaling pathways.

In the human epidermis, multiple Notch receptor and Jagged ligands are expressed. A positive feedback mechanism between Notch-IC and the ligand has been proposed to explain sustained Notch signaling [13]. Notch signaling has been shown experimentally to induce terminal differentiation in both murine and human skin [14], and to act as a tumor suppressor in mouse [15]. Downstream targets of Wnt signaling are known to maintain cell proliferation. The mammalian epidermis is composed of several layers. The basal layer is closest to the underlying dermis, and contains most proliferating keratinocytes. When kerationocytes initiate terminal differentiation, they migrate to the suprabasal layer. The cornified layer, which is located above the suprabasal layer, is mainly composed of dead cells.

The model of the mammalian epidermis proposed in our previous work consists of five identical keratinocytes, each of them representing one layer of the epidermis. We define a set of requirements to ensure that the fate of each cell of the model corresponds to the histological observations for the corresponding layer. In the first cell, which represents the basal layer, the level of the targets of Wnt signaling must be higher than the level of the targets of Notch signaling. In the last two cells, which represent the supra-basal layer, the level of the targets of Notch signaling must be higher than the level of the targets of Wnt signaling. The model we obtain has a total of 6561 infinitely visited states (stable states) that all satisfy this requirement, as well as additional requirements derived from experimental data.

In this work, we show how we can set more restrictive requirements to lower the number of infinitely visited states, and how the iterative improvement approach leads to the suggestion of a positive interaction between targets of the Wnt signaling pathway and the Jagged ligand. This result has been independently proposed and experimentally tested [16] while our modeling work was in progress. A detailed version of this study is available as the first author's Master's thesis [17].

2 Methods

In this section, we first describe the Qualitative network formalism and the iterative improvement approach that we use to build and analyze our model of the mammalian epidermis. We then describe the model itself, as well as the specifications related to cell fate. This section focuses on methods that are directly used in the analysis of the model presented herein. Additional details about the Qualitative network formalism, the iterative improvement approach, the symbolic algorithms and the model construction can be found in [6].

2.1 Qualitative Networks

A Qualitative $Q(C, T, N)$ consists of a set C of biological components, such as genes and proteins, and a list of *target functions* T that define the interactions between the components. The state of a component $c_i \in C$ can be any integer value between 0 and N. For the purpose of modeling the mammalian skin, we use $N = 3$. An active component can thus have three distinct (active) qualitative levels of activity: *low*, *medium* or *high*. A target function $target_i \in T$ is associated with each component c_i. This function computes the discrete level towards which the component moves at the next timestep. Any function on the state of the whole system can be used as a target function:

$$target_i : \{0, ..., N\}^{|C|} \rightarrow \{0, ..., N\} \tag{1}$$

At each discrete time step, the value of the state of a component can only increase or decrease by a single level. The value of each component at the next time step is therefore computed as follows:

$$c_i(t+1) = \begin{cases} c_i(t) - 1 & \text{if } target_i(S(t)) < c_i(t) \\ c_i(t) + 1 & \text{if } target_i(S(t)) > c_i(t) \\ c_i(t) & \text{if } target_i(S(t)) = c_i(t) \end{cases} \tag{2}$$

Qualitative networks can be constructed directly from the information available in the diagrammatic models that are commonly used by biologists. A diagrammatic model can be represented as a graph $G(V, E)$, in which each node $v_i \in V$ corresponds to a biological component. A node v_i will therefore directly corrspond to a component c_i in the Qualitative network. An edge $e_{ij} \in E$ from node v_i to node v_j has a weight α_{ij}, corresponding to the effect of the component represented by v_i on the component represented by v_j. Activation is specified by a positive value of α_{ij}, inhibition by a negative value. The model of mammalian skin uses equal weights for all interactions ($\alpha_{ij} \in \{-1, 0, 1\}$). Target functions can be derived from the graph. For each component c_i, we separately compute the weighted amount of activation act_i and the weighted amount of inhibition inh_i on the component. Both of these values range from 0 to N.

$$\begin{aligned} act_i(s) &= \frac{\sum_{\alpha_{ji}>0} \alpha_{ji} c_j}{\sum_{\alpha_{ji}>0} \alpha_{ji}} \\ inh_i(s) &= \frac{\sum_{\alpha_{ji}<0} \alpha_{ji} c_j}{\sum_{\alpha_{ji}<0} \alpha_{ji}} \end{aligned} \tag{3}$$

When there is no inhibition, the target function corresponds to the amount of activation. When there is inhibition, the value of the target function decreases.

