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Targeting the Notch pathway to prevent rejection

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Abbreviations: APC, CSL, DC, Dll1, Dll3, Dll4, DNMAML, DP, GSI, GVHD, ICN, IFNγ, MAML, T_{eff}, TF, Th1, Th2, TNFα, T_{reg}

Abstract

Immune rejection is mediated by a complex interplay of cellular and humoral mechanisms. Current therapeutic strategies, which rely on global immunosuppression, can result in serious complications and are not completely effective. Notch signaling is a cell-to-cell communication pathway that plays an important role during T cell development and in the regulation of peripheral immune responses. Initial work, performed mainly through gain-of-function approaches, paradoxically identified Notch as an inducer of tolerance. However, recent studies using loss-of-function approaches in mouse models of transplant rejection and graft-versus-host-disease have clarified an important role for Notch as a central mediator of T cell alloreactivity. Short-term inhibition of individual Notch ligands in the peri-transplant period had long-lasting protective effects. In a vascularized heart allograft model, blockade of Delta-like Notch ligands dampened both cellular and humoral rejection. Here, we summarize current knowledge on the role of Notch signaling during allograft rejection, and provide an overarching mechanism through which Notch acts to promote T cell pathogenicity and allograft damage. We propose that targeting elements of the Notch pathway could provide a new therapeutic approach to prevent allograft rejection.

First identified in Drosophila close to a century ago, the Notch pathway has received increasing recognition for its functions in mammalian biology, as well as its effects in health and disease (1, 2). Notch plays an essential role at early stages of T cell development in the thymus, as Notch ligands expressed by thymic epithelial cells induce commitment of thymocyte progenitors to the T lineage program (3). Notch also regulates subsets of innate lymphoid cells, B cells and dendritic cells (3). In addition, recent studies uncovered major functions of Notch signaling in the control of antigen-driven immune responses in the periphery (4, 5). These effects of the Notch pathway are prominent in T cell alloimmunity and highly relevant in the context of allograft rejection (6-14). In this minireview, we introduce aspects of Notch regulation that are critical for its functions in immunobiology, and discuss emerging evidence showing that Notch signaling could be an attractive new therapeutic target to prevent organ rejection.

Introduction to Notch signaling. Notch signaling is a highly conserved cell-to-cell communication pathway triggered by Notch ligand-receptor interactions between adjacent cells (**Fig. 1**) (1, 2). In mammals, four Notch receptors (Notch1-4) have been identified in addition to

five Notch ligands of the Jagged (Jagged1/2) and Delta-like families (Dll1/3/4). Jagged1/2 and Dll1/4 have agonistic properties, while Dll3 functions as a natural antagonist of the pathway (1). Notch ligand-receptor interactions induce sequential proteolytic cleavage of the receptor by an ADAM family metalloprotease (ADAM10) and by the γ-secretase complex, ultimately releasing intracellular Notch (ICN) into the cytoplasm (1, 2). ICN migrates into the nucleus where it interacts with a DNA-binding transcription factor referred to as CSL (CBF1/Suppressor-of-hairless/Lag-1) or RBP-Jκ (encoded by *Rbpj*). Upon Notch activation, CSL and ICN nucleate a large transcription activation complex that recruits a member of the Mastermind-like (MAML) family and other co-activators to mediate transcriptional activation of Notch target genes (1, 2, 15). Recent studies in Notch-driven cancer cell lines detected binding of CSL and ICN at thousands of genomic sites (16). However, only a fraction (<10%) of these sites appeared dynamically regulated by Notch signaling. Regulated sites were enriched for concomitant binding of specific transcription factors, suggesting context-specific regulation of the Notch target gene landscape by cooperating factors. Moreover, the majority of dynamic Notch-binding sites were located in distant elements with superenhancer features, suggesting that Notch is involved in long-range chromatin regulation (17). Additional studies about the molecular mechanisms of Notch-mediated transcriptional activation will be essential to understand the context-specific effects of the Notch pathway. Of note, little is known so far about the nature and regulation of Notch transcriptional targets in mature T cells.

