Role of the adaptor protein, beta-arrestin1, in the Notch signaling pathway

by

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Abstract

The Notch receptor is part of a highly conserved signaling pathway shared in *Drosophila*, *C. elegans* and mammals. Extensive studies of Notch signaling have revealed its participation in the development of diverse organ systems including brain, blood cells, blood vessels, gut, and skin. Many genetic modifiers of the Notch signaling pathway have been identified, including some which act at the membrane and others in the nucleus. One such member is Deltex, an E3 ubiquitin ligase, which was originally identified as a modifier of Notch in a Drosophila genetic screen. In early lymphoid development, Deltex has been demonstrated functionally to antagonize Notch signaling but the precise molecular mechanism for this functional antagonism between Notch and Deltex is not understood. However, in *Drosophila*, recent data supports the formation of a trimeric complex between Deltex, Kurtz and Notch that promotes Notch ubiquitin-mediated proteosomal degradation. Beta-arrestin1 is one of the closest mammalian homologues of Kurtz and functions as an adaptor protein in a variety of cellular processes such as endocytosis, ubiquitination and nuclear shuttling. We hypothesize that a similar interaction occurs in mammalian cells between Notch, beta-arrestin1 and Deltex to negatively modulate the Notch signaling pathway. Our data reveal a physical interaction between beta-arrestin1 and the Notch receptor. We could not, however, detect an interaction between Deltex and beta-arrestin1 by co-immunoprecipitation. We also demonstrate that Notch and beta-arrestin1 physically associate with both a membrane-bound form of activated Notch, as well as the intracellular form of Notch after membrane cleavage. Using RNA interference, as well as overexpression of beta-arrestin1, we demonstrate that beta-arrestin1 negatively regulates a Notch/CSL dependant reporter assay. We also show that the presence of Deltex enhances the negative modulation of the Notch signaling pathway mediated by beta-arrestin1. Therefore, we reveal a new Notch interacting protein and a novel role for beta-arrestin1 in the Notch signaling pathway.
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<th>Description</th>
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<tr>
<td>293T/HEK</td>
<td>Human Embryonic Kidney cells</td>
</tr>
<tr>
<td>AGM</td>
<td>aortic-gonadal mesonephros</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid progenitor</td>
</tr>
<tr>
<td>CCP</td>
<td>Clathrin-coated pit</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DN</td>
<td>Double-negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELP</td>
<td>Early lymphoid progenitor</td>
</tr>
<tr>
<td>ETP</td>
<td>Early thymic progenitor</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FoB</td>
<td>Follicular B-cells</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescence Protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-coupled protein receptor</td>
</tr>
<tr>
<td>GSI</td>
<td>Gamma-secretase inhibitor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
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<tr>
<td>HECT</td>
<td>Homology to E6-AP carboxy terminus</td>
</tr>
<tr>
<td>HES</td>
<td>Hairy/Enhancer of Split</td>
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<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>ICN</td>
<td>Intracellular Notch</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani bacteria culture medium</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MVB</td>
<td>Multi-vesicular bodies</td>
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<tr>
<td>MZB</td>
<td>Marginal B-zone cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NaCl:</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NK:</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NP-40:</td>
<td>Ipegal</td>
</tr>
<tr>
<td>PBS:</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR:</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI:</td>
<td>Propidium Iodine</td>
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<tr>
<td>PMSF:</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF:</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RING:</td>
<td>Really Interesting New Gene</td>
</tr>
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<td>RNA:</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI:</td>
<td>Roswell Park Memorial Institute</td>
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<td>RT-PCR:</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<td>SDS:</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE:</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA:</td>
<td>small interference RNA</td>
</tr>
<tr>
<td>SOP:</td>
<td>Sensory organ progenitor</td>
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<tr>
<td>T-ALL:</td>
<td>T-Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>TACE:</td>
<td>TNF-alpha converting enzyme</td>
</tr>
<tr>
<td>TAD:</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TBS:</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TCR:</td>
<td>T-cell receptor</td>
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<tr>
<td>TE:</td>
<td>Tris-EDTA</td>
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<td>Th:</td>
<td>T helper</td>
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À grand-papa,
je t'avais promis d'essayer...
Chapter one
Introduction

1.1 The Notch signaling pathway plays a significant role in normal hematopoiesis and T-cell development

1.1.1 Hematopoiesis

Hematopoiesis is the process by which immature precursor cells differentiate into mature blood cells. It is currently thought that all cell lineages constituting blood originate from only one pluripotent stem cell referred to as the hematopoietic stem cell (HSC). The earliest blood cells to develop during embryogenesis are red blood cells; they are present in the yolk sac of the mouse embryo, as early as embryonic day (E) 7.5-11. Between E11-16, blood progenitor cells of the yolk sac migrate to the liver and the spleen, where some of the cells commit to the myeloid lineage and start differentiating into granulocytes. At later time points during gestation, the bone marrow cavity is colonized by hematopoietic progenitors, and become the primary active hematopoietic site. At birth, the spleen and the liver no longer produce blood cells and the bone-marrow (BM) of flat bone (like the sternum, vertebrae, iliac bones and the ribs) assure the production of blood cells. In adulthood, long bones exclusively (like the humerus and the femur) have the capacity to produce blood cells. However, the liver and the spleen's hematopoietic capacities can be activated in situations where the bone marrow is damaged, or if the blood demand is exceedingly high called extramedullary hematopoiesis.

Blood cells are divided into two different lineages: 1) the myeloid cells, involved in erythrocytes formation, primary defense, innate immunity and blood clotting, and 2) the lymphoid cells, involved in adaptative immune responses. The initial differentiation of HSCs begins with their commitment to either the myeloid or the lymphoid lineages in response to certain growth factors and cytokines. Once the differentiation process begins, stem cells lose their self-renewal ability and they mature to either the myeloid...
cell lineage (to give rise to erythrocytes, neutrophils, eosinophils, basophils, monocytes, mast cells and platelets) or to the lymphoid cell lineage (to generate B-cells, NK cells or T-cells).

1.1.2 Lymphopoiesis

All lymphoid committed precursors must go through a series of maturation steps, including the acquisition of phenotypic and functional characteristics of their cell lineage, before being considered mature B-cells or T-cells. In addition, the maturation of lymphoid precursors is highly dependent on the surrounding environment. Stromal cells of the bone marrow and the thymus secrete cytokines and growth factors as well as express cell surface ligands that affect the maturation of progenitors.

Multiple genes regulate the passage of stem cells to committed B-cell precursors. Expression of genes specific to B-cell development drives B-cell precursors toward the pro-B cell stage of maturation. The pro-B stage is a critical step for precursors as it is the step in which they acquire their functional immunoglobulin. Expression of Rag genes in pro-B cells begins a process called somatic recombination, involving enzymatic deletion and re-ligation of immunoglobulin gene segments within genomic DNA. Somatic recombination leads to rearrangement of immunoglobulin heavy chain and eventually to functional immunoglobulin signaling. Pro-B cells that fail this maturation step die by apoptosis. Cells carrying a functional immunoglobulin are then called pre-B cells. As they mature, pre-B cells undergo negative selection, a process in which B-cells that recognize antigen of the self and die quickly by apoptosis. These cells are dangerous to the body as they can lead to autoimmunity. Finally, mature non auto-reactive B-cells exit the bone-marrow to populate peripheral lymphoid organs such as the spleen or the lymph nodes, where they lie dormant until stimulated and activated by a non-self antigen (Abbas et al, 2003).

The development of the T-cell lineage is somewhat different than that of the B-cell lineage. Thymic precursors migrate from the bone marrow to the thymic cortex, where they interact with thymic stromal cells. The nature of the precursors entering the
thymus is not fully characterized. In the past, thymic precursors were believed to give rise exclusively to T-cells, but recently strong \textit{in vitro} data suggest that precursors entering the thymus are not fully committed to the T-cell fate and can still generate NK and dendritic cells (Carlyle et al., 1998; Ikawa et al., 2004). These thymic progenitors display all characteristics of T-cell progenitors but cannot give rise to B-cells, suggesting that the B-cell potential is lost before entering the thymus. Several regulatory genes guiding T-cell lineage decisions are expressed in thymocyte precursors after the loss of B-cell potential, but none of them are master regulators of a specific T-cell lineage. Notch signaling is well known for its capacity to promote T-cell development in addition to blocking the commitment of thymic precursors to B-cells (Pui et al., 1999). The T-cell fate is not the end point of a failed commitment to B-cell fate and Notch signaling plays a crucial role in the acquisition of T-cell characteristics by thymic precursors. During the pro-T cell differentiation process, T-cell precursors undergo a Notch-dependent expansion. During this expansion period, thymic precursors still have the potential to commit to either NK or dendritic cell (DC) lineages. Eventually, along the T-cell maturation path, pro-T-cells lose their NK/DC potential and dedicate themselves exclusively to the T-cell fate (David-Fung et al., 2006). In summary, T-cell commitment of precursors entering the thymus can be divided into three main steps 1) loss of B-cell lineage commitment, 2) Notch-dependent expansion with NK/DC potential, 3) loss of NK/DC potential.

Similar to B-cell maturation, T-cell maturation begins with the expression of rag genes at the pro-T cell stage. The rag gene products trigger the somatic recombination of the T-Cell Receptor (TCR) genes (αβ vs γδ chains). The first somatic recombination begins with the TCR beta chain rearrangement. Thereafter, pre-T cells carrying a rearranged TCR beta chain must associate with a surrogate light chain called pre-T-alpha to deliver a signal through their functional TCR. This signal is critical for survival of T-cell precursors and hence, thymocytes failing to express a functional pre-TCR complex apoptose. This checkpoint step is called beta-selection and is necessary to ensure that the first somatic recombination of the beta chain was successful. As they mature, pre-T cells undergo a second somatic recombination event to rearrange their alpha chain and go through positive selection: the recognition of self-MHC.
Thymocytes unsuccessfully recognizing self-MHC inevitably fail to recognize an antigen presented in the context of MHC by an antigen-presenting cell (APC). Therefore, those thymocytes are not selected for further maturation steps and apoptose. Subsequently, the pool of thymocytes capable of recognizing self-MHC undergoes negative selection (see above) to mature toward the last thymic maturation step that occurs in the thymus, the CD4 vs CD8 specification. Thymocytes that have successfully completed the beta-selection process will enter into a stage where both CD4 and CD8 markers are expressed at the surface of their membrane. Eventually some of them will differentiate into CD4+ T helper while others will adopt a CD8+ cytotoxic fate. As they achieve their final maturation state, they leave the thymus to migrate to peripheral lymphoid organs where they are eventually activated by an APC.

1.1.3 Role of Notch signaling in development

The Notch signaling pathway was first identified in *Drosophila melanogaster* in a genetic screen for mutations causing phenotypic changes in wing development. An indentation at the margin of the fly wing blades was induced by a partial loss-of-function mutation of the *notch* gene. It was later found that this gene encodes a membrane-tethered transcription factor (Wharton et al., 1985). In the beginning of the 1990’s, extensive characterization of the Notch signaling pathway led to the conclusion that Notch receptor was highly involved in fly embryo development and played a pivotal role in cell-fate decisions (Artavanis-Tsakonas et al., 1999). In *Drosophila*, enhancing Notch signaling induces lateral bristle inhibition by forcing neural progenitors to adopt an epidermal cell fate over a neuronal fate. In a cluster of pro-neural equipotent cells, only one of the cells mature into sensory organ progenitors (SOPs), to become a neuron (Schweisguth and Posakony, 1992). The SOP also induces Notch signaling in the neighboring cells, causing the suppression of neural gene expression, thus resulting in an epidermal cell fate. The potential of the Notch signal to induce lateral inhibition in non-committed cells has also been confirmed in Notch loss-of-function experiments. Knocking-down the Notch signal causes the loss of epidermal fate and drives the differentiation of progenitors toward a neural cell fate (Lai, 2004). This lateral inhibition was the first indication of a negative role of Notch signaling on progenitor cells.
because it blocks their differentiation. Nevertheless, Notch signaling also plays a positive role in SOP differentiation: it directs several binary cell fate choices leading one SOP to differentiate into four different cells (socket, hair, sheath, neuron) constituting the assembly of the bristle. Finally, a third proposed function for Notch signaling is its role in terminal cell differentiation as revealed by studies on human and mouse skin. Notch signals induce the expression of early differentiation markers and cell cycle arrest (Rangarajan et al., 2001). Together, these findings highlight not only the importance of Notch signaling in cell fate but also shed light on the context-dependent manner in which Notch signaling exerts its effect.

1.1.4 Involvement of Notch signaling in several steps during hematopoiesis

1.1.4.1 Notch signaling may be involved in hematopoietic stem cell maintenance

The first murine hematopoietic stem cell appears in the extra-embryonic yolk sack at E7.5 and the first long-term repopulating HSC population is believed to originate from a bi-potential precursor called hemangioblast. That precursor cell has the potential to commit either to an endothelial cell fate or a hematopoietic cell fate (Haar and Ackerman, 1971). It is not entirely clear whether primitive cells from the yolk sac migrate to another region called the aortic-gonado mesonephros (AGM) or repopulate directly the liver (Traver and Zon, 2004). However, the AGM region is well characterized as an importance site also showing of early HSC activity. The AGM region is believed to be a potential site for the localization of hemangioblasts because cells from the AGM region have the capacity to reconstitute the major arterial regions of the embryo as well as the entire murine hematopoietic cell system (Godin et al., 1993). In addition, the reconstitution activity of hemangioblasts in the AGM region is believed to depend on Notch signaling. In fact, cells derived from the AGM at E9.5 cannot differentiate in vitro when Notch signaling is blocked, but the suppression of Notch signaling in cells derived from the AGM at E10.5 has no effect on their in vitro hematopoietic differentiation (Kumano et al., 2003). Not only do these findings illustrate how Notch signaling is required for a very short period of time, but they also allow speculation that Notch may drive the bi-potent hemangioblasts towards
hematopoietic lineage commitment at the expense of the endothelial lineage. This is reminiscent of Drosophila neurogenesis where Notch signaling leads to epidermal cell fate at the expense of neuronal cell fate.

Several lines of evidence also demonstrate the role of Notch signaling in adult hematopoietic stem cell maintenance. In vivo, Notch overexpression in murine bone-marrow progenitors leads to an increase in HSC renewal and immortalization of hematopoietic cells having the potential to differentiate either into myeloid cells or lymphoid cells (Varnum-Finney et al., 2000). In vitro, murine or human HSCs co-cultured with immobilized or soluble Notch ligands show an enhanced level of stem cell renewal, possibly mediated by the expression of the well-known Notch target gene HES-1 (Hairy Enhancer of Split-1), a co-transcriptional repressor (Carlesso et al., 1999; Davis and Turner, 2001). In addition, in vitro Notch1 loss-of-function studies have shown an increased level of HSC differentiation (Duncan et al., 2005). These experiments confirm the role of Notch signaling in the maintenance of undifferentiated HSCs (Duncan et al., 2005). However, no robust Notch1 genetic loss-of-function studies can support this paradigm. Indeed, conditional CSL (Su(H)/CBF1/Lag-1) knock-out mice lacking all Notch family member transcriptional signaling as well as conditional Notch1 and Notch2 knock-out mice, do not show any abnormal HSC phenotypes (Han et al., 2002; Radtke et al., 1999). Nevertheless, Notch is well proven to play critical roles in cell differentiation and maturation at different times of embryonic development. More elaborate characterization of HSCs at each stage of differentiation will eventually lead to a better understanding of the role of Notch in HSC biology.