If the amount of inhibition is higher than the amount of activation, the value of the target function is zero. This assumes that a given component has at least one incoming activating edge. If all incominng edges of a component are inhibitory, then we consider that this component has the maximal possible value in the absence of any inhibition. When the amount of inhibition increases, the value of the target function of the component decreases. We obtain the following definition of the target functions:

$$target_i(s) = \begin{cases} \max(0, act_i(s) - inh_i(s)) & \text{if } max(\alpha_{ji}) > 0 \\ N - inh_i(s) & \text{if } max(\alpha_{ji}) < 0 \end{cases} \quad (4)$$

Since the target function can be any function of the current state of the system, it is possible to combine target functions that are directly derived from the diagrammatic model using Equation 4 with target functions specially designed to model the particular behavior of a component. This is the case for the Notch cleavage process in the mammalian skin model. The cleavage of the Notch receptor, which releases Notch-IC, requires binding between Notch and its ligand. Therefore both Notch and the sensed level of ligand are limiting factors of the interaction. The target function of component Notch-IC is most accurately modeled as the minimum between the level of the Notch receptor and the level of the ligand.

2.2 Model Analysis Methodology

We use an iterative improvement process in order to suggest a model that is consistent with the laboratory experimentation performed on the actual biological system. Figure 1 provides an overview of this process.

1. An initial, putative, model is constructed based on the current mechanistic understanding of the biological process.
2. A set of formal requirements are derived from prior experimental data, including the experiments that were used to suggest the current mechanistic model.
3. Formal verification techniques, such as simulation and model checking [2] are then used to exhaustively verify that all possible executions of the model adhere to the requirements.
4. If this is not the case, then the verification algorithm produces a trace of an execution of the system in which a requirement is violated. This counterexample can be used to formulate new hypothesis about the interactions between the biological components of the system. The verification algorithm is then applied to a modified model that incorporates the new hypothesis. Steps 3 and 4 are repeated until there is no contradiction between any execution of the model and the specifications.
5. The new hypotheses that become part of the improved model can then be validated experimentally.

6. If the experiment shows that the hypothesis is not correct, then additional requirements can be derived from the new biological evidence. The iterative improvement process (steps 3-5) needs to be repeated in order to obtain a model that is compatible with the new requirements.

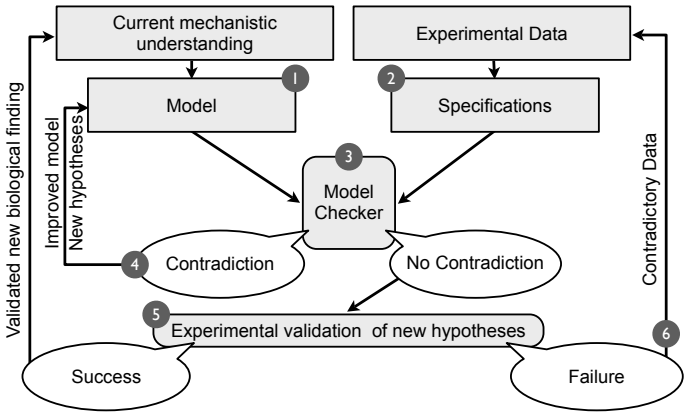

Fig. 1. The iterative improvement process. Numbers correspond to the steps described in sect. 2.2

In this work, we focus on a specific kind of specifications: properties that must hold for all infinitely visited states. A state is said to be infinitely visited if there is an execution of the model in which the state appears infinitely often. In a determinsitic system, such as Boolean and Qualitative networks, loops of infinitely visited states are called attractors. We consider that the infinitely visited states correspond to the stable states of the biological system. States that are not visited infinitely often are considered to be unstable. If the system is in such a state, it will evolve towards an attractor in a bounded number of steps, and then remain in the attractor indefinitely. This definition of stable states is useful for analyzing a biological system for which no definition of initial states is available. We consider that a model adheres to a requirement if the requirement holds for any infinitely visited states, given that any state can be initial. Since the number of initial states is exponential in the number of components, approaches that exhaustively enumerate all states are intractable even for very small models. In our previous work, we have introduced an efficicient symbolic algorithm using Binary Decision Diagrams [18] and a partition reduction approach that is able to scale to complex multicellular models with more than 10^{86} initial states [6].

A model that satisfies all requirements can be *queried* to obtain additional insights into the biological process. There are two possible ways for querying a model: the analysis of an altered model, and the use of additional requirements.