Evolution selected multiple mechanisms to ensure tight control over Notch signaling, as Notch receptors can deliver powerful signals with profound effects on cellular differentiation and function. Active ICN is rapidly targeted for degradation, ensuring that Notch signals are short-lived in nature, unless receptors are repetitively engaged (1, 2). Notch receptors are relatively ubiquitous, although the *Notch1-4* paralogues are differentially expressed by specific cell types. In contrast, individual Notch ligands are expressed in a highly controlled fashion within defined anatomical niches. This regulatory arrangement ensures tight control over temporal and spatial delivery of Notch signals. For example, thymic epithelial cells express high levels of Dll4 under control of the *Foxn1* gene, generating an intrathymic niche that delivers strong Notch signals to T lineage progenitors while restricting normal T cell development to a single anatomical site (18). Evidence for restricted and regulated expression of Notch ligands within secondary

lymphoid organs has also emerged (19). Jagged and Delta-like ligands were reported to induce different functional outcomes in several contexts (20). Based on these considerations, individual Notch ligands and receptors can have distinct biological effects. These distinct effects offer opportunities for the development of therapeutic targeting strategies that provide specific benefits, while avoiding the systemic side effects of pan-Notch inhibition (7, 21, 22).

Early work on Notch signaling in T cell alloreactivity and tolerance. Initial studies exploring a potential role for Notch signaling in mature T cell function and alloreactivity relied heavily on gain-of-function strategies. Lamb and coworkers were the first to spark interest in a role for Notch as an inducer of tolerance (23). While studying T cell responses against a house dust mite protein, they engineered mouse dendritic cells (DCs) to overexpress the Notch ligand Jagged1. Adoptive transfer of antigen-pulsed Jagged1-overexpressing DCs led to antigen-specific hyporesponsiveness. Building on this concept, the Brenner group tested the ability of Jagged1-overexpressing antigen-presenting cells to modulate responses to alloantigens or viral antigens (24, 25). Using Jagged1-transduced Epstein Barr Virus-transformed lymphoblastoid cell lines, they observed decreased T cell reactivity and evidence for transferrable suppressive effects. Similar outcomes were described in an *in vivo* cardiac allograft model, when Dallman and colleagues adoptively transferred mouse L cell fibroblasts engineered to overexpress MHC alloantigens and the Notch ligand Dll1 (26). Although Dll1-overexpressing L cells delayed allograft rejection in a $CD8^+$ T cell-dependent manner, it is unclear whether the effects were the result of direct engagement of Dll1 with Notch receptor in T cells. Similar observations were reported recently upon *in vivo* transfer of a Jagged1-transduced dendritic cell line in combination with CD40 blockade (27). Altogether, these studies suggested that inducing artificially high Notch signals in T cells could generate a state of antigen-specific tolerance.

In parallel, other laboratories observed that expression of specific Notch ligands could be induced by innate stimuli in professional antigen-presenting cells (APCs) (20, 28). In coculture systems, Delta-like or Jagged Notch ligands within APCs was reported to promote skewing of T cell differentiation towards the T helper 1(Th1) vs. Th2 lineage, respectively (20, 29), although dichotomous inductive effects of Delta-like and Jagged Notch ligands were not detected in subsequent studies (30). Collectively, while helpful for recognizing an important role for Notch

in T cell alloreactivity, the use of artificial *ex vivo* conditions and overexpression models led to conclusions that were contradictory and have to be interpreted with caution. Subsequent *in vivo* loss-of-function studies on mature T cells identified an even broader range of effects of Notch on both $CD4^+$ and $CD8^+$ T cell reactivity and function (31-33), many of which are discussed later in the "Mechanistic Considerations" section of this review.

***In vivo* studies of Notch signaling in allograft rejection.** In recent years, several groups have used genetic and pharmacological loss-of-function approaches to evaluate the *in vivo* effects of Notch signaling in alloreactive T cell responses, both in the setting of allograft rejection and in graft-versus-host disease (GVHD) (**Table 1**) (6-9, 11-14). An emerging consensus across these studies indicates that Notch signaling is a major pro-inflammatory pathway in T cell alloimmunity, and that Notch inhibition can dampen both allograft rejection and GVHD. Thus, the true *in vivo* functions of Notch signaling appear to be diametrically different from the tolerogenic effects first detected using artificial gain-of-function strategies (23-26). Furthermore, these studies identify Notch inhibition as a new promising therapeutic approach to mitigate the damaging consequences of T cell alloreactivity.