1.1.4.2 Role of Notch in T-cell fate lineage choices

The best-characterized role for Notch signaling in hematopoiesis is its regulation of the binary cell fate choice of early lymphoid progenitors (ELP), as well as, in more committed T-cell progenitors. The established role for Notch signaling in T-cell development, especially in promotion of T-cell fate over B-cell fate, has been demonstrated by several means. First, inducible inactivation of Notch signaling in BM progenitors causes a block in intrathymic T-cell differentiation and maturation, as well
as, the generation of intrathymic B-cells (Radtke et al., 1999). Moreover, conditional CSL loss-of-function inhibits T-cell development and generates intrathymic B-cells in BM progenitors (Han et al., 2002). These results establish a unique role for Notch1 in T-cell commitment of BM progenitors by demonstrating that Notch 1 pushes the cell lineage choice of BM progenitors toward a T-cell fate through a CSL-mediated pathway. In agreement with the latter results, gain-of-function studies performed by retroviral transduction of BM progenitors with the intracellular subunit of Notch (ICN), have shown inhibition of B-cell development as well as increased production of T-cells in the bone marrow (Pui et al., 1999).

It is still not clear at what level of commitment precursors exit the bone marrow to enter the thymus, since the nature of the bone marrow progenitors entering the thymus is not well characterized. In the past, the paradigm was that common lymphoid progenitors (CLPs) entered the thymus with the potential to become either T-cells or B-cells and the T-cell fate would be induced, upon induction of Notch signaling, at the expense of the B-cell fate. However, the characterization of CLP surface markers revealed that CLPs could not be detected in the thymus or give rise to the T-cell lineage. This suggests that a more primitive type of progenitor enters the thymus and differentiates into the T-cell lineage (Allman et al., 2003) upon activation of the Notch signal. The existence of such a multipotent lymphoid precursor has not yet been identified at the single cell level. However, it is also possible that, at the population level, these early T-lineage progenitors (ETPs) enter the thymus and produce NK, B, myeloid or dendritic cells depending on the dosage of Notch signaling and the level of commitment of T-cells precursors (Schwarz and Bhandoola, 2004). \textit{In vitro}, it has been demonstrated that high stimulation of Notch signaling suppresses the non T-cell phenotype whereas mild stimulation of Notch signaling (induced by exogenous Notch inhibitors) allows T-cell precursors to commit to cell lineages like NK and dendritic cells (De Smedt et al., 2005).
1.1.4.3 Role of Notch in T-cell maturation

Maturation of pro-thymocytes can be monitored by their expression of surface markers. When entering the thymic cortex, immature thymocytes do not express the mature T-cell surface markers TCR, CD3, CD4 or CD8. First, immature thymocytes enter the thymus at the DN1 stage and are not yet committed to the T-cell fate. Maturation events occurring during the DN1 to DN3 transition of immature thymocytes are TCR signaling independent. However the final commitment to the T-cell lineage and the transition from the DN3 stage to the DN4 stage is solely depend on successful signaling of a functional pre-TCR complex (David-Fung et al., 2006). The role of Notch signaling is critical in the two major maturation events of the DN3-DN4 passage. First, it promotes the formation of the TCR-αβ complex. The thymus of mice transplanted with mixed Notch +/- and Notch +/+ BM progenitor cells show a decrease in TCR-αβ expression in the Notch +/- population versus the expression of TCR-αβ expression in the Notch +/+ population. This result is specific to the TCR-αβ as there was no significant change in the levels of expression of another mature TCR complex, TCR γδ (Washburn et al., 1997). Hence, Washburn’s study suggests that the loss of Notch signaling does not affect TCR-γδ formation but does severely block TCR-αβ development. This hypothesis has been confirmed genetically by a Notch1 conditional knock-out mouse in which the level of expression of TCR γδ was not significantly increased (Wolfer et al., 2002). However, CSL conditional knock-mice revealed moderate (2-fold) increases in the expression of TCR-γδ (Tanigaki et al., 2002). These two genetic models strengthen the hypothesis that the lack of Notch signaling severely blocks the formation of TCR-αβ but does not promote the formation of TCR-γδ at the expense of TCR-αβ formation.

Secondly, Notch signaling is critical at the DN3 stage since it participates in the beta-selection process by inducing the transcription of the pre-Talpha gene necessary for the formation of a functional pre-TCR complex. As thymocytes mature to the DN3 stage, they start rearranging their TCRβ and, with the help of preTalpha, they assemble a functional pre-TCR signaling complex. Successful pre-TCR signaling leads to the passage of the thymocytes to the next maturation stage, whereas thymocytes failing...
the pre-TCR signaling apoptose. Notch is also believed to initiate apoptosis of failed thymocytes since Notch loss-of-function studies reveal compromised steps of VDJ rearrangement when Notch signaling is downregulated, causing an accumulation of dysfunctional thymocytes in the thymus (Wolfer et al., 2002). Thus, Notch likely has a role in inducing apoptosis of failing thymocytes as well as in αβ-δγ TCR lineage commitment and the transcription of pre-Talpha gene.

Thymocytes complete their maturation by adopting either a CD4 T-helper or a CD8 T-cytotoxic cell fate. In the past, Notch was believed to play a role in this lineage fate decision favoring the CD8+ phenotype over the CD4+ phenotype (Robey et al., 1996). These experiments were performed in an overexpression context and were difficult to interpret since it has been demonstrated since that constitutive activation of Notch signaling causes a block in differentiation and the induction of leukemia. Later, genetic studies (Notch 1 conditional knock-out mice and CSL conditional knock-out mice) showed no favoring to CD8+ thus confirming that Notch does not influence lineage commitment in the case of CD4 vs CD8 cell fate (Tanigaki et al., 2002; Wolfer et al., 2001).

1.1.4.4 Role of other Notch family members in lymphoid differentiation

In mammals, Notch has four family members (Notch 1 to 4) that are expressed in a variety of tissues and are mostly involved in cell lineage commitment. All 4 Notch family members are expressed in lymphocytes; Notch 1 and 3 are expressed in T-cells whereas Notch 2 is predominantly expressed in B-cell progenitors (Saito et al., 2003). Notch 4 is also present in T-cells, but at almost undetectable levels; its expression is more prominent in endothelial vasculature (Hainaud et al., 2006). While Notch 1 plays a crucial role in T-cell development, Notch 2 is actively involved in B-cell development. The highest level of Notch 2 expression is in the B-cell population of the spleen where it has been demonstrated that Notch 2 drives the differentiation of B-cell progenitors toward a specific subset of splenic B-cells called marginal zone B-cells (MZB), at the expense of follicular B cells (FoB), another subset of splenic B-cells. Conditional loss of Notch 2 or its transcriptional activator, CSL, leads to a significant decrease in MZB
cells in the spleen, but only a minor increase in FoBs (Tanigaki et al., 2002). However, to date, it is not entirely clear if MZB and FoB cells are derived from the same progenitors as a significant increase in FoB cells has not been reported in the absence of Notch2. It is also noteworthy that conditional Notch 2 loss-of-function does not show an abnormal B-cell precursor development in the bone-marrow suggesting that Notch 2 may not be involved in maturation of these early progenitors (Saito et al., 2003).

Notch 3 is also expressed in T-cells but its role is not yet clarified. In thymocytes, Notch 3 is up-regulated and seems to trigger thymocyte differentiation through its interaction with Jagged1 and 2 ligands (Felli et al., 1999). Also, following activation by an antigen, naive CD4+ T helper precursors differentiate into either T-helper 1 (Th1) or T-helper 2 (Th2). Maekawa and colleagues have shown that Notch 3, through its interaction with the Delta 1 ligand, promotes Th1 cell fate over Th2 (Maekawa et al., 2003). The role of Notch 3 in directing bipotential cell fate is better understood in other systems such as in the development of the gut or the pancreas (Apelqvist et al., 1999; Sander and Powell, 2004). Nevertheless, there is still a lot of work remaining to be done in order to characterize fully the role of Notch 3 in hematopoiesis.

Notch 4 is structurally different from the other Notch members and is barely detectable in thymocytes. However, RT-PCR experiments have identified the presence of Notch4 mRNA in both CD34- and CD34+ stem cells from human bone marrow. When constitutively activated, Notch4 enhances stem cell activity, causing a block in B-cell differentiation, thereby altering lymphoid development (Vercauteren and Sutherland, 2004). The level of functional redundancy between Notch members is not yet fully understood, but there is a clear and distinct role for Notch 4 in hematopoiesis that does not seem to overlap with other Notch family members.
1.2 The Notch signaling pathway

1.2.1 Notch gene

The human Notch 1 gene, originally identified as TAN-1 in mammals, was discovered from a chromosomal translocation (t (7;9)(q34;q34.3)) in a case of T-acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). At the time, cytogenic studies revealed a frequent site of chromosomal translocation on chromosome 7 between the positions 34 and 34.3 of the “q” arm that was causing neoplasia. Sklar’s group first cloned the DNA breakpoint of the translocation and could identify the TCRβ locus on chromosome 7. However, a locus of the chromosome 9, originally referred as TCL-3, contained most of the cDNA sequence of a gene highly homologous to the Drosophila Notch gene. Sklar’s group went further, looking at the tissue distribution of mammalian Notch1. Notch1 was mostly present in normal human and mouse tissue but its expression was more prominent in lymphoid tissues. That was the first insight that Notch1 could play an important role in normal lymphoid development (Ellisen et al., 1991).

1.2.2 Notch receptors

The Notch protein family is composed of four members in mammals (Notch 1-4). Notch 1 forms a heterodimer receptor composed of two subunits: a highly glycosylated extracellular domain and a transmembrane intracellular domain. The extracellular domain is composed of 36 N-terminal epidermal growth factor (EGF) repeats, necessary for ligand binding, followed by 3 LNR (Lin12/Notch repeats) repeats and an additional 103 residues forming the C-terminal tail of the extracellular domain. The region comprising the LNR repeats and the C-terminal tail is called the negative regulatory region (NRR) and is responsible for the maintenance of the heterodimer association between the extracellular and the membrane tethered subunits (Sanchez-Irizarry et al., 2004). The membrane-tethered subunit of Notch contains an extracellular, short series of residues rich in cysteine, followed by a transmembrane region and the intracellular domain (ICN). The ICN is composed of a RAM domain, a
110 amino acid region that contains high affinity binding sites for an important DNA binding transcription factor of the Notch signaling pathway called Su(H)/CBF1/Lag-1 (CSL). Downstream of the RAM domain, seven ankyrin repeats are necessary for Notch functions and are known to mediate protein-protein interactions. At the C-terminus to the ankyrin repeats, 219 amino acids code for a transcriptional activation domain followed by two nuclear localization signals and a PEST domain that is directly involved in rapid degradation of ICN (Aster et al., 2000). The other Notch receptors share the highest degree of homology in the ankyrin repeats whereas the highest degree of divergence is located in the PEST domain (Aster et al., 2000). Also, Notch1 and Notch2 contain a transcriptional transactivation domain that hasn’t been identified yet in Notch3 and Notch4 (Radtke et al., 2005).

1.2.3 Notch ligands

To be activated, Notch1 receptor needs to bind its ligand expressed on the surface of neighboring cells. Notch1 receptor can bind up to five different ligands and all of them have been well characterized. Delta-like 1, 3 and 4 (mammalian homologue of Delta in Drosophila) and Jagged1, 2 (mammalian homologue of Serrate in Drosophila) activate Notch1 receptor in a highly context and time-dependent fashion. Notch ligands are transmembrane proteins containing EGF-like repeats capable of binding the EGF repeats of Notch receptors. However, the intracellular domains of Notch ligands are poorly conserved and variations in their intracellular domains could be responsible for context dependent Notch signaling. In fact, downregulation or deletion of Notch ligands leads to a loss-of-function phenotype. Also, the binding of Notch ligands to the extracellular subunit of Notch receptor not only leads to the activation of the receptor but also to the transendocytosis of the ligand with the extracellular subunit of the Notch receptor in the neighboring cell (Parks et al., 2000). Once endocytosed, Notch ligands are eventually degraded or recycled to the surface of the plasma membrane (Le Borgne et al., 2005).
1.2.4 Notch signaling pathway

Notch receptors are first translated as a large 300kD peptide transiting to the Golgi apparatus before reaching the surface membrane. In the Golgi, the peptide is cleaved into two subunits by a furine-like protease (S1 cleavage) (Selkoe and Kopan, 2003). This cleavage allows the heterodimerization of the receptor and its further glycosylation by the glycosyltransferase Lunatic Fringe. After glycosylation, Notch traffics to the surface membrane ready to be activated by its ligands on neighboring cells, often referred to as sending-cells. Upon ligand binding, the endocytosis of the ligand which is still associated with the extracellular domain of Notch, creates a physical pulling force that exposes a specific cleavage site for proteases of the TACE family. TACE proteases cleave Notch receptor (S2 cleavage) causing the dissociation of the extracellular subunit from the receptor. On the receiving-cell, the intracellular subunit of Notch remains tethered to the membrane until the formation of an enzymatic complex called gamma-secretase is formed and cleaves the intracellular subunit of Notch (ICN) from the membrane (S3 cleavage) (Fortini, 2002). Recent insights have further characterized the cleavage mechanism of the Notch receptor at the membrane. Gupta-Rossi et al. have demonstrated that Notch is monoubiquitinated on a critical lysine residue (K1749) immediately following the TACE cleavage and this ubiquitination event is necessary for the endocytosis of the Notch receptor. This group also showed that the membrane bound Notch can recruit inactive members of the gamma-secretase complex and that the gamma-secretase cleavage requires endocytosis to be effective. Therefore, they suggest a model in which Notch receptor needs to be monoubiquitinated at lysine 1749 to be further endocytosed in the receiving cell. The newly formed endocytotic vesicles would then fuse with an early endosome where the low pH activates the gamma-secretase enzymatic complex. Consequently, Notch would be cleaved off the membrane of these vesicles to translocate toward the nucleus and not the plasma membrane as previously thought (Gupta-Rossi et al., 2004).

Whether or not Notch is cleaved directly from the plasma membrane or from endocytic vesicle membranes, the intracellular subunit of Notch (ICN) is free to translocate to the nucleus. Once ICN enters the nucleus, it associates with the DNA binding transcription factor CSL (Kao et al., 1998), (Zhou et al., 2000) and promotes the recruitment of
transcriptional co-activator proteins such as Mastermind-like-1 (Nam et al., 2003) and CBP/p300 proteins (Fryer et al., 2002) to the promoter of Notch target genes.