Altering a refined model is useful to simulate alterations to the normal behavior of the studied system, such as disruptions in the biological mechanisms, and mutations. For example, the robustness of models of redundant biological pathways to disruptions can be tested by disabling interactions between two components. Additional requirements can be added based on the observation of the executions of the model, for example to avoid situations which appear to be unrealistic even though they were compatible with the original requirements. New requirements might require repeating the iterative improvement process, and thus lead to new hypotheses. It can therefore also be useful to add requirements in order to see what new hypotheses would allow explaining a more precise, or different, behavior of the system.

2.3 Model

The model we study consists of five identical keratinocytes, representing the mammalian epidermis. Each cell represents one layer. In this section, we first describe the interaction between components in a single cell, then how the cells interact in the complete model, and finally the specifications that are used in the complete model. Figure 2 represents the schematic view of a single cell (A), and the interactions between all the cells in the model (B).

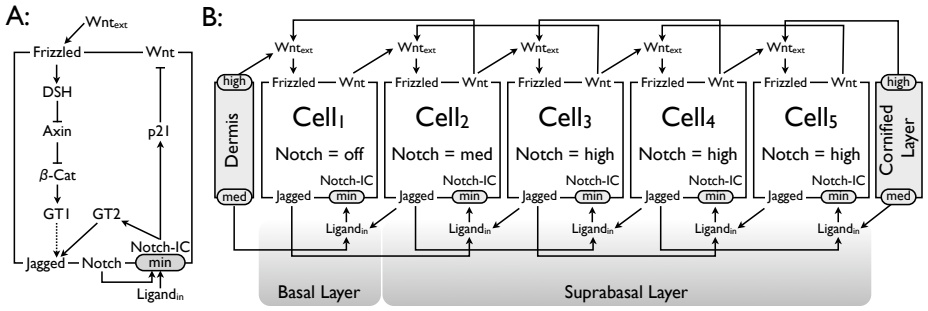

Fig. 2. A: Single cell of the model. The dashed line represents the new suggested interaction. B: Schematic view of the multicellular model.

The Wnt pathway starts with variable Wnt_{ext}, which represents the amount of Wnt protein in the immediate neighborhood of the cell. This variable is used for cell-cell interactions, as described in the next paragraph. Variable $Frizzled$ represents the amount of activation of the Frizzled receptor, to which Wnt proteins bind. This interaction leads to an increase in the intracellular level of *DSH*. DSH inhibits the level of the scaffolding protein *Axin*. Axin is part of the degradation complex of *β-Catenin*, and there is therefore an inhibition-like interaction from Axin to *β-Catenin*. Variable *GT1* represents all the downstream targets whose transcription is activated by increased levels of *β-Catenin*. The Notch

pathway is based on the interaction between the Notch receptor and the ligand expressed on the surface of immediate neighboring cells. Variable $Notch$ represents the amount of receptor on the cell surface. This variable is a constant depending on the position of the cell. The amount of ligand sensed by the receptor is represented by variable $Ligand_{in}$. This variable depends on the neighboring cells, as described in the next paragraph. Interactions between the ligand and the receptor leads to the cleavage of the Notch protein. The level of the intracellular part in the cell is represented by variable *Notch-IC*, and corresponds to the minimum between the amount of ligand and the amount of receptor. Similarly as for the Wnt pathway, we use a single variable ($GT2$) to represent the level of all downstream targets of Notch-IC. One of these targets is *p21*, which we represent separately for clarity. *p21* represses the amount of *Wnt* emmited by the cell, thus providing an interaction between both pathways. The amount of Jagged ligand expressed on the surface of the cell (and sensed by the neighboring cells) is represented by variable *Jagged*. *Jagged* is activated by *GT2*, which creates a positive feedback loop in the Notch signaling pathway. Both the *Jagged* and the *Wnt* variables are used for cell-cell communication.

Five keratinocytes (named $Cell_1$ to $Cell_5$) in a row form the complete model (Fig. 2.B). The left side of the model represents lower layers of the skin, the right side higher layers. The leftmost cell ($Cell_1$) is part of the basal layer, in which the level of Notch receptor is lower than in the supra-basal layer. Therefore we set constant *Notch* to *off* in $Cell_1$, to *medium* in $Cell_2$ and to *high* in $Cell_{3-5}$. Cell-cell communication is modelled by connecting the Wnt_{ext} and $Ligand_{in}$ variables of a cell to the *Wnt*, respectively *Jagged* variables of the immediate neighboring cells. The sensed level is maximal when the produced level is *high* in both neighboring cells. The level of Wnt molecules emitted by the dermis (which is immediately left of $Cell_1$) is known to be *high*, whereas there is no Wnt signaling comming from the cornified layer (which is immediately right of $Cell_5$). We set the level of *Wnt* on the border of the model accordingly. The border levels of *Jagged* are set to *medium*, since the ligand is expressed in the whole epidermis.