Riella and coworkers used monoclonal antibodies to target the Notch ligand Dll1 in a MHC-mismatched cardiac allograft transplantation model (9). Systemic anti-Dll1 antibodies delayed allograft rejection when provided in conjunction with costimulatory blockade in *Cd28*-deficient mice or in recipients treated with CTLA4-Ig. Protection was associated with decreased production of IL-2, interferon gamma (IFNγ), IL-6 and IL-17 by donor-specific T cells, but with increased production of the Th2 cytokines IL-4 and IL-5. In this model, the protective effects of anti-Dll1 antibodies were lost when transplantation was performed in STAT6-deficient recipients or upon concomitant IL-4 neutralization, indicating that increased IL-4 production was important to delay rejection. This paper was the first to demonstrate a pathogenic effect of Notch signaling and a therapeutic benefit of Notch inhibition in allograft rejection *in vivo*, in stark contrast to earlier literature using artificial gain-of-function systems. Because this study was performed in the presence of costimulatory blockade and only examined the effect of Dll1 inhibition but not other Notch ligands, it was unclear if similar outcomes would be observed without costimulatory blockade or upon more complete Notch inhibition. Due to the systemic nature of Dll1 inhibition,

it could not be determined if the protective effects of anti-Dll1 antibodies were related to their direct effects on T cells and/or on other cell types.

To achieve a higher level of Notch inhibition in alloreactive T cells, Wood et al. studied a MHC-mismatched heart allograft model in mice expressing the pan-Notch inhibitor dominant negative Mastermind-like1 (DNMAML) specifically in T cells (14). DNMAML blocks transcriptional activation downstream of all Notch ligands and receptors (15). In *Cd4-Cre x ROSA26*^{DNMAML} mice, DNMAML expression first arises in $CD4^+CD8^+$ double positive (DP) thymocytes without interfering with early Notch-dependent stages of T cell development (34). Thus, mature $CD4^+$ and $CD8^+$ T cells develop normally from DP thymocytes in these mice, but cannot respond to Notch signals during subsequent T cell responses due to DNMAML expression. This strategy is highly effective in capturing the overall effects of Notch signaling in T cell immunity, irrespectively of the individual Notch ligands and receptors involved (6, 8). DNMAML mice rejected MHC-mismatched hearts in a delayed fashion (14). Although the delay was relatively modest in the absence of other interventions, it was observed in the absence of costimulatory blockade, suggesting that complete Notch inhibition in T cells could achieve higher protection from rejection than the level of protection seen upon partial Notch inhibition with anti-Dll1 antibodies (9, 14). Importantly, upon concomitant CD8 depletion prior to transplantation, DNMAML expression led to markedly enhanced protection, with a median allograft survival of >40 days. These findings suggested that $CD4^+$ alloreactive T cells were particularly sensitive to Notch inhibition. Mechanistically, DNMAML led to decreased production of both IFNγ and IL-4 by donor-reactive T cells, decreased immune cell infiltration and an increased regulatory T cell (T_{reg})/effector T cell (T_{eff}) ratio within the graft. DNMAML recipients also showed delayed appearance of donor-specific alloantibodies, suggesting a role for Notch in T cell help to allospecific B cell responses.

Building on their observations from studies using genetic pan-Notch inhibition in T cells, Wood et al. assessed the impact of humanized anti-Dll1 and anti-Dll4 antibodies, alone or in combination, on allograft rejection (14). This approach was chosen given the effects of Dll1 in transplant rejection (9) and the dominant role of Dll1/4 Notch ligands in GVHD (7, 12). Anti-Dll1/4 antibodies had high therapeutic activity in allograft rejection. Both anti-Dll1 and anti-Dll4

antibodies by themselves induced significant protection, indicating that these two Notch ligands were involved non-redundantly in the rejection process. Combined administration of anti-Dll1 and anti-Dll4 antibodies was the most effective strategy tested, enabling long-term engraftment in CD8-depleted recipients and markedly delayed rejection even in CD8-replete hosts. Surprisingly, systemic Dll1/4 blockade provided a higher degree of protection from allograft rejection than DNMAML-mediated pan-Notch inhibition in T cells. Enhanced protection was associated with a persistent decrease in donor-specific alloantibody titers, plasma cell numbers and complement deposition in the graft. These findings suggest that the therapeutic activity of anti-Dll1/4 antibodies is related both to their effects on T cells (preventing acute cellular rejection) and to their effects on the B cell response (preventing chronic rejection at least in part through humoral mechanisms). Furthermore, long-term protection was observed upon short-term Dll1/4 blockade in the peri-transplant period, similar to findings in allogeneic bone marrow transplantation and GVHD (7, 14).