1.2.5 Notch target genes

Few genes have been identified as direct Notch targets; some of them are actively involved in cell differentiation, others in cell survival and in hematopoietic decisions. However, these distinct effects are highly context dependent. One of the best-identified direct Notch targets is the Hairy/Enhancer of Split (HES) (Davis and Turner, 2001). HES genes are often described as the canonical genes of the Notch signaling pathway. HES-1 and 5 are beta-helix-loop-helix transcription factors known to recruit co-repressors to HES target genes. They participate in various differentiation events such as neurogenesis, vasculogenesis and lymphocyte development (Davis and Turner, 2001). Even though HES-1 transcription is upregulated by Notch signaling, HES-1 proteins are not necessary for T lineage commitment. Overexpression of HES-1 in the absence of Notch signaling, in vitro, does not rescue T-cell development (Maillard et al., 2005). Pre-Talpha (Reizis and Leder, 2002) and Deltex are other Notch target genes that are representative of the context-specificity of the Notch signaling pathway. Pre-T-alpha transcription is needed to undergo beta-selection and only occurs upon Notch signaling in immature T-cells. Deltex1, the most potent Notch direct target is also expressed in lymphoid cells, but to date its role still remains unclear. In mammals, a high level of Deltex1 transcription was found upon Notch translocation to the nucleus (Deftos et al., 1998). Deltex1 acts as a negative regulator in mammals and it is suggested that Deltex participates in a negative feedback loop to downregulate the level of Notch signaling in T-cells (Storck et al., 2005). Recently, a number of genes involved in cell survival have also been identified as transcriptional targets of Notch. For examples, C-myc has been identified as an important transcriptional target of ICN, directly contributing to cell survival of leukemic cell lines (Weng et al., 2006). It has also been reported that constitutive activation of Notch signaling activates several genes of the NF-κB pathway (Vilimas et al., 2007). This is important as the NF-κB pathway plays an important role downstream of pre-TCR signaling, serving as a selective survival signal. These findings indicate a possible role
for Notch regulation in cell death via an NF-κB dependent pathway and further strengthen the role of the Notch signaling pathway in cell survival.
1.3. The modulation of the Notch signaling pathway

The Notch signaling pathway is crucial in several pivotal steps of development. Its activation is highly context dependent and time specific. Therefore, its regulation must be tightly controlled in order to ensure, for example, proper embryonic development and the proper cell maturation and differentiation patterns. Post-translational modifications of Notch such as glycosylation have been identified to explain how Notch signaling is regulated in sequential differentiation events like cell lineage commitment. Also, Notch post-translational modifications play an important role in appropriately regulating the level of Notch signaling in cell proliferation and cell survival.

1.3.1 Notch glycosylation

One of the identified mechanisms by which Notch receptors can successfully signal in a time dependent manner is by glycosylation of the EGF repeats of the extracellular subunit. In the Golgi, the glycosyltransferase Fringe links N-acetylglucosamine molecules to some of the EGF repeats which enables the receptor to differentially bind to Notch ligands at the cell surface. Differential glycosylation of the Notch EGF repeats regulates the specificity of Notch ligand binding and signaling. For instance, glycosylation of Notch’s EGF repeats inhibit Jagged-mediated Notch signaling whereas glycosylation facilitates Delta-mediated Notch signaling (Haltiwanger and Stanley, 2002). Therefore, the regulation by glycosylation induces cell sensitivity of Notch only in the appropriate cell context and at the correct time during development.

1.3.2 Notch phosphorylation

Phosphorylation is also another way by which Notch signaling is regulated. Although phosphorylation of the receptor upon ligand binding and activation is not well characterized, the phosphorylation of the intracellular domain is critical for Notch degradation. Mastermind-like1 proteins have been shown to recruit the co-activator CBP/P300 to activate the transcription of the well-known Notch target gene, HES-1
(Fryer et al., 2002). Moreover, Fryer et al. have shown that Mastermind also induces phosphorylation of CBP/P300 and the hyperphosphorylation of the TAD and the PEST domain of ICN by physically binding the CDK8 protein kinase. The Mastermind/CDK8 complex is believed to participate in the Notch degradation process, through the ubiquitination of ICN in the nucleus. These finding were confirmed by a study showing inhibition of Notch transcriptional activity at the HES1 promoter when the CDK8 kinase is overexpressed (Fryer et al., 2004).

1.3.3 Notch regulation by ubiquitination

Two other levels of Notch regulation have been extensively studied over the years, 1) the TACE/ gamma-secretase cleavage events at the plasma membrane that allow ICN to translocate to the nucleus (discussed in section 1.2.4) and 2) Notch ubiquitination. The regulation of Notch by ubiquitination is highly specific because ubiquitination reactions are mediated by the nature of the E3-ligase involved in the ubiquitin transfer and the location of the Notch proteins in the cell (membrane vs cytoplasm vs nucleus).

1.3.3.1 The ubiquitination pathway

The ubiquitination pathway catalyzes the transfer of a highly conserved 76 amino acid polypeptide called ubiquitin to a substrate. Ubiquitin is covalently linked to its substrate by three proteins, E1, E2 and E3, organized in a hierarchical organization and acting together in an ATP-dependent enzymatic reaction. E1 enzymes have very low affinity for ubiquitin and these molecules first need to be activated by Mg-ATP to form a ubiquitin adenylate intermediary that serves as a donor of ubiquitin to a specific cysteine in the E1 active site. The ubiquitin adenylate forms a thiol ester on the E1 cysteine and is eventually transferred to the E2 enzyme. E2 enzymes are limited in number and they bind very weakly to free ubiquitin or to free E1 enzymes. In contrast, they associate tightly to E3- ligases and this cooperation leads to the transfer of ubiquitin either onto the E3 or directly to the substrate depending on the nature of the E3- ligase E2 it binds to. However in every case, E2 enzymes do not change their
conformation upon binding to either E1 or E3, thus suggesting that the transfer of ubiquitin is most probably mediated by a group of residues located in the E1 or the E3 catalytic sites (Pickart, 2001).

E3 enzymes, also called E3-ligases, are numerous in the cell and they confer substrate specificity during the ubiquitin transfer. E3 enzymes have ligase activity which catalyses a two-step enzymatic reaction: 1) the binding of E3 with its substrate and 2) the covalent ligation of one or multiple ubiquitin molecules it is bound to. The latter step is dependent on the nature of the C-terminus domain that confers a ligase activity to the E3 enzyme. HECT (homology to E6-AP carboxy terminus) E3s are modular and interact with the substrate via their N-terminal domains, whereas they mediate the E2 binding and the chemistry of ubiquitination by their C-terminal HECT domain. They also act as a physical intermediate between the E2 ligase and the substrate by first loading ubiquitin and then transferring the ubiquitin moiety onto the substrate (Huibregtse et al., 1993). RING (really interesting new gene) E3s were discovered in the early 1990's and to date, it is thought that every E3 that does not have an HECT domain has a default RING finger domain (Weissman, 2001). RING finger domains include eight metal-binding residues that coordinate two zinc ions arranged by an interval pattern. Notably, the spacing between each interval pattern is conserved more than any primary sequence among the RING family (Pickart, 2001). Like for HECT E3s, the residues on or proximal to the RING domain facilitate the E2-dependent ubiquitination whereas other domains outside the RING domain recognize the substrate. Some RING E3s, like HECT, mediate ubiquitin molecule transfer and substrate recognition on their own. Such is the case for the oncoprotein MdM2 ubiquitinating P53 and for c-Cbl ubiquitinating growth factor receptors. On the other hand, other RING E3s can still mediate the E2-dependent ubiquitination, but they need to form a multi-protein complex with at least one protein from the Cullin family (Cul1, Cul2, Apc2) and with other adaptor proteins in order to recognize the substrate (Pickart, 2001). However, RING E3s do not form a thiolester ubiquitin intermediate, therefore do not take up of ubiquitin. This suggests that RING E3s act simply as docking sites for the E2, which passively transfer the ubiquitin residues directly onto the substrate without physically interacting with the RING E3-ligase (Borden, 2000).
E3 ligases are key regulators of the ubiquitination pathway as they specify both the timing and the substrate selection in the ubiquitination process. Nevertheless, the fate of the substrate is not defined by the type of E3-ligase conducting the ubiquitination but by the linkage pattern of ubiquitin on the substrate. Substrates can be poly-ubiquitinated with a tail of ubiquitin molecules linked with specific lysines on the protein, mono-ubiquitinated by one ubiquitin molecule or they can be multi-ubiquitinated with several ubiquitin single peptides linked to different lysines on the substrate. Polyubiquitinated substrates carrying a tail of ubiquitin linked by their lysine 11 (K11), lysine 29 (K29) or lysine 48 (K48) act as a potent recognition signal by the 26S subunit of the proteasome and are targeted for degradation (Weissman, 2001). However, ubiquitin molecules linked by their lysine 63 (K63) mediate endocytosis of some plasma membrane proteins as well as endosomal sorting (Hicke and Dunn, 2003).

1.3.3.2 Notch ubiquitination in the nucleus

Notch ubiquitination was first identified and characterized in the nucleus where Sel-10, an E3-ligase, mediates the ubiquitination and the degradation of the ICN. Sel-10 is an F-box protein of the Cdc4 family in which the F-box/WD-40 repeats are characteristic of HECT E3-ligases and the WD-40 repeats bind to the target proteins in a phospho-dependent manner. Sel-10 has been shown to specifically interact with the nuclear form of Notch ICN, after a nuclear phosphorylation event to promote the polyubiquitination of the PEST domain of ICN. (Gupta-Rossi et al., 2001) (Oberg et al., 2001) As well, treatment with the proteasome inhibitor MG132 greatly enhances the stability of Notch proteins in the presence of Sel-10, suggesting that, upon Sel-10 mediated-ubiquitination, ICN is likely degraded via the proteasomal pathway (Wu et al., 2001).

1.3.3.3 Notch ubiquitination in the cytoplasm

Three different E3 ligases have been identified as participants in the regulation of the Notch level in the cytoplasm: Numb, c-Cbl and Deltex1. Numb, has been highly described in Drosophila as a Notch modulator in neural progenitors. Upon
asynchronistic division, one daughter cell inherits the Numb protein, and displays a neural phenotype (sign of weak Notch signaling), whereas the other daughter cell remains an epithelial cell (the consequence of strong Notch signaling). Numb, with the help of adaptor proteins of the endocytotic pathway, such as AP-2 and Spodo, promotes Notch internalization and degradation (Fischer et al., 2006). In mammals, overexpression of Numb proteins has been reported to promote Notch ubiquitination of the membrane-bound Notch1 receptor. Ubiquitination studies have also revealed an increase in the ubiquitination of both the unprocessed (300kD) and the heterodimeric form of Notch (120kD) suggesting that Numb ubiquitimates Notch prior to ligand activation. However, unlike Sel-10 mediated- ubiquitination, the phosphotyrosine domain of Numb does not need to associate with the PEST domain of Notch to promote Notch ubiquitination and degradation (McGill and McGlade, 2003).

C-Cbl RING E3-ligase is another potential candidate that could play a role in the ubiquitination of membrane-tethered Notch molecules. C-Cbl is critically involved in the ubiquitin-dependent downregulation of activated receptor tyrosine kinases like EGF-R and PDGF-R (Pickart, 2001). Thus far, only one study has shown that c-Cbl can promote Notch ubiquitination in the myoblast cell line, C2C12. Jehn et al. revealed that c-Cbl ubiquitinates the membrane-bound form of Notch and targets it to the endosomal/lyzosomal pathway to be degraded (Jehn et al., 2002). Mono-ubiquitinated transmembrane receptors are endocytosed and then traffic from the early endosome to multivesicular bodies (MVBs), where MVBs eventually fuse to a lysosome and degrade the mono-ubiquitinated receptor. However, to date, there exists little evidence demonstrating that Notch is located in MVBs after activation by its ligand.

Deltex is a RING E3-ligase well-conserved among the Notch target genes first identified in a Drosophila genetic screen for loci suppressing the lethal phenotype caused by certain mutations in the Notch locus (Xu and Artavanis-Tsakonas, 1990). Further genetic studies have revealed that overexpression of mutated Deltex results in the same phenotype as the one caused by constitutively active Notch, identifying a role for Deltex as a positive regulator of the Notch signaling pathway (Matsuno et al., 1995). The deltex gene codes for a three domain protein; the N-terminus domain comprising
two WWE tandem repeats mostly mediating protein interaction with Grb2 (Matsuno et al., 1998) and p300 (Yamamoto et al., 2001), a central region rich in proline motifs and the C-terminus domain carrying the E3-ligase activity. In *Drosophila*, Deltex localization in the cell is mostly cytosolic, more specifically located near the plasma membrane. However, in mammals, a significant fraction of Deltex has been detected in the nucleus (Yamamoto et al., 2001). In mammals, the role of Deltex is not entirely clear; it is believed to be a negative regulator of the Notch signaling pathway in lymphoid cells. Two different Deltex knock-out mice have revealed a normal immune system development without any defect in T-cell differentiation and maturation (Lehar and Bevan, 2006; Storck et al., 2005), therefore reclassifying Deltex as a subtle modulator of the Notch signaling pathway. However, an overexpression study in common lymphoid progenitors showed that Deltex could antagonize Notch signaling and redirect progenitors toward a B-cell fate (Izon et al., 2002). The Deltex crystal structure has demonstrated for the first time the Deltex-Notch interaction by showing that the ankyrin repeats of *Drosophila* Notch physically bind the WWE domains of *Drosophila* Deltex (Zweifel et al., 2005). Many studies have tried to show a clear mechanism in which Deltex would suppress Notch signaling and new evidence is pointing toward the E3-ligase activity of Deltex and its potential to ubiquitinate Notch. In *Drosophila*, Deltex was shown to physically associate with a novel protein called Kurtz, one of the closest homologues to human non-visual beta-arrestin1, to promote Notch polyubiquitination and degradation through the proteasome. Kurtz acts as a scaffold protein, helping Deltex in its E3-ligase function to transfer ubiquitin molecules onto Notch. Also, the trimeric complex formed by Notch, Deltex and Kurtz has been found to colocalize in cytosolic vesicles. However, these vesicles do not correspond to any of the common endocytic pathway vesicles identified to date in mediating degradation (Mukherjee et al., 2005). These results shed new light on the regulation of Notch regulation and signaling mediated by Deltex.
1.3.4 Non-visual beta-arrestins

Arrestins have only recently been linked to the Notch signaling pathway in *Drosophila* where the closest homologue of beta-arrestin1 (Kurtz) is thought to participate in the degradation process of the Notch receptor. Involved in several cytosolic events like endocytosis, endosome sorting, protein degradation and nuclear translocation, arrestins have become highly interesting to study in the context of Notch signaling.