This model is consistent with the experimental results in the work of Nicolas *et al.* [15] on the Notch pathway in mouse keratinocytes, and the work of Devgan *at al.* [12] on the role of p21. In order to ensure that the model is consistent with the observed histology, we design specifications about the fate of individual cells in the model. The downstream targets of Wnt signaling (represented by variable *GT1*) maintain the cells in a proliferating stage, whereas the downstream targets of Notch signaling (*GT2*) initiate terminal differentiation. We can therefore derive the cell fate from the levels of *GT1* and *GT2*: a cell is *proliferating* if $GT1 > GT2$, *in transition* to differentiated when $GT1 = GT2$ and *differentiated* when $GT1 < GT2$. We want $Cell_1$, which represents the basal layer, to have a proliferating fate, whereas $Cell_{4-5}$ are differentiated. Furthermore, we want to have a single change from proliferating to differentiated cell fates. This means that cells in a layer above a differentiated cell cannot be proliferating. This leads to the following requirements:

- R1: $GT1 > GT2$ in $Cell_1$
- R2: $GT1 < GT2$ in $Cell_{4-5}$
- R3: $GT1 = GT2$ in $Cell_i \Rightarrow \forall j > i$, $GT1 \leq GT2$ in $Cell_j$
- R4: $GT1 < GT2$ in $Cell_i \Rightarrow \forall j > i$, $GT1 < GT2$ in $Cell_j$

All stable states of the model described in this section satisfy these requirements. We call this initial model M_1.

3 Results

The set of infinitely visited states of the model consists of 6561 states which are all consistent with the requirements defined in the previous section. These requirements do not fix the fate in $Cell_2$ and $Cell_3$. We observe that, for $Cell_2$, all three possible cases appear in some stable states. Furthermore, we observe that there are attractors in which the status of $Cell_2$ changes. A possible explanation would be that this cell is in transition between the two cell fates. Here we consider using a more constraining set of specifications, and apply iterative improvement to see if we can find a model in which all infinitely visited states adopt the same pattern. We add the requirements that $Cell_3$ must be differentiated, and $Cell_2$, must be in transition from proliferating to differentiated:

- R5: $GT1 > GT2$ in $Cell_3$
- R6: $GT1 = GT2$ in $Cell_2$

Since there are infinitely visited states for which this is not true, we have a contradiction and thus we need to formulate a new hypothetical model M_2 for which all requirements would hold, and then test if this is indeed the case. Furthermore, the new model still needs to satisfy all the requirements derived from the experimental work. We find that adding an activating edge from *GT1* to *Jagged* (represented as a dashed arrow on Fig. 2) results in a model which has a single infinitely visited state S. This state is consistent with all requirements. Figure 3 provides a graphical representation of the level of each component in state S.

The fact that this model has a single stable state is of particular interest when considering the dynamical evolution of this model at the cell level. When the system is in a stable state, $Cell_1$ is proliferating, and will, at some point, divide. Since cells of the model represent layers, we need to consider this process from a higher level point of view: cells in the basal layer will divide, then migrate to the suprabasal layer where they terminally differentiate, while cells at the top of the suprabasal layer move to the cornified layer, where they die. We can model this process as follows: All cells move one layer to the right of the model, $Cell_5$ moves to the cornified layer and is therefore removed from the model and $Cell_1$ and $Cell_2$ are identical (which mimics the division of the cells in the basal layer). More formally, we build a new state S' corresponding to the state of the system after the division. The values of the variables of $Cell_{2-5}$ in S' correspond to the value of the same variables in, respectively, $Cell_{1-4}$ in S. Border conditions, and