Together, Dll1/4 Notch ligands play dominant roles in the regulation of alloimmunity, but the role of Jagged ligands remains unclear. To start addressing this question, Riella and coworkers used a Jagged2-specific antibody in mouse heart allograft rejection models (10). This antibody was previously shown to specifically bind Jagged2, but was suggested to facilitate forward Notch signaling in an *in vitro* coculture system through unknown mechanisms (35). Jagged2-specific antibodies induced accelerated rejection in two heterotopic heart transplantation models. Accelerated rejection was associated with complex immunological changes, including increased production of IL-6 and Th2 cytokines, and increased T_{reg} expansion. These findings suggest that Jagged2 can have a proinflammatory role in allograft rejection, but interpretation is challenging, as the biochemical impact of the Jagged2-specific antibody used in these studies is not fully characterized. Future work using genetic approaches and other pharmacological reagents could clarify the role of Jagged ligands in transplant rejection.

Although there are differences in experimental approaches, particularly in terms of global Notch inhibition in T cells vs. selective systemic targeting of Notch pathway components, studies of allograft rejection and *in vivo* Notch inhibition delineate elements of an emerging consensus: 1) Notch signaling is a major pathway that promotes inflammation and opposes tolerance in

allograft transplantation; 2) Notch signaling controls alloreactive T cell immunity, but may also regulate non-T cell subsets that contribute to the pathogenesis of organ rejection; 3) Targeting the Notch pathway has therapeutic potential to prevent allograft rejection, with short-term blockade of Delta-like Notch ligands in the peri-transplant period capable of inducing long-term effects.

In vivo studies of Notch signaling in graft-versus-host disease. Although the focus of this minireview is on allograft rejection, it is useful to highlight for comparison other recent findings about the role of Notch signaling in graft-versus-host disease (GVHD) (**Table 1B**) (6-8, 11, 12). Using DNMAML expression or *Rbpj* inactivation to block all canonical Notch signals in T cells, Zhang et al. first reported major protective effects of Notch inhibition in mouse models of acute GVHD (6). Notch inhibition led to markedly increased survival of transplant recipients. Notch-deprived alloreactive T cells showed decreased production of multiple inflammatory cytokines (including IFNγ, Tumor Necrosis Factor alpha, IL-17 and IL-4) and increased expansion of preexisting T_{regs} (6, 8). Decreased cytokine production was observed in both $CD4^+$ and $CD8^+$ T cells, and was associated with features of acquired hyporesponsiveness in alloreactive T cells (8). Individual T cell effector functions were affected to a variable extent by Notch inhibition, as *in vivo* T cell proliferation and expansion were preserved in irradiated recipients. T cell cytotoxic functions were also largely maintained in the absence of Notch signaling, leading to the preservation of potent graft-versus-tumor effects. Using a genetic strategy to inactivate the *Notch1* or the *Rbpj* gene only in T_{regs}, Chatila's group reported that Notch negatively regulates T_{reg} numbers and function *in vivo*, and that Notch inhibition in Tregs alone conferred therapeutic benefits in acute GVHD (13). Thus, Notch inhibition may exert beneficial immunomodulation in conventional $CD4^+$ and $CD8^+$ T_{eff} as well as in T_{regs}.

Therapeutically, γ-secretase inhibitors (GSI) were shown to be effective in a mouse model of alloimmune bone marrow injury (11). However, in acute GVHD models involving lethal irradiation, systemic pan-Notch inhibition with GSI was poorly tolerated because of on-target toxicity in the gut (7). To bypass this toxicity, the role of individual Notch ligands and receptors was investigated using genetic models and paralog-specific monoclonal antibodies (7, 12). Notch1/Notch2 receptors and Dll1/4 Notch ligands accounted for all the effects of Notch

signaling in alloreactive T cells during GVHD, with dominant roles for Notch1 and Dll4. Dll1/4 blockade emerged as the most promising therapeutic approach to prevent GVHD while avoiding system side effects of pan-Notch inhibition. Interestingly, transient early Dll1/4 inhibition was essential and sufficient to confer long-term GVHD protection (7). Altogether, clear parallels are emerging between the functions of Notch signaling in acute GVHD and allograft rejection. In both cases, early Dll1/4-mediated Notch signals exert profound and durable pro-inflammatory effects, such that transient Dll1/4 inhibition provides long-lasting therapeutic benefits.