1.3.4.1 The function of non-visual beta-arrestins in biology

Non visual beta-arrestins have been well-characterized with respect to their role in internalization and downregulation of a variety of G-coupled-protein receptors (GPCRs). Upon activation and phosphorylation of GPCRs, non-visual beta-arrestins bind to the receptor and act as adaptor proteins to recruit components of the clathrin-coated pit (CCP), such as clathrin, and the adapter protein-2 (AP-2) (Krupnick and Benovic, 1998). Nonetheless, the scope of beta arrestin’s role in biology is much broader than simply trapping receptors in CCPs, as they are also involved in cytosolic and nuclear trafficking, Src (Luttrell et al., 1999) and MAPK activation (DeFea et al., 2000) as well as in signaling events and gene expression.

1.3.4.2 The structure of non-visual beta-arrestins.

Non-visual beta-arrestins comprise two isoforms, beta-arrestin1 and beta-arrestin 2. They share approximately 78% homology at the amino acid level, with the highest discordance lying in their C-terminus. The crystal structure of beta-arrestins has revealed two major domains, the N-domain and the C-domain, attached together by a polar core of buried salt bridges (Hirsch et al., 1999). Also, a flexible linker of the C-domain connects with a C-tail that is highly involved in the postendocytic fate of receptors (Hirsch et al., 1999). The N-domain is known to mediate the interaction with the receptor targeted to the CCPs whereas the C-domain is in charge of recruiting and binding components of the CCP. The two isoforms may share overlapping functions.
since the single arrestin1 or 2 knock-out mouse is viable (Conner et al., 1997) while the arrestin double knockout mouse is lethal (Kohout et al., 2001). These results suggest that the two isoforms have their own distinct functions as well as overlapping functions.

1.3.4.3 Non-visual beta-arrestins as adaptor proteins of the endocytic pathway

The best characterized role of non-visual beta-arrestins is its involvement in the endocytic pathway. At the plasma membrane, arrestin isoforms bind transmembrane receptors and the nature of the binding determines the fate of the receptor. Beta-arrestin1 is characterized by its tight and prolonged binding to poorly recycling transmembrane receptors; upon activation and phosphorylation of these receptors, beta-arrestin1 bound receptors are endocytosed. Beta-arrestin1 remains linked to the activated receptor on the surface of the endocytic vesicles targeting the receptor to multivesicular late endosomes, where it is eventually degraded by the lysosome. Beta-arrestin2 does not have a higher affinity for recycling transmembrane receptors, but is a key player in their endocytotic processing. Receptor activation and phosphorylation triggers a rapid translocation of beta-arrestin2 near the plasma membrane, where beta-arrestin2 transiently associates with the activated receptor and targets it to the CCPs. Once in the CCPs, beta-arrestin2 quickly dissociates from the receptor immediately before endocytosis (Oakley et al., 2000; Zhang et al., 1999). Overall, the strength and the stability of beta-arrestin binding to the transmembrane receptor influences later arrestin functions in the endocytic pathway.

1.3.4.4 Scaffolding role of non-visual beta-arrestins in ubiquitination of transmembrane receptors

Another function of beta-arrestins is to act as a scaffolding protein in endocytosis. Several modifications of the beta-arrestin's C-tail, such as ubiquitination, leads to post-endosomal sorting to the lysosomal degradation pathway. To date, IGF-1R, Toll-like/interleukin-1 (Wang et al., 2006), TGF-β (Chen et al., 2003) and LDL (Wu et al., 2003) are some of several transmembrane receptors in which beta-arrestins have been shown to mediate ubiquitination and/or internalization. For instance, in BE cells (human melanoma cells), beta-arrestin1 binds to the E3-ligase MdM2 to mediate
IGF-1r ubiquitination \textit{in vitro}. Moreover, knocking-down the expression of beta-arrestin1 by siRNA leads to a decrease of IGF-1R internalization and ubiquitination in these cells (Girnita et al., 2005). In \textit{Drosophila}, Kurtz, the closest homologue to beta-arrestin1, associates with Deltex1 to promote ubiquitination and degradation of the Notch receptor (Mukherjee et al., 2005). In addition to its participation in degradation and endocytosis, ubiquitination modifications are also highly involved in endosomal sorting. It is possible that Kurtz and Deltex could be involved in the intracellular trafficking of the Notch receptor as ubiquitination plays a major role in endosomal sorting. The participation of beta-arrestin in the ubiquitination process of transmembrane receptors adds yet another regulatory role to beta-arrestin's multiple functions.
1.4 Thesis objectives

Notch signaling is a highly conserved pathway among species and plays a pivotal role in cell differentiation and maturation. Notch signaling requires a high level of regulation and one way by which Notch signaling is kept under control is by a post-translational modification called ubiquitination. Deltex is an E3 ubiquitin ligase which was originally identified as a modifier of Notch in a *Drosophila* genetic screen (Xu and Artavanis-Tsakonas, 1990). Several studies performed in animals and cell lines (Izon et al., 2002) have demonstrated that Deltex functionally antagonizes Notch signaling but the precise molecular mechanism for this functional antagonism is not well understood. The Deltex crystal structure has revealed a direct physical interaction between Deltex and Notch (Zweifel et al., 2005), suggesting that Deltex may target Notch for ubiquitin-mediated proteosomal degradation. The direct evidence of Notch ubiquitination by Deltex was revealed in *Drosophila* in which Deltex, Notch and kurtz (*Drosophila* homologue of human beta-arresin1) form a trimeric complex leading to ubiquitination of Notch and its degradation by the proteosomal degradation pathway (Mukherjee et al., 2005).

**First objective. Demonstrate the presence of a complex between Notch-Deltex and beta-arrestin1 in mammals by co-immunoprecipitation.** The first objective of this project was to demonstrate the presence of a trimeric complex between Deltex, beta-arrestin1 and Notch in mammals. The formation of this complex has been well demonstrated in *Drosophila* (Deltex-Kurtz-Notch) but while Kurtz and beta-arrestin1 are close homologues, they are not identical proteins. Besides, the role of Deltex in the Notch signaling pathway is highly variable, depending on the cellular context. For instance, Deltex has been identified as a positive regulator of Notch signaling in *Drosophila* (Matsuno et al., 1995) whereas it is a negative regulator of Notch signaling in mammals (Izon et al., 2002). Therefore, it was important to confirm the presence of this complex in mammalian cells.
Second objective. Demonstrate the role of beta-arrestin1 in the Notch signaling pathway. The second objective of this study was to characterize the functional role of beta-arrestin1 in the Notch signaling pathway. Beta-arrestin1 is involved in several cellular processes such as endocytosis, endosomal sorting, ubiquitination and degradation. Therefore, better characterization of the functional interaction between Notch and beta-arrestin1 would provide a better understanding of beta-arrestin1’s role in the cellular regulation of Notch signaling.
Chapter 2
Materials and Methods

2.1 Cell culture and reagents

Human Embryonic Kidney cells, 9E10 hybridomas and U2OS cells were grown in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2mM glutamine (Invitrogen) and 1X penicillin-streptomycin. T-ALL cells were grown in RPMI medium (Invitrogen) supplemented with 10% FBS, 1X penicillin-streptomycin and 1mM sodium pyruvate (Invitrogen). Stable T-ALL-hDeltex1 cells were grown in T-ALL culture medium supplemented with 300 ug/mL of G418 (Invitrogen).

2.2 Cloning

**MigR1 HX-Flag-beta-arrestin1**

**Flag Tag.** Oligos containing the FLAG tag sequence (Integrated DNA Technology) were hybridized together to subsequently be cloned into a MigHX retroviral vectors. In addition to the FLAG sequence, we also included a BamHI site in 5' and NotI in 3' of each oligos. Oligos were resuspended at a final concentration of 100μM in 1XTE. 22.5 μL of each oligo were annealed together in 1M NaCl, 100 mM Tris pH 7.5, 10 mM EDTA solution in a final volume of 50 μL using the BioRad ICYCLER PCR machine (94°C with a ramp of -0.5°C/min). The annealed Flag tag was cloned into the linearized vector MigR1 HX with 200U of T4 DNA ligase (New England Biolab).

(Fwd 5'CGCGGATCCACCATGGATTACAAGGATGACGACGATAAGGCAGCAAGATCTGGC-3')

(Rev 5'- GGCCGCCAGATCTTGCTGCCTTATCGTCGTCATCCTAATCCATGGTG-3').

**MigHX-Flag Beta-arrestin1.** Beta-arrestin1 isoform A full length cDNA (Openbiosystems) was amplified by PCR with High-Fidelity Platinium Taq polymerase (Invitrogen) (95°C 2 minutes, 95°C 30 seconds, 65°C 30 seconds, 68°C 30 seconds for
30 cycles, 68°C 7 minutes, hold at 4°C) with oligos carrying a 5' NotI and 3' XhoI restriction cloning sites. The newly synthesized DNA was digested with NotI and XhoI and cloned into the linearized vector MigRI-HX-Flag.

(5'-AAATATCGGCCGCGGCGACAAAGGGACCCGAGTG-3')
(5'-CCGCTCGAGTCTGTTGTTGAGCTGTGGAGAGCC-3')

2.3 Transfections

Transfection studies. 293T cells were seeded the night prior to transfection at 1.2x10^6 total cells in a 60 mm culture dish. 4 μg total of DNA (pcDNA3 deltaE +C (L), pcDNA3 TAN-1 (full-length hN1), MigICNX, Migdelta E, MigHX-Flag-beta-arrestin1, pcDNA3 hDtx1-myc) were transfected into the cells using Lipofectamine and Plus reagent (Invitrogen). 7.5 μL of Plus reagent and 15 μL of Lipofectamine were incubated with the DNA according to the manufacturer's protocol. 293T cells were incubated for 3 to 5 hours with the DNA-liposome complexes and then refed with new complete DMEM for 48 hours.

2.4 Protein extraction

293T cell overexpression studies. 293T cells were detached from the culture dish by trypsinization and lysed in 500 μL of 1% NP-40 lysis buffer (50 μM Tris-HCl ph 7.4, 1% Ipegal (Sigma), 150 mM NaCl, 2 mM NaF, 2 mM NaOVanadate, 100 μM PMSF and 1X of protease inhibitor cocktail III (Calbiochem)) for 30 minutes on ice. Lysates were centrifuged at 4°C, 14 000 rpm for 10 minutes in a refrigerated centrifuge and kept on ice.

TALL cells, level of Notch protein expression. 1E6 TALL cells were lysed in 250 μL of 1% NP-40 lysis buffer (50μM Tris-HCl ph 7.4, 1% Ipegal, 150 mM NaCl, 2 mM NaF, 2 mM NaOVanadate, 100 μM PMSF and 1X of protease inhibitor cocktail III) for 30 minutes on ice. Lysates were spun at 4°C, 14 000 rpm for 10 minutes and kept on ice.
TALL cells endogenous co-immunoprecipitation. 15E6 of HPBALLs were treated with 0.01% DMSO or 1 μM of Compound E (Calbiochem) for 24 hours. Cells were lysed in 500 μL of 1% NP-40 lysis buffer (50 μM Tris-HCl ph 7.4, 1% Ipegeal, 150 mM NaCl, 2 mM NaF, 2 mM NaOVanadate, 100 μM PMSF and 1X of protease inhibitor cocktail III) and submitted to the same treatment as TALL cells above.

2.5 Protein quantification

Cell lysates were diluted 1:3 in 1% NP-40 lysis buffer. 2 μL of the dilution were diluted with 800 μL of PBS pH 7.4 and incubated for 5 minutes with 200 μL of Bradford Reagent (Sigma). Samples were read at 595 nM with a BioRad spectrophotometer.

2.6 Co-immunoprecipitation

20 μL of a 50% protein G bead suspension (Calbiochem and Pierce) were washed twice with 1X PBS pH7.4. Washed protein G beads were incubated overnight at 4°C with 5 μL of anti-beta-arrestin1 K16 (Santa Cruz Biotechnology) or 5 μL of anti-Flag (Rockland) and 500 μL of 1X PBS pH 7.4. Protein G beads were washed 3 times with cold 1% NP-40 lysis buffer prior to incubation with cell lysates. Between 400 μg and 800 μg of total proteins were incubated with beads (conjugated either with beta-arrestin1 or anti-FLAG) overnight at 4°C. The day after, protein G beads were washed 5 times with cold1% NP-40 lysis buffer and eluted with 30 μL of 2X sample loading buffer (8% SDS, 40% glycerol, 0.5M Tris pH 6.8)

2.7 SDS-PAGE/Western Blot

In overexpression studies, 20 μg of whole cell lysate were loaded on an SDS-PAGE gel as input control. For the endogenous study, 50 μg of whole cell lysate were loaded on the gel as input control. Input and protein G beads were boiled for 3 minutes at 95°C, run on 8% SDS-PAGE gels and transferred onto PVDF membranes (Millipore). Following transfer, membranes were incubated in blocking solution containing 5% skim milk in 1XTBST (1X TBS-0.1% Tween 20) or in Pierce Super Blocker solution for 1
Membranes were incubated with primary antibodies, i.e., anti-Notch C20r, 1:200 (Santa Cruz Biotechnology), anti-beta-arrestin1 K16, 1:200 (Santa Cruz Biotechnology), anti-Flag M2, 1:1000 (Sigma), anti-HA HA.11 (Covance) anti-myc (5 mL of 9E10 culture supernatant) dilute in western blocker solution (Sigma and Pierce) rocking gently 0/N at 4°C. Membranes were washed 3 times with 1X TBST for 10 minutes at 23°C and incubated for 1 hour at 23°C with a secondary antibody conjugated to a horse radish peroxidase goat anti-rabbit, 1:5000 (Invitrogen), rabbit anti-mouse, 1:2500 (Rockland), rabbit anti-goat, 1:2500 (Rockland), True Blot anti-goat, 1:1000 (EBioscience). Membranes were washed 3 times with 1X TBST and revealed with a Super Signal West Pico Chemiluminescence kit and Kodak Biomax films.

2.8 RT-PCR

**Beta-arrestin1 expression in TALL cells.** Total RNA was isolated from 5E6 T-ALL cells using Trizol (Invitrogen). 2 µg of total RNA and 50 µM of oligo (dT)2012-18 were used to perform a reverse transcriptase reaction (Invitrogen) using 200U of Superscript II Reverse Transcriptase. Newly synthesized cDNA was diluted 1:10 in RNase-free water and 5 µL of diluted cDNA were used to amplify human beta-arrestin1. (FW 5'- GTGAAGCTGGTGGTGTCTCG-3', RV 5'-AGAGCCGGTACCATCCTCCTC-3') and human beta-arrestin2 (FW 5'-ATGGGGGAAGCCGGGACC-3', RV 5'-GGGGTGGGTTGGGCACCCG-3'). Human actinB was used as an internal control. 5 µL of a 1:50 dilution of cDNA were used to amplified human actinB (FW 5'-CGCGAGAAGATGACCCAGAT-3', RV 5'- GATAGCACAGCCTGGATAGCAAC-3'). DNA was amplified using an ICYCLER PCR machine and the following program: 95°C 2 minutes, 95°C 30 seconds, 60°C 30 seconds (beta-arrestin1 and hactin B) and 66°C (beta-arrestin2), 72°C 30 seconds for 30 cycles, 72°C for 7 minutes and hold at 4°C. Samples were then run on a 2% agarose gel.