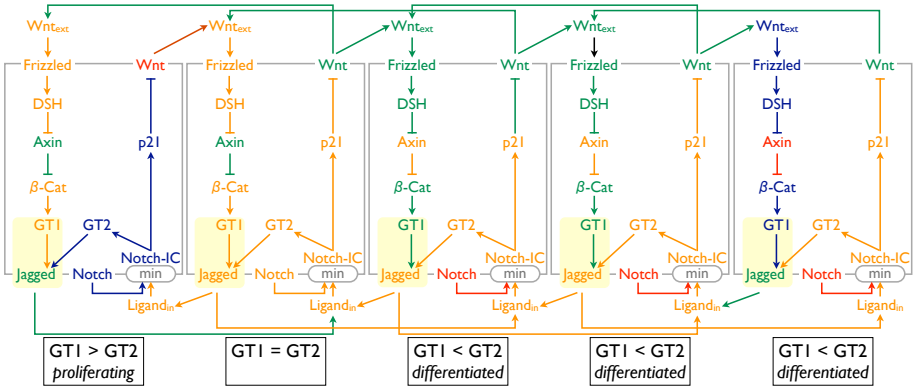

Fig. 3. The single stable state of the modified model. Colors are used to indicate the level of the components: blue corresponds to off, green to low, orange to mediun and red to high. The new interaction is highlighted in beige.

the level of Notch (which is a constant depending on the layer) are not modified. We now consider the execution of the model starting from state S'. The creation of state S' does not change modelres M_2. We can therefore directly conclude that the execution will necessarily converge to the unique infinitely visited state S in a bounded number of steps. We analyze the sequence of states between S' and S. We find that there is a single change in the fate of $Cell_3$ from *in transition* to *differentiated*, and a single change in the fate of $Cell_2$ from *proliferating* to *in transition*. Model M_2 is thus able to reproduce the dynamic behavior of the system at the cell layer level.

4 Discussion

The interaction between the Wnt signaling pathway and the Jagged ligand has been independently proposed and experimentally demonstrated by Estrach *et al.* [16]. They observe a very tight correlation between β-catenin levels and Jagged 1 expression in mice hair follicles. Their analysis of the mouse, rat and human Jagged promoter region reveals the existence of multiple binding sites of Tcf/Lef, the DNA-binding protein which becomes an activator when the canonical Wnt pathway is activated. Furthermore, a bioinformatics search for Tcf/Lef binding sites in the various kinds of Notch ligands [19] finds that such binding sites exist in Jagged 1, and that this binding site is conserved across multiple species. Evolutionary conservation indicates that this interaction plays an important role in the crosstalk between the Notch and Wnt pathway. These two results, which were not used in the construction and analysis of our model (and were indeed not known when this model was first built and analyzed), correspond to the hypothesis we made in the iterative improvement approach. This shows

that the construction and analysis methods we propose allow finding pertinent biological interactions.

The Qualitative networks framework allows to build a model at a similar level of abstraction seen in diagrammatic models commonly used in biology. Hypotheses formulated in the iterative improvement process have therefore a clear biological meaning. Here, we can use this mechanistic overview to conceptualize the implication of the new mode of interaction in the context of the interplay between the Notch and Wnt signaling pathways. We first observe that there is a positive interaction from the canonical Wnt signaling pathway towards the Notch signaling pathway. A potential implication of this interaction could be that if, for some reason, the level of Wnt signaling rises sharply, then the level of Notch signaling will also increase. This mechanism would make it less likely that abnormal rises in Wnt signaling lead to hyperproliferation. The interaction from the Notch signaling pathway to the Wnt signaling pathway is negative. This makes it possible for the cells to adopt a differentiated cell fate, since the activation of the targets of Notch signaling (which trigger terminal differentiation) coincide with the repression of the targets of Wnt signaling (which would maintain proliferation). Combining the two interactions creates a negative feedback loop for both Notch and Wnt signaling. Negative feedback loops allow the system to avoid excessive variations in the activity level of a pathway. Therefore, the refined model provides a control mechanism that is able to limit the activity of both pathways, and is more resistant against both excessive proliferation (through excessive Wnt signaling) and depletion of the proliferating cell pool (through excessive Notch signaling).

In our previous work, we have introduced the Qualitative networks framework, which allows to build executable models of biological system at a level of abstraction similar to the discrete results of laboratory experiments, and clearly separates the conceptual model from its implementation. The iterative improvement approach uses formal verifications method to build a model that is compatible with a set of requirements derived from laboratory experimental results. Efficient symbolic algorithms allow an exhaustive analysis of all stable states of the system. In this work, we show how an executable model of the crosstalk between the Notch and Wnt pathways can be analyzed, and further improved by using more constraining requirements. During the improvement process, we suggest that the Jagged ligand is a downstream target of the canonical Wnt pathway. This interaction has been demonstrated using both experimental and bioinformatics methods in independent studies that were conducted in parallel to our modeling work. This result illustrates that the analysis of a Qualitative network can lead to biologically meaningful results and suggest new avenues to explore experimentally.

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