Mechanistic considerations. The molecular mechanisms of Notch action in mature T cells remain under active investigation. The most relevant observations are and will continue to be derived from *in vivo* experiments that evaluate physiological levels of Notch signaling in defined immunological contexts. Along these lines of investigations, Notch was recently reported to regulate specific functions of $CD4^+$ and $CD8^+$ T cells, including *in vivo* survival and metabolism, responsiveness to CD28-mediated costimulatory signals, and $CD8^+$ T cell differentiation (29, 31, 33, 36-38). An important overarching theme is that Notch does not appear to function as a lineage-specific regulator, but instead as a regulator of T cell reactivity and function. In T cell alloimmunity, multiple investigators observed that Notch inhibition tips the balance between inflammatory T_{eff} and suppressive T_{reg} functions (**Fig. 2**) (6, 13, 14). Notch-deficient T_{eff} cells appear defective in their production of multiple inflammatory cytokines, while Notch-deficient T_{regs} accumulate in higher absolute or relative numbers and may have enhanced suppressive ability. Furthermore, selective blockade of Notch1 receptor in a fully MHC-mismatched model reduced T_{eff} and increased T_{regs}, significantly prolonging allograft survival (Riella, unpublished work). T_{regs} were not only expanded in number but also had enhanced suppressive function in the absence of Notch1 signaling. The dual effects on both T_{eff} and T_{regs} likely account for the prolonged impact of transient Notch inhibition. Of note, key downstream effects of Notch signaling are likely to be mediated by canonical CSL/MAML-dependent transcriptional mechanisms (6, 13), but the functionally essential targets of Notch signaling in T_{eff} and T_{regs} remain to be identified.

Therapeutic implications and future directions. Based on available preclinical data, we propose that Notch signaling is an attractive new therapeutic target to prevent allograft rejection.

Short-term inhibition of Notch signaling exerts a longstanding beneficial impact by dampening the alloimmune response, highlighting the promise of transient Notch inhibition strategies in the peritransplant period (7, 14). Beyond allograft rejection, Notch inhibition could also be beneficial in other T cell-mediated immune disorders, including GVHD and autoimmunity (7, 39, 40). In practice, targeting individual Notch ligands and receptors with specific monoclonal antibodies currently appears to be the most promising therapeutic approach to target Notch signaling in alloimmune cells, while avoiding the systemic side effects of pan-Notch inhibition (7, 9, 14). As for other strategies, an important challenge will be to translate these findings from preclinical mouse models into more advanced preclinical models (e.g. non-human primates) and into humans. Given that Notch is an ancient and highly conserved signaling pathway, it is tempting to speculate that key features of its effects will be conserved, although this needs to be investigated systematically. Promising observations such as the significant enhanced Notch signaling in T_{eff} upon kidney transplant rejection in humans compared to non-rejectors provide optimism in this translational approach (Riella, unpublished work). Advances in our understanding of Notch's immunobiological effects and carefully designed translational investigations could unravel the full therapeutic potential of Notch inhibition in allograft rejection and other immune-mediated disorders.

Figure legends

Figure 1. Overview of Notch signaling.

Mammalian Notch signaling is initiated by interactions between Notch receptors (Notch1-4) and Notch ligands (Delta-like 1,3,4; Jagged 1,2). Ligand-receptor binding triggers two sequential proteolytic cleavages of the Notch receptor by the ADAM10 metalloprotease and by the γ-secretase complex, releasing the intracellular domain of Notch (ICN) into the cytoplasm. Upon entry into the nucleus, ICN forms a transcriptional activation complex with the transcription factor (TF) CSL (CBF1/Suppressor-of-hairless/Lag-1), a member of the Mastermind-like (MAML) family, and other coactivators such as p300. ICN/CSL transcriptional complexes often assemble adjacent to other TFs, and can regulate Notch target gene expression proximally through promoter binding or distally through enhancer binding and long-range interactions.

Figure 2. Emerging model of Notch signaling as a central regulator of alloreactivity vs. tolerance.