2.9 siRNA

**siRNA Transfection.** Beta-arrestin1 siRNA duplex (5'AAAGCCUUUCUGCGCGGAAU-3') and random siRNA (ALLSTAR) were synthesized by Qiagen and tagged on the 3'
end of the sense strand with ALEXA 647. 24 hours prior to transfection, U2OS cells were plated in 10% DMEM without antibiotics at 8,3E4 cells/well (24-well plate). On the day of transfection, cells were refed with 218 μL of 10% DMEM without antibiotics. 100nM of either beta-arrestin1 siRNA or ALLSTAR siRNA were diluted in 218 μL of plain DMEM. In a separate tube, 1.4 μL of Dharmafect DUO transfection reagent were diluted in 218 μL of plain DMEM. Diluted siRNA and diluted transfection reagent were mixed together and incubated for 20 minutes at 23°C. 350 μL of 10% DMEM without antibiotics were added to the complex and incubated with U2OS cells overnight at 37°C. 24 hours post-transfection, cells were trypsinized and 2/3 of the total number of cells were transferred into a new 24-well plate for luciferase reporter transfection (see below). The other extra 1/3 of the cells was analyzed by flow cytometry to assess the siRNA transfection efficiency and cell viability. To determine the level of transfection efficiency, transfected cells were scanned through a FACS Calibur and ALEXA 647 fluorescence emission peak (680nm) was read in the channel FL4. To determine cell viability, transfected cells were stained with propidium iodide at a concentration of 2 ng/μL and the fluorescence emission peak of propidium iodide (617nm) was read in the channel FL3.

Transfection of luciferase reporter in siRNA treated U2OS cells. 30 hours post-siRNA transfection, U2OS were transfected with 40 ng of pGL2-JH23A, 0.8 ng of phRL-TK and various amounts of pcDNA3 deltaE C(L) or MigICNX (depending on the assay) using the Lipofectamine transfection reagent. 0.5 μL of Plus reagent and 1 μL of Lipofectamine were incubated with the DNA according to the manufacturer’s protocol. U2OS cells incubated for 3 hours with the DNA-liposome complexes at 37°C. Cells were refed with complete DMEM for 48 hours.

Beta-arrestin1 expression in U2OS cells treated by siRNA. A 24-well of U2OS cells (about 3.2x10^6 cells) was transfected with either 100 nM of siRNA against beta-arrestin1 or 100 nM of random siRNA. 48 hours later, U2OS cells were trypsinized and RNA extracted using Trizol reagent. 2 μg of RNA were been used to perform a reverse-transcriptase reaction using 50μM of oligo (dT)20 and 200 U of Superscript II Reverse Transcriptase. Newly synthesized cDNA was diluted 1:10 in RNAse-free
water and 5 μL were used to amplify human beta-arrestin1 and human actin B (primer sequences above). DNA was amplified using an ICYCLER PCR machine and the following program: 95°C 2 minutes, 95°C 30 seconds, 60°C 30 seconds, 72°C 30 seconds for 20 cycles, 72°C for 7 minutes and hold at 4°C.

2.10 Luciferase Assay

60 hours post-siRNA transfection cells were lysed in 50 μL of 1X Passive Lysis Buffer for 10 minutes at 23°C. Luciferase assays were performed with 10 μL of cell lysate, 50 μL of LAR reagent and the reaction was stopped with 40 μL of Stop’n Glo solution (Promega). Firefly luciferase and Renilla luciferase were read using the E&EG Berthol luminometer. The level of Firefly luciferase expression was normalized against the level of expression of Renilla luciferase.
Chapter 3
Results

3.1 Beta-arrestin1, Deltex and Notch protein interactions

Overexpression studies in S2 Drosophila cells have shown that Deltex, Kurtz and the Notch receptor interact together to form a trimeric complex that promotes Notch polyubiquitination and degradation (Mukherjee et al., 2005). In mammals, Notch and Deltex have previously been identified as interacting partners. The first study to reveal an interaction between Deltex and Notch in COS cells demonstrated that the first three ankyrin repeats of Notch were responsible for Deltex1 binding (Yamamoto et al., 2001). Crystal structure studies of the Deltex protein further confirmed this interaction (Zweifel et al., 2005). Although the negative effect of Deltex on Notch signaling has been well described in cell lines and animal studies (Izon et al., 2002), the mechanism by which Deltex down-modulates Notch signaling remains unclear. In light of the data concerning Deltex in the fly model, we elected to ascertain whether a similar trimeric complex exists between beta-arrestin1 (the closest human homologue of Kurtz), Deltex1 and Notch in mammalian cells (Figure 3.1.1). As the Deltex and Notch interaction has already been shown by co-immunoprecipitation in cell lines and by crystal structure, we decided to specifically address the putative interaction between either beta-arrestin1 and Deltex or beta-arrestin1 and Notch.

![Diagram](image.png)

Figure 3.1.1: Putative trimeric complex between beta-arrestin1, Notch and Deltex.
3.1.1 Deltex1 and beta-arrestin1 do not co-immunoprecipitate in 293T cells

In our first set of experiments, we asked whether Deltex1 and beta-arrestin1 could be coimmunoprecipitated. We co-transfected 293T cells with a full-length human Deltex1 cDNA tagged at the C-terminus with a myc epitope along with a full-length human beta-arrestin1 cDNA tagged at the N-terminus with a FLAG epitope. Previous in vitro biochemical studies, (unpublished data), revealed the partial insolubility of Deltex in solution. For this reason, we used a lysis buffer very similar to the lysis buffer used by other investigators to reveal the interaction between Deltex and Kurtz (Mukherjee et al., 2005) and the interaction between Notch and Deltex (Yamamoto et al., 2001). Both Deltex1 and beta-arrestin1 proteins were strongly expressed in the whole cell lysate with sizes of 75kD and 55kD, respectively (Figure 3.1.2, lane 1). We co-immunoprecipitated beta-arrestin1 with an anti-FLAG antibody and western blot analysis with an anti-FLAG antibody revealed beta-arrestin1 (lanes 3 and 4, bottom panel), but no Deltex was detected upon incubation with an anti-myc antibody (lane 3, top pannel). We used non-transfected 293T cells as a negative control for non-specific binding (lanes 2, 4). Our anti-FLAG antibody also detected a strong band at 50 kD (lanes 3, 4) representing IgGs from the antibody conjugated to protein G beads.
Figure 3.1.2: Beta-arrestin1 and Deltex do not physically interact together. 293T cells were transfected with pcDNA3-Deltex-myc and MigHX-FLAG-beta-arrestin1. 400 µg of whole cell lysate were co-immunoprecipitated with an anti-FLAG antibody (Rockland) conjugated to Protein G beads. Deltex1 and beta-arrestin1 expression were revealed by Western blot with 20 µg of whole cell lysate (lane 1) with an anti-myc antibody (9E10 hybridomas supernatant) and an anti-beta-arrestin1 antibody (K16). Beta-arrestin1 was successfully immunoprecipitated (lane3) but no Deltex proteins were detected by Western blot analysis upon incubation with an anti-myc antibody (lane 3). Non-transfected 293T whole cell lysate was used as a negative control (lane 2,4). The anti-myc and anti-Flag blots were electrophoresed separately and aligned together for better visualization.
As Deltex binding to beta-arrestin1 may be dependent on the presence of Notch, we tested this hypothesis by co-transfecting Deltex, beta-arrestin1 and Notch in 293T cells. To do this, we used a constitutively active form of Notch receptor lacking the ligand binding domain called deltaE. Delta E, once translated, traffics to the surface membrane, undergoes TACE and gamma-secretase cleavages and then translocates to the nucleus. The membrane processing of delta E is very similar to the membrane processing of the full length Notch receptor upon ligand binding. In this experiment, Notch and beta-arrestin1 were highly expressed in the whole cell lysate with an expected size of 120kD (Figure 3.1.3, lane 1). We immunoprecipitated beta-arrestin1 with an anti-FLAG antibody and Western blot analysis using an anti-FLAG antibody (lanes 3, 4 bottom panel), but no Deltex was revealed upon incubation with an anti-myc antibody (lane 3, top panel). We used non-transfected 293T cells as a negative control for non-specific binding (lanes 2, 4). Our anti-FLAG antibody also detected a strong band at 50 kD (lanes 3, 4) representing IgGs from the antibody conjugated to protein G beads.
Figure 3.1.3: In the presence of Notch, beta-arrestin1 and Deltex do not co-immunoprecipitate together. 293T cells were transfected with pcDNA3-Deltex-myc, MigHX-FLAG-beta-arrestin1 and pcDNA3deltaE. Notch and Deltex expression were revealed with 20 µg of whole cell lysate by Western blot analysis with respectively an anti-Notch (C-20r) antibody and an anti-myc (9E10 hybridomas supernatant) antibody (lane1). 400 µg of whole cell lysate were co-immunoprecipitated with an anti-FLAG antibody (Rockland) conjugated to Protein G beads. Beta-arrestin1 expression was revealed by Western blot using an anti-FLAG (M2) antibody (lanes 3 and 4, bottom panel) but no Deltex proteins were detected by Western blot analysis upon incubation with an anti-myc antibody (lane 3, top panel). Non-transfected 293T whole cell lysate was used as a negative control (lane 2 and 4). The anti-myc, anti-Flag and anti-Notch Western blots were run separately and aligned together for better visualization.
As the myc-tag on the C-terminus of the Deltex may be preventing its binding to beta-arrestin1, we made use of a Deltex stable T-cell line (TALL1-HADeltex) expressing Deltex tagged at the N-terminus with an HA-tag. In addition to stably expressing HA-Deltex, TALL1-HADeltex cells show constitutive Notch signaling and endogenously express beta-arrestin1. In these cells, we successfully revealed the endogenous Notch expression by Western blot using an anti-Notch antibody (Figure 3.1.4, lanes 1 and 2, top panel). We immunoprecipitated HA-Deltex with an HA-agarose resin and revealed its expression by Western blot using an anti-HA antibody (lane 2, bottom panel). We next examined if we could co-immunoprecipitate beta-arrestin1 and Deltex with an anti-beta-arrestin1 antibody. Western blot analysis revealed beta-arrestin1 expression at an expected size of 55 kD, but no Deltex was detected (lane 4, top panel). We used the parental TALL1 cells as a negative control for non-specific binding (lanes 1, 3). Our anti-beta-arrestin1 antibody also detected a strong band at 50 kD (lanes 3, 4) representing IgGs from the antibody conjugated to Protein G beads.
Figure 3.1.4: The position of the tag-epitope on Deltex protein does not explain the failure of Deltex to co-immunoprecipitate with beta-arrestin1. Notch expression was revealed in 50 μg of TALL1-HA-Deltex or TALL1 whole cell lysate by Western blot (lanes 1, 2) using an anti-Notch (C-20r) antibody. HA-Deltex expression was detected by immunoprecipitation of TALL1-HA-Deltex whole cell lysate with an anti-HA agarose resin and revealed on a Western blot with an anti-HA (HA.11) antibody (lanes 1, 2, bottom panel). 400 μg of TALL1-HA-Deltex or TALL1 cell lysates were incubated with Protein G beads conjugated with an anti-beta-arrestin1 (K16) antibody. Beta-arrestin1 was successfully conjugated to Protein G beads (lanes 3, 4) but no Deltex co-immunoprecipitation was revealed by Western blot analysis using an anti-myc antibody (lane 4, top panel). TALL1 parental cells were used as a negative control (lanes 1, 3). The anti-HA, anti-beta-arrestin1 and anti-Notch Western blots were run separately and aligned together for better visualization.
3.1.2 Notch and beta-arrestin1 physically interact in 293T cells

We next attempted to reveal an interaction between Notch and beta-arrestin1. To examine this, we used our deltaE construct expressing the membrane-bound form of Notch1 and the human beta-arrestin1 cDNA tagged at the N-terminus with a FLAG epitope. As mentioned previously, the deltaE construct codes for a protein lacking the ligand binding domain of Notch (Figure 3.1.7), but still retains the transmembrane domain that must be cleaved by TACE proteases (S2 cleavage) and gamma-secretase (S3 cleavage) in order to release Notch from the membrane for translocation to the nucleus. Thus, the use of this construct allowed us to overexpress a Notch receptor that undergoes similar processing to the endogenous receptor after ligand stimulation.

We overexpressed MigRI-deltaE with MigHX-FLAG-beta-arrestin1 in 293T cells. Notch protein was detected in the whole cell lysate as a strong band at approximately 120kD corresponding to the size of a membrane-anchored Notch receptor (Figure 3.1.5, lane 1, top panel). Several other bands of smaller sizes were also present. These smaller bands may be degradation products of Notch or the result of different transcriptional start sites in the vector. Beta-arrestin1 protein was detected in the whole cell lysate by Western blot with an anti-beta-arrestin1 antibody at an expected size of 55kD (lane 1, bottom panel). We immunoprecipitated beta-arrestin1 and Western blot analysis revealed an interaction between Notch and beta-arrestin1 via the presence of a strong band at approximately 120kD (lane 3, top panel). We used non-transfected 293T cells (lanes 2 and 4) and beads non-conjugated to antibody (lanes 4 and 5) as negative controls for non-specific binding.
Figure 3.1.5: Beta-arrestin1 and Notch physically interact. 293T cells were cotransfected with MigHX-FLAG-beta-arrestin1 with MigRI-delta E. Notch and beta-arrestin1 were detected in 5 µg of whole cell lysate (lane 1). 400 µg of cell lysate was immunoprecipitated with an anti-beta-arrestin1 (K-16) antibody and Notch was detected by Western blot analysis with an anti-Notch (C-20r) antibody (lane 3). Protein G beads without anti-beta-arrestin1 antibody were incubated with the whole cell lysate (lane 5) as a negative control for non-specific binding. Non-transfected 293T whole cell lysate was used as a negative control (lane 2 and 4). The Notch and beta-arrestin1 blots were run separately and aligned together for better visualization. Those results represent three independent experiments.
To further elucidate the relationship between Notch and beta-arrestin1, our next objective was to determine whether the interaction between Notch and beta-arrestin1 occurred before or after Notch activation. To accomplish this, we overexpressed beta-arrestin1 with either the full-length Notch protein or the intracellular subunit of Notch (ICN). As Notch activation requires cleavage of full-length Notch and translocation of ICN to the nucleus, an interaction between full-length Notch and beta-arrestin1 would indicate a pre-activation relationship, whereas an interaction between ICN and beta-arrestin1 would indicate a post-activation relationship.

We cotransfected 293T cells with pcDNA3-hNotch FL or MigICN constructs with MigHX-beta-arrestin1-FLAG. pcDNA3-hNotch FL construct codes for both the extracellular and intracellular domain of the Notch receptor. In the absence of ligand, the Notch full-length receptor does not undergo the cleavage steps leading to its translocation to the nucleus and remains bound to the surface membrane. MigICN codes for the intracellular subunit of the Notch receptor. It does not undergo membrane processing and translocates directly to the nucleus after translation. Both the full-length Notch protein and ICN showed high levels of expression in the cell lysates, as revealed by bands at approximately 120 kD and 100kD, respectively (Figure 3.1.7 lanes 2 and 4). We immunoprecipitated beta-arrestin1 and Western blot analysis revealed beta-arrestin1 expression by a band at 55kD (lanes 6, 7, 8, 9 and 10). We did not detect any interaction between full-length Notch and beta-arrestin1 (lane 7 top panel). However, we did detect a weak interaction between ICN and beta-arrestin1 (lane 9, top panel). The weakness of this interaction may be due to several reasons like 1) the fact that ICN is quickly degraded after its translocation to the nucleus, 2) the lysis buffer used to perform our co-immunoprecipitation may not have been strong enough to lyse the nuclei and we lost most of the ICN retained in the nucleus or 3) that the co-immunoprecipitation efficiency was very low. The delta E and beta-arrestin1 interaction was used as a positive control for the interaction between Notch and beta-arrestin1 (lanes 1 and 6). We used non-transfected 293T cells (lanes 3, 5, 8 and 10) and beads non-conjugated to antibody (lane 11) as negative controls for non-specific binding. The difference in protein level expression between Notch full-length, deltaE and ICN in the whole cell lysate leads us to be prudent about the conclusions that we
draw from our results. Our results suggest that beta-arrestin 1 physically associates with Notch after activation of the receptor and, refining the method by which we obtained those results will allow us to assert our conclusions.
Figure 3.1.6: Beta-arrestin1 binds to Notch receptor after ligand activation. 293T cells were cotransfected either with pcDNA3-hNotch FL or MigICN and MigHX-FLAG-beta-arrestin1. Notch FL and ICN expression were revealed by Western blot in 5μg of cell lysate (lanes 2 and 4) using an anti-Notch (C-20r) antibody. 400 μg of cell lysates were incubated with Protein G beads loaded with an anti-beta-arrestin1 (K16) antibody. ICN was detected by Western blot analysis (lane 9) but no Notch FL was revealed upon incubation with an anti-Notch (C-20r) antibody (lane 7). Beta-arrestin1 expression was revealed by Western blot analysis with an anti-beta-arrestin1 (K-16) antibody (lanes 6,7,3,9 and 10). Co-immunoprecipitation of deltaE with beta-arrestin1 was used as a positive control (lanes 1 and 6). Non-transfected 293T cells were used as a negative control for non-specific binding (lanes 3, 5, 8 and 10). Protein G beads incubated with whole cell lysates but without anti-beta-arrestin1 antibody were used as negative controls for non-specific binding (lane 11). The full-length and the ICN blots were run separately and aligned for better visualization. Those results represent two independent experiments.
The activation of the Notch receptor is not straightforward and can not be seen simply as non-active (full-length) vs active (ICN). TACE and gamma-secretase cleavage events at the membrane (S2 and S3 cleavages) transform the full-length receptor into several Notch intermediates before releasing ICN from the membrane. To determine if beta-arrestin1 is recruited to a membrane-tethered intermediate of Notch (after ligand binding) and is not exclusively interacting with ICN in the cytoplasm, we used a gamma-secretase inhibitor (compound E) to block the cleavage of Notch at the surface membrane.

Figure 3.1.7: Graphic representation of Notch intermediate proteins and their different sizes (adapted from (Weng et al., 2003))

We overexpressed deltaE and beta-arrestin1 in 293T cells, and treated these cells with either 0.01% DMSO or 1μM GSI for 24 hours. Consistent with our previous results, Notch binding to beta-arrestin1 was revealed by a band at 120kD in cells treated with DMSO (Figure 3.1.8, lane 3). However, GSI-treated cells also revealed a strong band at 120kD (lane 4), indicating that blocking Notch signaling, in an overexpression context, caused an accumulation of Notch protein associated to beta-arrestin1 at the membrane. Together, these results show that beta-arrestin1 interacts with a membrane-tethered intermediate of Notch after ligand binding.
Figure 3.1.8: The Notch and beta-arrestin1 interaction is not dependent on gamma-secretase in 293T cells. 293T cells were cotransfected with MigdeltaE and MigRI-FLAG-beta-arrestin1. 24 hours post-transfection, 293T cells were treated with either 0.01% of DMSO or 1 uM of GSI for 24 hours. Notch expression was revealed with 5 pg of whole cell lysate by Western blot analysis using an anti-Notch (C-20r) antibody (lanes 1 and 2). 400 pg of cell lysate were incubated on Protein G beads loaded with an anti-beta-arrestin1 antibody and Notch expression was revealed by Western blot using an anti-Notch (C-20r) antibody (lanes 3 and 4). Beta-arrestin1 expression was revealed by Western blot using an anti-beta-arrestin1 (K-16) antibody (lanes 3 and 4, bottom panel). Protein G beads without antibody were incubated with the whole cell lysate and used as negative controls for non-specific binding (lanes 5 and 6).
3.1.3 Endogenous Notch1 and beta-arrestin1 interact in HPBALL cell line

We chose a mammalian T-cell model to test the nature of the interaction between Notch and beta-arrestin1. T-cell acute lymphoblastic leukemia (T-ALL) cell lines carry mutations in the heterodimerization domain and/or the PEST domain of the Notch receptor, resulting in ligand-independent Notch signaling and/or increased stability of the Notch protein, respectively. Notch signaling is vital for T-ALL cell growth and survival. Furthermore, Deltex as a Notch target gene is also highly expressed in T-ALL cells. The use of these cell lines allowed us to perform endogenous co-immunoprecipitation studies in a more suitable environment without exogenous Notch expression.

To confirm that beta-arrestin1 and beta-arrestin2 were expressed in T-ALL cell lines at the transcription level, we extracted RNA from four T-ALL cell lines (ALLSIL, TALL-1, Jurkat, and HPBALL) as template for cDNA synthesis with primers against either human beta-arrestin1 or beta-arrestin2. Primers were designed to discriminate beta-arrestin transcript variant isoform A from its splice variant isoform B, with the former missing an exon in the C-terminus. As shown in Figure 3.1.9 (panel A), all T-ALL cell lines, with the exception of HPBALL, expressed both isoforms A and B of beta-arrestin1 or beta-arrestin2 (top band). HPBALL was the only T-ALL cell line in which isoform B was not detectable. As isoform B of beta-arrestins is poorly characterized, we can only speculate that the lack of isoform B may give a certain transcriptional, growth or survival advantage to HPBALL tumour cells.

We also determined the level of Notch protein expression in T-ALL cell lines. Using an antibody against the C-terminus of the Notch protein, we detected a band at approximately 120kD for every cell line tested (Figure 3.1.9, panel B). HPBALL and ALLSIL showed an extra band at around 110 kD, likely owing to their PEST deletions which ensure a longer protein half-life.
Since HPBALL cells expressed the well-characterized isoform A of beta-arrestin1 and had high Notch protein expression, we used this cell line to confirm the endogenous interaction of Notch with beta-arrestin1.

Figure 3.1.9: Beta-arrestin1, 2 and Notch are expressed in T-ALL cell lines. Panel A: RNA from T-ALL cells was extracted with Trizol and 2 μg were used for cDNA synthesis. Human beta-arrestin1 and beta-arrestin2 cDNAs were amplified by PCR using primers annealing to either human beta-arrestin1 (left panel) or human beta-arrestin 2 (right panel). Primers were designed to discriminate the longest transcript (isoform A, top band) from the splice variant (isoform B, bottom band) of beta-arrestins. cDNAs were also amplified with primers against human actin B and used as a loading control. Panel B: 50 μg of T-ALL cell lysate were loaded on a Western blot and Notch expression was detected with an anti-Notch (C-20r) antibody.
By Western blot analysis, we showed that HPBALL whole cell lysate had strong Notch protein expression (Figure 3.1.10, lane 1). We then incubated these cell lysates on Protein G beads loaded with an anti-beta-arrestin1 antibody and probed for Notch with an anti-Notch antibody. We found that Notch and beta-arrestin1 co-immunoprecipitated together, as shown by the presence of a band at approximately 110kD (lane 3) (GSI experiment explained below). As mentioned previously, Notch undergoes several cleavage steps at the membrane before translocating to the nucleus. The size of these intermediates varies depending on the cleavage step and the potential post-translational modifications associated with the receptor prior to gamma-secretase cleavage. The 110kD band detected by co-immunoprecipitation may represent the intermediate between the S2 and S3 cleavage (membrane-tethered form of Notch) or a post-translationally modified form of ICN.

Figure 3.1.10: Endogenous interaction of Notch and beta-arrestin1 in HPBALL cells. HPBALL cells were treated for 24 hours prior to lysis with either 0.01% DMSO (vehicle) or 1 μM of GSI (compound E). Notch expression was revealed with 50 μg of whole cell lysate and detected by Western blot analysis using an anti-Notch (C-20r) antibody (lanes 1 and 2). 800 μg of each cell lysate were co-immunoprecipitated with an anti-beta-arrestin1 (K-16) antibody and Notch was detected by Western blot with an anti-Notch (C-20r) antibody (lanes 3 and 4). Beta-arrestin1 expression was detected with an anti-beta-arrestin1 antibody (lanes 3 and 4, bottom panel) and Protein G beads without antibody were incubated with the whole cell lysate and used as negative controls for non-specific binding (lanes 5 and 6, top and bottom panel). Those results represent three independent binding experiments.
In Section 3.1.2, we showed that beta-arrestin1 was recruited to a membrane-tethered Notch receptor generated after ligand binding but not exclusively interacting with ICN in the cytoplasm. To further assess the endogenous association between beta-arrestin1 and Notch, we used a gamma-secretase inhibitor (GSI) to trap the Notch receptor at the membrane. The Notch species generated by TACE proteases (S2 cleavage) is about 110 kD. Under normal conditions, the S2 cleaved Notch peptide is rapidly processed by gamma-secretase to generate ICN (S3 cleavage). Blocking Notch signaling with GSI traps the S2 cleaved form of Notch at the surface membrane and prevents the release of ICN into the cytoplasm (Figure 3.1.7).

We next treated HPBALL cells with 1 pM of GSI (compound E) for 24 hours at 37°C. Cells treated with GSI showed a higher level of Notch expression due to the S3 cleavage blockage. The presence of a second Notch species of approximately 110 kD likely represents the Notch intermediate generated by the S2 cleavage (Figure 3.1.10, lane 2). The S2 cleaved Notch peptide is very transient as this intermediate is normally cleaved immediately by the gamma-secretase. In this case, cells treated with GSI showed an accumulation of the S2 cleaved form of Notch at the membrane (lane 2). The presence of the S2 cleaved form of Notch is a good indication that a 24-hour drug treatment is sufficient to trap Notch at the membrane. When HPBALL cells were treated for 24 hours with 1 μM of GSI, the interaction between beta-arrestin1 and Notch1 was almost entirely lost (lane 4), indicating that the binding is dependent on the capacity of gamma-secretase to cleave Notch off the membrane. If beta-arrestin1 was recruited to a membrane-bound form of Notch, the GSI treatment would have caused an accumulation of the S2 cleaved form of Notch at the membrane. These results suggest that the association between beta-arrestin1 and Notch is dependent on gamma-secretase cleavage, and that beta-arrestin1 is more likely to associate with ICN rather than membrane-bound Notch protein in T-ALL cells.
Our overexpression studies showed that beta-arrestin1 was recruited to the membrane likely after dissociation of the extracellular subunit from the Notch receptor. We showed that beta-arrestin1 associates with a membrane-tethered form of Notch and this association is not dependent on gamma-secretase activity. We also confirmed that beta-arrestin1 does not interact exclusively with ICN. These results suggest that beta-arrestin1 is recruited after activation of the Notch receptor but before its release in the cytoplasm. However, in HPBALL cells, beta-arrestin1 endogenously interacts with Notch only upon gamma-secretase secretase cleavage. These results suggest that in these cells, beta-arrestin1 binds exclusively to ICN. Together, these results suggest that beta-arrestin1 may be recruited differently by the Notch receptor depending on its level of expression in the cell.
3.2 Functional role of beta-arrestin1 in the Notch signaling pathway

In *Drosophila*, Kurtz (*Drosophila* homologue of beta-arrestin1) acts as a negative regulator of the Notch signaling pathway by participating in the degradation process of Notch protein. To assess the contribution of beta-arrestin1 to the Notch signaling pathway in mammals, we first made use of a loss-of-function approach and decreased the intracellular level of beta-arrestin1 protein with RNA interference (RNAi). Using a Notch luciferase assay reporter, we compared the level of Notch signaling in cells treated with a random siRNA compared to cells treated with siRNA directed against beta-arrestin1 (Ahn et al., 2003). We also used a gain-of-function approach by overexpressing beta-arrestin1 and, using the same Notch luciferase reporter assay, we looked at the modulation of Notch signaling by beta-arrestin1.

3.2.1 Beta-arrestin1 protein level is knocked-down by siRNA in U2OS cells

To begin our functional studies, we chose to use U2OS cells to perform our Notch reporter assay since U2OS are known to have minimal endogenous Notch and Deltex expression. To test the efficiency and the specificity of our siRNA, we used two different approaches to determine the level of endogenous beta-arrestin1 knock-down in U2OS cells. First, we co-transfected U2OS cells with the coding sequence of beta-arrestin1 cloned into an IRES-GFP vector in combination with either 100nM siRNA against beta-arrestin1 or 100nM of random siRNA. After 60 hours, we monitored the level of green fluorescence by flow cytometry. We successfully knocked down the bicistronic RNA message of beta-arrestin1-GFP by approximately 65%, and we could not detect a significant effect of the random siRNA control on GFP expression (Figure 3.2.1, panel A). However, this flow cytometry data only confirmed a successful knock-down of GFP expression, which is a surrogate marker of the beta-arrestin1 expression knock-down. To confirm that our siRNA successfully targeted the beta-arrestin1 message and not the GFP message, we assessed the efficiency of the endogenous knock-down of beta-arrestin1 by RT-PCR.
Endogenous beta-arrestin1 protein levels in U2OS cells are too low to be detected by Western blot. Therefore, it would be necessary to immunoprecipitate beta-arrestin1 in order to visualize its expression by Western blot. As quantitative immunoprecipitation can be quite challenging, we decided to bypass this technical matter by extracting RNA from U2OS cells transfected with 100nM of siRNA against beta-arrestin1 or 100nM of random siRNA. Two days post-transfection, we performed RT-PCR to detect the presence of beta-arrestin1 mRNA in the cell. The RT-PCR reaction revealed a decrease in the mRNA level of endogenous beta-arrestin1 isoform A in U2OS cells (Figure 3.2.1, panel B). However, the RT-PCR reaction did not show a successful knock-down of the isoform B. It is worth mentioning that all our overexpression experiments (biochemical and functional data) were done with a construct exclusively expressing the isoform A of beta-arrestin1 and our endogenous studies were conducted in HPBALL cells having no isoform B expression. We acknowledge that the RT-PCR is not a quantitative measurement of the knock-down of beta-arrestin1 in U2OS cells, but by relative comparison to the isoform B (not influenced by the siRNA against beta-arrestin1) we concluded that the knock-down of the isoform A of beta-arrestin1 was successful. These data confirmed, by two different assays, that our siRNA could successfully decrease the level of beta-arrestin1 expression in U2OS cells with minimal non-specific side effects. We could therefore use this functional siRNA targeting beta-arrestin1 in our Notch reporter assay to investigate the effect of beta-arrestin1 knock-down on the Notch signaling pathway.
Figure 3.2.1: Knock-down of exogenous and endogenous beta-arrestin1 expression by siRNA. Panel A: U2OS cells were transfected with either 100 nM siRNA against beta-arrestin1 or 100 nM of random siRNA and MigRI-beta-arrestin1-FLAG. 60hrs post-transfection, GFP expression was analyzed by flow cytometry. Panel B: U2OS cells were transfected with either 100 nM of siRNA against beta-arrestin1 or 100 nM of random siRNA. 48 hrs post-transfection, RNA was collected and human beta-arrestin1 was amplified from the cDNA. Human Actin B was used as loading control. Water was used as a negative control and we also included an ALEXA-647 fluorophore at the 5'end of the siRNA as a transfection control. These results are representative of 2 independent experiments.
3.2.2 The Notch signaling pathway is upregulated in the absence of beta-arrestin1

To test the effect of beta-arrestin1 knock-down on Notch signaling, we chose a commonly used Notch luciferase reporter (JH23a) composed of four copies of the CBF1 promoter (Hsieh and Hayward, 1995). As demonstrated by Western blot analysis, Notch transient expression (under MSCV or CMV promoters) is high. To test the saturation level of our luciferase assay, we first titred the amount of deltaE and ICN needed to obtain a Notch reporter stimulation falling into an increasing linear range of luciferase expression. We found that more than 40ng of delta E caused the saturation of our luciferase reporter. However, in the case of ICN, we did not seem to reach the saturation level of our luciferase reporter. We chose 5ng of deltaE and 10ng of ICN to pursue our beta-arrestin1 knock-down studies (Figure 3.2.2).