Notch drives T cell pathogenicity during allotransplantation by enhancing pathogenic functions in effector T cells (T_{eff}), while decreasing numbers and beneficial immunosuppressive functions of $FoxP3^+$ regulatory T cells (T_{regs}). Interfering with the Notch pathway can reverse this imbalance by dampening proinflammatory cytokine production by T_{eff} cells and enhancing both T_{reg} function and numbers. Importantly, short-term Notch inhibition in the peri-transplant period can confer long-lasting immunological benefits.

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Figure 1

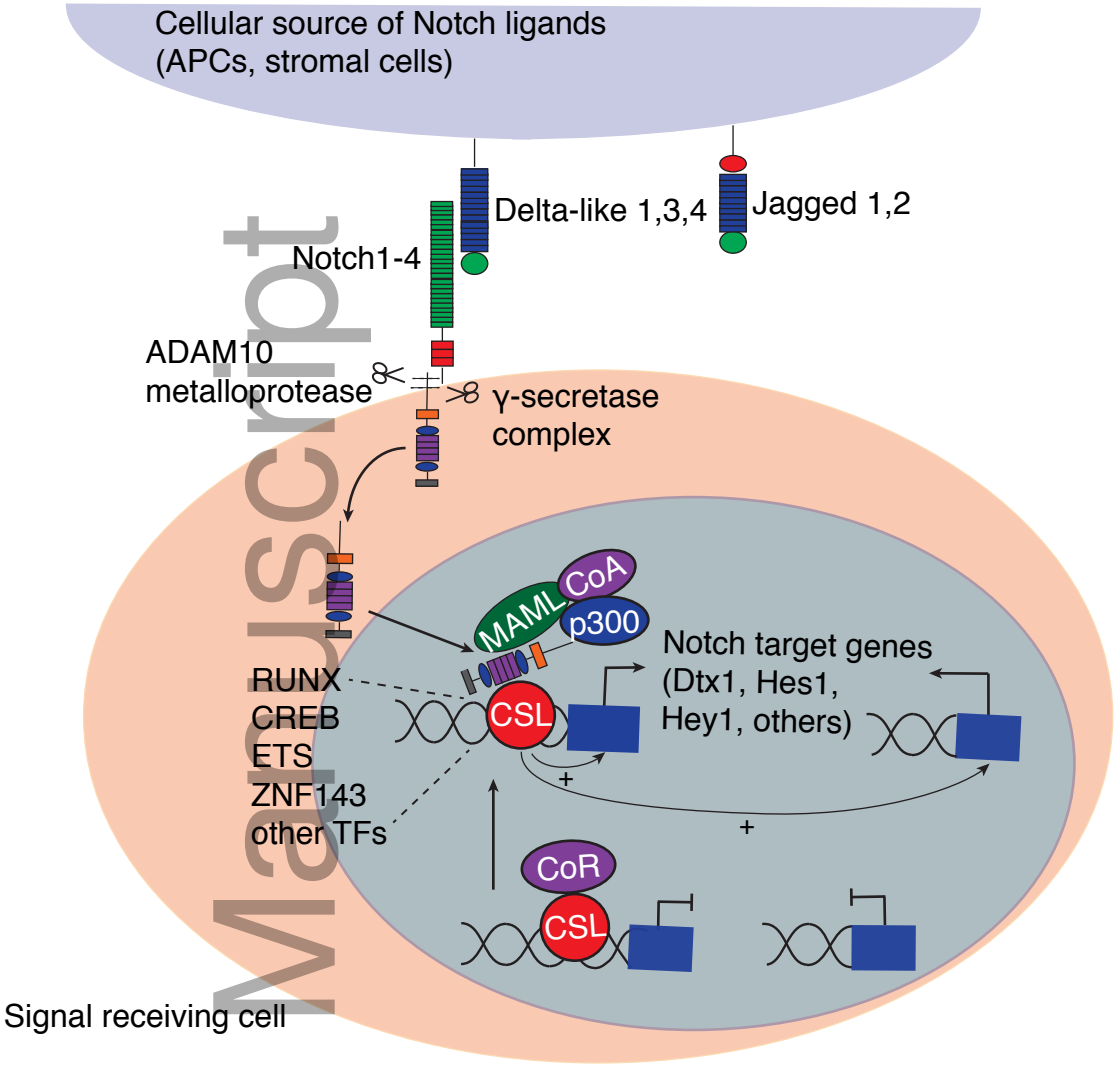

Figure 2

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