Figure 3.2.2: Notch titration assay. U2OS cells were transfected with the Notch reporter pGL2-JH23a, phRL-TK and increasing amounts of pcDNA3 deltaE and MigICN. 48hrs post-transfection, cells were lysed and Firefly and Renilla luciferase expression was monitored with a luminometer. The Firefly luciferase readings were normalized against the Renilla luciferase readings. DeltaE and ICN assays were done in triplicate.
We transfected U2OS cells with 100nM of siRNA against beta-arrestin1 in combination of 10ng of ICN or 5ng of deltaE. We observed a significant increase in Notch signaling (P value= 0.027) when U2OS cells were transfected with 10ng of ICN. However, when U2OS cells were transfected with deltaE, we did not detect a significant increase in Notch signaling (P value= 0.2295) (Figure 3.2.3). These results are ambiguous as deltaE undergoes the S2 and S3 cleavage to become ICN (see section 3.1.2). We would thus expect to see the same effect observed for both deltaE and ICN upon the reduction of beta-arrestin1 protein levels in U2OS cells. This issue will be discussed more extensively in the Discussion (see Chapter 4). We transfected U2OS cells with 100nM of random siRNA and either 10ng of ICN or 5ng of deltaE and we showed non-significant variation in Notch signaling in cells transfected with ICN or deltaE. We used U2OS cells transfected without siRNA as a negative control. We also stained U2OS cells with propidium iodide staining and assess cell viability by flow cytometry. Thus, the significant increase of Notch signaling was specifically due to the diminution of beta-arrestin1 protein level in U2OS cells and not to non-specific siRNA effects. These results show that the lack of beta-arrestin1 positively contributes to Notch signaling. These results also demonstrate that beta-arrestin1 may exert its regulatory effect exclusively on the intracellular subunit of Notch (ICN).
Figure 3.2.3: Knocking-down beta-arrestin1 upregulates Notch signaling. U2OS cells were transfected with either no siRNA or 100nM of random siRNA or 100nM of siRNA against beta-arrestin1 using Dharmafect transfection reagent. 30hrs later, U2OS cells (transfected with Dharmafect transfection reagent) were split into triplicate and transfected with the Notch reporter pGL2-JH23a reporter, phRL-TK and either 5ng of pcDNA3-deltaE or 10ng of MigICN using the Lipofectamine transfection reagent. 60hrs post-Dharmafect transfection, cells were lysed and Firefly luciferase and Renilla luciferase expression detected with a luminometer. Firefly luciferase readings were normalized against Renilla luciferase readings. The P value was determined using an unpaired two-tailed T test. These results are representative of two independent experiments.
We further confirmed the negative role of beta-arrestin1 in the Notch signaling pathway by looking at the negative effects of beta-arrestin1 overexpression on Notch signaling. We transfected U2OS cells with 80ng of ICN and a range of MigHX-beta-arrestin1-FLAG amounts (from 40 to 160 ng). As determined in our Notch titration assay (Figure 3.2.2), stimulation of the Notch reporter caused by 80ng of ICN was still in the linear range of the luciferase assay. Small doses of beta-arrestin1 (40 to 80ng) did not have a significant effect on Notch signaling. However, at a higher dose (160ng), beta-arrestin1 decreased the stimulation of the Notch reporter by about 40% (P value= 0,0423) (Figure 3.2.4). These results demonstrate that a high level of beta-arrestin1 is necessary to have a significant negative effect on Notch signaling.

![Figure 3.2.4: Overexpression of beta-arrestin negatively modulates Notch signaling. U2OS cells were transfected in triplicate with pGL2-JH23a, phRL-TK, 80ng of ICN and increasing amounts of beta-arrestin1 (40 to 160ng). 48hrs post-transfection, cells were lysed and Firefly and Renilla luciferase expression detected with a luminometer. Firefly luciferase readings were normalized against Renilla luciferase readings. The P value was determined using an unpaired two-tailed T test. These results are representative of two independent experiments.](image-url)
3.2.3 Deltex enhances the negative modulation of Notch signaling by beta-arrestin1

The absence of the appropriate E3-ligase may explain why low amounts of beta-arrestin1 are not sufficient to significantly decrease Notch signaling. To verify if the presence of a more suitable E3-ligase would augment the negative effect of beta-arrestin1 on Notch signaling, we overexpressed beta-arrestin1 in the presence of Deltex in U2OS cells. We chose to use 80ng of beta-arrestin1 to perform our overexpression experiment. As demonstrated in Figure 3.2.4, 80ng of beta-arrestin1 did not cause a significant change in Notch signaling. Thus, we eliminated the possibility that the negative modulation of Notch signaling by beta-arrestin1 was the result of both its interaction with Deltex and its non-specific interaction with other E3-ligases present in the cell. We transfected U2OS cells with 80ng of MigICN, 80ng of MigHX-beta-arrestin1-FLAG and 40 ng of pcDNA3-Deltex-myc. Beta-arrestin1 overexpression had no significant effect on Notch signaling. Deltex overexpression decreased Notch signaling by approximately 65% (P value <0.01). The presence of Deltex enhanced the negative effect of beta-arrestin1 by decreasing Notch signaling by a further 50% (P value <0.05) (Figure 3.2.5). These results suggest that Notch signaling is downregulated by beta-arrestin1 and that Deltex is a major contributor to this phenomenon. All these findings support our hypothesis that beta-arrestin1 and Deltex functionally interact to negatively regulate Notch signaling.
Figure 3.2.5 Deltex enhances the negative modulation of Notch signaling by beta-arrestin1. Panel A: U2OS cells were co-transfected with the Notch reporter pGL2-JH23a, phRL-TK, 80ng ICN, 80ng of beta and 40ng of Deltex1 in triplicate. 48hrs post-transfection cells were lysed and Firefly and Renilla luciferase expression detected with a luminometer. Firefly luciferase readings were normalized against Renilla luciferase readings. The assay was done in triplicate and the $P_1$ value ($P_1<0.01$) was determined by a one-way ANOVA followed by a Dunnett's post-test. The $P_2$ value ($P_2<0.05$) was determined by a one-way ANOVA followed by a Bonferroni's post test. Panel B: Same assay done with all controls. The $P_1$ value ($P_1<0.05$) was determined by a one-way ANOVA followed by a Dunnett's post-test. The second $P$ value ($P_2<0.05$) determined by a one-way ANOVA followed by a Bonferroni's post test.
Chapter 4  
Discussion

Originally, the *Drosophila* homologue of beta-arrestin1, Kurtz, was identified as an interacting partner of Deltex. The formation of a trimeric complex between Notch, Deltex and Kurtz was shown to promote Notch ubiquitination and degradation. These results provided useful insights into the mechanism by which Deltex exerts its negative regulation on the Notch signaling pathway. However, even if extensive studies have been conducted in mammals to demonstrate the negative role of Deltex in the Notch signaling pathway, no one to date has provided a mechanism for this inhibition.

4.1 New findings in the role of beta-arrestin1 in Notch signaling.

In this study, we wanted to pursue the formation of this trimeric complex in a mammalian system and reveal its negative effect on the Notch signaling pathway. Our results show a physical interaction between beta-arrestin1 and the Notch receptor. Beta-arrestin1 interacts with both the membrane-bound and the intracellular forms of Notch, and the interaction occurs after Notch activation. In addition, we show that beta-arrestin1 levels inversely correlate with Notch signaling. Finally, although we could not demonstrate that beta-arrestin1 and Deltex co-immunoprecipitate (as discussed below), the presence of Deltex enhances the negative effect of beta-arrestin1 on Notch signaling. Our findings identify a new Notch interacting partner as well as a novel role for beta-arrestin1 in the Notch signaling pathway. Our study also validates the negative role of Deltex on Notch signaling and may provide interesting insights about the mechanism by which Deltex, in collaboration with beta-arrestin1, negatively regulates the Notch signaling pathway in mammals.

Some aspects of the regulation of the Notch signaling pathway such as cleavage events and nuclear degradation have been extensively studied in the past. Nonetheless, there are still many studies to perform in order to fully understand the cytosolic regulation of Notch. The fact that beta-arrestin1 and Notch physically bind
together bring numerous new possible mechanisms in which beta-arrestin1 could exert a negative effect on Notch signaling. Ubiquitination is most likely responsible for beta-arrestin1's negative role on Notch signaling. Beta-arrestin1 has been shown to act as a scaffold protein to promote ubiquitination and downregulation of several transmembrane receptors like the IGF-1R, Toll-like receptors and TGF-beta. It is not excluded that Notch undergoes ubiquitination upon ligand binding since it has been demonstrated that Notch needs to be monoubiquitinated and endocytosed to be cleaved by gamma-secretase. However, our study does not clarify if beta-arrestin1 is involved in the ubiquitination process of the Notch receptor but strongly suggests that beta-arrestin1 could be involved in a degradation process of the Notch protein.

Beta-arrestins are also involved in endosomal sorting. In Drosophila, Notch colocalizes with Deltex and Kurtz in yet to be identified endocytic vesicles. Those studies were done by overexpression but still raise the possibility that somehow, a fraction of Notch receptors does not get cleaved by gamma-secretase. What is the fate of uncleaved Notch receptors? How do Notch receptors traffic in the cell? Are they recycled at the plasma membrane or are they degraded by the lysosomal pathway? Given the fact that beta-arrestin1 interacts with a membrane-bound form of activated Notch receptor, this may suggest that Notch is also undergoing endosomal sorting after endocytosis. Beta-arrestin1 could play a key role is this scenario by dictating the fate of the uncleaved Notch receptors after activation and endocytosis.
4.2 Hypothetic role of beta-arrestin1 in the Notch signaling pathway

Beta-arrestin1 is involved in a variety of key cellular functions. Therefore it is difficult to speculate what exact role beta-arrestin1 plays in the Notch signaling pathway. We propose a model, based on our biochemical and functional assay data, which may explain how beta-arrestin1 negatively affects the Notch signaling pathway.

Specifically, we propose a model in which beta-arrestin1 plays a bi-functional role in the negative regulation of Notch signaling by being involved in endosomal sorting and in degradation of Notch receptors. We also propose that the role of beta-arrestin1 in these two cellular events may be dependent on the intracellular concentration of Notch. At physiological levels of Notch activation (low intracellular level), beta-arrestin1 could mainly take charge of Notch degradation in the cytoplasm whereas at high levels of Notch activation (elevated intracellular level), beta-arrestin1 could also be involved in Notch endosomal sorting. This bi-functional role of beta-arrestin1 would thus contribute to controlling the amount Notch proteins translocating to the nucleus under normal conditions and would shut down Notch signaling when there is a sudden rise of Notch receptor activation (Figure 4.2.1).

Figure 4.2.1 Hypothetical model of the role of beta-arrestin1 in the Notch signaling pathway. Graphic representation of a hypothetical model for the role of beta-arrestin1 in the Notch signaling pathway.
4.2.1 The possible role of beta-arrestin1 in endosomal sorting

In the model we propose, beta-arrestin1 could be recruited to the activated Notch intermediate resulting from the TACE cleavage (before the gamma-secretase cleavage) to facilitate the endosomal sorting of the Notch receptor. Gamma-secretase cleavage is an enzymatic reaction and may limit the release of ICN into the cytoplasm. Therefore, at the endosomal membrane location in which Notch remains tethered to the membrane (because of a lack of available gamma-secretase), beta-arrestin1 could act as a scaffold protein for an E3-ligase (Deltex or others) to ubiquitinate Notch and direct the uncleaved receptors to the MVBs/lysosome pathway (since Notch is not known to be recycled).

We were able to demonstrate that beta-arrestin1 physically interacts with the Notch receptor in heterologous cells as well as endogenously in T-cells. In 293T cells, our overexpression studies reveal an interaction between beta-arrestin1 and the membrane-tethered form of Notch (~120kD) but not with the full-length receptor. Moreover, when we overexpressed Notch and beta-arrestin1 in 293T cells under GSI treatment which blocks Notch cleavage from the membrane, cells treated with GSI showed an accumulation of the complex beta-arrestin1-Notch trapped at the surface membrane. This suggests that beta-arrestin1 is recruited to an activated membrane-bound form of Notch receptor and the interaction is not dependent on the gamma-secretase cleavage.

4.2.2 The possible role of beta-arrestin1 in Notch degradation

We also propose in our model that beta-arrestin1 associates with ICN in the cytoplasm, and facilitates Notch ubiquitination and degradation in collaboration with Deltex (Figure 4.2.1). Our biochemical data support this model since we showed that beta-arrestin1 binds to ICN in 293T cells. Moreover, in our endogenous co-immunoprecipitation studies we revealed the loss of the interaction between Notch and beta-arrestin1 upon GSI treatment in HPBALL cells. These results suggest that only ICN recruits beta-arrestin1 endogenously in T-cells. Given that ICN is about 100kD, we
hypothesize in our model that the 110kD Notch species revealed by co-immunoprecipitation may represent ICN post-translationally modified monoubiquitination and endocytosis of the Notch receptor. Our functional data also support this model since knocking-down the intracellular level of beta-arrestin1 by siRNA significantly increased Notch signaling mediated by ICN but had no statistically significant effect on Notch signaling mediated by deltaE (Figure 3.2.3) (as discussed below). Further confirming the effect of beta-arrestin1 knock-down, beta-arrestin1 overexpression assays showed that Notch signaling is downregulated in a Notch-CSL dependent luciferase assay. Our functional data also demonstrate that Deltex may be involved in this negative regulation. Although we could not reveal the co-immunoprecipitation between beta-arrestin1 and Deltex, the presence of Deltex enhances the negative effect of beta-arrestin1 on Notch signaling. These results suggest that Deltex could be at least a functional interacting partner of beta-arrestin1.

4.2.3 The dual role of beta-arrestin1 may be dependent on the level of Notch activation

In our model, we propose that the dual role of beta-arrestin1 (endosomal sorting and degradation) may be dependent on the level of Notch activation. If our model is correct, when Notch is activated at concentrations close to physiological levels, beta-arrestin1 would mainly play a role in ICN degradation but a very minimal role in endosomal sorting. Under steady state conditions, it is possible that the role of beta-arrestin1 in endosomal sorting remains minimal since there is no massive amount of Notch receptors activated and endocytosed. As demonstrated in our endogenous T-cell co-immunoprecipitations, the interaction between beta-arrestin1 and Notch occurs after gamma-secretase cleavage and suggests that beta-arrestin1 associates only with ICN. T-ALL cells carry mutations allowing constitutive Notch signaling but the level of Notch protein expression in these cells is much lower than in cells expressing Notch protein under a viral promoter. Therefore, in T-ALL cells, the number of endocytosed Notch receptors may be small enough so there is no limitation imposed by gamma-secretase and most of the endocytosed Notch receptors are cleaved off the membrane. Thus, beta-arrestin1 would almost exclusively be recruited to ICN in the cytoplasm to promote its degradation.
However, in a Notch overexpression context, the role of beta-arrestin1 in endosomal sorting would become more prominent. Notch overexpression in 293T cells caused the recruitment of beta-arrestin1 to an activated membrane-bound form of Notch and GSI treatment did not cause the loss of the interaction between beta-arrestin1 and Notch. These results suggest that beta-arrestin1 is also recruited to the Notch receptor at the membrane (in addition to ICN). As GSI treatment does not affect the beta-arrestin1- Notch interaction, we can assume that beta-arrestin1 is recruited to the membrane where a large number of Notch receptors are endocytosed without being cleaved by gamma-secretase. Thus, beta-arrestin1 could participate in the endosomal sorting of uncleaved Notch receptors and may target them to the MVB/lysosomal degradation pathway since Notch is not known to be recycled at the membrane. Nonetheless, in addition to its role in endosomal sorting, beta-arrestin1 could also participate in Notch degradation in the cytoplasm. Endosomal sorting could be a way by which the cell quickly throws away extra Notch receptors before new gamma-secretase complexes become available and allows the extra Notch receptors to translocate to the nucleus.

In summary, in our model we propose that beta-arrestin1 could have two roles in the Notch signaling pathway depending on the level of Notch activation 1) sorting non-cleaved Notch receptors to prevent them from gamma-secretase cleavage and 2) sending Notch receptors cleaved by gamma-secretase to the degradation pathway.

This model does not explain all our results. Firstly, the endogenous co-immunoprecipitation study was only conducted in the HPBALL cell line, which was specifically chosen for its higher expression of beta-arrestin1 as well as its higher level of Notch protein expression. We could not observe co-immunoprecipitation in another T-ALL cell line (ALLSIL) in which the level of beta-arrestin1 and Notch expression were more comparable to other T-ALL cell lines.

Secondly, we showed that knocking-down the endogenous expression of beta-arrestin1 protein in U2OS did not have a statistically significant effect on Notch signaling when delta E (membrane-bound form of Notch) was overexpressed. As
explained in section 3.1.2 (Figure 3.1.7), ICN does not traffic to membranes but directly translocates to the nucleus after translation. Thus, the only difference between deltaE and ICN is their membrane processing. Therefore, when we knocked-down the protein level of beta-arrestin1, we expected to see similar upregulation in Notch signaling whether it was mediated by deltaE or ICN.

One explanation could be that Notch signaling is highly sensitive to dosage. In normal biology, a variety of cellular mechanisms ensure that an adequate amount of ICN translocates to the nucleus. One of these mechanisms is the regulation of the cleavage events occurring at the membrane. Gamma-secretase activity, location and availability may be a limiting factor in the membrane processing of the Notch receptor. When deltaE is overexpressed, the release of ICN into the cytoplasm may be limited by the activity and the availability of the gamma-secretase complex. Therefore, the amount of ICN translocating to the nucleus is elevated over endogenous levels but constant. However, when ICN is overexpressed in cells, it is not subject to any membrane regulation. Therefore, the amount of ICN present in the cytoplasm becomes suddenly very high compared to endogenous levels. It is possible that a sudden burst of ICN in the cytoplasm triggers a massive recruitment of proteins containing degradation machinery (including beta-arrestin1) to shut down Notch signaling. This reaction may occur to a much lesser extent when cells overexpress deltaE since the release of ICN into the cytoplasm would be more gradual. This would explain why the diminution of beta-arrestin1 protein level does not cause a significant increase in Notch signaling when deltaE is overexpressed. It is thus possible that beta-arrestin1 may modulate regulation by reacting against a sudden rise of Notch signaling.

Also, beta-arrestin1 seems to exert its negative effect on Notch signaling only in a certain cellular context. In fact, at equimoles amounts of Notch and beta-arrestin1 (80ng) were transfected into U2OS cells, beta-arrestin1 overexpression caused a very minor decrease in luciferase expression of the Notch reporter (Figure 3.2.4). One possibility is that our overexpression study was done in heterologous cells and it is possible that the appropriate E3-ligase was not present in U2OS cells to promote Notch degradation. This would explain why small DNA amounts (40 and 80 ng) of beta-
beta-arrestin1 transfected in U2OS cells caused a mild diminution of the luciferase expression. On the other hand, high amounts of DNA transfected into U2OS cells (160ng of beta-arrestin1) were sufficient to decrease the luciferase expression of our Notch reporter by about 30% compared to the control (Figure 3.2.4). It is possible that beta-arrestin1 is present in high amounts and associates non-specifically with other E3-ligases present in the cytoplasm to target Notch to the degradation pathway.

Finally, we showed that the negative effect of beta-arrestin1 on Notch signaling was enhanced by the presence of Deltex. However, we did not reveal an interaction between beta-arrestin1 and Deltex by co-immunoprecipitation and several biological and technical reasons can explain our negative results. Deltex and beta-arrestin1 may indirectly associate through their respective binding to the Notch receptor. Notch may be acting as a linker between beta-arrestin1 and Deltex in order to form a trimeric complex between Notch, beta-arrestin1 and Deltex (figure 4.2.2). In this case, Deltex and beta-arrestin1 would each be binding to Notch protein separately, but would not be physically interacting.

Figure 4.2.2 Hypothetical representation of a trimeric complex between beta-arrestin1, Notch and Deltex.
Secondly, the role of Deltex in the Notch signaling pathway is highly cell context dependent. In *Drosophila*, it was first identified as a positive regulator whereas in mammals, it was clearly demonstrated as a negative regulator of the Notch signaling pathway. The cells (293T) in which we performed our co-immunoprecipitation studies may not have provided the right cellular environment for an association between Deltex and beta-arrestin1. However, we tried to co-immunoprecipitate Deltex and beta-arrestin1 in a more relevant cell line (T-ALL cells) in which Notch and Deltex were endogenously expressed (Figure 3.1.4). T-ALL cells are blocked at the double-positive stage of thymocyte differentiation. It is possible that the negative regulation of Deltex on Notch signaling is minimal at this step of maturation and thus, the association between Deltex-beta-arrestin1 and Notch did not occur. This could explain why we could not co-immunoprecipitate Deltex and beta-arrestin1 in T-ALL cells.

Conversely, it is possible that Deltex and beta-arrestin1 may not interact. Although beta-arrestin1 is one of the closest human homologue of Kurtz, they share only 60% identity and 73% similarity at the amino acid level. It is possible that beta-arrestin1 may associate with a different E3-ligase than Deltex to promote Notch ubiquitination in mammals, or that Deltex uses another adaptor protein to ubiquitinate Notch. Our functional data may refute this hypothesis since we show that beta-arrestin1 participates in the negative regulation of Notch signaling, and the presence of Deltex enhances this effect.

Deltex is a RING E3-ligase and is believed to participate in the cascade of reactions leading to the transfer of ubiquitin onto a substrate. Enzymatic reactions are generally quick and transient and so, studying such protein-protein interactions brings several technical challenges. Some E3-ligases are known to associate with their substrate very transiently and this phenomenon could explain why the Deltex-beta-arrestin1 interaction was impossible to reveal by co-immunoprecipitation. If Deltex quickly associates with beta-arrestin1 to ubiquitinate Notch for instance, it would be almost impossible to capture the dynamic interaction by co-immunoprecipitation. Also, the association between Deltex and beta-arrestin1 may be very weak so that protein solubilization may destroy the interaction. We anticipated this problem and we chose a
lysis buffer based on several studies either showing a Deltex-Notch or a Deltex-Kurtz interaction. Although we tried to stay within the range of mild non-denaturating lysis buffers, we did not test other lysis buffers, such as those available commercially which are specifically designed for more delicate co-immunoprecipitation studies. It is therefore possible that our co-immunoprecipitation conditions were sub-optimal for a Deltex and beta-arrestin1 interaction while they were optimal for the beta-arrestin1-Notch interaction.

In addition to weak and transient interaction, Deltex and beta-arrestin1 may not co-immunoprecipitate because of antibody steric hindrances. The anti-FLAG antibody conjugated to Protein G beads may interfere and/or block binding sites involved in beta-arrestin1 interaction with Deltex. Besides, using fusion proteins constituted another technical challenge. There are no specific antibodies available commercially against Deltex. That forced us to rely on a N- or C-terminus tag epitope to visualize Deltex by Western blot analysis. Even though, we moved the tag from the C-terminus to the N-terminus of Deltex, we did not detect any interaction with beta-arrestin1. Also, it is possible that the presence of the epitope tag may have changed the conformation of Deltex at some binding sites involved in its interaction with beta-arrestin1.
4.3 Future directions

The model we propose to explain our results also raises other questions regarding the nature of beta-arrestin1 protein interactions as well as the other functions in which beta-arrestin1 is involved. For instance, beta-arrestins are involved in endocytosis of transmembrane receptors. So far, there are no studies explaining how Notch endocytosis may be mediated by a clathrin-dependent mechanism, what events trigger Notch endocytosis, or what endocytotic machinery is recruited to allow Notch endocytosis. It would be interesting to fully understand the steps by which Notch is endocytosed especially since all commercial inhibitors target only Notch cleavage events and have side effects rendering them useless for in vivo studies. Also, GPCR studies have revealed that beta-arrestin recruitment to the plasma membrane depends on phosphorylation of G-coupled receptors. In this study, we show that Notch and beta-arrestin1 interact together and these findings may indirectly suggest that Notch is phosphorylated in the cytoplasm. So far, little is known about Notch phosphorylation events in the cytoplasm. Does Notch activation trigger phosphorylation events at the plasma membrane? Does Notch phosphorylation have an impact on the fate of Notch receptor or on the downstream cascade of events following gamma-secretase cleavage?

4.3.1 Notch ubiquitination studies

Studying Notch ubiquitination would be the next step in this research project. It would be important to first determine which of the Notch intermediates are ubiquitinated and the type of ubiquitination modifications these intermediates undergo (e.g. mono- or multi- or polyubiquitination). Ubiquitination assays can be quite challenging and many attempts have been made during this study to demonstrate Notch ubiquitination. Once the ubiquitination of Notch intermediates is shown, it would be interesting to determine whether modulation of the cellular level of beta-arrestin1 has an impact on Notch ubiquitination. Using an RNA interference (siRNA) approach would be a way to prove that beta-arrestin1 participates to Notch ubiquitination. Because of the multi-functional
role of beta-arrestin1 in the cell, these results could greatly contribute to our understanding of the fate of Notch receptors after ligand activation.

4.3.2 Identity of the E3 ligase that interacts with beta-arrestin1

As hypothesized in our model, it is not impossible that different E3-ligases use the same adaptor protein (in this case beta-arrestin1) to ubiquitinate Notch, depending on its location or its cellular concentration. Identifying the E3-ligase(s) involved in the different ubiquitination steps in which beta-arrestin1 participates would be another interesting question to answer. Notch is highly context and dosage-dependent, and these characteristics of the Notch signaling pathway are likely to increase the challenges of these experiments. Here, TALL cell lines could be used as a model to avoid Notch exogenous expression and to provide the right cellular context for proteomic studies. Using more sophisticated proteomic methods, we could isolate protein complexes formed by Notch, beta-arrestin1 and other interacting proteins under non-denaturating conditions to identify the members of the complex by mass spectrometry. Hopefully this approach would generate a list of potential E3-ligase candidates that could be linked to beta-arrestin1 and Notch interactions. These experiments would help to characterize how Notch is modified by ubiquitination and would elucidate the fate of Notch receptors upon ubiquitination modifications. Identifying the E3-ligases responsible for each of these ubiquitination events would bring us closer to understanding the mechanism of Notch regulation through the ubiquitination pathway.

4.3.3 Notch endocytosis studies

Notch regulation at the membrane is mostly known through studying Notch cleavage events (S2 and S3 cleavages). Little evidence demonstrates the endocytosis and the trafficking of uncleaved Notch receptor. It would be interesting to better understand how Notch is endocytosed and if beta-arrestin1 participates in this process. Our T-ALL cells endogenously express beta-arrestin1 and they carry mutations allowing ligand independent Notch signaling. Therefore, T-ALL cells display a high rate
of Notch endocytosis. We could address this question by using cell biology techniques like immunocytochemistry and confocal microscopy analysis to characterize the participation of beta-arrestin1 in Notch endocytosis.

4.3.4 Notch phosphorylation studies

Beta-arrestin1 is normally recruited to phosphorylated receptors. No clear evidence has shown the phosphorylation of Notch upon activation. Therefore, it would be interesting to know if Notch is phosphorylated at the membrane and if beta-arrestin1's binding is dependent on the phosphorylated level of the Notch receptor. To do this, we could co-immunoprecipitate beta-arrestin1 and Notch with or without phosphatase inhibitors and look at the fate of the interaction when Notch is phosphorylated vs non-phosphorylated. These results would highlight the potential role of phosphorylation in the cellular regulation of the Notch receptors.

In conclusion, a further understanding of the intracellular regulation of Notch is paramount given the fact that Notch has a pivotal role in the development of the embryo, in cell proliferation and in cell survival. Here we provide new evidence that beta-arrestin1 interacts with the Notch receptor and plays a role in the regulation of the Notch signaling pathway.
References


Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* **18**, 699-711.


Appendix

Raw luciferase data

A) siRNA against beta-arrestin1 experiment. Figure 3.2.3

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B) Overexpression of beta-arrestin1 in the presence of Deltex Figure 3.2.5

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